LHRH Peptides as Female and Male Contraceptives

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Preface

A widespread and, unfortunately, an erroneous belief is that the population problem is now less serious than it was ten years ago. This belief has been encouraged by articles in the lay and the popular press that purport to show that the large number of births occurring in the decade of the sixties did not occur also in the decade of the seventies. While it is true that population growth during the past decade did not increase as rapidly as demographic projections in the early sixties predicted, the rate of increase was, and still is, sufficiently high to double the world's population to eight billion or more by the middle of the next century, if not sooner. Indeed, the current situation of continually expanding population growth is perhaps even more critical in 1980 than it was in 1970, for there exists a huge number of children born during the past twenty years who are now entering their reproductive years, and during the 1980s this bulge of reproductive age women and men will continue to increase. Estimates of the total number of women at risk of pregnancy on a worldwide basis have increased from about 500 million in 1971 to over 600 million in 1979. Generally, only about 20% to 25% of these women and their partners use some form of recognized fertility control.

There are many constraints, both real and imagined, that prevent effective family planning programs. Significant among these constraints are the inadequacies and nonacceptability of presently available methods of fertility regulation. The existing methods have significant limitations with respect to the development of side effects and long-term safety concerns. Other major limitations include the reliance on medical delivery systems, actual use-efficacy of a method, and the ever-important costs to the consumer as well as to a family planning program. These limitations have severely curtailed the widespread availability and use of all presently existing methods. Extensive differences in cultural, religious, and personal attitudes, health status, and socioeconomic situations absolutely necessitate as wide a range of fertility control methods as is technologically possible. These methods must be personally and culturally acceptable, convenient to use, safe, inexpensive, easy to distribute and store, and they must accommodate a variety of changing life situations in the same couple as the partners progress through their reproductive years.

Recognizing the profound and beneficial impact of contraceptive technological advances in the success of family planning programs, the Agency for International Development (AID) has, over the past ten years or so, supported a variety of research programs with the objective of improving the technology of fertility control. One of these programs is the Program for Applied Research on Fertility Regulation (PARFR), which was established in 1972 at the University of Minnesota with the financial and technical assistance of AID. In 1975, PARFR moved to its present location at the downtown Chicago campus of Northwestern University. The primary objective of PARFR is to provide financial, technical, and scientific assistance to both United States and foreign institutions for applied research with the goal of developing safe, effective, and acceptable methods of fertility regulation for both men and

women. Since the inception of PARFR, approximately 150 innovative projects have been supported at some 100 institutions in many developed and developing countries. Despite many administrative and research constraints and obstacles, PARFR has been responsible for the development of a significant number of promising new or improved fertility regulation methodologies for men and women.

As one of its major objectives, PARFR reviews and disseminates the findings of applied research in the field of fertility regulation. This objective is accomplished through a variety of mechanisms, including PARFR workshops. The present workshop is the ninth in a series of PARFR workshops bringing together scientists, physicians, and international family planning experts to consider significant and timely issues in the field of fertility regulation. Earlier conferences focused on the following topics: Hysteroscopic Sterilization; Control of Male Fertility; Advances in Female Sterilization Techniques; Risks, Benefits, and Controversies in Fertility Control; Reversal of Sterilization: Pregnancy Termination: Procedures, Safety, and New Developments; Vaginal Contraception: New Developments; and Research Frontiers in Fertility Regulation. PARFR also supported a workshop sponsored by the National Academy of Sciences/Institute of Laboratory Animal Resources, on Animal Models for Research on Contraception and Fertility. Proceedings of these workshops have been published as the PARFR Series on Fertility Regulation.

The intent of these workshops is to inform PARFR and other interested agencies and institutions of advances in various methods of fertility regulation. The workshops also provide a forum for technical review of new approaches in order to provide guidance to PARFR, AID, and other agencies for the directions of future research. We believe that these workshops have provided a most valuable function in bringing scientific thinking up to date on each of the selected topics.

For the past five years there has been a continual increase of scientific and public interest in the potential of LHRH agonists and antagonists as reversible contraceptives for both women and men. In fact, articles in the lay press already have heralded these substances as the first "unisex" contraceptive, and these same articles have predicted that the new contraceptives soon will become commercially available. As with other recent technological advances, expectations often overshoot reality, and there is little or no public recognition of the tremendous effort still required even to establish the contraceptive feasibility of these peptides, let alone to develop them into effective and safe pharmaceutical products for the regulation of human fertility.

Accordingly, PARFR organized a workshop that was held in Chicago in May, 1981, and invited 70 scientists and researchers in this exciting new field to report their findings and describe their work in progress. The objectives of the workshop were to provide an overview of current research efforts and to update the potential use of these LHRH peptides as female and male contraceptives. The proceedings of this three-day meeting, presented here, include the formal papers and summaries of discussion sessions. Of special interest are the two panel discussions at the end of the volume, which address what is needed for the further development of LHRH analogs as contraceptive agents for women and men.

Gerald I. Zatuchni, M.D., M.SC.

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LHRH Peptides
as Female and Male Contraceptives

PARFR Series
on Fertility Regulation

PHARMACOLOGIC DEVELOPMENT OF LHRH AND ANALOGS

1

Pharmacology of LHRH and Analogs

JOHN M. STEWART

The last decade has seen tremendous advances in our understanding of the control mechanisms that regulate reproduction and sexual function in animals. With this increased knowledge comes the ability to enhance or inhibit fertility with concomitant new possibilities for contraceptive development.

The most important development in this area has been the discovery of the structure of luteinizing hormone releasing hormone (LHRH). This is the hypothalamic peptide that stimulates the release of luteinizing hormone and follicle-stimulating hormone (FSH) from the anterior pituitary.

Since the structure of LHRH was found to be a decapeptide, chemists could readily synthesize this material in large quantities for physiologic and pharmacologic studies. In subsequent years, programs of research have led to the synthesis of very potent agonist and antagonist analogs of LHRH and their application to regulation of fertility and reproductive endocrine functions. Today clinical use of these peptides is already a reality, and preliminary studies under way may soon lead to wide-scale development and use of drugs based on LHRH.

CHEMISTRY OF LHRH

The structure of LHRH, as determined in the laboratories of Schally[17] and of Guillemin[4] from porcine and ovine hypothalamic tissues, respectively, is that of the decapeptide <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Fig. 1-1). This structure is common to all mammalian species that have been investigated. Owing to the availability of the rapid and elegant solid-phase method of peptide synthesis introduced by Merrifield,[19,28] synthesis of this peptide was accomplished rapidly in several laboratories.[16,21,26] The use of automatic instruments for solid-phase peptide synthesis, benzhydrylamine resins for synthesis of peptide amides,[23] and rapid methods of peptide purification (such as countercurrent distribution and high-performance liquid chromatography) have made the synthesis of peptides related to LHRH a relatively easy task and have provided large quantities of these materials for study. At present, more than a thousand analogs of LHRH have been synthesized and evaluated biologically.

Essential to accurate regulation of the circulating levels of any hormone are mechanisms for rapid inactivation of the molecule. At first inspection the

1. LHRH: Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
2. Super Agonist: Glu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-NH-C_2H_5
3. Potent Antagonist: Acetyl-Ala-D-ClF-D-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2

Fig. 1-1. Structures of LHRH and potent analogs. (D-ClF = D-*p*-chlorophenylalanine).

LHRH molecule appears to be designed for long life—since it has the pyroglutamic residue blocking the amino end of the molecule to protect it from degradation by aminopeptidases and the amide function to protect the carboxyl end of the peptide from degradation by carboxypeptidases. In actuality, LHRH has only a short half-life.

It was learned early that the primary site of inactivation of LHRH is at the Gly^6-Leu^7 bond, which is broken by a brain enzyme.[12,15] A subsidiary site of inactivation is at the carboxyl end, where the glycineamide residue is removed by an enzyme which also inactivates oxytocin and vasopressin. Peptide synthetic chemists routinely insert D-amino acids into the molecules of peptide hormones in an effort to block enzymatic degradation. In the case of LHRH, a D-amino acid is acceptable in position 6 without reduction of potency of the hormone; in fact, potency is markedly enhanced, owing to protection from degradation.[20] At present, all the potent agonist and antagonist analogs of LHRH have a D-aromatic amino acid in this position.

Exchange of a D- for an L-amino acid, particularly in the center of the peptide molecule, makes a large change in the range of conformational possibilities available to the molecule; this change usually has a great effect on the way the hormone interacts with its receptors. Of all the amino acids, glycine, having no side chain, is not optically active and can assume a pseudo-D or pseudo-L conformation in peptides. In the case of LHRH, the peptide evidently binds to receptors with the glycine residue in the pseudo-D conformation, since a D-amino acid is acceptable at this position. This is by no means universally true in peptide hormones. The only other instance in which substitution of a D-amino acid for glycine is acceptable and enhances potency is in the enkephalin peptides. In other cases, insertion of a D-amino acid decreases potency drastically. In peptide hormones, the small glycine residue apparently usually serves primarily as a "spacer" to hold the more important amino acid side chains in the proper spatial relationship for interaction with receptor-binding sites. This appears to be the case with LHRH, since the critical sites for receptor interaction are near both ends of the peptide chain. In addition, there does not appear to be a restriction in the space available to the peptide at this site when it combines with receptors. Amino acids with large, bulky side chains and even polymers (attached to the side chains of lysine or glutamic acid residues) can be placed in position 6 of LHRH with retention of good potency.

A useful modification of the carboxyl end of LHRH was introduced by Fujino, who showed that removal of the glycine and termination of the peptide at the proline residue with an ethylamide (Fig. 1-1) greatly enhanced the potency of LHRH agonists.[8] Yet, this modification does not appear to en-

hance the potency of LHRH antagonists. At present, then, potent LHRH agonists have a D-aromatic residue at position 6 and the Fujino modification at position 10.

Very soon after the structure of LHRH was known, the Guillemin laboratory group discovered that the key to intrinsic activity in LHRH peptides was the histidine residue in position 2. Omission of that residue or substitution of it by other small amino acid residues led to analogs with weak but significant inhibitory potency.[29] Somewhat later, the Wyeth group[25] discovered that insertion of a D-aromatic amino acid residue in position 2 created antagonists with much enhanced potency, and they described the first antagonist with *in vivo* activity.[32] Further enhancement of antagonist potency came with the discovery that the amino acid residues in positions 1 and 3 could also have the D-configuration. The potency of antagonists was increased even further with the discovery that the pyroglutamic acid residue in position 1 could be replaced by an acylated amino acid, either D- or L-.[7] The last compound in the table in Figure 1-1, incorporating all these modifications, is the most potent LHRH antagonist known. Such antagonists have extremely high affinity for LHRH receptors and appear to be useful in contraceptive studies.

Structure–activity relationships in a hormone cannot be studied without adequate assays to evaluate the effect of structure modifications. Straightforward and reproducible *in vitro* and *in vivo* assays are necessary. *In vitro* assays can give the most accurate information about combination of the hormone with its receptors and activation of functional responses in the target organ tissue. *In vivo* assays have superimposed upon these considerations the additional factors of drug absorption, transport, and metabolism. In LHRH work, receptor binding can be studied with membrane preparations from rat anterior pituitary tissue.[10]

Binding potency of LHRH analogs is studied by competition with a radioiodinated superagonist for membrane receptors. Even in this *in vitro* system, degradation of the radioactive standard is significant. It is thus important to use an enzyme-resistant superagonist rather than LHRH itself. Both the superagonists and the most potent antagonists presently available show much higher affinity for LHRH receptors in this assay than does LHRH itself.

Although high-affinity specific binding to LHRH receptors is a prerequisite for biological function, it is by no means sufficient. It is therefore necessary to use an assay in which the normal biological function of LHRH—namely release of luteinizing hormone (LH) and FSH—is measured. Formerly the standard *in vitro* assay for this function was incubation of excised hemipituitaries with the analog in question. Antagonists are studied by examining their effect on release of LH and FSH induced by a standard dose of LHRH. More recently, better results have been obtained by using dispersed anterior pituitary cells in culture and applying agonist and antagonist peptides to these cultures. The LH and FSH that are released are measured by radioimmunoassay. These *in vitro* assays serve as the primary guide for study of structure–activity relationships in LHRH analogs today.

The ultimate answer, of course, is functioning of the peptides *in vivo*. LHRH agonists have most often been studied by measuring the increase in LH and FSH concentration in the blood of mature, ovariectomized rats pre-

treated with estrogen and progesterone.[24] Antagonists can be examined in this system by injection of a mixture of the presumed antagonist with LHRH or a suitable superagonist.

To further contraceptive development, inhibition of endogenous LHRH function in an intact normal animal should be studied. The most widely used assay has been inhibition of ovulation in the normally cycling rat.[7] Female rats maintained on a fixed light schedule ovulate every fourth day. At approximately 2 p.m. on the day of proestrus, a burst of LHRH is released from the hypothalamus, followed by the preovulatory surge of LH and FSH at about 5 to 6 p.m. Ovulation occurs about midnight. The next day the animals are killed and examined for the presence of shed ova by flushing the tubes and uterus and counting the ova that are present. If an adequate dose of a potent LHRH antagonist is injected at noon on the day of proestrus, ovulation will be inhibited. Since the potent antagonists are generally insoluble in water, these peptides are often administered in corn oil solution or suspension; this may actually improve the response by prolonging the release of peptide from the oil depot. When assayed in this way, compound 3 in Figure 1-1 shows an ID_{50} of approximately 5 μg peptide per rat. Inhibitors with potency of this order of magnitude may already be useful for many purposes, although doses required for inhibition of human ovulation may still be prohibitively large.

ROLES OF LHRH IN THE ENDOCRINOLOGY OF REPRODUCTION

Successful outcome of the reproduction process requires precise interdigitation and interaction of the various links in a long chain of events. These events take place in at least five distinct loci in the body:

1. Higher brain centers
2. Hypothalamus
3. Anterior pituitary
4. Gonadal steroidogenic cells
5. Gonadal gametogenic cells.

Correct functioning of each of these steps depends absolutely upon the presence of the appropriate stimulus from the organs involved in the preceding steps, with overall control of the system being regulated by complex positive and negative feedback mechanisms.

Table 1-1 lists the steps leading to the production of ova and sperm, along with a summary of what is known about the nature of the messengers produced at each step and the mechanisms by which production of these messengers is regulated. It is apparent that LHRH is the actor at the focal point in the control chain. As far as is known, LHRH is the sole—or at least principal—stimulatory substance produced by the hypothalamus, and is the uniquely adequate stimulus for gonadotropin release from the anterior pituitary. All of the other steps in the chain involve multiple messengers at each step.

TABLE 1-1. Control of Reproduction

LOCUS	MESSENGER PRODUCED	PRODUCTION STIMULANT	PRODUCTION INHIBITOR
Higher brain centers	Norepinephrine	Estrogen	Estrogen
	Dopamine	Other factors	ACTH (?)
	Endorphins		Other factors
Pineal	Melatonin	Darkness	Light
Hypothalamus	LHRH	Norepinephrine	Endorphins
		Progesterone	Melatonin
		Estrogen	Estrogen, androgen
			Dopamine, serotonin
Anterior pituitary	LH, FSH	LHRH	Estrogen, androgen
	FSH	FSHRH (?)	Inhibin
		Estrogen, androgen	
Gonads			
Steroidogenic cells	Estrogen	FSH	LHRH (?)
	Androgen	LH	
	Progesterone*		
Gametogenic cells	Ova	Estrogen	
	Sperm	Androgen	

*In corpus luteum.

The hypothalamus serves as an integration point for the various factors that regulate reproduction. The higher brain centers impose both circadian and ultradian rhythms upon the hypothalamic secretion of LHRH.

The pineal gland appears to be a major regulator of the circadian rhythm of endocrine regulation, although the exact mechanisms involved are poorly understood.[31] As far as is known, the principal regulatory substance produced by the pineal gland is melatonin, which is synthesized from serotonin by a series of reactions that are regulated by light (Fig. 1-2). In some lower species the pineal gland itself appears to contain the light-sensing mechanism, but in mammals the eye serves as the light sensor.

The pineal is innervated by nerve fibers from a branch of the optic nerve, which synapse in the superior cervical ganglion. These fibers release norepinephrine (NE) at their terminals in the pineal. The level of NE rises in the dark and falls during light hours. The released NE acts upon β-receptors to increase the level of cyclic AMP, which then stimulates the synthesis of serotonin acetyl transferase, the enzyme that catalyzes the first step in the conversion of serotonin to melatonin. The second enzyme, hydroxyindole O-methyl transferase, may also be influenced by similar mechanisms. Thus the amount of melatonin produced in the dark increases markedly and appears to exert its effect mainly by inhibiting hypothalamic function. It is not clear whether this effect on the hypothalamus may involve hormones other than LHRH. This physical-neuronal-biochemical-humoral chain may explain, at a simplistic level, the fact that female rats placed in constant light remain in constant estrus, that male rats in constant darkness perform poorly sexually, and that blind men frequently are impotent.

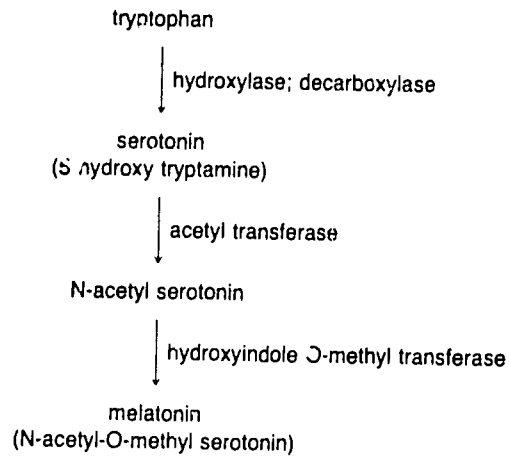

FIG. 1-2. Synthesis of melatonin in the pineal gland.

Additional innervation of the pineal gland by fibers originating in the habenula has been described recently. This provides a connection of the pineal with the limbic system and may explain the effects of emotional factors upon sexual function. The pineal body also contains large amounts of LHRH. It is not clear whether LHRH is produced in the pineal or whether it is delivered there by axonal transport from LHRH neurons in the hypothalamus. LHRH serves as a potent stimulus for mating behavior in rodents,[22] this effect most likely being mediated via the septum or amygdala,[13] other limbic brain centers known to be intimately involved in sexual behavior.

Light and darkness are by no means the only factors that affect LHRH release through the action of higher brain centers. Sleep is known to have a profound effect on LH levels. LH appears to be secreted primarily during periods of slow wave sleep (deep sleep). This is particularly striking at the onset of puberty, when large sleep-associated surges of LH occur and evidently play an important role in initiating the processes that lead to sexual maturity.[30]

Recent interest in opioid peptides in the brain has led to some insight into the mechanisms whereby opiates affect sexual functioning. It has long been known that drug addicts show depressed sexual function, although it has not been clear that this was not solely a behavioral effect. Recently, however, it has been shown that opiates and opioid peptides do in fact cause decreased secretion of LHRH and consequently lowered levels of circulating sex steriods.[8] Low doses of opiates or opioid peptides inhibited mating behavior in normally active male rats, and the morphine antagonist naloxone stimulated mating behavior in normally inactive male rats.[9] Furthermore, there is a significant release of enkephalin from the hypothalamus just before the peak of LHRH release on the day of proestrus in rats.[6] Apparently enkephalin inhibits LHRH release, and when the enkephalin level falls precipitously on the day of proestrus LHRH is released.[14,18]

Gonadal steroids have both stimulatory and inhibitory effects on LHRH release from the neurosecretory cells of the hypothalamus. The preovulatory surge of LHRH in rodents appears to be induced, to a large degree, by the rising level of estrogen produced by the maturing ova. On the other hand, very high levels of estrogen actively inhibit release. It has been suggested that these opposing effects of estrogen on LHRH secretion are mediated by different classes of neurons in the hypothalamus.[18] The stimulatory action of estrogen is thought to be exerted primarily on LHRH-producing cells in the anterior hypothalamus, whereas the inhibitory action is apparently mediated primarily through more caudally located cells in the arcuate region of the hypothalamus. The inhibitory action of estrogen has been exploited on a very wide scale in the use of oral contraceptives.

Underlying all the other effects on LHRH secretion is a basic ultradian rhythm having a cycle time of 90 to 120 minutes. Many body functions that involve control by the central nervous system are regulated by this rhythm; sleep is a notable case that has been studied better than most. This rhythm in LHRH release can be seen best in castrated animals, where the inhibitory effect of gonadal estrogen or androgen is lacking and LHRH release is greatly enhanced. As discussed later, this pulsatile release of LHRH is critical for normal functioning of the reproductive system. Though a preovulatory peak of LHRH in rats can be demonstrated under appropriate conditions,[27] in other species, notably humans, evidence for this peak is lacking, and the preovulatory burst of LH and FSH may be released principally in response to a greatly increased sensitivity of pituitary cells to the normal pulsatile cycles of LHRH, which apparently continue without major change.

Many LHRH-producing neurons in the hypothalamus appear to be innervated by NE-producing neurons.[18] The cell bodies of these NE neurons lie in the midbrain, principally in the area of the locus ceruleus. NE appears to stimulate release of LHRH from both of the classes of hypothalamic cells discussed above. This innervation by NE-producing cells, in conjunction with other less well-understood dopaminergic innervation, probably accounts for the marked but poorly understood effects of emotional factors upon normal sexual and reproductive functioning.

Action of LHRH upon the Anterior Pituitary

LHRH stimulates the gonadotrophs of the anterior pituitary to secrete both LH and FSH. The exact nature and number of steps involved in the process between combination of LHRH with its receptors on the external membrane of the pituitary cells and ultimate release of LH and FSH into the circulation are not known. In common with most secretory processes, the ultimate and critical factor appears to be an increase in intracellular calcium concentration. An earlier step in the process may be an increase in the intracellular level of cyclic AMP, although this is less clear.

The actual process of regulation of LH and FSH release is far more complex than indicated in this portion of text. The mechanisms for regulation of the differences in relative rates of secretion of LH and FSH occurring in the different phases of the human menstrual cycle are not understood. Although estrogen and progesterone are known as major regulators of these differ-

ences, the ultimate mechanisms at the hypothalamic level are not understood. There may be a separate FSH-releasing hormone that has not yet been purified and characterized. Although most of the LH and FSH produced by the anterior pituitary appears to arise from a single type of gonadotroph that is stimulated by LHRH, this does not allow selective release of the gonadotropins. According to P. K. Nakane,* in rat pituitary there are other classes of cells, some containing only LH, and others, only FSH. It may well be that these cells are the ones responsible for the differential release of LH and FSH.

The concept of down-regulation of receptor concentration in target organs by sustained high levels of a hormone was originally introduced to explain some phenomena in the action of insulin. It also appears to be very important in regulating the action of LHRH on the anterior pituitary.[2] Early experiments with LHRH superagonists showed that with continued administration of the peptide, initial stimulation of gonadotropin release was followed by a return to baseline levels or below. This frustrated attempts to induce reproductive function in hormonally deficient individuals. Receptor-binding studies have shown that the effect is due to a decrease in the number of receptors on the cell and not to an alteration of the affinity of the receptors for LHRH. This phenomenon has diverse consequences. In the first place, it means that for a continued stimulation of pituitary function, LHRH must be delivered in a pulsatile fashion, similar to that which occurs normally. Thus LHRH superagonists must be administered carefully and in controlled doses. It is of interest for animal experimentation that osmotic minipumps have now been adapted to provide pulsatile release of substances *in vivo*. The prompt and marked down-regulation of receptors by continued agonist administration means that a superagonist can serve effectively as an antagonist. This has now been demonstrated amply in both animal and human studies.[11] As detailed in later chapters, the use of superagonists as contraceptives is already at the stage of clinical trial.

Although the production of unacceptable side-effects may prevent use of the peptides alone, it may be possible to use them in conjunction with added steroid to provide effective and acceptable contraceptives. The mechanisms by which gonadal steroids regulate anterior pituitary sensitivity to LHRH are less well understood and need further investigation.

Actions of LHRH at the Gonads

The recent discovery of LHRH receptors on gonadal cell membranes has opened new possibilities for fine-tuning of endocrine regulation.[1] Interaction of LHRH with these gonadal receptors appears to cause inhibition of certain steps in the biosynthetic pathway for the conversion of cholesterol to the sex steroid hormones. It was originally suggested that the inhibitory effects seen with long-term chronic administration of LHRH superagonists might be mediated at the gonadal level, but it now seems more likely that these effects are mediated via down-regulation of LHRH receptors on the pituitary. The

*Personal communication.

role of the gonadal LHRH receptors is not clear. Further information appears in later chapters. Much additional research is required to clarify the interactions between all the different actors in the complex play of reproduction.

MEDICAL APPLICATIONS OF LHRH AGONISTS AND ANTAGONISTS

Development of a new contraceptive based on LHRH was a major goal of reproduction researchers even before the hormone was characterized and synthesized in 1971. As soon as the key for synthesis of antagonists was discovered, a large effort was directed toward increasing the potency of such antagonists. As mentioned, antagonists that may be potent enough to provide useful contraceptive drugs are available, but there is not adequate information on relative potency of the best LHRH antagonists in humans. Early studies reported by Schally have been disappointing, although they were not done with antagonists having the potency available today. The search for more potent antagonists is continuing in several laboratories.

During the entire decade of LHRH research, some pessimistic researchers have insisted that an acceptable contraceptive based on LHRH could not be developed, since such a peptide drug would require not only administration by injection but precise timing of its administration. These objections have been largely overcome. Satisfactorily therapeutic levels of LHRH agonists and antagonists can be achieved by routes other than injection. Such peptides are effective when administered intranasally, although much larger doses are required than when administered by injection. This decreased efficacy was prohibitive with low-potency analogs. Effective clinical work is now being done with LHRH analogs by intranasal administration. If ongoing research provides new analogs having greatly increased potency, intranasal administration should be eminently practical. Since much of the enhanced potency of LHRH analogs is due to incorporation into the peptides of features that make them resistant to degradation by enzymes, the problem of precise timing of peptide administration also becomes less serious. Sustained effectiveness can be achieved in humans with single daily applications of potent agonists or antagonists.

LHRH research took its most surprising turn with the discovery of the profound inhibition produced by sustained administration of potent LHRH agonists. Since the agonists available at that time appeared to have greater net potency than the antagonists, they were immediately exploited as potential contraceptives. In the male, these LHRH superagonists have proved to be almost too effective, and apparently require some additional modification in administration. At the same time, additional research into the effects of chronic administration of potent LHRH antagonists is necessary. The antagonists may also produce effective down-regulation of LHRH receptors; if that proves to be the case, they may ultimately be more satisfactory than the agonists. The side-effects accompanying prolonged treatment with either class of peptides are as yet unknown.

In addition to their use as potential new contraceptives, the LHRH analogs are proving valuable in other human disorders. When administered by ap-

propriate routines, LHRH superagonists have proved to be effective in both males and females for inducing fertility in subfertile individuals. The use of LHRH superagonists to reverse the undesirable features of precocious puberty in children offers promise for alleviating not only the physical but also the psychological problems involved. Indeed, LHRH research stands at the threshold of a new era of research in reproduction.

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2

GnRH Analogs: Structure–Activity Relationships

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Because of the anticipated physiologic role and importance of the decapeptide GnRH in the control of reproductive functions, with possible applications of similar molecules as investigative tools or contraceptive agents, hundreds of analogs of this peptide have been made and tested *in vitro* and *in vivo* for agonist and antagonist activity.

Two classes of analogs have been developed: agonists, which are equal to or several hundred times more potent than GnRH itself, or antagonists. Agonists are nonapeptides with the following features:

1. A D-amino acid[1,2,3] at position 6 (preferably aromatic and hydrophobic), where it might fulfill two receptor requirements:
   a. Stabilizing a preferred backbone conformation
   b. Exposing a hydrophobic moiety capable of interacting and binding within a hydrophobic receptor pocket possibly through π–π interaction
2. A proline C-terminus ethylamide.[4]

The antagonists have modifications involving the three N-terminal amino acids, resulting in compounds having reduced or no intrinsic activity and high binding affinity.

Stepping stones in the development of such antagonists were the synthesis of des-His^2-GnRH,[5] the first analog to be demonstrated to exert inhibitory

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activity. This was followed by the synthesis of [D-Ala^2]-GnRH, a weak antagonist,[12] and [D-Phe^2]-GnRH,[17] which is about three times as potent as des-His^2-GnRH. Substitution of Trp^3 by D-Trp^3 was shown by Coy and associates[3] to be more effective in an antiovulatory test. [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH was later shown to be long-acting and to inhibit ovulation in the rat at a single dose of 250 μg.[26] Introduction of pClD-Phe at position 2 in conjunction with an aromatic D-amino acid at positions 1, 3, and 6 [Ac-D-Phe^1,pClD-Phe^2,D-Trp^{3,6}]-GnRH was found to be even more potent[4] (100% inhibition of ovulation at 40 to 60 μg per rat).

Recently, we reported on the synthesis and biologic evaluation of a series of GnRH antagonists that were designed to test the applicability of the manual method of Topliss to the general Hansch approach.[29] A series of GnRH agonists was also synthesized in order to test this method.

The rationale for this approach was based on two observations:

1. An apparent correlation between biological potency of GnRH superagonists containing a D- aromatic amino acid at position 6, with their overall hydrophobic character as measured by their behavior on high pressure liquid chromatography (HPLC) at physiological *p*H.
2. The failure of similar experiments to correlate potencies of a homologous series of GnRH antagonists. The implication is that the receptor requirements for recognition and binding may be selective in more than one way (hydrophobicity, localized electronic density of substituents, and steric effects).

These results led us to believe that more potent analogs could be obtained by a systematic approach to optimize these parameters.

A convenient and well-understood way of altering the electronic and lipophilic properties of an active chemical species is to manipulate substituents on an aromatic ring. Topliss offered an efficient solution to the problem of optimizing substitution.[29] What he called a manual method for applying the Hansch approach to drug design is a decision-making tree that takes into consideration the essential parameters most commonly found in Hansch-type correlations.

This chapter reports the latest results in the development of agonist and antagonist GnRH analogs. Our efforts have relied not only on Topliss' systematic approach, but also on working hypotheses—such as those of Grant and Vale[7]—that lead to a series of cyclic GnRH peptides endowed with significant agonistic and antagonistic activities.

EXPERIMENTAL PROCEDURES

D, L-phenylalanine analogs and β(2 Naphthyl) D, L-alanine were made according to Herr and co-workers[8] and enzymatically resolved according to Turk and co-workers.[30] Peptide amides were synthesized using a benzhydrylamine resin.[22] Peptide ethylamides were synthesized on a chloromethylated resin followed by ammonolysis in redistilled monoethylamide overnight at 0°C. Protecting groups were t-Boc for the α-amino functions, tosyl for the imidazole and guanidine functions, benzyl ether for serine, and 2,6-dichlorobenzyl for the phenolic hydroxyl of tyrosine. Crude peptides obtained after

HF cleavage were first purified on CMC (linear gradient 0.05 → 0.25 M NH_4 OAc *p*H 6.5 in H_2O/MeOH: 1/1) followed by partition chromatography on Sephadex G-25F.

Two systems were used: butanol, acetic acid, water (4:1:5) for the agonists, and n-butanol, 0.1N acetic acid (1:1), for the antagonists. Peptides were characterized by amino acid analysis and optical rotation, and they were checked for homogeneity by reverse phase high pressure liquid chromatography.[24] Peptides were generally found to be more than 95% pure. If not, they were further purified by reverse-phase HPLC using an acetonitrile gradient in 0.1% TFA.

In vitro, the peptides were tested for their ability to inhibit GnRH-mediated luteinizing hormone (LH) secretion by cultured pituitary cells.[32] Potencies of GnRH antagonists are expressed relative to a standard shown at the top of each table.

In binding studies, the K_D for [D-Ala^6,N$^\alpha$MeLeu^7,Pro^9-NEt]-GnRH (taken as standard) was determined from a Scatchard analysis to be approximately 0.3 nM. All the other K_Ds were calculated from the potencies of the analogs (relative to the standard) determined from displacement data.[14,15]

The *in vivo* (ovulation) test was carried out as described by Corbin and Beattie.[2] Results are expressed in terms of the ratio of GnRH antagonist-treated rats ovulating over vehicle-administered controls. The peptides were dissolved in corn oil and injected subcutaneously in a volume of 200 µl at noon on the day of proestrus. Other studies directed toward a better understanding of the role of GnRH in reproductive functions in male and female rats have been reported elsewhere.[18-21] The prolonged action of these analogs *in vivo* was demonstrated, thus allowing for chronic studies.

RESULTS

GnRH Agonists

Table 2-1 gives biological potencies *in vitro* and retention times at two *p*H values (acidic, *p*H 2.25; physiologic, *p*H 7.30) of five GnRH superagonists of the general formula: pGlu-His-Trp-Ser-Tyr-X-Leu-Arg-Pro-Gly-NH_2. This

TABLE 2-1. Effect of Substitutions at Position 6 on Biological Potencies and Retention Times* of GnRH Agonists

PEPTIDE	RETENTION TIMES *p*H 2.25 (sec)	RETENTION TIMES *p*H 7.30 (sec)	POTENCY *IN VITRO*†
X = D-Lys	93	87	17
X = D-Leu	689	331	15
X = D-Tyr	287	175	68
X = D-Trp	1277	541	144
X = D-His (im-Bzl)	230	579	200

*HPLC at two *p*H values

†Potencies are relative to GnRH = 1.0

TABLE 2-2. Effect of D-Phe^6 Ring Substitutions on Biological Potencies and Retention Times (HPLC) of GnRH Agonists

PEPTIDE	POTENCY *IN VITRO**	RETENTION TIMES *p*H 7.30 (sec)
[D-Phe^6,Pro^9-NEt]-GnRH	1.00	319
[4OMeD-Phe^6,Pro^9-NEt]-GnRH	1.70	299
[4CH$_3$D-Phe^6,Pro^9-NEt]-GnRH	1.80	459
[4ClD-Phe^6,Pro^9-NEt]-GnRH	0.49	545
[3,4Cl$_2$D-Phe^6,Pro^9-NEt]-GnRH	0.75	919
[4NO$_2$D-Phe^6,Pro^9-NEt]-GnRH	1.82	
[4Ac-NHD-Phe^6,Pro^9-NEt]-GnRH	0.46	

*Potencies are relative to [D-Phe^6,Pro^9-NEt]-GnRH = 1.0

formula can be considered part of a homologous series (X = D-Lys, D-His (im-Bzl), D-Tyr, D-Leu, D-Trp). Different acetonitrile concentrations were used at each *p*H so that elution of all components could be achieved under isocratic conditions. No correlation was found at an acidic *p*H. Even though a better fit is obtained at physiologic *p*H, other factors—such as aromaticity—will have to be introduced if experimental data are to correlate. Indeed only the three peptides having X = Tyr, D-Trp, and D-His (im-Bzl) have higher biological potencies correlating with higher retention times. Unfortunately, similar data shown in Table 2-2 suggest that this correlation may be only fortuitous and that the operating factor may be one which is dependent on the electronic properties and location of the substituents on the aromatic ring.

It is noteworthy that [D-His^6(im-Bzl),Pro^9-NEt]-GnRH was designed to have the very characteristics that it displays (high solubility under mild acidic conditions while retaining high biological activity), thus allowing for the making of highly concentrated medical preparations.

Antagonists

On the basis of the observation that several position 1 substitutions were compatible with the high potency of GnRH antagonists,[26] other D- and L-amino acids—which were either blocked (acetyl) or unblocked, with or without functional side chains, aliphatic or aromatic—were introduced at position 1 of the basic structure [X-D-Phe^2,D-Trp^{3,6}]-GnRH^{1,9} (Table 2-3).

These systematic studies showed that the Ac-dehydro^{3,4}Pro^1(Δ^3Pro) substitution was clearly superior to any other 1 position substitution tested by us or by others.[1,9] While those studies were being conducted, substitutions at the 2 positions were also evaluated (Table 2-4). None of the substitutions tested (excluding Phe derivatives) led to peptides with higher biological potencies than that attained with D-Phe^2 substitution originally reported by Rees and others.[17] Coy and associates[4] made the observation that introduction of pClD-Phe at position 2 in conjunction with an aromatic amino acid at position 1 would yield peptides with greatly enhanced biological potency. We confirmed those results by making the corresponding [Ac-D-Phe^1,pClD-Phe^2,D-

TABLE 2-3. GnRH Antagonists with Position 1 Substitutions

ANTAGONISTS	*IN VITRO* POTENCIES*	μg	*IN VIVO* RAT OVULATING
[D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH	1.00	250	0/10
[pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.16	1000	4/10
[Gly^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.35	250	8/10
[β-Ala^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.24	750	1/10
[GABA^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.22	250	9/10
[Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	1.29	500	2/10†
[D-Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	3.70	250, 100	0/10, 4/10
[For-D-Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	1.60	500	4/10
[Ac-D-Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	2.10	500	0/10†
[Bzl-D-Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	1.25	250	3/10
[β-Ac-Naphthyl-D-Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	2.80	250	9/10
[D-pGlu-D-Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.21	250	9/10
[D-Leu^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.50	750	10/10
[D-Trp^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.60	750	9/10
[D-Lys^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.03	250	9/10
[D-His^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.20	750	3/10
[D-Arg^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.05	750	5/10
[Z-OBzlD-Ser^1,D-Phe^2,D-Trp^{3,6}]-GnRH	0.60	1000	9/10
[Ac-Δ^{3}Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH‡	5.20	20	5/7
[Ac-Δ^{3}D,L-Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH‡	9.60	100	0/10

*Potencies are relative to D-pGlu^1,D-Phe^2,D-Trp^{3,6} = 1.0.

†*In vivo* data from the laboratories of Drs. Folkers[9] and Steward.[1]

‡Δ^{3}Pro = dehydro^{3,4} Proline.

Trp^{3,6},Pro^9-NEt]-GnRH and [Ac-D-Phe^1,pClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH both highly potent *in vitro* and *in vivo* (Table 2-5). We therefore introduced the pCl-D-Phe^2 substitution in conjunction with our best substitutions at positions 3 and 6 while still trying to improve positions 1 and 7 (Table 2-5). The Ac-Δ^{3}Pro^1 substitution proved compatible with the pClD-Phe^2 substitution and was significantly better than any other modification tested. The N$^{\alpha}$MeLeu^7 modification first reported by Ling and Vale,[10] however, does not

TABLE 2-4. GnRH Antagonists with Position 2 Substitutions

ANTAGONISTS	*IN VITRO* POTENCIES*	μg	*IN VIVO* RAT OVULATING
[D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH	1.000	250	0/10
[D-pGlu^1,3MeHis^2,D-Trp^{3,6}]-GnRH	0.070		
[D-pGlu^1,D-His^2,D-Trp^{3,6}]-GnRH	<0.010	500	3/10
[D-pGlu^1,D-Trp^2,D-Trp^{3,6}]-GnRH	0.210	500	4/10
[D-pGlu^1,Trp^2,D-Trp^{3,6}]-GnRH	0.130	250	9/10
[D-pGlu^1,D-Tyr^2,D-Trp^{3,6}]-GnRH	0.017		
[D-pGlu^1,Tyr^2,D-Trp^{3,6}]-GnRH	0.050		
[D-pGlu^1,D-Orn^2,D-Trp^{3,6}]-GnRH	<0.010	500	9/10
[D-pGlu^1,N$^{\alpha}$MePhe^2,D-Trp^{3,6}]-GnRH	0.140	500	9/10

*Potencies are relative to [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH = 1.0

TABLE 2-5. GnRH Antagonists with Positions 1 and 7 Substitutions

ANTAGONISTS	*IN VITRO* POTENCIES*	K_D	μg	*IN VIVO* RAT OVULATING
[D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH	1	0.54	250	0/10
[Ac-Δ^{3}Pro^1,D-Phe^2,D-Trp^{3,6}]-GnRH	10–20	0.17	20	5/7
[Ac-D-Phe^1, 4ClD-Phe^2,D-Trp^{3,6},Pro^9-NEt]-GnRH	45		30	2/10
[Ac-D-Phe^1, 4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	75		25	1/8
[Ac-D-Phe^1,D-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH			100	6/10
[Ac-Δ^{3}Pro^1,4ClD-Phe^2,D-Trp^{3,6}]-GnRH	70–125	0.29	7.5	0/10
[Ac-Δ^{3}Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	30–52	0.19	20	0/10
[Ac-Thz^1,4ClD-Phe^2,D-Trp^{3,6}]-GnRH	21		25	3/10
[Ac-Thz^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	23		25	9/10
[Ac-D-Pro^1,4ClD-Phe^2,D-Trp^{3,6}]-GnRH	16	0.32	20	3/8
[Ac-D-Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	11			

*Potencies are relative to [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH = 1.0

seem compatible with bulky amino acids at position 6, possibly because of steric hindrance, thus preventing optimal backbone conformation.

The receptor requirements for optimal binding at position 1 were further evaluated by changing the nature of the acylating substituent on dehydroPro^1 (Table 2-6). The acrylyl substituent appears slightly better than the acetyl group; larger substituents (butanoyl, heptanoyl), however, are not compatible with high biological potency.

At this stage, since the pCl substitution of D-Phe^2 had resulted in such a leap in biological potency, we decided to investigate other D-Phe analogs at position 2. The results of those extensive studies are reported in Table 2-7.

The first five structures are those suggested in the Topliss approach to structure–activity relationship studies. The order of potency (4Cl > 3,4 Cl_2 ≥ 4 CH_3 > 4H ≥ 4 OMe) indicates that the probable operative potency dependent parameters were either $2\pi - \pi$ or $\pi + \sigma$. If $2\pi - \pi^2$ was the operating determinant, greater potency would be predicted for the 4BrD-Phe^2 analog. If $\pi + \sigma$ was the operating determinant, then the 2,4Cl_2- or 4CF_3- D-Phe^2 analogs should be more potent. On the other hand, if "other effect" were operative, then the 4FD-Phe^2, 4NO_2D-Phe^2, or 4Ac-NHD-Phe^2

TABLE 2-6. Effect of Acylating Group at Position 1 on Biological Potencies of GnRH Antagonists

ANTAGONISTS	*IN VITRO* POTENCIES*	K_D	μg	*IN VIVO* RAT OVULATING
[Ac-Δ^3 Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	1.0	0.19	10	4/8
[Acr-Δ^3 Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	3.0	0.25	10	1/10
[Pro-Δ^3 Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	1.8		10	3/3
[But-Δ^3 Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	0.6		20	4/9
[Hep-Δ^3 Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH	≤0.1		10	9/10

Ac = acetyl; Acr = acrylyl; Pro = propiolyl; But = butanoyl; Hep = heptanoyl.
*Potencies are relative to [Ac-Δ^{3}Pro^1,4ClD-Phe^2,D-Trp^{3,6},N$^{\alpha}$MeLeu^7]-GnRH = 1.0.

TABLE 2·7. Effect of Substitutions at Position 2 on Biological Potencies of a Series of [Ac-Δ^3Pro^1,X^2,D-Trp^{3,6}]-GnRH Antagonists

ANTAGONISTS	*IN VITRO* POTENCIES*	K_D	μg	*IN VIVO* RAT OVULATING
[Ac-Δ^3Pro^1,4ClD-Phe^2, D-Trp^{3,6}]-GnRH	1.00	0.29	5	2/11
[Ac-Δ^3Pro^1,D-Phe^2, D-Trp^{3,6}]-GnRH	0.14	0.17	20	5/7
[Ac-Δ^3Pro^1,4OMeD-Phe^2, D-Trp^{3,6}]-GnRH	0.12	0.23	10	3/10
[Ac-Δ^3Pro^1,4CH$_3$D-Phe^2, D-Trp^{3,6}]GnRH	0.34	0.23	5	9/9
[Ac-Δ^3Pro^1,3,4Cl$_2$D-Phe^2, D-Trp^{3,6}]-GnRH	0.38	0.47	5	1/10
[Ac-Δ^3-Pro^1,2,4Cl$_2$D-Phe^2, D-Trp^{3,6}]GnRH	0.44	0.70	5	4/7
[Ac-Δ^3Pro^1,4CF$_3$D-Phe^2, D-Trp^{3,6}]-GnRH	0.22	3.16	5	7/9
[Ac-Δ^3Pro^1,4FD-Phe^2 D-Trp^{3,6}]-GnRH	0.70	0.13	5	0/10†
[Ac-Δ^3Pro^1,4NO$_2$D-Phe^2, D-Trp^{3,6}]-GnRH	0.70	0.12	5	2/10
[Ac-Δ^3Pro^1,4BrD-Phe^2, D-Trp^{3,6}]-GnRH	1.50	0.21	5	5/7
[Ac-Δ^3Pro^1,4MeSD-Phe^2, D-Trp^{3,6}]-GnRH	0.50	0.32	5	7/10
[Ac-Δ^3Pro^1,4Ac-NHD-Phe^2, D-Trp^{3,6}]-GnRH	0.03	0.92	10	9/10
[Ac-Δ^3Pro^1,(CH$_3$)$_5$D-Phe^2, D-Trp^{3,6}]-GnRH	0.04	3.70	10	7/10
[Ac-Δ^3Pro^1,C^{t}Me$_4$ClD-Phe^2, D-Trp^{3,6}]-GnRH	0.33	1.25	10	2/10
[Ac-Δ^3Pro^1,3FD-Phe^2, D-Trp^{3,6}]-GnRH		0.34	10	5/7

*Potencies are relative to [Ac-Δ^3Pro^1,4ClD-Phe^2,D-Trp^{3,6}]-GnRH = 1.0.
†Results for this analog have varied from 0 to 3/10 at the 5-μg dose.

should be more potent. In fact, the most potent analogs *in vivo* were found to be the analogs having the 4Cl-D-Phe^2, 3,4Cl$_2$D-Phe^2, 4FD-Phe^2 and 4NO$_2$D-Phe^2 substitutions, whereas the 4BrD-Phe^2 substitution was superior *in vitro*. Thus, even though the Topliss approach has led us to synthesize very potent GnRH analogs, it is not yet evident which parameters are predominantly operative, because of the close similarity in potency of a significant number of analogs. This in some ways limits the value of this particular approach. Yet, systematic substitution of aromatic rings with moieties having different electronic or steric characteristics has demonstrated the validity of the concept that more potent compounds can be designed by optimizing local and global electronic and steric properties of the GnRH analogs.

Other amino acids in the basic antagonist structures that could be substituted following the guidelines of Topliss are the two D-tryptophan residues of positions 3 and 6 and the tyrosine residue at position 5. Before proceeding, we decided to test the β(2 Naphthyl)-D-Ala substitution at positions 3 and 6. The use of this unnatural amino acid as a tryptophan analog in the GnRH field is limited to three reports.

1. Yabe and co-workers[11] introduced the L-isomer at position 3 of GnRH and obtained an analog one-third as potent as GnRH.
2. Prasad and associates[16] also introduced the same L-isomer in position 3 in conjunction with the des-His^2,D-Ala^6 substitutions and obtained a weak GnRH antagonist.
3. The Syntex group[13] introduced the D-isomer at position 6 and obtained a potent GnRH agonist.

Quantitative comparison of the potency of this analog with other known GnRH agonists is not available, but claims to the effect that this analog was very potent intrigued us and led us to systematically substitute D-Trp^3 and D-Trp^6 by β(2 Naphthyl)D-Ala. Ring substituted analogs of this amino acid should be easier to synthesize than their tryptophan homologs. Comparative biological data on the three analogs versus [Ac-Δ^{3}Pro^1,4FD-Phe^2,D-Trp^{3,6}]-GnRH are shown in Table 2-8.

It is noteworthy that the three analogs have similar potencies. Further studies including introduction of the β(1 Naphthyl)D-Ala as opposed to β(2 Naphthyl)D-Ala at positions 3 and 6 should give us a better appreciation of the receptors' requirement for tight binding. In fact, [β(1 Naphthyl)L-Ala^3]-GnRH was reported by Yabe[34] to be approximately twice as potent as GnRH. These latest results (Table 2-8), which yield analogs one hundred times more potent than those available two years ago, demonstrate the feasibility of the step-by-step optimization approach to the design of GnRH analogs.

Cyclic GnRH Agonists

Our efforts in designing cyclic GnRH analogs go back several years. At that time, we produced some Cys-containing GnRH agonists[25] and cyclic [Glu^4,D-Ala^6,Orn^7]-GnRH[5] to test the hypothesis of the existence of a postulated βII bend encompassing residues 4 through 7[11] and to obtain a conformationally restricted biologically active analog that could be studied using conventional spectroscopies such as NMR and CD. It was hoped that such studies will lead to a better understanding of the receptor requirements for recognition. *In vitro* potencies of those cyclic Cys-containing GnRH agonists are reported in Table 2-9 along with those of two backbone cyclized analogs subsequently reported by Seprodi and co-workers.[28] Potencies of those analogs are relatively low even though they exhibit full intrinsic activity.

Cyclic GnRH Antagonists

Our observation that GnRH antagonists with more drastic substitutions in positions 1, 2, and 3 would still exhibit high affinity for the GnRH receptor[14]

TABLE 2-8. Effect of β (2 Naphthyl) D-Ala Substitution at Positions 3 and 6 on Biological Potency

ANTAGONISTS	*IN VITRO* POTENCIES*	K_D	μg	*IN VIVO* RAT OVULATING
[Ac-Δ^{3}Pro^1,4FD-Phe^2,D-Trp^3,D-Trp^6]-GnRH	1.0	0.13	7.5	0/10
[Ac-Δ^{3}Pro^1,4FD-Phe^2,β (2 Naphthyl)D-Ala^3,D-Trp^6]-GnRH	1.7	0.16	5	0/10
			2.5	3/10
[Ac-Δ^{3}Pro^1,4FD-Phe^2,β(2 Naphthyl)D-Ala^3,β(2 Naphthyl)D-Ala^5]-GnRH	2.3	0.72	5	0/10
			2.5	0/10
[Ac-Δ^{3}Pro^1,4FD-Phe^2,D-Trp^3,β(2 Naphthyl)D-Ala^6]-GnRH	3.1	0.22	5	0/10
			2.5	2/10

*Potencies are relative to [Ac-Δ^{3}Pro^1,4FD-Phe^2,D-Trp^{3,6}]-GnRH = 1.0.

TABLE 2-9. *In vitro* Potencies of Cyclic GnRH Agonists

AGONISTS	RELATIVE POTENCY
LRF	100.00
[Cys^{4,8},D-Ala^{6}]-GnRH	<0.01
[Cys^{4,9}]-GnRH	<0.01
[Cys^{4,10}]-GnRH	<0.01
[desamino-Cys^{1},D-Lys^{6},Cys^{10}]-GnRH	0.2 (0.09–0.8)
[Cys^{2,10},D-Ala^{6}]-GnRH	0.09
Cyclic [β-Ala^{1},D-Ala^{6},Gly^{10}]-GnRH*	1.20 (0.92--1.56)
Cyclic [6-Aminohexanoic^{1},D-Ala^{6},Gly^{10}]-GnRH*	0.65 (0.48—0.89)

*From Seprodi J, Coy DH, Vilchez-Martinez *et al:* Cyclic analogues of luteinizing hormone-releasing hormone with significant biological activities. J Med Chem 21:993–995, 1978.

led us to consider making cyclic GnRH antagonists. Their primary structure and relative biological potency is reported in Table 2-10. Several conclusions can be drawn from those results. Contrary to what was observed in the series of linear GnRH antagonists, introduction of the 4ClD-Phe^{2} substitution in [D-Cys^{1},D-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH does not result in an increase of biological activity. In the acetylated peptide [Ac-D-Cys^{1},D-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH, it is apparent that the increasing order of potency with 4 positions substitution on the phenylalanine ring goes from H < Cl < F.

TABLE 2-10. Cyclic GnRH Antagonists*

STRUCTURE	POTENCY (ICR_{50})†	μg	*IN VIVO* RAT OVULATING
[D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-GnRH	3/1	250	0/10
[D-Cys^{1},D-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH	9/1		
[Ac-D-Cys^{1},D-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH	50/1		
[D-Cys^{1},4ClD-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH	63/1		
[Ac-D-Cys^{1},4ClD-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH	28/1		
[Ac-D-Cys^{1},4ClD-Phe^{2},D-Trp^{3,6},N$^{\alpha}$CH$_3$Leu^{7},Cys^{10}]-GnRH	27/1		
[Ac-Cys^{1},4ClD-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH	63/1		
[Ac-Cys^{1},4FD-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH	19/1	500	10/10
[Ac-Cys^{1},4FD-Phe^{2},D-Trp^{3,6},D-Cys^{10}]-GnRH	70/1	500	10/10
[Ac-D-Cys^{1},4FD-Phe^{2},D-Trp^{3,6},Cys^{10}]-GnRH	17/1	500	9/10
[Ac-D-Cys^{1},4FD-Phe^{2},D-Trp^{3,6},D-Cys^{10}]-GnRH	25/1	500	10/10
[CycloΔ^{3}L-Pro^{1},4ClD-Phe^{2},D-Trp^{3,6},N$^{\alpha}$MeLeu^{7},βAla^{10}]-GnRH	3.5/1		

*Preliminary results were reported in Vale W, Rivier C, Perrin M *et al:* Pharmacology of GnRH, a model regulatory peptide. In Martin JB, Reichlin S, Bick KL (eds): Neurosecretion and Brain Peptides: Implications for Brain Functions and Neurological Diseases, pp. 609–625. New York, Raven Press, 1981.

†Potencies as ICR_{50} are expressed as the concentration ratio [antagonist/GnRH] required to reduce the amount of GnRH-induced LH secreted by 50%.

In the acetylated series in which the stereo chemistry at positions 1 and 10 was studied [Ac-L or D-Cys[1],4FD-Phe[2],D-Trp[3,6],L- or D-Cys[10]]-GnRH, it was found that the most favorable conformation was that encompassing the L- or D-isomer at position 1 in conjunction with the L-isomer only at position 10. The most potent cyclic antagonist that was tested was found, however, to be [CycloΔ[3]Pro[1],4ClD-Phe[2],D-Trp[3,6],N[α]MeLeu[7],βAla[10]]-GnRH, a noncysteine-containing analog that was cyclized through the backbone. Similar analogs are presently being synthesized that should exhibit even greater potency and be even more restricted in their terriary conformation.

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3

The Role of Peptide Backbone Modifications in Increasing Biological Stability of LHRH Analogs

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The potential usefulness of both agonistic and antagonistic analogs of LHRH for regulating fertility in males and females has been established. Problems remaining to be solved include the synthesis of structures with increased potencies—especially among antagonists—and the need for further insights about dosage, duration, and mode of administration. In most respects, the solution to each of these problems is intimately related to the successful synthesis of peptides with improved biological stabilities. Certainly an ideal polypeptide would be safe, effective, easily and relatively inexpensively synthesized, and capable of oral administration. However, few peptide analogs have been prepared, either of LHRH or others, that can be orally administered and survive passage through the gastrointestinal tract, in addition to being absorbed without drastic losses in potency.

Most approaches to increased enzymic stability have involved buttressing the weak link in the peptide "backbone," namely the amide bond. Other backbone modifications not involving the amide bond itself have also proven useful, such as D-amino acids, dehydro residues, and α-methyl-substituted or α-aza analogs. In addition, cyclic peptides have been suggested as contributing to increased rigidity and thus improved resistance to degradation by

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common proteolytic enzymes. Of these many types of substitutions, the use of D-amino acids in place of glycine of L-residues has been most prevalent, largely because of the commercial availability of most common D-amino acids. However, relatively few of the resulting monosubstituted diastereomeric derivatives yield peptides of increased potencies. In LHRH, for example, only position 6 of the decapeptide is known to be rendered more active by D-amino acid incorporation, and the placement there is for the *achiral* glycine group (Table 3-1).[21] For the tetradecapeptide somatostatin, only two of the 14 possible D-substituted analogs were found to be more active than the parent hormone.[21] None of the nine possible oxytocin D-substituted analogs are as active as oxytocin.[1]

With any backbone replacement, the conformational implications as well as the presumed enzymatic stabilization must be considered. Clearly, D-amino acids have been as valuable for their conformational stabilizing properties as for their intractability to enzyme degradation. If still other methods might be found which could increase the notoriously short biological half-lives of peptides, while simultaneously permitting a more subtle manipulation of the stereochemistry of the backbone, the result might be a new and generally useful adjunct to D-amino acids for structure–function studies.

With such considerations in mind, we initiated a program that incorporated several new kinds of readily synthesized amide bond replacements within LHRH and its antagonists.[18] Recent work has involved the synthesis of *chiral* amide bond surrogates that contain one or more asymmetric centers within the amide bond-replacing unit. A major objective has been to investigate the potential of these sulfur-based surrogates as nearly isosteric replacements for the peptide link, and to determine to what extent the resulting new asymmetric center might lead to variable biologic activities, much as D- and L-amino acids contribute quite different but often exploitable variations in bioactivity profiles.

The general synthetic strategy has been to prepare analogs of dipeptides that already contain the amide replacements and to then incorporate these "δ-amino acids" within the peptide chain by traditional solution or solid phase

TABLE 3-1. LHRH Derivatives with D-Amino Acids Substituted at Positions 1–10

POSITION	SUBSTITUENT	*IN VITRO* ACTIVITY
—	LHRH	100.0
1	D-pGlu	8.0
2	D-His	<1.0
3	D-Trp	0.1
4	D-Ser	7.0
5	D-Tyr	0.1
6	D-Ala (Gly)	500.0
7	D-Leu	1.0
8	D-Arg	0.3
9	D-Pro	—
10	D-*Pro* (Gly)	10.0

methods.[20] Once the physical characteristics of such dipeptide analogs have been established, such as their R_f values on thin layer chromatography, or their HPLC or amino acid analysis retention times, the resulting products can be characterized in nearly identical manner to their amide counterparts. In several cases, the use of selective degradation, derivatization and mass spectrometric analysis has proven useful in unambiguously establishing the presence of these pseudopeptides.

The amide bond replacements discussed in this study include the following sulfur-based moieties; CH_2S, CH_2SO, $CHCH_3S$, and $CHCH_3SO$. All but the first contain at least one new chiral center. In addition, the carbon hybridization at the pseudocarbonyl position is changed from sp^2 to sp^3 in each of these analogs, not unlike "transition state" analogs that have been proposed as enzyme inhibitors.

NOMENCLATURE

A simplified nomenclature for peptide backbone amide bond replacements is proposed. The symbol ψ has been previously used to refer to the methylene thio (CH_2S) replacement[20] but now refers to *any* amide bond replacement followed by brackets, in which the elements substituting for the normal CONH group are specified. In the case of *chiral* amide bond replacements, or with configurational isomers of the Z,E type, the configurations are described parenthetically. Thus a dipeptide analog of the sequence Phe-Leu, which contains a $CHCH_3S$-replacing element, would be named as Pheψ[$CHCH_3S$]Leu, or, if the configuration is known, as Pheψ[(*R*)-$CHCH_3S$] Leu (Fig. 3-1). (In this chapter all amino acids except glycine have the L- or S-configuration, unless otherwise specified.) Other examples are shown in Table 3-2.

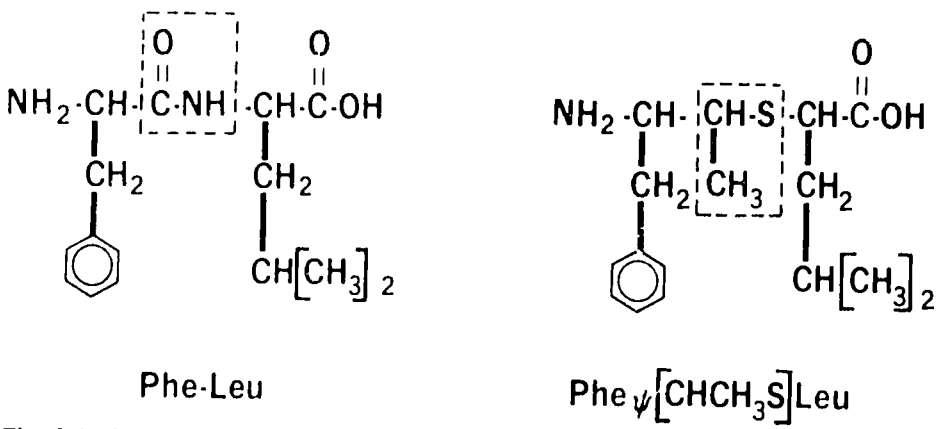

Fig. 3-1. Comparison of the structure Phe-Leu with representative pseudodipeptide equivalent, Phe-ψ[$CHCH_3S$]Leu.

TABLE 3-2 Examples of Proposed Nomenclature for Amide Bond Replacements: ψ[ ]

REPLACING UNIT FOR AMIDE BOND	NOMENCLATURE SYMBOL	EXAMPLE
CH_2S	ψ[CH_2S]	Glyψ[CH_2S]Phe
NHCO	ψ[NHCO]	Pheψ[NHCO]D-Phe
CH=CH	ψ[CH=CH]	Alaψ[Cis-CH=CH]Gly
$CHCH_3S$	ψ[$CHCH_3S$]	Glyψ[(S)-$CHCH_3S$]Tyr
CH_2NH	ψ[CH_2NH]	Pheψ[CH_2NH]Pro
$SOCH_2$	ψ[$SOCH_2$]	D-Valψ[(R)-$SOCH_2$]Lys

LHRH AGONISTS

When this work was initiated, the findings of Marks and Stern provided substantial evidence that the weak links in LHRH (with respect to hypothalamic degradation) were the amide bonds between residues 6–7 and 9–10 (Fig. 3-2).[13] Further support was furnished by the extraordinarily high activity found for such analogs as [D-Trp^6,Pro-NHEt^9]-LHRH with protection at both of these sites.[23] We thus undertook the synthesis of several ψ[CH_2S]-containing analogs including structures with Glyψ[CH_2S]Leu$^{6-7}$ and Proψ[CH_2S]Gly-NH_2^{9-10} replacements. The biologic activities for these structures, based on *in vitro* hemipituitary and dispersed cell culture assays, are summarized in Figure 3-3.

These results demonstrate the range of activities and substantial site variability to be expected for structurally isomeric compounds. All of the agonist modifications are based on the parent LHRH molecule rather than on partially modified peptides containing position 6 or 9 "Fujino" modifications (ProNHEt).[8] Although the resulting structures are likely to yield compounds with lower biological activities, this is the only way to assess the effect of a new structural perturbation unambiguously.

Nevertheless, the three compounds range in activity from less than 0.1% the potency of LHRH (for [Glyψ[CH_2S]Leu$^{6-7}$]-LHRH) to structures with diminished but still relatively high potencies such as [Tyrψ[CH_2S]Gly$^{5-6}$]-LHRH and [Proψ[CH_2S]Gly-NH_2^{9-10}]-LHRH. The similar activities of these latter compounds suggested that there might very likely be a conformational requirement for rigidity at the 6–7 bond, but that internal as well as C-terminal positions could be equally well substituted without necessarily sacrificing biological potencies. It appeared significant that several proposed models for LHRH postulated a bend in the region of the 6–7 residues,[14] and the stabil-

```
                                  ↓           ↓
LHRH:pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2
        1   2   3   4   5   6   7   8   9   10
Modifications:                D-Trp6    Pro-NHCH2CH3 9
```

Fig. 3-2. Vulnerable sites of LHRH toward hypothalamic proteolytic extracts and useful protective modifications.

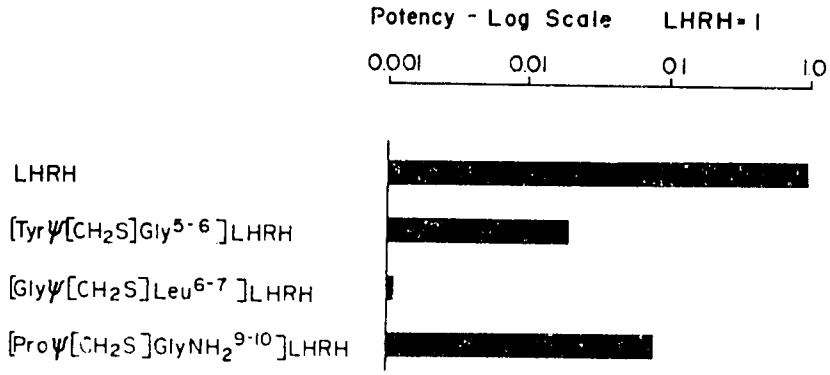

Fig. 3-3. *In vitro* activities of LHRH agonists containing $\psi[CH_2S]$ amide replacements at selected sites.

izing influence of large hydrophobic D-configuration groups at corners of β-turns might explain their increased activities.[16] However, a formal hydrogen bonded structure, comprising residues 5–8, which could be at least one contributing structure of the likely set of low energy conformers for LHRH, can probably be ruled out as an essential receptor-active form. The 5% to 10% activity of the Tyrψ[CH_2S]Gly$^{5-6}$ analog is incompatible with a strict requirement for such a bonding feature.

More recent work on the stability of LHRH to endogenous proteases indicates that, in addition to the 6–7 and 9–10 bonds, other vulnerable sites include the pGlu^1-His^2, His^2-Trp^3, and Tyr^5-Gly^6 bonds.[10-12] The latter bond (as well as the Trp^3-Ser^4 bond) is known to be susceptible to chymotrypsin cleave. Thus, the synthesis of any peptide derivative with prolonged activity would require protection from a wide variety of peptidases while retaining a reasonable profile of adsorptivity and biological activity parameters.

LHRH ANTAGONISTS

While the synthetic work leading to LHRH pseudopeptide agonists was in progress, the synthesis of analogs of known LHRH antagonists with amide bond replacements at varying sites was also begun. Initially, the similarity between the C-termini of both agonists and antagonists allowed the incorporation of

Typical N-terminal residues		Typical C-terminal residues
Agonist	pGlu-His-Trp	Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2
Antagonist	D-pGlu-D-Phe-D-Trp	

pseudodipeptides within both structures. Figure 3-4 summarizes the *in vitro* activity of five analogs containing amide bond replacements at positions 1–2, 6–7, and 9–10. Once again, the least active structure is that with the amide bond replaced between the Gly^6–Leu^7 residues. The hypothesis that undue flexibility may be the problem is supported by the similar lack of activity found for a D-Serψ[CH_2S]Leu$^{6-7}$ which has a D-residue at position 6, often leading to increased potency for both agonists and antagonists.

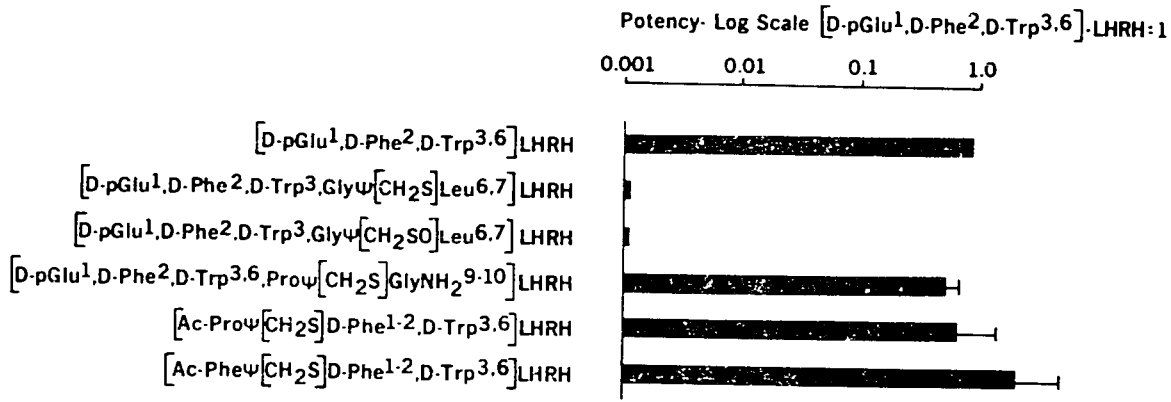

Fig. 3-4. *In vitro* activities of LHRH antagonists containing $\psi[CH_2S]$ amide replacements at selected sites.

More recently, our synthetic effort has concentrated on modifications of the N-terminus of LHRH, inasmuch as all of the structural changes which contribute to inhibition appear to be localized in the first three residues. Figure 3-5 contains *in vitro* activities for 11 pseudopeptides with position 1–2 modifications. These include replacements of the ψ[CH_2S]-type as well as the chiral amide bond surrogates ψ[CH_2SO], ψ[$CHCH_3S$], and ψ[$CHCH_3SO$].

As a result of the gradual evolution of more potent antagonists as our work progressed, our model compounds have varied. We initially used [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH as a model, reported by Vale and Rivier to be fully active in the rat antiovulatory assay at the level of 250 μg per animal.[15] Our most recent pseudopeptides have been based on the highly potent antagonist [Ac-Gly[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH, which we reported to be fully active at the 25-μg per rat level and highly active at 10 μg per rat.[17] In addition to the achiral Gly[1] modification—useful for synthesis of N-terminal amide bond replacements—this structure contains the activity-enhancing N-terminal acetyl and position 2 D-*para*-chlorophenylalanyl modifications reported by Channabasavaiah and Stewart[3] and Coy,[5] respectively. The potential utility of this simplified antagonist is detailed later.

The structures in Figure 3-4 are thus related (on a logarithmic scale) to the [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH structure (relative antagonist potency = 1); Figure 3-5 compares the *in vitro* potencies of a wide variety of pseudopeptides with [Ac-Gly[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH, which is approximately 50-fold more active than [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-LHRH in the dispersed pituitary cell culture assay.[7]

In general, all of the LHRH analogs containing the ψ[CH_2S] link have proven highly active *in vitro* but inactive as antiovulatory agents *in vivo*. We noted that the behavior of these compounds on HPLC was always characterized by increased lipophilicity, as expected for replacement of a polar amide bond by the nonpolar thiomethylene moiety. The pattern of biological activity was reminiscent of the lack of correlation seen between *in vitro* and *in vivo* results for another relatively lipophilic series in which the D-pGlu[1] group of a series of antagonists was replaced by a cyclopentylcarboxyl group.[2] In this case, the amide group of the cyclic pyroglutamyl ring is formally replaced by a dimethylene (CH_2CH_2) group, also representing a considerable increase in lipophilicity. Extensive biological characterizations of these compounds revealed that, while the cyclopentycarboxyl structures were inactive when administered via a subcutaneous injection, these same analogs were active *in vivo* when administered intravenously in the male rat and the male chimpanzee, in terms of completely inhibiting LH release in these models.

These results can be understood in terms of impaired absorption if the structures lie beyond the crest of optimal solubility in the Hansch formulation.[9] In fact, all of the LHRH pseudopeptide antagonists are considerably more retentive on reverse phase HPLC than any of the corresponding LHRH agonists.

These results thus led to the synthesis of a modified group of LHRH analogs containing the more polar amide bond replacement, ψ[CH_2SO]. We reasoned that these structures should possess the virtues of enzymatic stability, decreased flexibility, and improved solubility characteristics with HPLC retention times virtually identical to their amide counterparts. If these com-

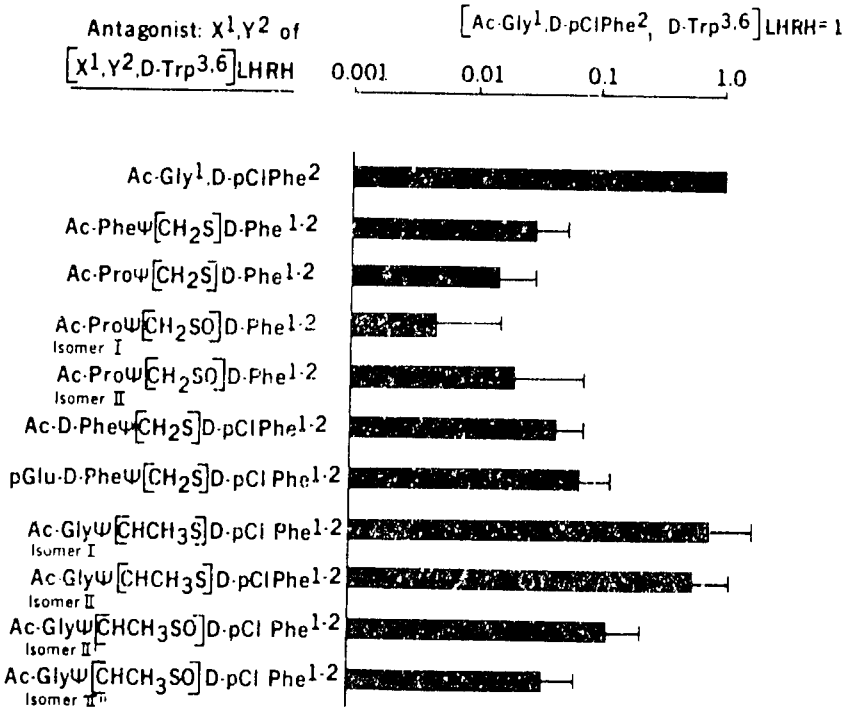

Fig. 3-5. *In vitro* activities of LHRH antagonists containing amide replacements at position 1-2.

pounds retained their *in vitro* activities, the *in vivo* results should provide a clear test of the lipophilicity hypothesis. However, as Figure 3-5 indicates, the sulfoxide derivatives have proven consistently less active than their ψ[CH_2S] precursors. The compounds were also inactive *in vivo* but this could be readily understood and anticipated from their *in vitro* behaviors.

With the formation of sulfoxides came the intriguing prospect of contrasting the biologic activities of structures that contain a chiral center at the replaced amide site. These compounds are among the first of a new class of structures—chiral amide bond replacements. Furthermore, since sulfoxides are configurationally stable, these compounds should provide a test for stereochemically related activity variations by pairs of diastereomeric analogs. Unfortunately, this comparison was rendered somewhat less meaningful by the disappointing *in vitro* results. Moreover, at this point no absolute stereochemical assignments for any of the chiral sulfoxides can be made. Nevertheless, differences in potency between the sulfoxide isomeric analogs have been observed. Further, it is interesting to note that, in every case tested thus far, the more potent activity is seen for the less lipophilic isomer.

Attention was next shifted toward the synthesis of still another class of chiral amide bond replacements, those incorporating a ψ[$CHCH_3S$] linkage with an asymmetric carbon at the pseudocarbonyl position. These analogs were also prepared in the form of pseudodipeptides and incorporated as

TABLE 3-3. *In Vitro* and *In Vivo* Comparison of N-Terminal Chiral Amide Bond Replacements

N-TERMINAL AMIDE REPLACEMENT	*IN VIVO* (RAT)	*IN VITRO** RELATIVE POTENCY
Ac-Glyψ[CHCH$_3$S]D-pCl-Phe^{1,2} Isomer I	5/10 (250 μg)	0.11 (0.5–0.25)
Ac-Glyψ[CHCH$_3$S]D-pCl-Phe^{1,2} Isomer II	7/10 (250 μg)	0.089 (0.5–0.16)
Ac-Glyψ[CHCH$_3$SO]D-pCl-Phe^{1,2} Isomer II Sulfoxide I	10/10 (250 μg)	0.017 (0.009–0.033)
Ac-Glyψ[CHCH$_3$SO]D-pCl-Phe^{1,2} Isomer II Sulfoxide II	10/10 (250 μg)	0.005 (0.0028–0.0093)

*[Ac-Dhp^1,D-pF-Phe^2,D-Trp^{3,6}]-LHRH = 1 (IDR_{50} = 0.008/1).

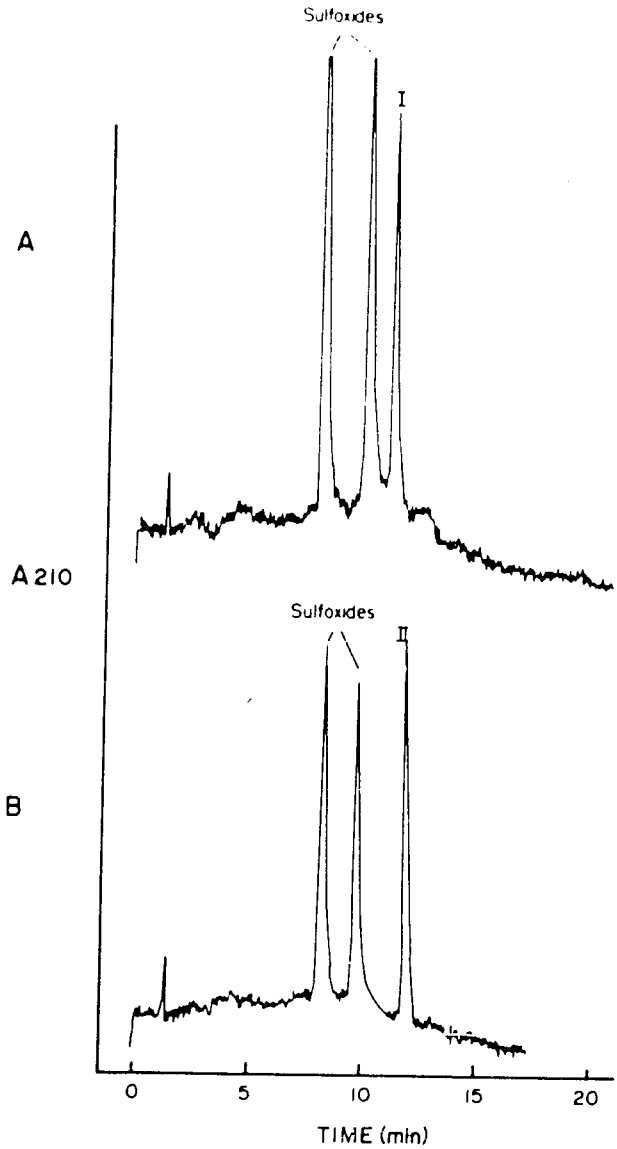

Fig. 3-6. HPLC separations of the partially oxidized diastereomers (I and II) of [Ac-Glyψ[CHCH$_3$S]D-pCl-Phe^{1,2},D-Trp^{3,6}]-LHRH to their corresponding sulfoxides (ψ[CHCH$_3$SO]).

protected "δ-amino acids." Thus far, synthetic routes have been devised for analogs of the Glyψ[CHCH$_3$S]X variety, and such dipeptide analogs as Glyψ[CHCH$_3$S]D-Phe, Glyψ[CHCH$_3$S]D-pCl-Phe, and Glyψ[CHCH$_3$S]Gly have been prepared. In view of the potent activity of [Ac-Gly^1,D-pCl-Phe^2,D-Trp^{3,6}]-LHRH, the synthesis of the N-terminal modified structures, "R" and "S" versions of [Ac-Glyψ[CHCH$_3$S]D-pCl-Phe$^{1-2}$,D-Trp^{3,6}]-LHRH was carried out.

As seen in Figure 3-5, the ψ[CHCH$_3$S] analogs have proven to be among the most successful of the various sulfur-based amide bond replacements thus far. In both the dispersed-cell and hemipituitary assays, these compounds were virtually as active as their amide counterparts in these *in vitro* systems. Furthermore, these were the first of the LHRH pseudopeptide analogs to exhibit *in vivo* antiovulatory activity, although once again, the *in vivo* potencies were considerably less than one would expect on the basis of the *in vitro* results.

The two diastereomers differed somewhat in their activities (Table 3-3). In each case, the less lipophilic compound (by HPLC) was more potent. If these ψ[CHCH$_3$S] analogs are oxidized to their sulfoxide counterparts, there would be four new diastereomeric products. By separation of the pair of ψ[CHCH$_3$S] isomers and separate oxidation (Figure 3-6), it was determined that the outer and inner pairs of peaks correspond to the methyl isomers I and II, respectively (again named in order of HPLC retention times: $t_I < t_{II}$ with k′ of 6.3 and 6.6). Thus far the inner sulfoxide pair II′ and II″ have been tested and their relative *in vitro* activities reveal the same dependence on k′. The pattern of the sulfoxides being less active than their ψ[CHCH$_3$S] precursors is repeated here. Neither of these sulfoxides showed any *in vivo* activity at 100 μg per rat. At this point, it cannot be ruled out that the sulfoxides could represent a less active metabolite leading to the disparity with the *in vitro* expectations.

The two ψ[CHCH$_3$S] isomers of [Ac-Glyψ[CHCH$_3$S]D-pCl-Phe$^{1-2}$, D-Trp^{3,6}]-LHRH were also tested for their ability to inhibit the LHRH–LH response in immature female rats (Table 3-4). The more lipophilic isomer was again somewhat less active, but both compounds effectively inhibited LH release in this *in vivo* system. The mode of administration was by IV injection; as with previously discussed cyclopentylcarboxylic acid derivatives, this finding is consistent with impaired absorption of the more lipophilic analogs when

TABLE 3-4. Inhibition of LHRH–LH Response by LHRH Analog Antagonists in Immature Female Rats

ANALOGS	LHRH μg IV	ANALOG* μg IV	LH ng/ml SERUM	± S.E.M.	*P* VALUE VS 2
Control			3.0	±0.5	<0.001
LHRH	0.1	0.1	26.8	±1.5	<0.001
[Ac-Glyψ[CHCH$_3$S]D-pCl-Phe$^{1-2}$, D-Trp^{3,6}]-LHRH Isomer I	0.1	10.0	7.6	±0.94	<0.001
[Ac-Glyψ[CHCH$_3$S]D-pCl-Phe$^{1-2}$, D-Trp^{3,6}]-LHRH Isomer II	0.1	10.0	10.5	±0.95	<0.001

*LHRH + analog injected IV and blood collected 15 minutes later.

given by the more traditional subcutaneous administration. Yet these compounds do appear to be active by IV injection, suggesting again that the amide bond replacements are fully compatible with receptor recognition and binding.

CONCLUSION

Various methods are available to the synthetic peptide chemist who desires to render polypeptides more enzymatically resistant. These include:

1. Conformationally restricted analogs, rendered less amenable to interacting with proteolytic enzymes by more compact structures
2. End-group modified peptides including pyroglutamyl, acetyl, or deamino structures
3. The use of nonpeptide templates to hold essential message elements of the hormone in relatively rigid, receptor-active configurations. (Morphine might be considered a naturally occurring example of a modified enkephalin using this approach.[7])
4. Use of peptide backbone modifications.

As considerable work by many groups has demonstrated, the latter approach is a viable and fruitful one in many instances.

One important benefit of such studies has been the determination of possible functional roles for peptide backbone elements, carbonyl oxygen and amide nitrogen. If any residual activity is discerned for an amide bond subjugated to replacement, this represents a strong argument *against* a functional role. Thus, in the LHRH analogs discussed, it appears unlikely that the 5–6 or 9–10 amide bonds are essential for activity. In the case of the antagonists, the 1–2 bond is likewise quite dispensable. On the other hand, no such conclusion can be reached for the 6–7 amide bond of either agonists or antagonists.

Amide bond replacements might also be of benefit where it has been established that some unusual amino acid that is important for potency might nevertheless prove toxic in its hydrolyzed state. It is quite conceivable that the potency might be retained, and the toxicity neutralized, by introducing the side chain in the form of a pseudodipeptide, which could not liberate the harmful free amino acid.

Among LHRH analogs, a number of *retro* amide bond replacements have been incorporated within LHRH analogs at the 1–2, 2–3, 5–6, 6–7 and 9–10 bonds.[4] None of these compounds retained more than 2–3% *in vitro* activity. Aza analogs have been prepared with α-carbons replaced by nitrogen at several residues including Gly-6 and Gly-10; the aza analogs of the C-terminal Fujino modification have proven at least as potent as the unmodified counterparts.[6]

In summary, the number and variety of peptide backbone modifications are on the increase. None of these has proved useful in all applications, but nearly all have shown potential in some instances. The sulfur-based analogs described here have the virtues of synthetic ease and considerable structural

versatility. The methyl-substituted analogs ψ[CHCH$_3$S] have proven active *in vivo* in at least two systems and offer promise for more successful applications. Other positions within LHRH agonists and antagonists lend themselves well to introduction of these replacements, and with further examples, the true potential of this promising approach should become more evident.

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4

Contraceptive Effects of Recent Potent LHRH Antagonist Analogs

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As a result of nine years of intensive analog work (which has recently been reviewed[14,15]), LHRH has undoubtedly become the most studied peptide from a structural standpoint. This effort has been fruitful and has already realized many of the goals originally contemplated, including the discovery of highly active and long-acting agonist analogs that exhibit good activity by routes other than parenteral, and the development of effective competitive inhibitors of endogenous LHRH binding. It is in the latter area that most synthetic studies are taking place. Although newly developed antagonist analogs are at last powerful enough to allow pharmacologic and clinical evaluations to proceed, much improvement in analog properties is desirable.

The rapid progress that has resulted in effective *in vivo* antagonist development began with the observation that [D-Phe^2]-LHRH was a far better antagonist than previously derived monosubstituted decapeptides.[13] Further improvements in activity *in vivo* were quickly achieved by making use of D-amino acid replacements for Gly in position 6 of the LHRH chain (Fig. 4-1) in conjunction with D-Phe^2. This work resulted in the first demonstrations of virtually complete inhibition of the gonadotropin surges in cycling rats and hamsters using multiple doses of [D-Phe^2,D-Ala^6]-LHRH[5] over the entire afternoon of proestrus and, more importantly, a single dose of the more active [D-Phe^2,Phe^3,D-Phe^6]-LHRH[9,10] given at 12 o'clock noon on proestrus. In rats, complete blockade of ovulation was achieved. The rat antiovulatory assay has become the standard, highly reproducible method for evaluating new compounds and has thus far given more realistic and useful data than cell culture or, more recently, receptor-binding techniques. Furthermore, as will be seen later, activities in the rat have fortunately extrapolated well to other primate and human systems.

Much of this research was funded from NIH NICHD Contract HD-0-2831.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10

Fig. 4-1. LHRH amino acid sequence.

Subsequent generations of LHRH antagonists have made use of several other key modifications, notably:

1. D-Trp in position 3[8]
2. Chain branching and dimerization through functional D-amino acids in position 6[7,16]
3. N^{α}-acyl-D-amino acids, particularly N-acetyl-D-Phe, in position 1.[3,4]

Many of these approaches have been used in combination. Certainly the most dramatic increase in inhibitory activity of late has been the result of incorporating D-pCl-Phe in position 2.[6,7] This has been found to generally increase activity by about a factor of ten over corresponding D-Phe[2] peptides. The next section describes some recent attempts to improve upon the biological properties of the most important member of this set of peptides—[N-Ac-D-Phe[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH which is fully active at a single dose of 60 μg in the rat[6,7] and which serves as a convenient standard for comparison.

RECENT APPROACHES TO IMPROVED ANTAGONIST DESIGN

Given the considerable effectiveness of pCl substitution on the benzene ring of certain D-Phe[2] analogs, it was projected that other substitutions might prove to be even more effective. Unfortunately, this has so far not proved to be the case. Early studies have already shown that pF and, particularly, pBr substituents were inferior to the original Cl atom.[6] Subsequently, in Table 4-1, neither pMe, pOH, or pNH_2 groups were beneficial. Likewise, the substitution of Cl at either of the other two ring positions gave no improvement in activity over the simple D-Phe[2] peptide. On the other hand, an o-Me-group appeared to be slightly beneficial. Thus, perhaps a 2-Me,4-Cl-D-Phe[2] analog might have interesting properties, although the actual synthesis of the amino acid would be time consuming. The 3,4-dichloropeptide was almost as active as the pCl compound. A larger aromatic ring system in the form of the indole nucleus of D-Trp resulted in an inactive peptide. It seems clear, then, that the electronic and steric requirements of the p-substituent are extremely critical and it may be impossible to improve upon the unique properties of the Cl atom in this type of analog.

Since the most active of the D-pCl-Phe[2] analogs initially contained Ac-D-Phe in the first position, a similar substitution strategy on its benzene ring was also carried out (Table 4-2). It was soon found that pCl resulted in a twofold improvement in inhibitory activity over the D-Phe[1] peptide. Para-F was equally effective but the larger Br and Me moieties were less so. The presence of a more polar OH group in the D-Tyr[1] compound resulted in no improvement. The most active in the series was the Ac-D-Trp[1] peptide; it is clear that the size of the amino acid side-chain is by no means as critical as

TABLE 4-1. Effect of Some Position 2 Modifications on Analog Antiovulatory Activities

LHRH ANALOG	DOSE (μg)	RATS OVULATING/ RATS TESTED
Ac-D-Phe^1,D-pCl-Phe^2,D-Trp^{3,6}	31	4/11
Ac-D-Phe^1,D-Tyr^2,D-Trp^{3,6}	31	10/11
Ac-D-Phe^1,D-pNH$_2$-Phe^2,D-Trp^3,D-Phe^6	62	9/10
Ac-D-Phe^1,D-pMe-Phe^2,D-Trp^{3,6}	31	8/10
Ac-D-Phe^1,D-o-Cl-Phe^2,D-Trp^{3,6}	31	10/10
Ac-D-Phe^1,D-o-Me-Phe^2,D-Trp^{3,6}	31	6/9
Ac-D-Phe^1,D-m-Cl-Phe^2,D-Trp^{3,6}	31	11/11
Ac-D-Phe^1,D-3,4-dl-Cl-Phe^2,D-Trp^{3,6}	31	4/8
Ac-D-Phe^1,D-Trp^{2,3},D-Phe^6	125	9/9

in the second position. In related studies, Ac-L-Phe was considerably less effective than its D-isomer (10% blockade of ovulation at a 31-μg dose).

The next increase in inhibitory activity was achieved by means of yet another D-amino acid substitution. Although some alterations to the Gly-NH$_2$^{10} residue in LHRH have produced superactive agonists, none had previously been found which improved antagonist activity. However, in an earlier series of analogs, D-Ala-NH$_2$^{10} present in [D-Phe^2,D-Trp^3,D-Phe^6,D-Ala^{10}]-LHRH resulted in retention of full inhibitory activity.[7] When D-Ala was incorporated into one of the more active sequences to give [Ac-D-Phe^1,D-pCl-Phe^2,D-Trp^{3,6},D-Ala^{10}]-LHRH (Table 4-3), another twofold increase in activity was found. A similar trend occurred in Ac-D-pCl-Phe^1 and Ac-D-Trp^1 peptides containing D-Ala^{10}. These two analogs, which gave significant blockade of ovulation at 5-μg doses (Table 4-3) represent perhaps the most powerful LHRH antagonists available. Some studies were also begun to elucidate the structural requirements of the position 10 residue in inhibitory analogs.

We previously reported that insertion of D-Phe^{10} into [D-Phe^{2,6,10},D-Trp^3]-LHRH produced a substantial loss of activity.[7] In the present series, a D-Ser^{10} analog was quite active, whereas the D-Leu^{10} peptide was virtually inactive at a 15-μg dose, which indicates that side-chain branching introduces steric problems. A D-N-Me-Ala^{10} analog also had much diminished activity which was expected in view of the loss of activity experienced with an earlier D-Pro^{10} peptide.[7] Spatola and Agarwal recently reported that Ac-Gly at the N-

TABLE 4-2. Effect of Some Position 1 Modifications on Analog Antiovulatory Activities

LHRH ANALOG	DOSE (μg)	RATS OVULATING/ RATS TESTED
Ac-D-Phe^1,D-pCl-Phe^2,D-Trp^{3,6}	31	4/11
Ac-D-pCl-Phe^{1,2},D-Trp^{3,6}	15	3/10
Ac-D-pF-Phe^1,D-pCl-Phe^2,D-Trp^{3,6}	15	2/10
Ac-D-pBr-Phe^1,D-pCl-Phe^2,D-Trp^{3,6}	15	6/12
Ac-D-pMe-Phe^1,D-pCl-Phe^2,D-Trp^{3,6}	15	4/8
Ac-D-Tyr^1,D-pCl-Phe^2,D-Trp^{3,6}	30	3/9
Ac-D-Trp^{1,3},D-pCl-Phe^2,D-Phe^6	15	1/10

TABLE 4-3. Effect of Some Position 10 Modifications on Analog Antiovulatory Activities

LHRH ANALOG	DOSE (μg)	RATS OVULATING/ RATS TESTED
Ac-D-Phe^{1},D-pCl-Phe^{2},D-Trp^{3,6},D-Ala^{10}	30	0/10
	15	2/6
Ac-D-pCl-Phe^{1,2},D-Trp^{3},D-Phe^{6},D-Ala^{10}	10	0/6
	7.5	1/8
	5	5/8
Ac-D-Trp^{1,3},D-pCl-Phe^{2},D-Phe^{6},D-Ala^{10}	10	2/7
	5	5/8
Ac-D-Ala^{1},D-pCl-Phe^{2},D-Trp^{3},D-Phe^{6},D-Ala^{10}	10	9/9
Ac-L-Ala^{1},D-pCl-Phe^{2},D-Trp^{3},D-Phe^{6},D-Ala^{10}	10	9/11
Ac-Gly^{1},D-pCl-Phe^{2},D-Trp^{3},D-Phe^{6},D-Ala^{10}	10	11/11
Ac-D-pCl-Phe^{1,2},D-Trp^{3},D-Phe^{6},D-Ser^{10}	15	2/8
Ac-D-pCl-Phe^{1,2},D-Trp^{3},D-Phe^{6},D-Leu^{10}	15	8/9
Ac-D-pCl-Phe^{1,2},D-Trp^{3},D-Phe^{6},N-Me-D-Ala^{10}	15	10/10

terminus was an effective modification despite the absence of a side-chain.[17] However, in the D-Ala^{10} series, it was considerably less effective than D-Phe or its active ring-substituted derivatives. Likewise, neither Ac-D-Ala^{1} or L-Ala^{1} analogs (Table 4-3) were significantly active in 15-μg doses.

Tyrosine in position 5 of the LHRH decapeptide is one of the majority of residues that have thus far not been modified to advantage in either agonist or antagonist analogs. Table 4-4 shows several analogs based on a [D-Phe^{2,6},D-Trp^{3}]-LHRH standard in which various new changes have been made to Tyr^{5}. Meta-Tyr has an OH group shifted from the p-position; this had little effect on biological activity. (The analog was assayed at a 2-mg dose in order to compensate for the presence of the inactive D-m-Tyr^{5} peptide.) Similarly, substitution of a Me group adjacent to the OH group was also without major effect. Tetrafluorotyrosine (F_4-Tyr) has an OH group whose acidity is increased to around that of a carboxyl group because of the strong electron-withdrawing effect of four F atoms. Its presence in position 5 created loss of antiovulatory activity at a 1-mg dose. Analogs in which the OH group was replaced by an F or Br, or in which Tyr itself was replaced by pentafluorophenylalanine (F_5-Phe) were inactive at a 1-mg dose and are, therefore, even less active than the Phe^{5} analog so that an increase in lipophilicity at this

TABLE 4-4. Effect of Some Position 5 Modifications on Analog Antiovulatory Activities

LHRH ANALOG	DOSE (mg)	RATS OVULATING/ RATS TESTED
D-Phe^{2,6},D-Trp^{3}	1.0	2/11
D-Phe^{2,6},D-Trp^{3},D,L-m-Tyr^{5}	2.0	3/9
D-Phe^{2,6},D-Trp^{3},D,L-3-Me-Tyr^{5}	2.0	4/9
D-Phe^{2,6},D-Trp^{3},F_4-Tyr^{5}	1.0	5/5
D-Phe^{2,6},D-Trp^{3},pF-Phe^{5}	1.0	8/8
D-Phe^{2,6},D-Trp^{3},pBr-Phe^{5}	1.0	8/8
D-Phe^{2,6},D-Trp^{3},F_5-Phe^{5}	1.0	8/8
D-Phe^{2,6},D-Trp^{3},D,L-α-Me-Tyr^{5}	2.0	9/9

position cannot be tolerated.[10] Alteration of the asymmetric center of Tyr is also detrimental to inhibitory activity since the α-Me-Tyr[5] analog was inactive at the doses tested.

SYNTHESIS

The solid-phase synthesis of LHRH antagonists—even those containing two or, in some instances, three acid-sensitive Trp residues—presents few problems in terms of the yields of desired materials. The peculiar solubility characteristics of the antagonists do, however, impose severe constraints on purification methods which can be used.

Until recently, elution on Sephadex G-25 in 50% acetic acid followed by silica gel chromatography in ethyl acetate:pyridine:acetic acid:water (20:5:1:3 by volume) was adequate to ensure a homogeneous product. The ever-increasing hydrophobicity and yet insolubility of the peptides in organic solvents made adsorption chromatography increasingly difficult and ineffective. Fortunately, the recent availability of intermediate HPLC packings in bulk at reasonable prices has made possible the development of the reverse-phase technique at a low cost on a truly preparative scale. All LHRH antagonists are now purified on columns of Whatman LRP-1 (13–24 μm particle size), which is a C_{18}-bonded silica. A buffer system has been developed which, unlike previously reported analytical systems, ensures high solubility of the analogs and excellent resolution. This consists of 0.1 M ammonium acetate in 25% acetic acid as the aqueous component with the application of various linear gradients of n-propanol.

Figure 4-2 shows a representative elution profile obtained with 300 mg of [Ac-D-pCl-Phe[1,2],D-Trp[3],D-pNO$_2$-Phe[6],D-Ala[10]]-LHRH on a 2.5 × 45-cm column pumped at a relatively low pressure of about 50 pounds per square inch but with a high enough flow rate to complete the run in just over 1 hour. Generally, the crude peptides, which have been previously partially purified on Sephadex, give major peaks containing material which is pure by analytical HPLC and thin layer chromatography (TLC). Contaminants in those peaks emerging close to the major component are usually barely resolved by TLC, although so far at least one out of our four regular TLC solvent systems gives an observable separation. The maximum load for this size of column will vary according to the difficulty of the separation required. We have loaded several grams of material with no observable loss of resolution.

RECENT PHYSIOLOGICAL STUDIES ON THE MOST ACTIVE ANTAGONISTS

Information on the basic physiological potential of LHRH antagonists in a variety of animal species, including primates, has slowly been accumulating but has been hindered by the large doses and short durations of action of early compounds. It now seems safe to predict that peptides such as [Ac-D-pCl-Phe[1,2],D-Trp[3],D-Phe[6],D-Ala[10]]-LHRH, which are fully active at single

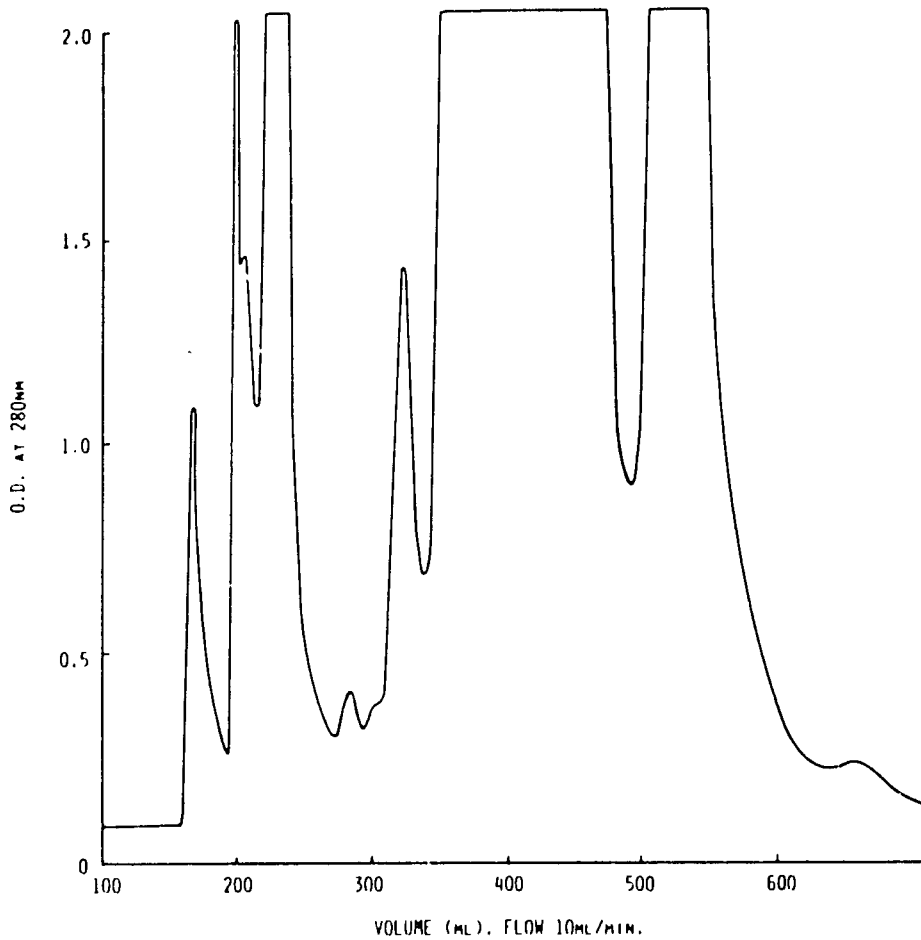

Fig. 4-2. Preparative high-performance liquid chromatography of 300 mg crude [N-Ac-D-pCl-Phe^{1,2},D-Trp^{3},p-NO_2-Phe^{6},D-Ala^{10}]-LHRH. The column (2.5 × 45 cm) containing Whatman RP-1 C_{18}-silica was eluted with a linear gradient of 25 to 60% n-PrOH in 0.1 M NH_4Ac made up in 25% acetic acid at a pressure of about 50 psi.

doses of 10 μg in the rat and exert an inhibitory effect for up to 18 hours after injection, will enable most contemplated studies to be done.

The prolonged activity of the new analogs is encouraging and is being investigated in some detail. In an extensive study using female rats, [Ac-D-pCl-Phe^{1,2},D-Trp^{3},D-Phe^{6},D-Ala^{10}]-LHRH was given at either a 15-μg or 150-μg dose on various days of the estrous cycle.[12] Oviducts were examined on the morning of the next expected estrus and for several days afterwards. At the same time, the ability of pituitary homogenates to bind iodinated [D-Trp^{6}]-LHRH, a superactive LHRH agonist, was also measured. The 15-μg dose was effective in blocking ovulation when given at the normal time of 12 o'clock noon on proestrus. However, this was primarily a simple delay in the cycle since most animals began to ovulate on diestrus I. In contrast, the 150-μg dose inhibited ovulation through diestrus I and, in 85% of the animals

examined, on diestrus II also. Indeed, only half of these animals had ovulated up to 4 days after injection. When 150 μg of the antagonist was given on either diestrus I or II, ovulation was blocked on the following estrus with the majority ovulating on diestrus I 3 to 4 days afterwards. Although this prolonged blockade of ovulation at the high-dose level might suggest extremely prolonged pituitary binding activity for the peptide, the situation may be rather more complicated. The extended inhibition produced by high doses was accompanied by a significant reduction in pituitary LHRH binding sites. Most strikingly, there were 90% fewer binding sites on estrus in the group treated with 150 μg than in control animals. The affinity constant was not significantly affected from diestrus II to estrus. Although ovulation was also blocked on estrus in animals receiving 15 μg of analog, no effect on receptor numbers could be seen. Thus the prolonged antiovulatory activity of high doses of the peptide is presumably directly linked to the large reduction in receptor numbers which, in turn, presumably only occurs when appreciable concentrations of the analog are present. It appears that ovulation resumes without an appreciable increase in pituitary LHRH receptors, indicating that they are present in excess in normal animals on proestrus. It will be interesting to determine whether the antagonists will exert a direct effect on testicular and/or ovarian LHRH receptors and even LH and prolactin receptors in a similar or opposite fashion to the "super" agonists discussed elsewhere in this book.

R.H. Asch,* in collaboration with us, supplied favorable data on the inhibitory effects of [Ac-D-Trp^{1,3},D-pCl-Phe^{2},D-Ala^{10}]-LHRH in various rhesus monkey models. The prolonged activity of the analog was demonstrated when blockade of LH and FSH release induced by LHRH injections in oophorectomized animals could be achieved from 2 up to 18 hours after injection of 1 mg of the antagonist. The same dose significantly lowered the elevated basal gonadotropin levels in oophorectomized monkeys within 2 to 4 hours of injection. However, the maximal inhibition took place only 12 to 24 hours later.

In another study again using oophorectomized animals, daily IM administration of 1-mg doses of analog, beginning on the day of surgery, completely blocked the gradual rise in gonadotropin concentrations.[1] This rise normally begins 3 to 4 days after operating and reaches a plateau from 8 to 22 days afterwards with three- to seven-fold concentration increases over preoperative values. After discontinuation of the peptide treatment, both gonadotropins rose to expected, elevated levels in a similar manner to control animals. The efficacy of [Ac-D-Trp^{1,3},D-pCl-Phe^{2},D-Phe^{6},D-Ala^{10}]-LHRH in these experiments has allowed rhesus monkey experiments to be extended to blockade of ovulation in normally cycling females.

Although various regimens of administration should eventually be examined, first attempts in which 1 mg of the analog was given daily from days 9 to 14 of the cycle were quite successful. Of six animals treated, ovulation was blocked completely in four, and in two it was delayed for 10 to 15 days.

Although previous studies with women have indicated that several of the

*Personal communication.

older analogs were indeed capable of preventing gonadotropin release in humans,[2,11] the far lower doses required with the new antagonists should allow such studies to be greatly extended. Already, Gonzalez-Barcena has found that IM administration of 5 mg of [Ac-D-Phe^{1},D-pCl-Phe^{2},D-Trp^{3,6},D-Ala^{10}]-LHRH blocked the high LH and FSH levels present in climacteric women for up to 18 hours after injection.[11] Zarate[2] found that the same dose of [Ac-D-pCl-Phe^{1,2}, D-Trp^{3},D-Phe^{6}, D-Ala^{10}]-LHRH administered on day 12 of the menstrual cycle blocked ovulation, as detected by measurement of gonadotropin and progesterone levels, in six of nine normal women.

CONCLUSIONS

There appear to be few significant obstacles to a full evaluation of LHRH antagonists as birth control agents. From a practical viewpoint, there is still a great need to develop more active analogs. Apart from the fertility control aspect, even the presently available analogs could well prove to be of value in the treatment of other conditions in which a lowering of gonadotropin levels is desirable. These might include postmenopausal problems, endometriosis, precocious puberty, and some forms of cancer.

The unusual solubility properties of these compounds have prevented a satisfactory delivery system from being developed. All the experiments described here were done with solutions of peptides in propylene glycol-saline, which is not ideal for human use. Many researchers use suspensions of peptides in corn oil, thereby introducing some possibility of error due to the use of nonhomogeneous mixtures. Much pharmaceutical research is required to extend delivery options. Certainly, many possible approaches for designing highly active yet adequately water soluble antagonists exist. Likewise structure–activity studies on requirements for intranasal and gastrointestinal absorption are possible. Some work along these lines has already begun in our laboratory.

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5

A Critique on Analog Antagonists of LHRH

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Ever since the isolation and identification of LHRH in 1971 by Schally and associates,[9,11] investigators in the United States have methodically attempted to develop the antagonists of this very important hormone for use as a new type of antifertility agent. The first LHRH analog antagonist, [des-His^2]-LHRH, developed by Vale and colleagues in 1972,[14] was weak in potency but nevertheless important in principle, the results indeed supporting the feasibility of the approach. Their effort was particularly important, besides, because of the unsuccessful attempt to develop a TRH analog antagonist over the preceding two years. Subsequently, in contrast to achieving the synthesis of potent LHRH analog antagonists, the development of only very weak TRH analog antagonists has been accomplished.[12] The first report by Beattie and Corbin and others demonstrated the antifertility properties of [D-Phe^2,D-Ala^6]-LHRH *in vivo* when milligram amounts of the analog were administered to spontaneously cycling female rats.[1] When the same *in vivo* assay[1] is used in current research, the antifertility effects of the current LHRH analog antagonists are able to be elicited with only a few micrograms.

A significant aspect in the development of LHRH analog antagonists has been the large number of structural changes that can occur while inhibitory activity is retained. The early LHRH analog antagonist of Rees and colleagues—[D-Phe^2]-LHRH—had only a single substitution at position 2.[10] As

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reviewed by Humphries and associates, a series of analogs with di-, tri-, or tetrasubstitutions followed at positions 2,6; 2,3,6; or 1,2,3,6.[8] At positions 2,3 and 6, substitutions of D-aromatic amino acids have been the most effective: this has not necessarily been true at position 1, where L- rather than D-amino acid substitutions have yielded antagonists that sometimes are much more potent. Even more complicated subtle structural relationships have become apparent with the discovery that [D<Glu^1,D-Phe^2,Pro^3,D-Phe^6]-LHRH produces less antiovulatory activity in rats than [<Glu^1,D-Phe^2,Pro^3,D-Phe^6]-LHRH; the reverse is true of the first position of D<Glu-substituted analog of a very closely related peptide, [D<Glu^1,D-Phe^2,D-Trp^3,D-Phe^6]-LHRH, which has considerably more antiovulatory activity than [<Glu^1,D-Phe^2,D-Trp^3,D-Phe^6]-LHRH.[7]

Although a variety of position-1 substitutions have been effective, the C-terminal modification (des-Gly^{10}-Proethylamide) of Fujino and others[6] in the LHRH analog agonists usually has not been favorable in the LHRH analog antagonists.[7] Also, in only certain LHRH analog antagonists is the activity enhanced by the C-terminal Az-Gly^{10} modification.* Coy and colleagues† have reported that the substitution of D-Ala^{10}NH$_2$ enhances the potency of some analog antagonists.

As reported by Friebel and co-workers, another dimension in the design of LHRH analog antagonists has evolved from the systematic synthesis of a series of analogs with a progressively increasing number of D-amino acid substitutions.[5] It is noteworthy that this occurrence could not be predicted from the biological results of the six intermediate analogs synthesized with varying numbers of D-amino acid substitutions at positions 4, 5, and 8; or that the final analog with six D-amino acid substitutions would be about two times more active than the parent analog with only three D-amino acid substitutions. The six D-amino acid substituted analog is N-Ac-Thr^1-D-Phe^2-D-Trp^3-D-Ser^4-D-Tyr^5-D-Trp^6-Leu^7-D-Arg^8-Pro^9-Gly^{10}-NH$_2$. This analog may be more resistant to biological degradation and closer to a peptide that could be utilized orally.

RESULTS AND METHODS

Table 5-1 demonstrates the significant progress accomplished in increasing the potency of the LHRH analog antagonists over the past seven years.[7] The *in vitro* LHRH inhibitory activity of the 1,2,3,6,10-pentasubstituted analogs is 100,000 times greater than the 2,3-disubstituted analogs, the 2,3,6-trisubstituted analogs having potencies somewhere between these two ranges of activity. Inhibition of ovulation in spontaneous cycling female rats is now accomplished with only a few micrograms.

Figures 5-1 through 5-6, and Figure 5-13, show the relationship between the *in vitro* and *in vivo* activity of LHRH analog antagonists in four different systems. As described,[2] the inhibition of the LHRH response was determined

*Bowers CY, Folkers K: unpublished data.

†Coy DH, Schally AV: unpublished data.

TABLE 5-1. Biological Perspective of LHRH Analog Antagonists

LHRH POSITION OF SUBSTITUTION 1	2	3	6	10	*IN VITRO* ACTIVITY ANALOG DOSAGE µg/ml	*IN VIVO* ACTIVITY AOA* DOSAGE µg/rat
	Leu	Leu			100.0	
	Leu	Leu	D-Ala		10.0	6000
	Phe	Leu	D-Ala		1.0	
	D-Phe	Leu	D-Phe		0.1	750
N-Ac-D-Thr	D-pCl-Phe	D-Trp	D-Trp	Az·Gly	0.001	6

*Antiovulatory activity.

in vitro using pituitaries of 21-day-old immature female rats and *in vivo* in adult mature male rats and adult male chimpanzees. For the latter studies, the LHRH analog antagonists and LHRH were administered together, the response was determined by measuring serum LH levels before and 10 to 30 minutes after the IV injection of the peptides. Antiovulatory activity in rats was determined by counting the number of ova shed on the fourth day of the estrous cycle after a single subcutaneous injection of the peptide, suspended in corn oil, at noon on proestrus. Results of a more extensive number of related LHRH analog antagonists have been reported previously.[2,7]

The results of the 2,3,6-trisubstituted analog, [D-Phe^2,Pro^3,D-Phe^6]-LHRH, show that the *in vitro* and *in vivo* results parallel each other. The LHRH

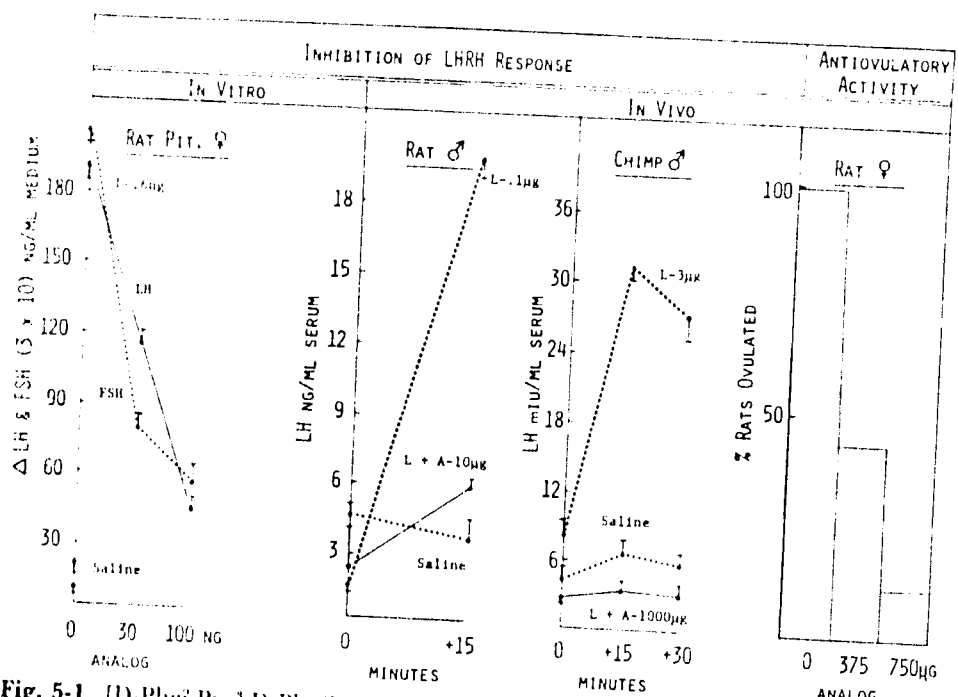

Fig. 5-1. [D-Phe^2,Pro^3,D-Phe^6]-LHRH antagonist activity *in vitro* and *in vivo*. Unless recorded, the *P* values are <0.001 of LHRH (L) vs L + Analog (A). Each result is the mean of 5–10 ± S.E.M.

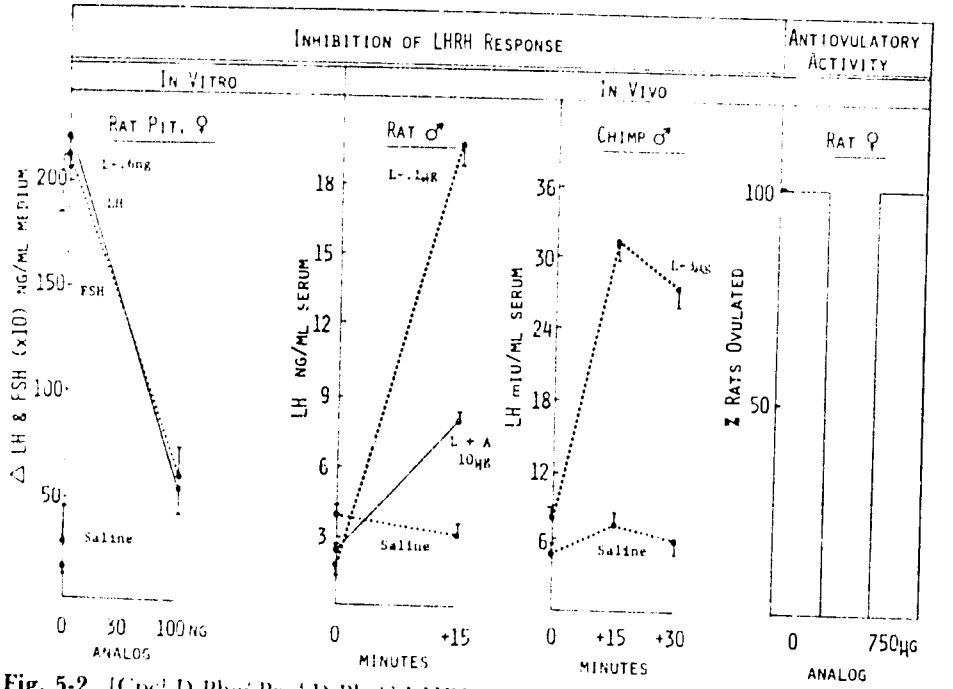

Fig. 5-2. [Cpc[1],D-Phe[2],Pro[4],D-Phe[6]]-LHRH antagonist activity *in vitro* and *in vivo*. Unless recorded, the *P* values are <0.001 of LHRH (L) vs L + Analog (A). Each result is the mean of 5–10 ± S.E.M. (Cpc = cyclopentane carboxylic acid.)

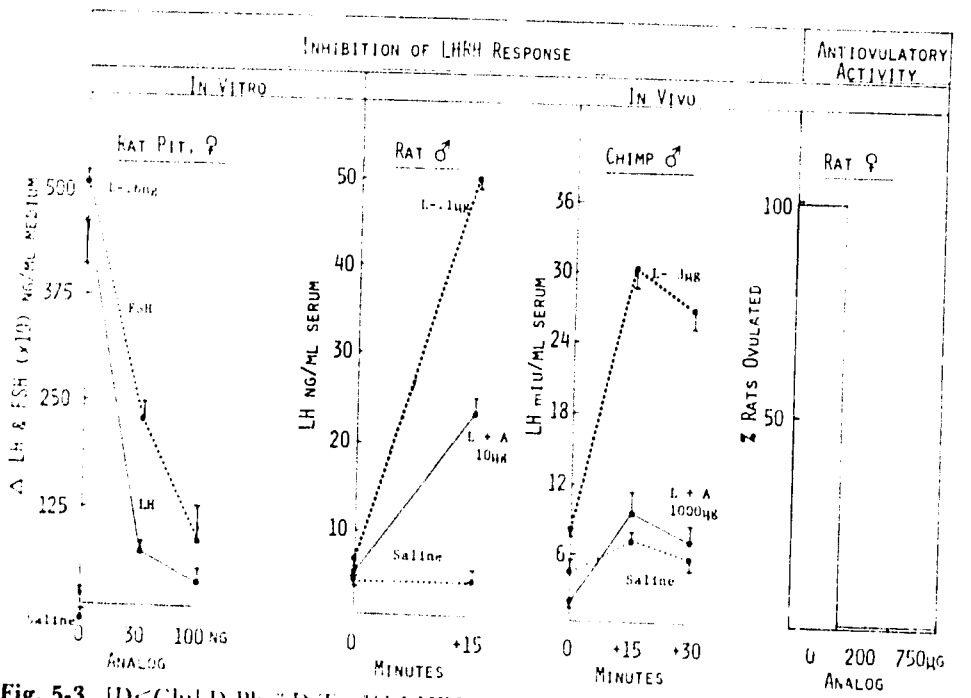

Fig. 5-3. [D-<Glu[1],D-Phe[2],D-Trp[3,6]]-LHRH antagonist activity *in vitro* and *in vivo*. Unless recorded, the *P* values are <0.001 of LHRH (L) vs L + Analog (A). Each result is the mean of 5–10 ± S.E.M.

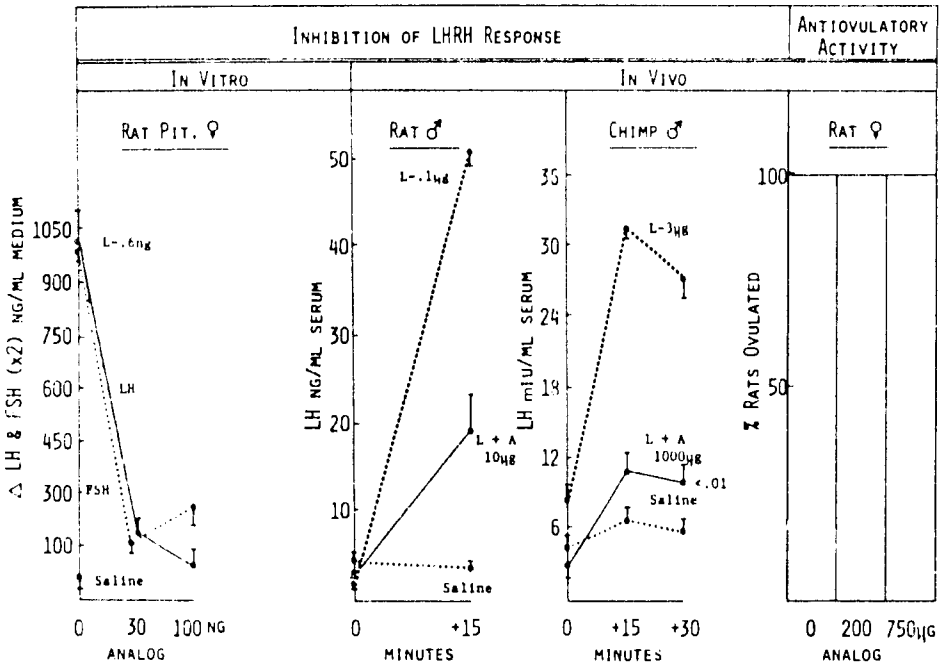

Fig. 5-4. [Cpc[1],D-Phe[2],D-Trp[3,6]]-LHRH antagonist activity *in vitro* and *in vivo*. Unless recorded, the *P* values are <0.001 of LHRH (L) vs L+Analog (A). Each result is the mean of 5–10 ±S.E.M. (Cpc = cyclopentane carboxylic acid.)

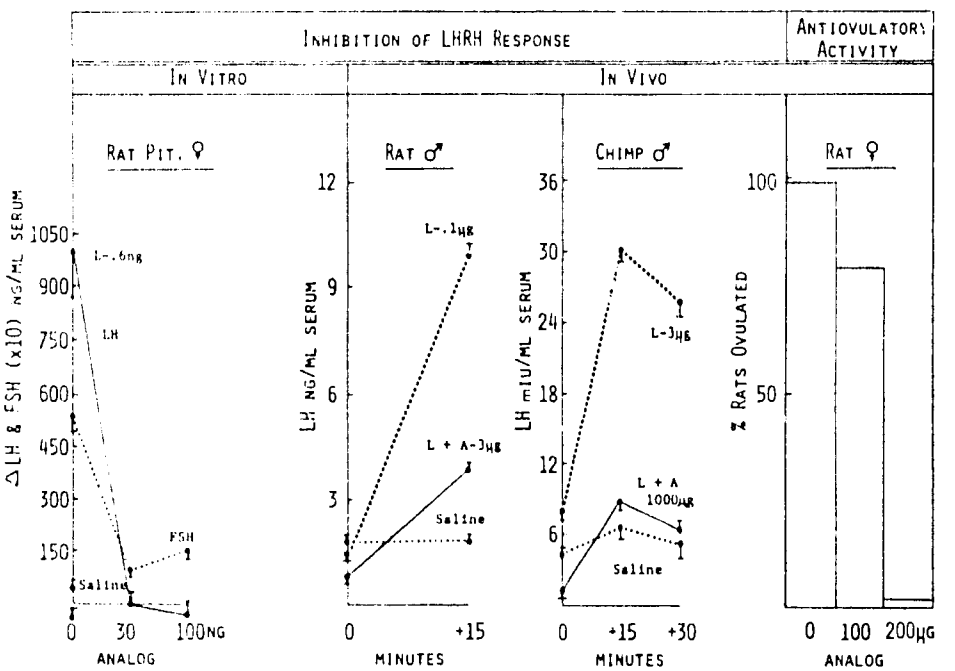

Fig. 5-5. [Ac-Pro[1],D-Phe[2],D-Trp[3,6]]-LHRH antagonist activity *in vitro* and *in vivo*. Unless recorded, the *P* values are <0.001 of LHRH (L) vs L+Analog (A). Each result is the mean of 5–10 ±S.E.M.

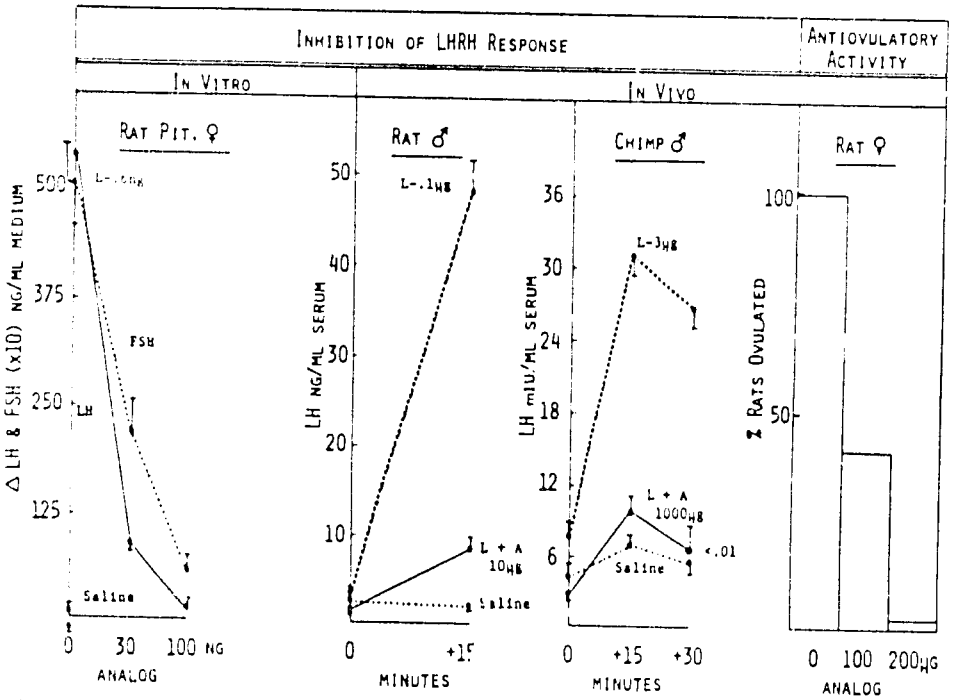

Fig. 5-6. [(<Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH antagonist activity *in vitro* and *in vivo*. Unless recorded, the *P* values are <0.001 of LHRH (L) vs L + Analog (A). Each result is the mean of 5–10 ± S.E.M.

response was completely inhibited *in vitro* at a dosage ratio of 166:1 (analog:LHRH) and *in vivo* at a dosage ratio of 100:1 in rats and 333:1 in chimpanzees. In addition, this analog completely inhibited ovulation in rats at 750 μg (Fig. 5-1).

Surprising results were obtained with the very closely related tetrasubstituted analog, [Cpc[1],D-Phe[2],Pro[3],D-Phe[6]]-LHRH (Fig. 5-2). Cpc represents a cyclopentane carboxylic acid residue that is less polar than the pyroglutamic acid residue normally present at position 1 since it no longer possesses the lactam or carbonyl group of the latter residue. This Cpc[1] analog had essentially the same potency *in vitro* and *in vivo* in inhibiting the LHRH response in the rat (chimpanzee not done) as the corresponding [D-Phe[2],Pro[3],D-Phe[6]]-LHRH analog; however, the Cpc[1] analog was completely inactive in inhibiting ovulation at 750 μg and the corresponding non-Cpc[1] analog completely inhibited ovulation at 200 μg. In further studies, the Cpc[1] analog appeared to be poorly absorbed from the subcutaneous injection site; presumably, it was this factor that was responsible for the inactive antiovulatory activity of this analog.[2]

Results in Figures 5-3 and 5-4 show a dissociation in *in vitro* and *in vivo* activity. [D<Glu[1],D-Phe[2],D-Trp[3,6]]-LHRH and [Cpc[1],D-Phe[2],D-Trp[3,6]]-LHRH were equally effective in inhibiting the LHRH response *in vitro* and *in vivo;* however, the Cpc[1] analog was inactive in the antiovulatory assay at 750 μg, while the corresponding D<Glu[1] analog completely inhibited ovulation in the rat at 200 μg. The same results were obtained with the [D-Phe[2],Pro[3],D-Trp[6]]-LHRH and [Cpc[1],D-Phe[2],Pro[3],D-Trp[6]]-LHRH pair of analogs. The former

completely inhibited ovulation in the rat at 750 μg, and the corresponding Cpc[1] analog was completely inactive at this dosage (not recorded).

In Figure 5-5, the N-terminal acetylated analog, [Ac-Pro[1],D-Phe[2],D-Trp[3,6]]-LHRH, inhibited the LHRH response *in vitro* and *in vivo*, as well as ovulation in the rat. This analog had about three times the *in vitro* and two times the antiovulatory activity of the corresponding nonacetylated analog.[7]

Extending the LHRH analog antagonists at the N-terminal end with additional amino acid residues also resulted in an effective antagonist. The 11-amino acid analog, [(γ-Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH, (Fig. 5-6) almost completely inhibited the LHRH LH response at 30 ng/ml medium *in vitro*, completely inhibited the LHRH response *in vivo* in the rat and chimpanzee, and completely inhibited ovulation in the rat at 200 μg. Furthermore, Wilks and colleagues demonstrated that this analog inhibited the preovulatory gonadotropin secretion of a rhesus monkey during a spontaneous menstrual cycle.[15]

Figure 5-7 shows the *in vitro* LHRH LH inhibitory results of another N-terminal extended undecapeptide, [(Ac-Pro-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH. The potency of the two related analog antagonists recorded was nearly the same at 30 ng/ml incubation medium. The D-pCl-Phe[2] for D-Phe[2] or Az-Gly[10] for Gly[10] substitutions did not enhance *in vitro* activity. In Figure 5-8, D-pCl-Phe[2],Az-Gly[10] and possibly D-pCl-Phe[2] were slightly more active than the parent analog *in vivo*.

The *in vitro* results in Figure 5-9 were obtained by the pituitary dispersed

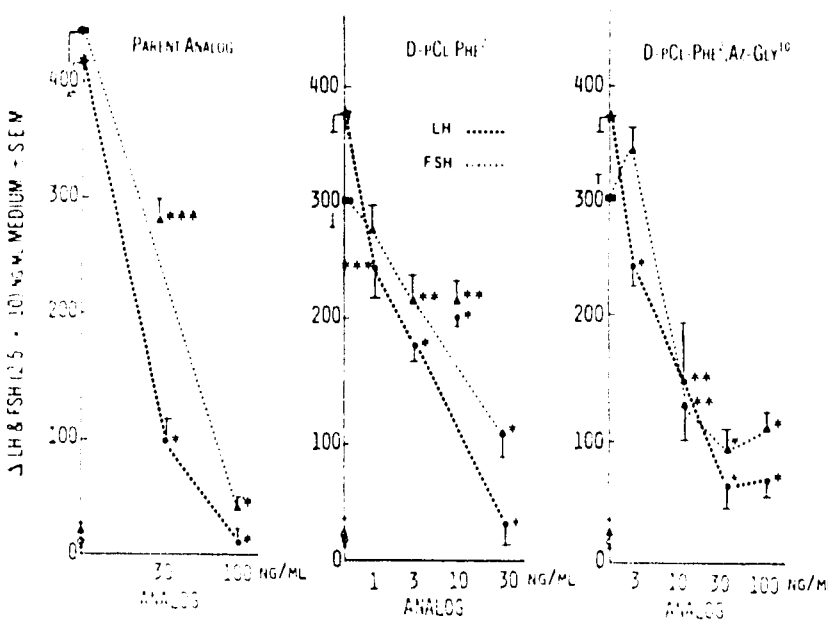

Fig. 5-7. *In vitro* LHRH antagonist activity of [(N-Ac-Pro-Pro)[1] D-Phe[2],D-Trp[3,6]]-LHRH (Parent Analog), [(N-Ac-Pro-Pro)[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH (D-pCl-Phe[2]), and [(N-Ac-Pro-Pro)[1],D-pCl-Phe[2], D-Trp[3,6],Az-Gly-[10]]-LHRH (D-pCl-Phe[2],Az-Gly[10]). The *P* values of LHRH (L) vs L + Analog are *<0.001, **<0.01 and ***<0.05. Each result is the mean of 6 ± S.E.M.

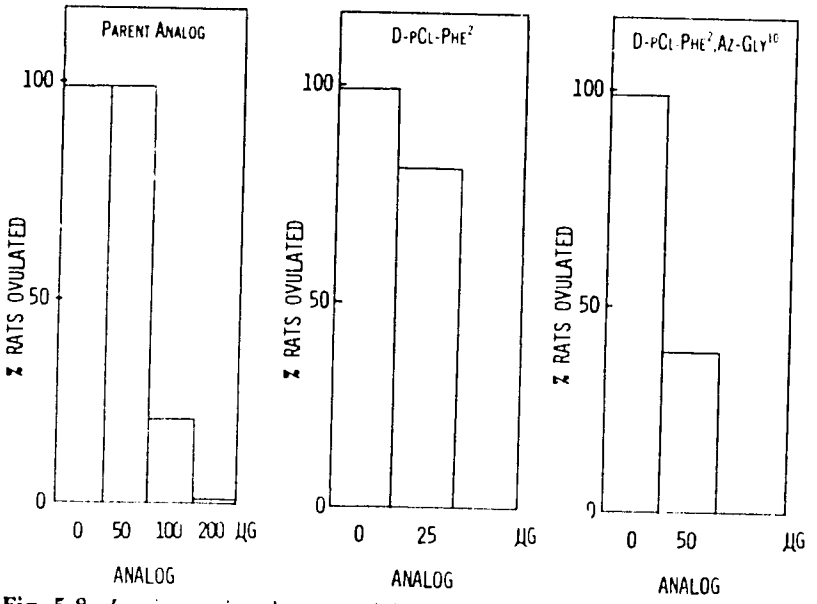

Fig. 5-8. *In vivo* antiovulatory activity of [N-Ac-Pro-Pro)[1], D-Phe[2], D-Trp[3,6]]-LHRH (Parent Analog), [N-Ac-Pro-Pro)[1], D-pCl-Phe[2], D-Trp[3,6]]-LHRH (D-pCl-Phe[2]), and [(N-Ac-Pro-Pro)[1],D-pCl-Phe[2],D-Trp[3,6],Az-Gly[10]]-LHRH (D-pCl-Pl -Gly[10]) in female rats.

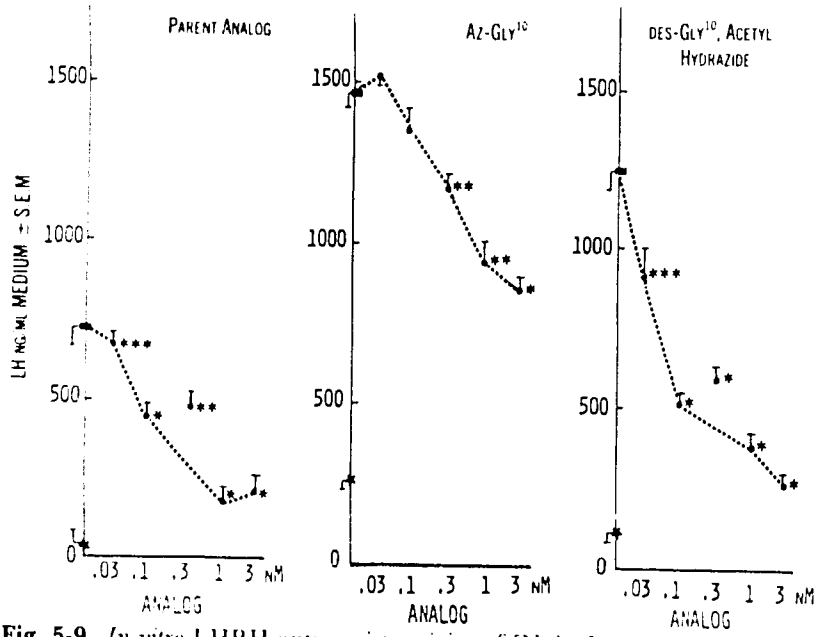

Fig. 5-9. *In vitro* LHRH antagonist activity of [N-Ac-D-pCl-Phe[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH (Parent Analog), [N-Ac-D-pCl-Phe[1],D-pCl-Phe[2],D-Trp[3,6],Az-Gly[10]]-LHRH (Az-Gly[10]), and [N-Ac-D-pCl-Phe[1],D-pCl-Phe[2],D-Trp[3,6],des-Gly[10],acetyl hydrazide]-LHRH (des Gly[10],acetyl hydrazide) in the pituitary dispersed cell culture assay of Vale and associates. The *P* values of LHRH (L) vs L+Analog are *<0.001, **<0.01 and ***<0.05. Each result is the mean of 5 ±S.E.M. The Parent Analog was synthesized by Dr. David Coy.

cell culture method of Vale and colleagues.[13] Both analogs were very potent *in vitro* since the inhibitory analog:LHRH dosage ratio was less than one. The analog with the acetyl hydrazide modification at position 10 was slightly more potent than the analog with the C-terminal Az-Gly[10] modification. Even though they were very potent *in vitro* in inhibiting LHRH LH response, neither analog was effective in inhibiting ovulation at 25 μg in the antiovulatory assay in rats (Figure 5-10).

The 1,2,3,6,10-pentasubstituted analog, [N-Ac-L-Ala[1],D-pCl-Phe[2],D-Trp[3,6], Az-Gly[10]]-LHRH is very active. For comparison (Fig. 5-11), the *in vitro* activity of one of Rivier's and Vale's currently most potent 1,2,3,6-tetrasubstituted analogs, [N-Ac-Δ[3]-Pro[1],D-pF-Phe[2],D-Trp[3,6]]LHRH was determined. Az-Gly[10] had about 70% of the potency of the latter analog *in vitro*. In the antiovulatory rat assay, Az-Gly[10] inhibited ovulation by 50% and N-Ac-Δ[3]-Pro[1]-analog by 100% at a dosage of 6 μg (Fig. 5-12).

Figure 5-13 shows results of the [N-Ac-D-Ala[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH and [N-Ac-L-Ala[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH analogs (both synthesized by Stewart and Channabasavaiah). N-Ac-D-Ala[1] was assayed *in vitro* and in the antiovulatory assay, and N-Ac-L-Ala[1] was assayed *in vivo* in the rat and chimpanzee. These analogs were very effective in all systems. N-Ac-D-Ala[1] partially inhibited LHRH response at 1 ng/ml medium, and only 20% of the rats ovulated after a single injection of 6.5 μg of the analog at noon on proestrus.

When D-Ser[4], D-Tyr[5], or D-Arg[8] was substituted into the LHRH analog

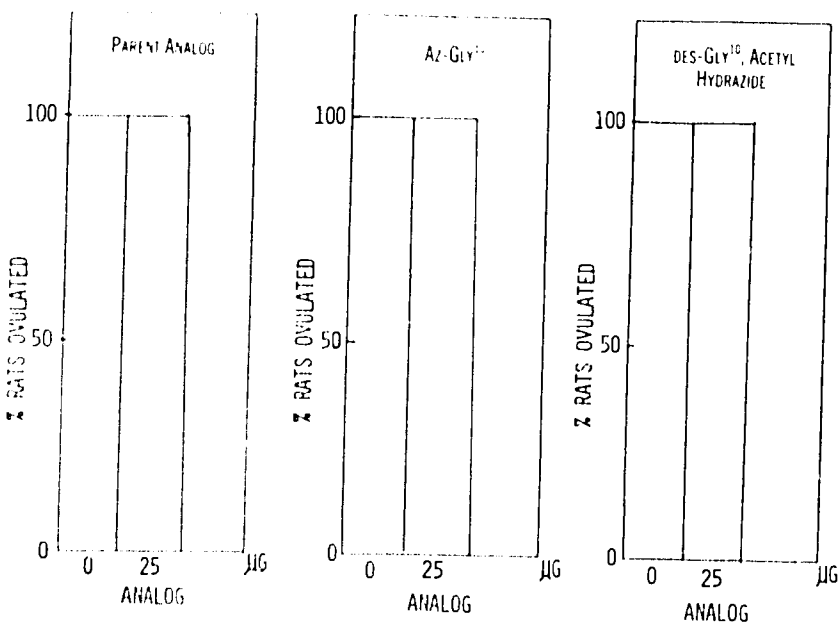

Fig. 5-10. *In vivo* antiovulatory activity of [N-Ac-D-pCl-Phe[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH (Parent Analog), [N-Ac-D-pCl-Phe[1],D-pCl-Phe[2],D-Trp[3,6], Az-Gly[10]]-LHRH (Az-Gly[10]), and [N-Ac-D-pCl-Phe[1],D-pCl-Phe[2],D-Trp[3,6],des-Gly[10],acetyl hydrazide]-LHRH (des-Gly[10],acetyl hydrazide) in female rats.

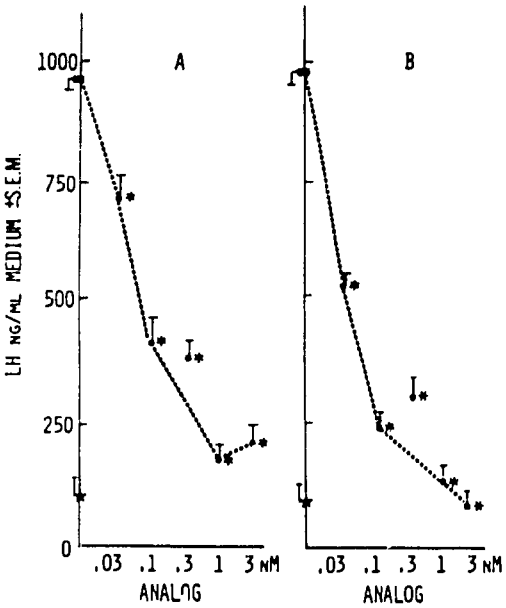

Fig. 5-11. *In vitro* LHRH antagonist activity of [N-Ac-L-Ala[1],D-pCl-Phe[2],D-Trp[3,6],Az-Gly[10]]-LHRH (*A*) and [N-Ac-Δ^5Pro[1],D-pF-Phe[2],D-Trp[3,6]]-LHRH (*B*) in the pituitary dispersed cell culture assay of Vale and associates. The *P* values of LHRH (L) vs L + Analog are *<0.001. Each result is the mean of 5 ± S.E.M. The Δ^5-Pro[1] analog was synthesized by Drs. J. Rivier and W. Vale.

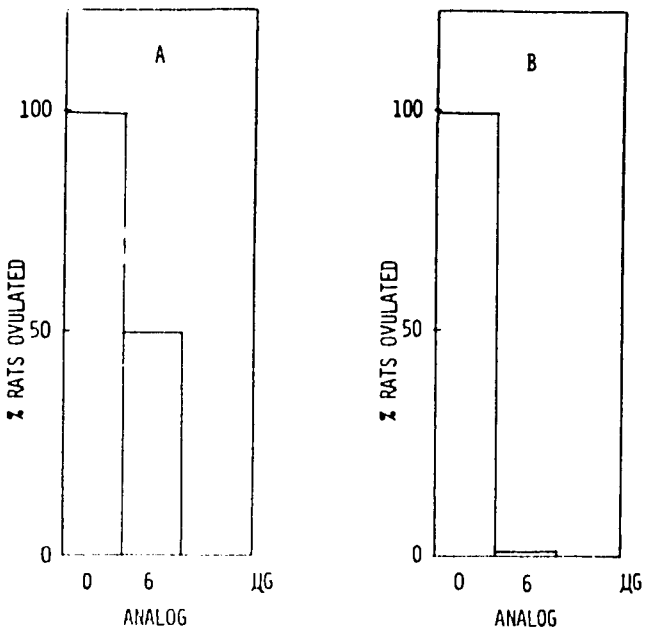

Fig. 5-12. *In vivo* antiovulatory activity of [N-Ac-L-Ala[1],D-pCl-Phe[2],D-Trp[3,6],Az-Gly[10]]-LHRH (*A*) and [N-Ac-Δ^5Pro[1],D-pF-Phe[2],D-Trp[3,6]]-LHRH (*B*) in female rats. The Δ^5-Pro[1] analog was synthesized by Drs. J. Rivier and W. Vale.

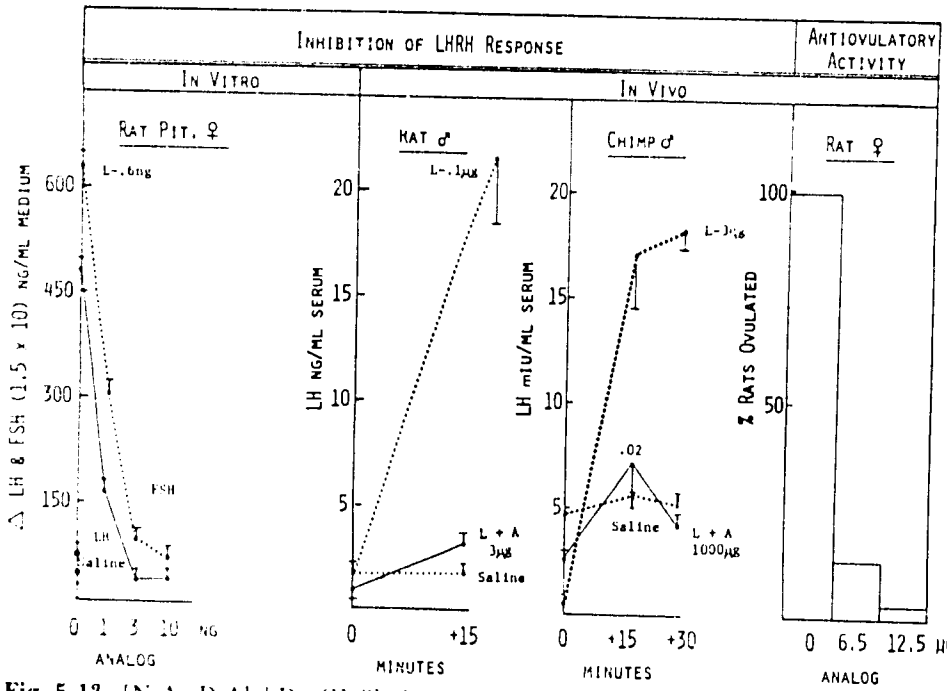

Fig. 5-13. [N-Ac-D-Ala[1],D-pCl-Phe[2], D-Trp[3,6]]-LHRH and [N-Ac-L-Ala[1],D-pCl-Phe[2],D-Trp[3,6]]-LHRH antagonist activity *in vitro* and/or *in vivo*. Unless recorded, the *P* values are <0.001 of LHRH(L) vs L + Analog (A). Each result is the mean of 5–10 ± S.E.M. These analogs were synthesized by Drs. J. Stewart and K. Channabasavaiah.

antagonist [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6]]-LHRH, each analog significantly inhibited the LHRH LH *in vitro* response (Fig. 5-14). D-Ser[4] was slightly more potent than D-Tyr[5] or D-Arg[8]. The D-Ser[4] analog had about the same potency as the above parent analog (not recorded). In the rat antiovulatory assay, these analogs were weak antagonists. With injections with 200 μg D-Ser[4], D-Tyr[5], or D-Arg[8] analogs, 75%, 100%, and 60% of the rats ovulated (Fig. 5-15). The parent analog was more effective in inhibiting ovulation; at 200-, 100-, and 50-μg dosages, 0%, 22%, and 44% ovulated, (not recorded).

Figures 5-16 and 5-17 show the results obtained from *in vitro* and *in vivo* studies by the analogs with pairs of D-amino acid substitutions at positions 4,5; 4,8; or 5,8 of the [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6]]-LHRH analog. *In vitro*, D-Ser[4], D-Tyr[5] was markedly more active than D-Ser[4],D-Arg[8] or D-Tyr[5],D-Arg[8], and was comparable in potency to D-Ser[4]. At 200 μg, these three analogs failed to inhibit ovulation (Fig. 5-17).

In contrast to these results, the analog with D-amino acid substitutions at 2,3,6 as well as 4,5,8—[N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Ser[4],D-Tyr[5],D-Arg[8]]-LHRH—resulted in a more potent analog both *in vitro* and *in vivo* than any of the other analogs (Figs. 5-14–5-17). Substitutions were at positions 4,5,8, and this analog was about two- to threefold more active than the parent analog without these 4,5,8 D-amino acid substitutions (Fig. 5-18). Whether

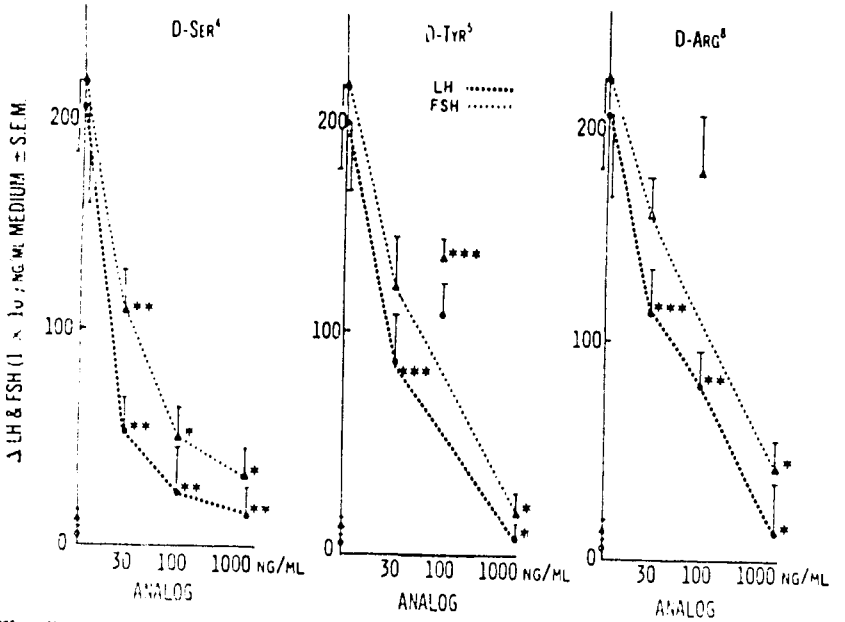

Fig. 5-14. *In vitro* LHRH antagonist activity of [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Ser[4]]-LHRH (D-Ser[4]), [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Tyr[5]]-LHRH (D-Tyr[5]), and [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Arg[8]]-LHRH (D-Arg[8]). The *P* values of LHRH(L) vs L + Analog are *<0.001, **<0.01 and ***<0.05. Each result is the mean of 6 ± S.E.M.

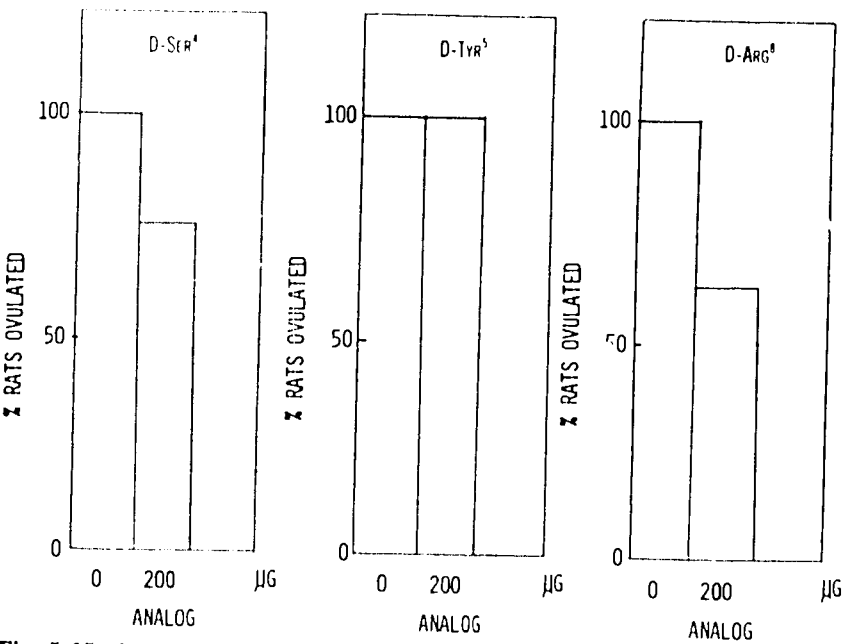

Fig. 5-15. *In vivo* antiovulatory activity of [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Ser[4]]-LHRH (D-Ser[4]), [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Tyr[5]]-LHRH (D-Tyr[5]), and [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Arg[8]]-LHRH (D-Arg[8]) in female rats.

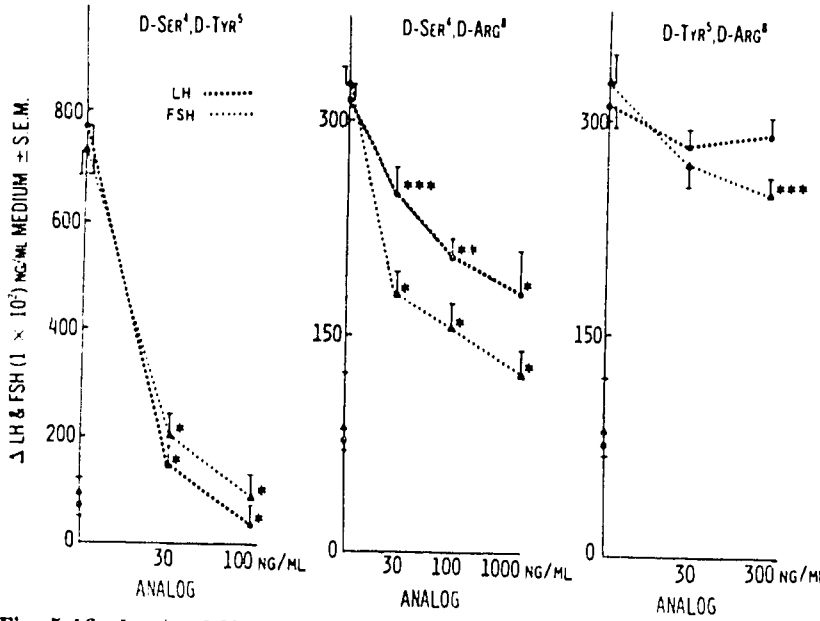

Fig. 5-16. *In vitro* LHRH antagonist activity of [N-Ac-Thr¹,D-Phe²,D-Trp³,⁶,D-Ser⁴,D-Tyr⁵]-LHRH (D-Ser⁴,D-Tyr⁵), [N-Ac-Thr¹,D-Phe²,D-Trp³,⁶,D-Ser⁴,D-Arg⁸]-LHRH (D-Ser⁴,D-Arg⁸), and [N-Ac-Thr¹,D-Phe²,D-Trp³,⁶,D-Tyr⁵,D-Arg⁸]-LHRH (D-Tyr⁵,D-Arg⁸). The *P* values of LHRH(L) vs L+Analog are *<0.001, **<0.01 and ***<0.05. Each result is the mean of 6 ±S.E.M.

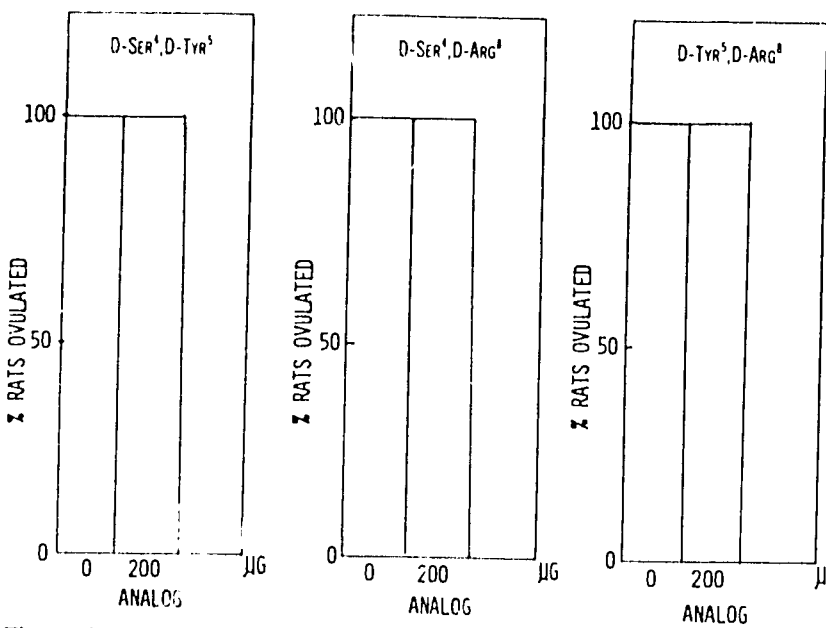

Fig. 5-17. *In vivo* antiovulatory activity of [N-Ac-Thr¹,D-Phe²,D-Trp³,⁶,D-Ser⁴,D-Tyr⁵]-LHRH (D-Ser⁴,D-Tyr⁵), [N-Ac-Thr¹,D-Phe²,D-Trp³,⁶,D-Ser⁴,D-Arg⁸]-LHRH (D-Ser⁴,D-Arg⁸), and [N-Ac-Thr¹,D-Phe²,D-Trp³,⁶,D-Tyr⁵,D-Arg⁸]-LHRH (D-Tyr⁵,D-Arg⁸) in female rats.

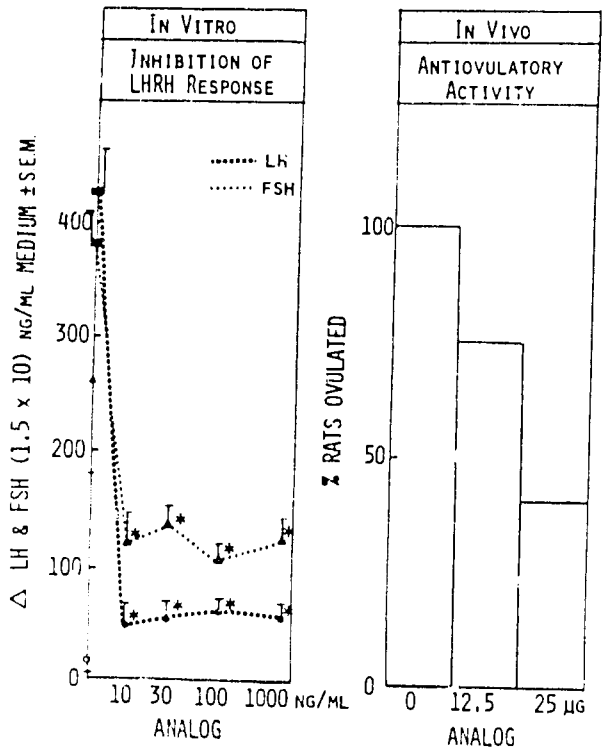

Fig. 5-18. *In vitro* LHRH antagonist and antiovulatory activity of [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6],D-Ser[4],D-Tyr[5],D-Arg[8]]-LHRH. The *P* values of LHRH (L) vs L + Analog are *<0.001. Each result is the mean of 6 ± S.E.M.

substitution of the D-pCl-Phe[2] residue into this analog can enhance its activity further has not yet been evaluated. However, the type of substitutions at 2,3,6 may in great part determine what might be most favorably substituted at 4,5,8 or the reverse. Also, as found for the 2,3,6-substitutions, the combination of substitutions at positions 4,5,8 may in great part be uniquely interdependent.

DISCUSSION

For most of the analogs, the *in vitro* and *in vivo* activities of the LHRH analog antagonists closely paralleled each other. This included inhibition of ovulation in rats and of LHRH LH and FSH responses *in vitro*, as well as the LHRH LH response *in vivo* in rats and chimpanzees. As the analogs became more active *in vitro*, they also became more active *in vivo*. However, if the LHRH inhibitory and antiovulatory potencies did not agree (*i.e.*, Cpc[1]), it is possible that some of the antagonists could be absorbed more slowly after subcutaneous injection.[2] The latter route of administration is performed in the antiovulatory, but not necessarily in the *in vivo* LHRH inhibitory, assay. Understanding more about the kinetics of absorption, metabolism, degradation, and excretion of some of the more potent antagonists could help to elucidate some of the problems. Development of radioimmunoassays (RIAs)

of some of the antagonists might be one of the most expeditious ways of accomplishing this goal.

An interesting problem has arisen as result of the utilization of two different types of *in vitro* assay systems. The LHRH analog antagonists have been assayed *in vitro* by our method of using intact pituitaries of 21-day-old immature female rats[2] and by the pituitary dispersed cell culture method of Vale[13] using the pituitaries of adult male rats. Results over the past eight years show that valid quantitative and consistent estimates of LHRH analog antagonist potency can be obtained by either method. Nevertheless, even though results parallel each other, potencies are consistently much greater when assayed by the dispersed pituitary cell culture method.

For example, the antagonist:LHRH dosage ratio of one of the more potent antagonists was 2.00 by the intact pituitary method and 0.01 by the dispersed cell method. The reason for the consistent difference in results is still unknown. It is recognized that by the dispersed pituitary cell method, we do indeed already have superactive LHRH analog antagonists; from the structural viewpoint, then, the potential for designing antagonists having many more-fold increases in potency is considerably less than if the present antagonists are not already superactive. By arbitrary definition, the antagonist: LHRH dosage ratio of a "super" antagonist would be less than 0.1. Also, if we already do have superactive antagonists by *in vitro* results and yet the analogs do not appear to be superactive by *in vivo* results, then the method of administering the antagonists is not yet optimal and more study is needed at this level.

In regard to structure–activity relationships, it is well established that using the sequence of LHRH as a starting structure, the most potent inhibitors to ovulation have the sequence [$DX^{2,3,6}$]-LHRH, where X is an aromatic amino acid. In this sequence, the position most well defined and most unchangeable seems to be D-Trp^3. Most modifications at this position seem to be less suitable to interaction at the receptor. The $DX^{2,6}$ positions appear to be more easily modified, as shown by the activity of the various groups of halogenated analogs in the second position[4] and the substitution of D-Phe for D-Trp in position 6.[8,9] Clearly, the many active inhibitors which retain the $DX^{2,3,6}$ modifications, but which have been changed at other positions in the LHRH sequence, indicate that the structural integrity of the molecule is defined principally by residues 2 through 6.

For example, at position 1 there is a wide variety of possible favorable substitutions, starting with the L<Glu to D<Glu change and continuing with the N-acetyl derivatives of L- or D-Ala; L- or D-Phe; L- or D-Pro; D-Trp; $\Delta^{3,4}$-Pro; Aib; Sar; L- or D-Thr; and Gly. A rigid conformational rule at position 1 is not evident, but there appears to be continuity in the requirement for the N-Ac group (8,3) and the observation that the N-terminal cannot be an amino or a charged group. The multiplicity of groups that can be substituted at position 1 is in sharp contrast to the LHRH agonist series, where <Glu is the prime choice for activity at the N-terminus.

Friebel and colleagues have contributed other findings that may eventually be helpful in better understanding these structure–activity relationships.[5] They observed that the additional substitutions of the respective D-amino acids alone or all together at position 4,5,8 into the [N-Ac-Thr^1,D-Phe^2,D-

Trp^{3,6}]-LHRH analog result in active antagonists.[5] The hexasubstituted D-amino acid analog position at 2,3,6 as well as at position 4,5,8 was more active *in vitro* and *in vivo* than any of the related analogs with one (position 4,5 or 8) or two (position 4,5; 4,8; or 5,8) D-amino acid substitutions, or even the parent analog without these additional substituted D-amino acids. From conformational energy calculations for the DX^{2,3,6} analogs, it is possible to incorporate these three 4,5,8 modifications into the molecule and obtain a structure closely similar to the starting structure.*

Finally, the tenth position can be studied for structural modifications of improvement. In most LHRH analog antagonists, the Fujino C-terminal modification des-Gly^{10},N-Proethylamide does not enhance antiovulatory activity.[7] However, in some analogs the Az-Gly^{10}, D-Ala^{10}, or D-Ser^{10} substitutions have been more effective. On the basis of a preferred conformation, the group in the tenth position will be in juxtaposition to the N-terminal residue. An interdependent relationship between the groups at the ends of the molecule may be vital for an effective antagonist. That is, large groups in one position may require small substituents at the tenth position for enhanced activity.

In conclusion, it is not yet clear from a structural point of view why the DX^{2,3,6} series makes the best inhibitors. It is apparent, however, from our results that the D-amino acid substitutions at 4,5, and 8 do not change the conformation of the DX^{2,3,6} analog, [N-Ac-Thr^{1},D-Phe^{2},D-Trp^{3,6}]-LHRH. Additionally, the structure–activity relationships of most of the LHRH analog antagonists paralleled each other in *in vitro* and *in vivo*.

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6

Discussion: Pharmacologic Development of LHRH and Analogs

CYRIL Y. BOWERS, MODERATOR

The potential of the new clinical approaches to the control of fertility and antifertility with the simple decapeptide hypophysiotropic hormone LHRH, which stimulates release of LH and FSH from the pituitary, is considerably greater than was initially envisioned. Results now show that in small carefully timed doses of LHRH, fertility is enhanced. However, in large doses, infertility is unexpectedly induced. This infertility effect is mediated not only in the pituitary, resulting in the decreased release of LH and FSH, but also, unexpectedly, at the direct gonadal site of action of LHRH, resulting in the decreased normal physiologic function of these organs.

The importance of LHRH or GnRH, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is indicated by the many analogs of this hormone that have been synthesized. The unique solid-phase method of peptide synthesis of Merrifield, along with improved purification methodology, has made it possible, over the last several years, to synthesize relatively rapidly more than a thousand highly purified analogs of LHRH. Substitution of a D-amino acid residue at position 6 of these analogs for the achiral Gly^6 residue of LHRH is unique, because by this modification, the activity of both the agonists and antagonists has been enhanced. Further structural changes of the agonists and antagonists, however, have not paralleled each other. In fact, amino acid substitution at sites other than position 6 rather markedly decreased the activity of the agonist, while often enhancing the activity of the antagonist. All the most potent antagonists have the sequence $[DX^{2,3,6}]$-LHRH, where X is an aromatic amino acid. The D-amino acid substitutions have not only helped to stabilize the analog conformation but have made it less susceptible to enzymatic degradation.

Superactive LHRH agonist analogs have been synthesized, as have highly active LHRH antagonist analogs. One of the most active LHRH agonist analogs is D-Trp^6, des-Gly^{10}, Proethylamide^9 ([D-Trp^6, Pro-$NHEt^9$]-LHRH). Its *in vitro* activity is 144 times greater than LHRH—a 36-fold increase as a result of the D-Trp^6 substitution, and a fourfold increase from the N-terminal Pro-NHEt modification. Also, [D-Trp^6, Pro-$NHEt^9$]-LHRH is much more active

Panelists: David H. Coy, Jean Rivier, Arno F. Spatola, John M. Stewart. *Discussants:* Linda Atkinson, Thomas M. Badger, Jose Balmaceda, Emil Steinberger

than LHRH *in vivo* in rats and humans. One of the most active LHRH antagonist analogs is [N-Ac-Δ^3Pro^1, D-p-F-Phe^2,D-Trp^{3,6}]-LHRH. In the dispersed pituitary cell culture system, it significantly inhibited the LHRH LH response, even when the antagonist:LHRH dosage ratio was 0.01. Also, when the N-Ac-Δ^3-Pro^1 antagonist was dissolved in corn oil and a single injection of 6.0 μg was administered to spontaneously cycling female rats at noon on proestrus, ovulation was completely inhibited.

The development of potent LHRH analog antagonists has gradually evolved from the sustained efforts of several different groups of investigators after intensive work on the problem since the elucidation of the chemical structure of LHRH in 1971. [des-His^1]-LHRH marked the initial success of these studies, because it was the first LHRH analog antagonist to be synthesized. This antagonist was weak in potency, but it did indicate the importance of a change at position 2 in the development of an antagonist. [D-Phe^2]-LHRH and [D-Phe^2,D-Trp^6]-LHRH are two other earlier antagonists of increasing potency. They emphasized the value of incorporating a D-aromatic amino acid residue in position 2, and also the value of incorporating a second D-amino acid, the propitious agonist–antagonist D-Trp^6 substitution noted above. The advantage of substitutions at positions 2, 3, and 6 were established next and again the optimum substitution at position 3 was a D-aromatic amino acid, seemingly specifically D-Trp^3 for optimal activity.

The next major increment of success came by modification of the N-terminus of the [DX^{2,3,6}]-LHRH antagonist. Noteworthy was that substitutions for the <Glu^1 residue were advantageous; however, structural requirements at position 1 were much less rigid than those at positions 2, 3, and 6. The possible number of favorable substitutions of position 1 started with L-<Glu to D-<Glu and gradually included the N-acetyl derivatives of L- or D-Ala, L- or D-Phe, L- or D-Pro, L- or D-Thr, D-Trp, Δ^3 Pro, Aib and even Gly. There was consistency in the requirement for the N-acetyl group and the observation that the N-terminal could not be an amino or a charged group. The C-terminal modifications, in comparison with the N-terminal modifications, have had a much smaller influence on the inhibitory activity of the antagonists. The des-Gly^{10}, Pro-NHEt^9 N-terminal modification has not been favorable, but the Az-Gly^{10} and the des-Gly^{10} acetyl hydrazide N-terminal modifications have been helpful when incorporated into select LHRH analog antagonists.

Structural refinements are now being more intensively made in the basic [X^1, DX^{2,3,6}]-LHRH analog antagonists. As described, the careful systematic step-by-step approach with additional minor modifications of this basic structure seems to be what is now needed for increasing the potency of the antagonist.

Currently, some of the following are the more favorable structural substitutions at positions 1, 2, 3, and 6. At position 1, N-acetyl-Δ^3Pro or N-acrylyl-Δ^3Pro are very good substitutions. At position 2, D-pCl-Phe and D-pF-Phe are indeed the most favorable in enhancing the inhibitory activity. So far, halogenation at the *para* position has been the optimal site and multiple halogenations have been less effective. Even though D-Trp at position 3 appeared to be the most unchangeable substitution, evidence has been obtained that the β(2 naphthyl)D-Ala may be slightly better. Also, at position 6, the

β(2 naphthyl)D-Ala substitution appeared to be more favorable than D-Trp, particularly when D-Trp and not β(2 naphthyl)D-Ala was substituted at position 3.

Synthesis of backbone cyclized analogs of LHRH has constituted another approach for developing improved agonists and antagonists. The agonist and antagonist LHRH analogs have been cyclized by disulfide bonds between the cysteine residues substituted at various sites in the peptides (*i.e.*, position 4,8;4,9;1,10), or have been cyclized through the backbone by a peptide bond between residues 1 and 10 in noncysteine-containing analogs. These conformationally more restricted peptides have the added advantage of being more amenable to physical–chemical studies which could help to better define the binding and activation of these peptides at the receptor. Although the cyclic LHRH agonists were weak in potency, some did elicit full intrinsic activity. Also, the cyclic LHRH antagonists were weak in potency. The most potent one was the noncysteine containing analog, [cyclo-Δ^3Pro^1,D-pCl-Phe^2,D-Trp^{3,6},N$^\alpha$Me-Leu^7,D-Ala^{10}]-LHRH. Although the D-pCl-Phe^2 or D-pF-Phe^2 substitutions at position 2 markedly increased the inhibitory activity of the linear LHRH analog antagonists, this was not true when they were substituted into the cyclic LHRH analog antagonists, [D-Cys^1,D-Phe^2,D-Trp^{3,6},Cys^{10}]-LHRH. These results may reflect the greater rigidity of the cyclic analogs and, thus, the more precise structural requirements of an analog if it is to bind with high affinity at the receptor. In principle, the synthesis of cyclic analogs seems like a good approach for learning more precisely about the structure activity relationships of these peptides, but also it appears to require more precision for which structural change can be introduced into the molecule.

Increasing numbers and varieties of peptide backbone modification have been introduced into the LHRH analogs, allowing another potentially unique approach to improving the biological stability of the analogs, possibly even to the extent that they eventually may be potent when administered orally. For example, these peptide backbone changes include not only *retro* amide bond replacements, which more directly assess the possible functional role of the carbonyl oxygen and the amide nitrogen of the peptide bond linkage, but also the replacement of the peptide bonds with nearly isosteric sulfur-based surrogates. These surrogates were first synthesized as pseudodipeptides before being incorporated into the peptide. The linkages included CH_2S, CH_2SO, $CHCH_3S$, and $CHCH_3SO$. All of these amide bond replacements except the first one contain at least one new chiral center and thus appear to have the potential of producing variable biological effects analogous to the effects induced when D-amino acids are substituted for L-amino acids.

All three agonist peptides with methylene thio (CH_2S) replacements at the 6-7, 5-6, or 9-10 peptide bond linkage LHRH were less active than LHRH. The very low activity of the 6-7 substituted peptide supports that a conformational rigidity is required at this site. The diminished, rather than low activity of the above other two peptides suggests that substitutions of a related type at the 5-6 and 9-10 linkages may not necessarily decrease the biological potency of LHRH. Evidence now indicates that not only are the above peptide bond sites vulnerable to endogenous protease cleavage, but so are the 1-2, 2-3, and 3-4 linkages, thus emphasizing the importance of the above approach.

In parallel studies, peptide backbone modifications of the LHRH analog antagonists also have been performed by incorporating the nearly isosteric sulfur-based surrogates. In addition, substitution of [CH_2S] or [CH_2SO] at the 6-7 linkage of an LHRH analog antagonist markedly decreased the activity, again indicating the requirement of more conformational rigidity at this site than is supported by these sulfur linkages. The 1-2 or 9-10 substituted CH_2S surrogate antagonists, in contrast, did not have full *in vitro* inhibitory activity, but the *in vivo* activity was definitely decreased. So far, the discrepancy between the *in vitro* and *in vivo* results has not been determined, even when analyzed in terms of the lipophilicity, diastereomerism, or different types of sulfur surrogate amide bond replacements. Since the oxidized (sulfoxides) in comparison to the nonoxidized sulfur surrogate linked antagonists had less inhibitory activity *in vitro*, the possibility arose that the sulfoxide metabolite may more readily form *in vivo* and, thus, may explain the discrepancies in the *in vitro* and *in vivo* potencies. Nevertheless, the results presented strongly support the value and intricacies of utilizing nearly isosteric sulfur-based surrogates for better understanding the functional role of peptide backbone atoms, the carbonyl oxygen and the amide nitrogen.

PHYSIOLOGY AND CONTRACEPTIVE EFFECTS OF LHRH AND ANALOGS IN FEMALE ANIMALS: AGONISTS

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6

Physiology and Contraceptive Effects of LHRH and Agonistic Analogs in Female Animals

ALAN CORBIN

FREDERICK J. BEX

In 1971, Schally and colleagues described the isolation, identification, and synthesis of LHRH.[43] Since that time, more than 1000 derivatives of LHRH have been synthesized.[10,15,17-20,44-46,49,51] The extensive chemical and pharmacologic advances made regarding LHRH and its antagonistic and agonistic analogs have provided a potentially new basis for the regulation of fertility.

From a historical perspective, the LHRH *antagonists* represented the first peptidic subclass to be synthesized and biologically identified, with the goal that such inhibitory analogs would prevent the critical surge of LH and of FSH, thereby blocking ovulation.[13,20,44,49] However, it is only recently that LHRH antagonists with potencies practical for more extensive pharmacologic, safety, and clinical evaluation have been identified. To date, while research continues in this area, progress in the use of the *agonists* ("super" LHRH analogs) has far exceeded that for antagonists.

The early availability of numerous, highly potent synthetic agonists has provided the foundation for a detailed reproductive pharmacologic evaluation of this peptide class.[18,20,44-46,49] Originally, it was thought that the LH-releasing and ovulation-inducing properties of LHRH observed in laboratory animals would offer a new therapeutic approach to clinical male and female infertility.[44,45] However, the collective effects observed with the commonly accepted profertility parent molecule (LHRH) and its potent agonists were contradictory to theory, and in numerous experimental models, were primarily and consistently antireproductive in character. This paradoxical antireproductive property is manifested by the ability of these agents to interfere with or terminate pregnancy pre- and postcoitally, reduce fecundity, retard puberty, and inhibit spermatogenesis in laboratory animals. In women

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and in farm animals, frequently these agents can produce luteal incompetence, despite their intended use to induce ovulation and establish viable pregnancies.[3-5,8,10,12,14-20,33]

From these studies has emerged a predictable reproductive relationship: in general, the greater the agonist potency, the greater the antireproductive property. Consequently, it was conceived that such LHRH agonists might represent a novel class of contraceptive agents for both sexes.

This chapter reviews investigations supporting the paradoxical antireproductive pharmacologic characteristics of LHRH and selected agonistic analogs in female animals. A brief note regarding safety studies and their relevance to the regulation of fertility in the clinic also appears.

BASIC LHRH AGONIST PROPERTIES

LHRH and agonistic derivatives share the property of inducing the release of pituitary LH and FSH and in inducing ovulation in a variety of animal models.[1,15,17,18,20,41-46,49] Numerous highly potent analogs have been synthesized by several laboratories[1,19,27,40,42,44-46,49,51] and include such compounds as D-Ala^6-des-Gly^{10}-Pro^9-ethylamide (NHEt)-LHRH (Wy 18,481); D-Trp^6-LHRH (Wy 42,462); D-Trp^6-des-Gly^{10}-Pro^9-NHEt-LHRH (Bachem 9022); D-Trp^6-N$^\alpha$Me-Leu^7-des-Gly^{10}-Pro^9-NHEt-LHRH (Wy 40,972); D-Leu^6-des-Gly^{10}-Pro^9-NHEt-LHRH (Wy 19,728), and D-Ser(TBU)^6-des-Gly^{10}-Pro^9-NHEt-LHRH (Wy 40,598; Buserelin). Several of these compounds have been compared in numerous biologic tests with the Wyeth compound, Wy 40,972.

The ovariectomized, steroid-blocked rat, which is highly sensitive to the LH-releasing activity of these compounds, represents a frequently used test for agonist classification (Fig. 7-1). A more direct, physiologically relevant, and equally reliable test is illustrated by Figure 7-2, which demonstrates the time course of LH release and subsequent ovulation with a "super" agonist (Wy 40,972) in proestrous rats whose spontaneous ovulatory mechanisms have been inhibited with Nembutal. Comparative ovulatory dose–response studies are presented in Figure 7-3. Figure 7-4 reveals that Wy 40,972, by 12 routes of administration, is effective in inducing ovulation.

ANTIFERTILITY EFFECTS OF LHRH AND AGONISTS IN FEMALE ANIMALS

Initial studies demonstrated that the parent molecule, LHRH, was capable of inhibiting pregnancy when administered pre- and postimplantationally to inseminated rats.[11] This paradoxical antireproductive effect became significant upon demonstration that a large array of agonists produced similar effects at considerably lower doses than LHRH. The ability of the two exemplary agonists, Wy 40,972 and Wy 18,481, to inhibit pregnancy in rats, both before implantation (claudogenic effect) and afterward (interceptive effect), is depicted in Figures 7-5 and 7-6, respectively.

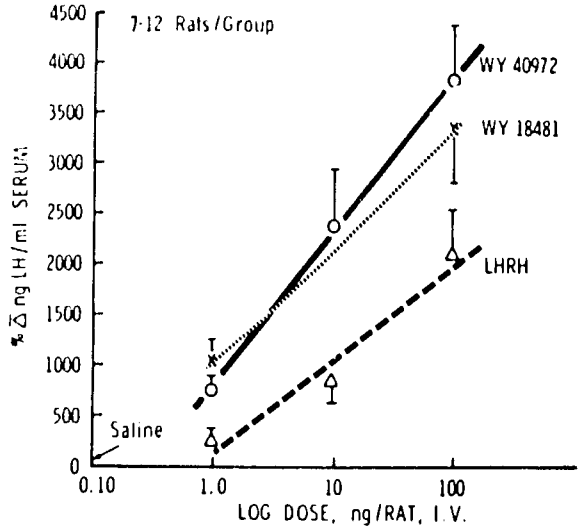


Fig. 7-1. Serum LH levels of ovariectomized steroid blocked rats treated with LHRH agonists. Each point is mean (±S.E.M.).

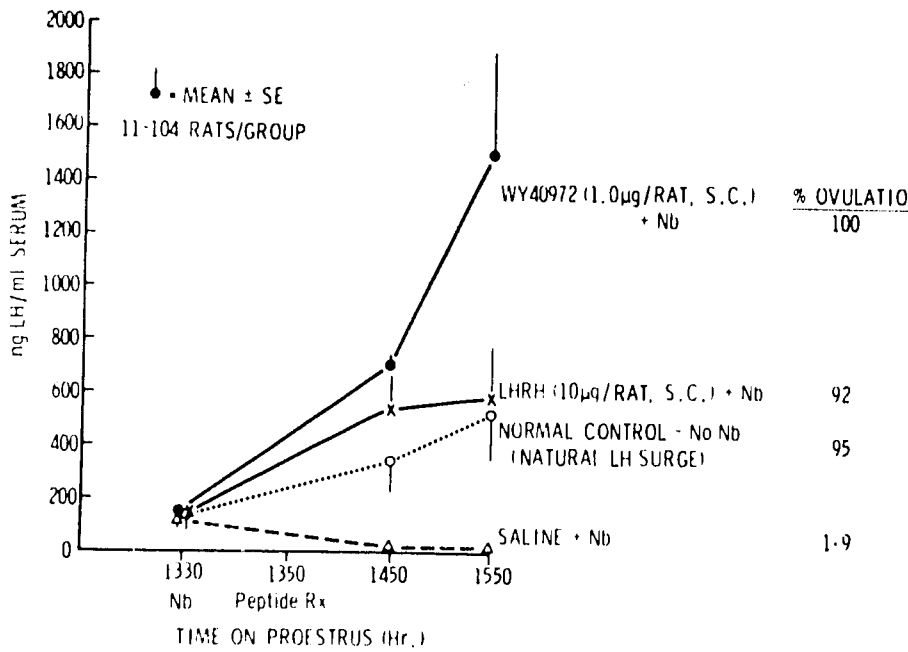


Fig. 7-2. Effect of LHRH and agonist on serum LH and ovulation in Nembutalized (Nb) proestrous rats. Each point is mean (±S.E.M.).

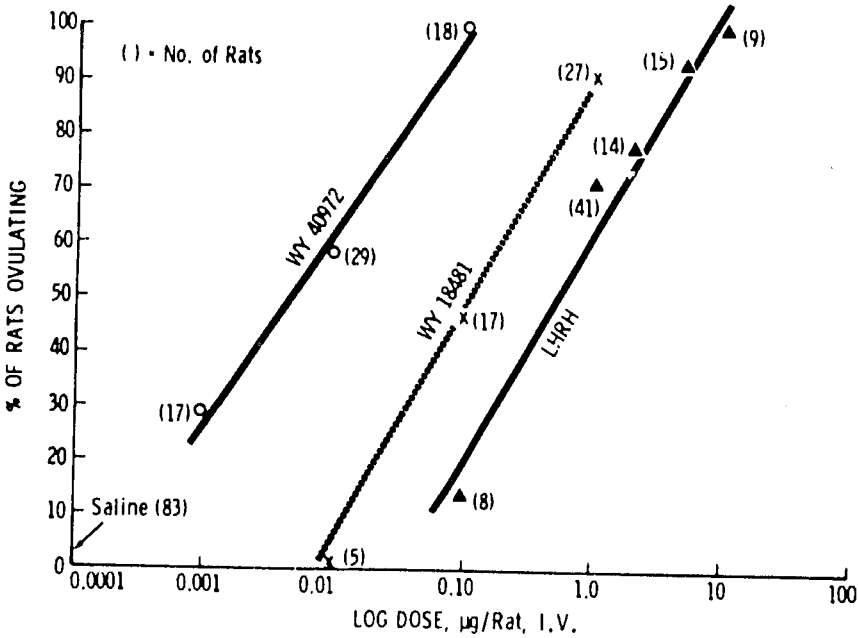


Fig. 7-3. Ovulation induction with LHRH agonists in the Nembutalized proestrous rat.

Extensive studies utilizing numerous agonists in several animal species established the fact that such agents can predictably produce postcoital contraceptive effects, such activity and potency being correlated with the basic agonist properties.[3,10,14-20,44-46,49] Table 7-1 summarizes for the D-Ala[6] and D-Trp[6] derivatives of LHRH the close association between basic agonistic activity, as represented by ovulation induction, and their pregnancy-inhibiting

TABLE 7-1. Agonist and Postcoital Contraceptive Profiles of the [D-Ala[6]]-LHRH and [D-Trp[6]]-LHRH Series

COMPOUND	AGONIST OVULATION INDUCTION MED100*, µg/RAT, IV	POSTCOITAL CONTRACEPTION CLAUDOGEN INTERCEPTIVE MED100, µg/DAY, SC	
LHRH	5.0	200–300	200–500
D-Ala[6]-LHRH	1.0	10.0	100.0
D-Ala[6]-des-Gly[10]-Pro[9]-NHEt-LHRH	1.0	1.0	25–50
D-Ala[6]-N[α]-Me-Leu[7]-des-Gly[10]-Pro[9]-NHEt-LHRH	0.10	1.0	1.0
D-Trp[6]-LHRH	0.10	10.0	1.0–10.0
D-Trp[6]-des-Gly[10]-Pro[9]-NHEt-LHRH	0.10	1.0	10.0
D-Trp[6]-N[α]-Me-Leu[7]-des-Gly[10]-Pro[9]-NHEt-LHRH	0.10	1.0	~1.0

*Approximate minimal effective 100% dose.

property. Furthermore, the antipregnancy effect, as evidenced for ovulation induction, is readily manifested via various routes of administration—subcutaneous (SC), intramuscular (IM), oral, aerosol, vaginal, and nasal.

These paradoxical antifertility effects have been extended and reinforced by the ability of this class of peptide to:

1. Retard puberty
2. Disrupt the estrous cycle (delay the onset of estrus and mating)
3. Induce premature ovulation
4. Induce luteolysis
5. Cause ovarian and uterine regression
6. Reduce fecundity in inseminated animals
7. Inhibit the ovarian/uterine stimulation produced by human chorionic gonadotropin.[1,15,18,26,38,45]

These effects are reversible, since once treatment has been withdrawn or the pregnancy terminated, normal breeding processes are restored rather quickly.[17,26] Thus, although these peptides had been shown to induce gonadotropin release and ovulation, and to be conceived as profertility agents originally, their most predominant property was one of an antireproductive nature. The plethora of data on the antifertility properties of these compounds provided the foundation for a novel approach to female contraception.

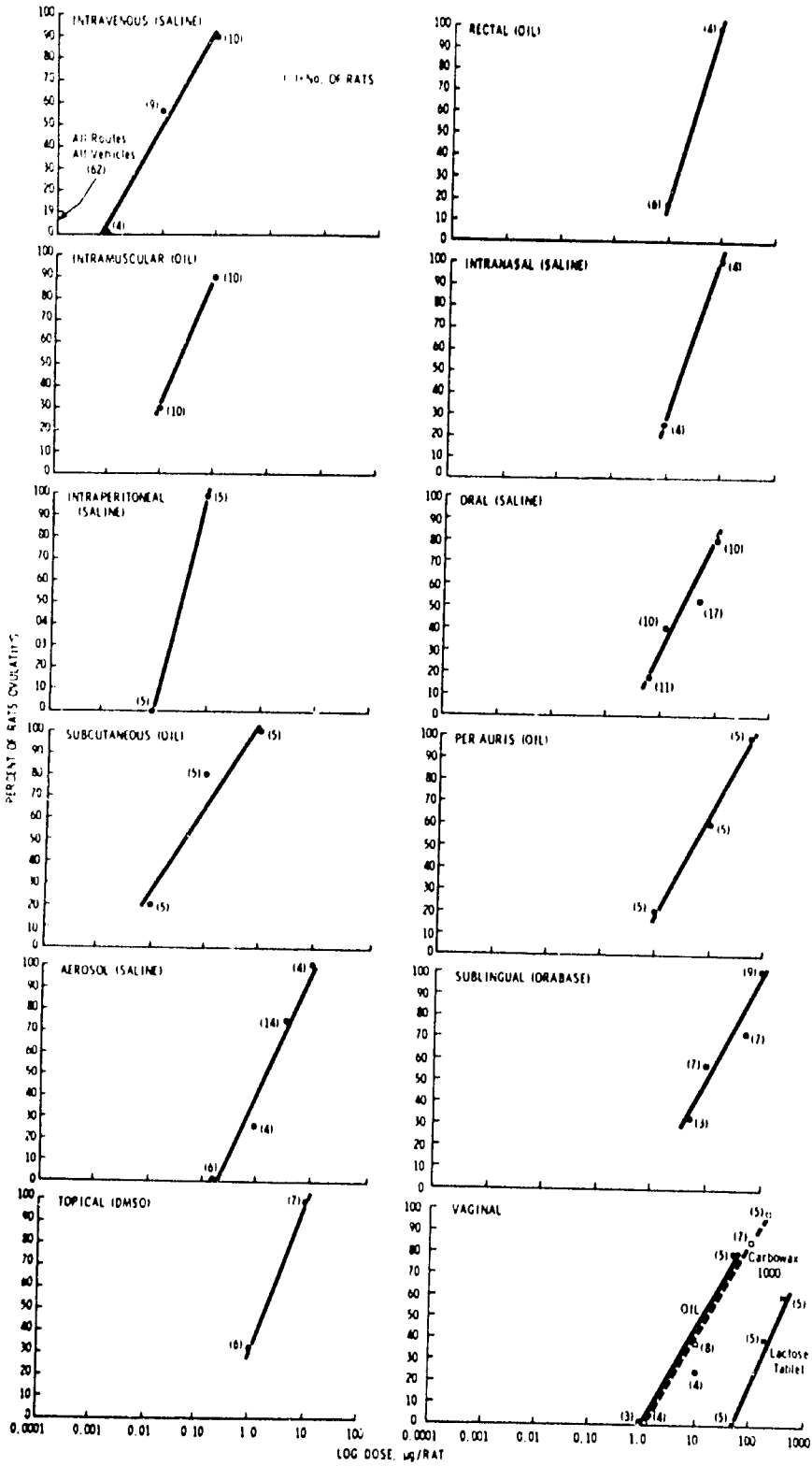
MECHANISMS OF ACTION

INDIRECT EFFECTS

It is noteworthy that in both sexes, a common mode of action subserves the antireproductive characteristics of LHRH and the agonists, expressed via the pituitary–gonadal target organ axis. Investigations utilizing varied dosing schedules and animal models have demonstrated that these peptides, by virtue of their traditional agonist properties, generate a series of hormonal events which eventually result in reproductive incompetence. In the female, these peptides (including LHRH) produce the following:

1. Large increments in serum LH levels
2. Distorted FSH patterns
3. Inappropriate prolactin secretion

Fig. 7-4. Wy 40,972: Ovulation induction in Nembutalized proestrous rats via various routes of administration.


INTRAVENOUS (SALINE)
( ) = No. OF RATS
All Routes
All Vehicles
(62)
RECTAL (OIL)
INTRAMUSCULAR (OIL)
INTRANASAL (SALINE)
INTRAPERITONEAL (SALINE)
ORAL (SALINE)
SUBCUTANEOUS (OIL)
PER AURIS (OIL)
AEROSOL (SALINE)
SUBLINGUAL (ORABASE)
TOPICAL (DMSO)
VAGINAL
Carbowax 1000
OIL
Lactose Tablet
PERCENT OF RATS OVULATING
LOG DOSE, µg/RAT
0.0001
0.001
0.01
0.10
1.0
10
100
1000

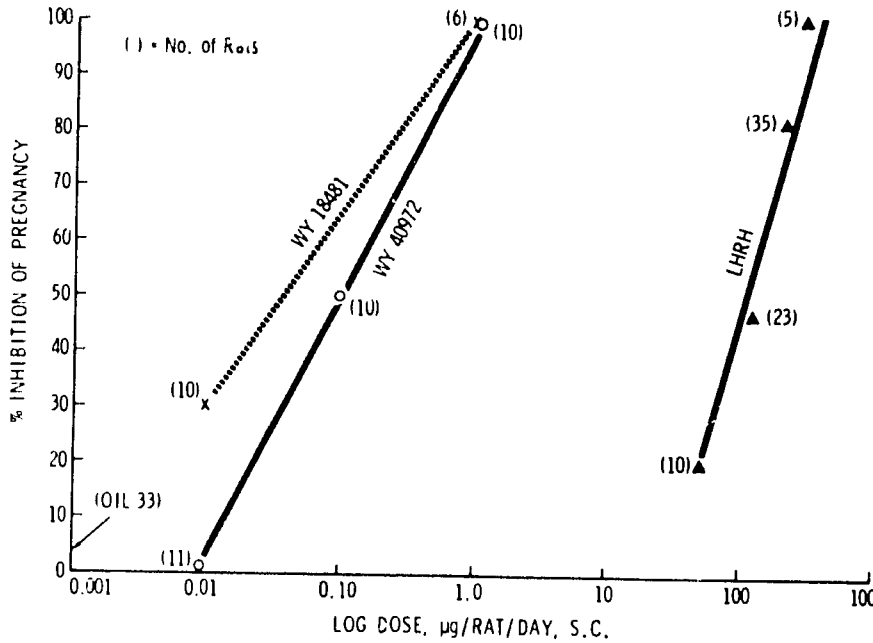


Fig. 7-5. Postcoital contraceptive effect of LHRH agonists in the rat: claudogenic; treatment given from days 1 through 7, subcutaneously.

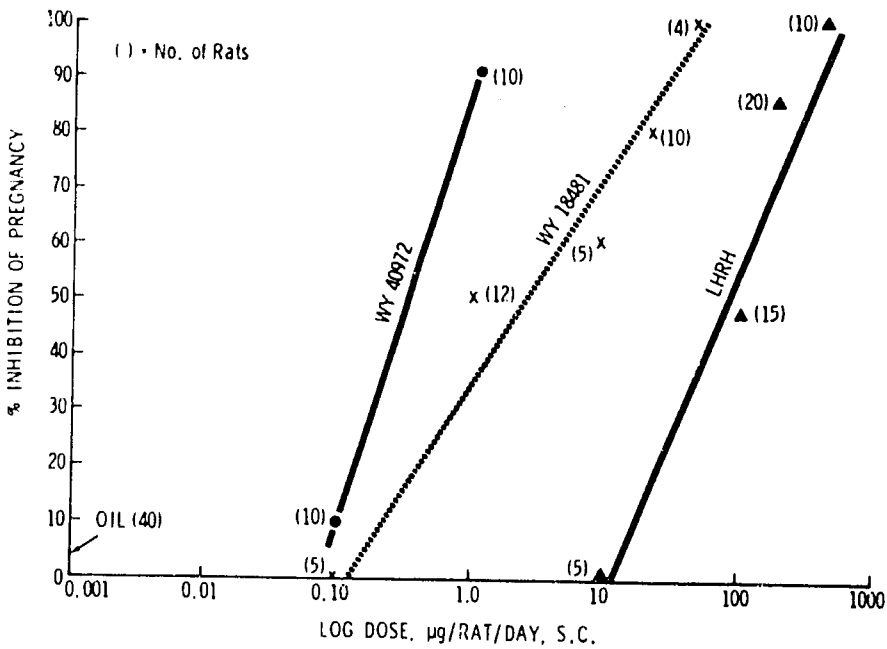


Fig. 7-6. Postcoital contraceptive effect of LHRH agonists in the rat: interceptive; treatment given from days 7 through 12, subcutaneously.

4. Decreased concentrations of ovarian LH, FSH, and prolactin receptors (desensitization/down-regulation). These effects result in histologic evidence of:
   a. Luteolysis
   b. Impaired steroidogenesis
   c. Delayed estrogen secretion
   d. Decreased progesterone secretion
   e. Elimination of uterine and/or placental support resulting in either infertile cycles or inhibition and/or termination of pregnancy.[4,8,9,15,17,18,44,45]

Figure 7-7 shows these effects in the pregnant rat subjected to LHRH intervention preimplantationally; similar results have been observed during the postimplantation period.[8] The agonists produce, in immature females, pubertal retardation, manifested by delayed vaginal canalization and gravimetric inhibition of the ovaries and uteri.[17,26,50] Chronic treatment can lead to attenuated pituitary responsiveness resulting in reduced gonadotropin output and inhibition of ovulation.[41,42]

EXTRAPITUITARY EFFECTS

Though the reproductive dysfunction subsequent to agonist treatment appears to take place mainly through the pituitary–gonadal axis, evidence has pointed to effects generated beyond the hypophysis.[10,18,47] In pregnant rats, hypophysectomy on day 12 or 13 of gestation does not interfere with the viability of pregnancy, since the placenta assumes the role of the pituitary gland at this stage.[9] Administration of LHRH or agonists to such hypophysectomized pregnant rats leads to pregnancy failure, implying a direct effect on either the ovary, the placenta, or both.[9,34,35] In those treated animals whose pregnancy is being terminated, blood levels of progesterone and rat placental lactogen (rPL) decline, thereby leading to fetoplacental wasting; replacement with progesterone reverses these effects. However, LHRH and agonist inhibition of rPL appears to be secondary to the disruption of progesterone secretion; this suggests that their extrapituitary action occurs through a direct effect on the ovary or by affecting placental hormones other than rPL (*e.g.*, rat chorionic gonadotropin) necessary for luteal support. Other studies have demonstrated the presence of LHRH in, and its synthesis by, the human placenta; moreover, placental progesterone secretion can be suppressed by LHRH. These data further implicate LHRH at a nonhypothalamic level.[28,52]

Additional studies revealed that LHRH and agonists can block follicular maturation and ovulation, and reduce ovarian FSH/LH receptors induced in hypophysectomized immature rats by PMSG or hCG stimulation.[25,53] The aromatization of androgens to estrogens by monolayer cultures of granulosa cells can be inhibited by LHRH and agonists.[25]

Further complicating factors are involved since unpublished data from this laboratory have demonstrated that LHRH and several of the aforementioned agonists can induce ovulation in hypophysectomized proestrous rats in a dose-related manner. Serum LH levels in these treated rats were below the level of detection, and such rats required higher doses to bring about ovulation than did intact proestrous rats; the dose–response curves were parallel. These

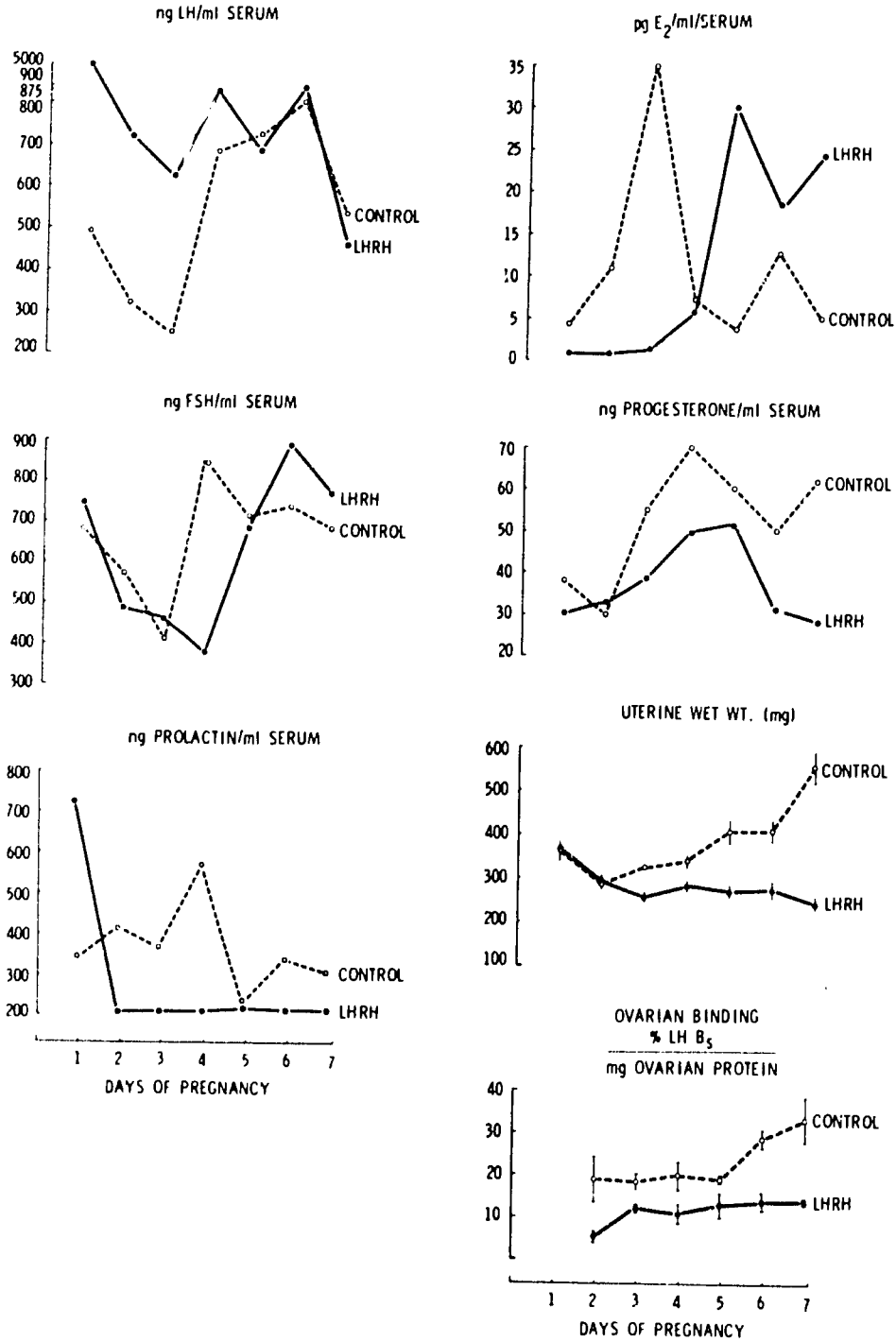


Fig. 7-7. Effect of LHRH on serum hormonal levels, uterine weight, and ovarian LH binding of pregnant rats treated from days 1 through 7 of gestation (200 μg/rat/day, subcutaneously).

results reinforce the plethora of reports supporting the recognition of these peptides by the ovary (as well as by the testes), and additionally attest to a direct stimulatory effect on the gonad. LHRH agonists can also stimulate oocyte meiotic maturation *in vitro*,[23] produce antisteroidal effects directly on the sexual accessory organs,[18] and produce antipregnancy effects unilaterally when instilled directly into a single, gravid uterine horn.[27]

The collective data support the contention that LHRH and agonists can produce antireproductive effects in the absence of the hypophysis. Such effects are manifested through direct actions on the placenta or gonads, adversely affecting secretion of both gonadotropin-like and steroid hormones. There is evidence that the placenta and gonads and perhaps the uterus possess receptors for LHRH, that a direct down-regulatory mechanism may be operating, and that the means by which this class of peptide produces its antifertility effects is more complex than previously appreciated. A summary of the antifertility effects produced by LHRH agonists is presented in Table 7-2.

SAFETY STUDIES: ANCILLARY PHARMACOLOGY AND PRELIMINARY TOXICOLOGIC EVALUATION

LHRH and several agonists have been subjected to a battery of tests to determine their toxicologic liability and their potential effects on nonreproductive organ systems in rats. The results of a preliminary acute intravenous toxicologic study in mature female rats are seen in Table 7-3; thyrotropin-releasing hormone (TRH), somatostatin (SRIH) and the LHRH antagonist, Wy 18,185, are included for comparison. The dosages required to produce any untoward effects are in considerable excess of those required for the primary agonist (ovulation induction) effect.

A more extensive evaluation in mature female rats (Table 7-4) relates the reproductive pharmacologic effects of Wy 40,972 to those observed in ancillary (nonreproductive) tests. Comparison of the utilitarian (agonist/contraceptive) properties of the analog with a number of nonreproductive parameters indicates that these laboratory results suggest large safety factors under proposed and realistic clinical dosing regimens.[18]

(*Text continues on p. 80*)

TABLE 7-2 Antifertility Effects of LHRH Agonists in the Female

1. Increased pituitary LH & FSH secretion; pituitary desensitization with chronic treatment
2. Termination of pregnancy
3. Inhibition of ovulation: premature ovulation
4. Down-regulation of gonadal receptors; decreased receptor numbers
5. Inhibition of gonadal steroidogenesis; interference with enzymes/steroidogenic pathways (inhibition of 17 hydroxylase and 17,20-desmolase; stimulation of reductases)
6. Luteolysis
7. Disruption of estrous cycle; ovarian and uterine regression; reduced fecundity
8. Shortening of menstrual cycle and early onset of menstruation
9. Retardation of puberty
10. Direct inhibitory effect at gonadal level in the absence of hypophysis
11. Direct inhibitory effect at level of uterus/placenta in absence of pituitary

TABLE 7-3. Acute Toxicology of LHRH Agonists in Mature Female Rats

COMPOUND	N	DOSE µg/kg, IV	% LETHALITY	REMARKS	AGONIST (OVULATION-INDUCING) DOSE MED 100* µg/kg, IV
LHRH	5	50,000	0	Slight ataxia	20.0
WY 18,185 [D-Phe^{2}-D-Ala^{6}]-LHRH	4	3,500	0	All rats appeared normal	—
WY 18,136 [D-Ala^{6}]-LHRH	5	400	0	All rats appeared normal	4.0
WY 18,481 [D-Ala^{6}-des-Gly^{10}-Pro^{9}-NHEt]-LHRH	3	50,000	33	Ataxia, respiratory depression, loss of muscle tone, loss of grasping and righting reflexes, cyanosis. Death within 9 minutes	4.0
WY 40,972 [D-Trp^{6}-N$^{\alpha}$-Me-Leu^{7}-des-Gly^{10}-Pro^{9}-NHEt]-LHRH	3	5,000	0	All rats appeared normal	0.40
WY 90,261 [D-Dihydro-Pgi^{6}-des-Gly^{10}-Pro^{9}-NHEt]-LHRH	5	3,130	0	Slight and transient hyperpnea; increased motor activity	0.40
TRH	4	4,000	0	Stimulation, increased motor activity, increased exploratory behavior, increased vibrissae movements, exaggerated startle response, fine tremors, straubtail, piloerection, stereotypy-sniffing, head bobbing, paw licking	—
SRIH	5	5,000	0	Transient ataxia, reduced grip strength, impaired righting reflex, constipation	—

*Minimum effective 100% dose.

TABLE 7-4. General Pharmacologic Profile of WY 40,972 in Rat

REPRODUCTIVE PHARMACOLOGY						PATHOLOGY/TOXICOLOGY		ANCILLARY PHARMACOLOGY					
									GI			CV	
Ovulation Induction		LH Release		Postcoital Conception				CNS general	Gastric acid secretion	Motility		Blood Pressure/Heart Rate	Renal
Total Ed_{100}* µg/kg						Total Dose (µg/kg)[I]		Total Dose µg/kg[I]					
IV	PO	IV	SC	IM	PO	IM	IP	IV	SC	SC	PO	IM	PO
0.40†	40†	0.004†	28‡	400† 280‡	5600–24000‡	Atrophy of uterus, cervix, vagina; reduced ovarian weight Acute IM—no death at 100,000	45,000§	1,000 (stimulation irritation)	>5,000 <10,000 General depression and death	No effect at 1,000	No effect at 10,000	Slight and transient increase at 1000	No diuretic effect at 100,000

*ED_{100} = Approximate minimum 100% effective dose.
†As single administration.
‡As multiple administration.
§Thirty-day study.
[I]Approximate minimum effective dose.

The inhibition and recovery of the reproductive organs observed following subacute, high-dose studies, and associated acceptable safety margins, have been demonstrated with LHRH[17,22] and several related agonists: Wy 19,728,[27] Wy 18,481[15,17] and Wy 40,598.[11] Furthermore, other investigations have indicated the lack of teratogenic liability for compounds of this type.[21] Collectively, the data in the literature have demonstrated that this class of compound is well tolerated and provides a wide and highly acceptable therapeutic ratio both in animal[24,39,41,42] and in chronic human investigations.[6,7,36]

CLINICAL OVERVIEW AND PROSPECTIVES

The clinical literature is burgeoning with the reproductive (more specifically, hormonal) effects of LHRH and agonists in humans. In addition to their diagnostic utility, these compounds have been used extensively in attempts to ameliorate a wide spectrum of gynecopathic (and andropathic) conditions. Though these agents clearly produce gonadotropin release, their effectiveness in inducing ovulation (and establishing subsequent pregnancy), and in normalizing such conditions as amenorrhea, hypogonadism, hypospermia, and retarded puberty has been marginal. In fact, it was originally hoped that the availability of the more potent agonists would overcome the difficulties encountered with the parent molecule—LHRH—based on the synthesis of a generation of LHRH congeners that would possess, in addition to greater potency, greater duration of activity and receptor binding properties, and increased resistance to enzymatic degradation. Though these chemical and pharmacologic criteria have been fulfilled, the traditional profertility application of these compounds still was encountered with frustration and disappointment. For years, it was presumed that these peptides would be useful fertility-inducing agents only if proper and carefully timed dosing regimens could be established. In spite of monumental manipulative efforts, associated with some small degree of success, the paradoxical antifertility effects remained primary in nature.

The abundance of animal data in both sexes has clearly defined the nature of this genre of compound and it appears to represent, specifically in the female, one of the major advances in contraceptive development since the advent of the steroidal oral contraceptives. Extension of the antireproductive animal data into the clinic has led to several investigations demonstrating the luteolytic and emmenagogic properties of LHRH and several of its agonistic analogs.[2,6,7,12,20,29,33,36,37] Quite appropriately and predictably, the doses chosen and routes of administration were based on the LH-releasing property of the compounds, the criterion that was previously employed in the attempted profertility approach. Short-term administration of several of these compounds during early and midsegments of the luteal phase results in rapid luteolysis, progesterone decline, and premature onset of menses.[12,20,29-32] This postovulatory luteal phase approach to potential contraception has been complemented with daily drug administration over either an entire menstrual cycle, or numerous cycles, leading to inhibition of ovulation, an inadequate luteal phase and subsequent menstruation.[2,6,7,20,36,37] Following withdrawal of treatment, normal ovulatory cycles return. Many of these studies have utilized

a nasal spray mode of drug delivery, thus providing a simple and practical means of administration.

The various safety studies reveal that no overt and serious liability would be anticipated for any troublesome effects on other organ systems at the efficacious doses employed in the animal studies and those already derived from a multitude of clinical experiences. In fact, the safety/efficacy margin appears to be outstanding, since it is foreseen that the clinical dose levels and their frequency of administration might be abbreviated, producing rapid contraceptive effects without any undue compromise to ensuing reproductive capabilities. It is further perceived, in view of these anticipated nonprotracted regimens (contrary to the case of the oral contraceptives), that no excessive stress would be placed on those organs associated with the reception, pharmacokinetics, metabolism, and disposition of these compounds.

Extensive trials are now required to establish contraceptive efficacy during unprotected intercourse employing various regimens. Of note is a recent report that D-Trp^{6}-des-Gly^{10}-Pro^{9}-NHEt-LHRH (up to 500 μg/woman/day, subcutaneously), given on two consecutive days, did not terminate pregnancy (4–6 weeks of gestation).[11] Although larger doses or longer dosing regimens of this or other agonists may be required in women to achieve the contragestational effects observed in a variety of animal models, the presence of hCG (whether exogenous to prolong the luteal phase of the menstrual cycle, or endogenous during pregnancy) may modify (*i.e.*, protect against) the luteolytic effects of the agonists.[11] Characterization of potential hCG/agonist interactions as well as elucidation of agonist luteolytic processes prior to implantation (during approximately days 1–12 postovulation) in inseminated women are major topics of ongoing research efforts.

A review of the clinical applications of LHRH and analog is presented in Table 7-5.

SUMMARY

The past ten years have witnessed dramatic advances in the development of the chemistry and pharmacology of LHRH and its peptidic analogs. Ap-

TABLE 7-5. Clinical Uses of LHRH and Analogs

1. Diagnostic
   a. Evaluation of integrity of hypothalamic–hypophysial–gonadal axis in reproductive hypofunction with agonists
   b. Pharmacologic identification: potency and activity identification in normal subjects with agonists and antagonists
2. Therapeutic (under investigation)
   Stimulation of reproductive function with agonists in clinical conditions such as amenorrhea, anovulation, hypogonadism, retarded puberty, nondescended testes; use of agonists in precocious puberty; use of agonists in gonadal steroid-dependent tumors
3. Contraceptive (under investigation)
   a. Inhibition of ovulation with LHRH antagonists
   b. Inhibition of ovulation; luteolysis, pregnancy termination with LHRH agonists
   c. Inhibition of spermatogenesis with LHRH agonists and antagonists

proximately 1000 compounds have been synthesized and evaluated for potential use as contraceptive agents, based on either their ability to inhibit gonadotropin secretion and consequently, ovulation, or to produce paradoxical antifertility effects in pregnant and nonpregnant animal models. The former class encompasses the LHRH antagonists, the latter, the LHRH ("super") agonists. The most promising developments in terms of scope of animal and clinical investigations have occurred with the agonists, whose basic LHRH-like properties of gonadotropin release and ovulation induction have resulted in dramatic alterations in the pituitary–gonadal axis, leading to contraceptive effects in both females and males. On the basis of their ability to induce luteolysis and terminate the secretion of progesterone, several LHRH agonists are presently undergoing extensive clinical evaluation for use as a potential luteal phase-inhibiting/menses-inducing approach to contraception.

The mechanisms by which the LHRH agonists produce their antireproductive effects include hypersecretion of LH, dysphasic FSH and distorted prolactin secretion, decrease in gonadal LH, FSH and prolactin receptors (down-regulation), inhibition of steroidogenesis, and eventual disruption of the reproductive continuum. Recent evidence additionally points to an extrapituitary site of action of LHRH and its agonists, in which these agents can modify gonadal and placental hormone secretion in the absence of the pituitary gland.

The spectrum of reproductive effects and the toxicologic, pathologic, and ancillary pharmacologic studies (*e.g.*, CNS, gastrointestinal, cardiovascular, pulmonary, renal), involving varied dosing regimens and routes of administration, have demonstrated the safety of selected LHRH agonists. Any undesirable side-effects thus far noted in any of the animal models employed for safety evaluation have occurred at doses far exceeding those necessary for practical and utilitarian purposes. As such, these compounds present a therapeutic margin (contraceptive dose vs. dose producing untoward side-effects) of considerable magnitude in laboratory studies. The long clinical record of safe, if relatively confined, use as profertility agents, and the rapidly increasing body of evidence for promising contraceptive utility, unencumbered by side effects, is most encouraging.

The collective animal and clinical data on the LHRH agonists have established their antifertility activity and, coupled with their demonstrated potency, reproducible efficacy, lack of significant secondary (nonreproductive) pharmacologic effects and no apparent compromise to safety under clinically realistic dosing protocols, clearly support their candidacy as potential contraceptives specifically in the female. Recent clinical reports on the nasal application of selected agonists, under varied dosing schedules, and their ability to terminate acutely the luteal phase of the menstrual cycle, reinforce this particular peptidic approach to the regulation of fertility.

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8

The Effects of LHRH Agonistic Analogs During the Luteal Phase of the Rhesus Monkey

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The discovery of gonadotropin-releasing hormones (GnRH) has been one of the major advances in the area of reproductive biology in this century. GnRH has been extremely useful during the last decade in understanding the neuroendocrine mechanisms of gonadotropin secretion by the pituitary, and has been an important tool in the diagnosis of different endocrine pathologies when used in dynamic tests.

Advancements in biochemistry have permitted the complete characterization and identification of the decapeptide LHRH as well as other hypothalamic hypophysiotropic hormones. Recently, several laboratories have developed agonistic and antagonistic analogs with different properties to the native LHRH as a result of changes in the potency and in the aminoacidic sequence.[12,27]

AGONISTIC ANALOGS

LHRH analogs of increased potency, relative to the native LHRH ("super" analogs), have been found to possess paradoxical antireproductive properties.[11,26] The antifertility effects of LHRH and agonists have been studied in the last year by several laboratories with interesting results that may open new approaches for fertility regulation.

Administration of LHRH or "super" analogs produces retardation of the onset of puberty and inhibition of weights of the adenohypophysis, ovaries, and uteri in the immature female rat.[20,30] Corbin and Beattie have reported that the administration of peptide analogs of LHRH produces an inhibition of the proestrus gonadotropin surge, ovulation, and pregnancy in the rat.[11]

Supporting the possible direct effects at the ovary, Hsueh and Erickson showed recently that LHRH and its agonistic analogs inhibited FSH-induced increases of estrogen and progesterone secretion *in vitro* by rat ovarian granulosa cells and *in vivo* when administered to hypophysectomized rats.[17]

These paradoxical pharmacologic properties of LHRH and agonistic de-

rivatives have also been observed in pregnancy. Administration of LHRH agonists results in termination of pregnancy in rats and rabbits prior to and after implantation.[1,16,19] Single injections resulted in complete resorption of implantation sites and retardation of nidation development. Restoration of fertility occurs in the subsequent cycle after analog administration has been discontinued. The mechanisms by which analogs induce termination of pregnancy are still unclear, but a possible luteolytic effect was suggested. These preliminary results stimulated clinical research trials to evaluate the possible future application of LHRH analogs in contraception.

Nillius and co-workers have shown that the administration of LHRH agonists inhibits the midcycle surge of estrogens and LH and subsequent ovulation in women.[21] Casper and Yen demonstrated that the subcutaneous injection of a long-acting analog of LHRH during the luteal phase in normally cycling women induced a short luteal phase and premature onset of menses.[9] The premature decline in progesterone and estradiol concentration during the treatment cycles suggests that luteolysis was induced by the LHRH analog.

This luteolytic effect of LHRH agonists may have obvious contraceptive applications. It is important, then, to clarify the mechanism by which luteolysis is induced by these drugs. The experiments described below were planned to establish if [D-Trp^6]-LHRH has a luteolytic effect in the rhesus monkey and to determine its mechanism of action.

THE RHESUS MONKEY AS AN EXPERIMENTAL MODEL

We have gained great experience in the timing of ovulation and physiology of the corpus luteum in the nonhuman primate.[3,8,25] The combined use of daily serum total estrogen values and laparoscopy allows us to determine the time of ovulation with an accuracy of ± 12 hours without interfering with the normal progression of the cycle. The use of microsurgical techniques has made it possible to obtain intact corpora lutea for *in vitro* studies without seriously damaging the animal's ovaries and with minimal manipulation of the tissue.

The results presented in this chapter have been obtained from experiments using the rhesus monkey (*Macaca mulatta*) as a model. This species of primate presents several advantages as a model for research in biology of reproduction. The monkey's menstrual cycle, like that of the human, is approximately one lunar month in duration. The relative lengths of the follicular and luteal phases are also similar to those in a woman's cycle. Endocrinologically, the quality of the hormonal relations between hypothalamus, hypophysis, and gonads is well established in the literature and presents no significant difference from what is known in women. For all these reasons, the rhesus monkey, perhaps being closest to the human in biology, provides a good model for studying the effects of LHRH analogs on the reproductive cycle.

EFFECT OF [D-Trp^6]-LHRH ADMINISTRATION DURING THE LUTEAL PHASE

The first group of experiments performed were planned to determine the effect of a single dose (10 mg) of [D-Trp^6]-LHRH administered on different

days of the luteal phase of the rhesus monkey.[4] Four animals received the drug on day 3 postovulation, five animals on day 5, and four on day 7. Daily serum estradiol, LH, and progesterone were determined in each animal in the study. Initiation of menses was determined by daily vaginal smears.

Figures 8-1, 8-2, and 8-3 present the results of [D-Trp^6]-LHRH administration on days 3, 5, and 7 after ovulation. The average luteal phase in our monkey colony lasted 15 ± 0.6 days (X ± S.E.M.). A significant shortening of the luteal phase was obtained in all the monkeys that received the drug on postovulatory day 3. Four of the five animals injected on day 5 had a shorter luteal phase. Serum progesterone values decreased significantly 48 hours after the administration of the drug in both groups of animals. No apparent shortening of the luteal phase was obtained in the monkeys injected on day 7 postovulation.

hCG AND THE LUTEOLYTIC EFFECT OF [D-Trp^6]-LHRH

In an experiment parallel to the ones described, the effect of hCG administration during the luteal phase of animals injected with [D-Trp^6]-LHRH was studied. The schedule used to administer hCG intramuscularly in increasing doses (30 IU on day 6, 60 IU on day 7, 90 IU on day 8, 180 IU on day 9, and 360 IU on day 10) mimics the secretion of mCG by the trophoblast during early pregnancy.[5]

Three animals received 10 mg of the analog on day 5 postovulation followed by daily IM injections of increasing doses of hCG from days 6 to 10. A second group of four monkeys received 10 mg of [D-Trp^6]-LHRH and hCG daily simultaneously from days 6 through 10 postovulation.

Administration of hCG prevented the [D-Trp^6]-LHRH shortening of the luteal phase and did not change the length of the luteal phase when given simultaneously. The serum progesterone and estradiol increase elicited by the administration of hCG were similar to those reported for untreated animals (Figs. 8-4 and 8-5).

Table 8-1 shows the results of both groups of experiments. Only the monkeys treated on day 3 or 5 postovulation showed a significant decrease in luteal phase length.

IN VITRO EFFECTS OF [D-Trp^6]-LHRH ON PROGESTERONE PRODUCTION BY CORPORA LUTEA

The preceding experiment confirmed the luteolytic effect of [D-Trp^6]-LHRH administered during the luteal phase of the rhesus monkey. An *in vitro* experiment was designed to determine whether [D-Trp^6]-LHRH had any direct effect on progesterone production by rhesus monkey dispersed corpora lutea cells.[5]

Corpora lutea were obtained from animals laparotomized between days 6 to 8 after ovulation. The tissue was then transported to the laboratory in cold Ham's F10-Hepes medium. Techniques of dispersion and incubation of corpus luteum cells were according to those reported by Stouffer and colleagues.[28] The corpora lutea cells were incubated in separate tubes with

(*Text continues on p. 91*)

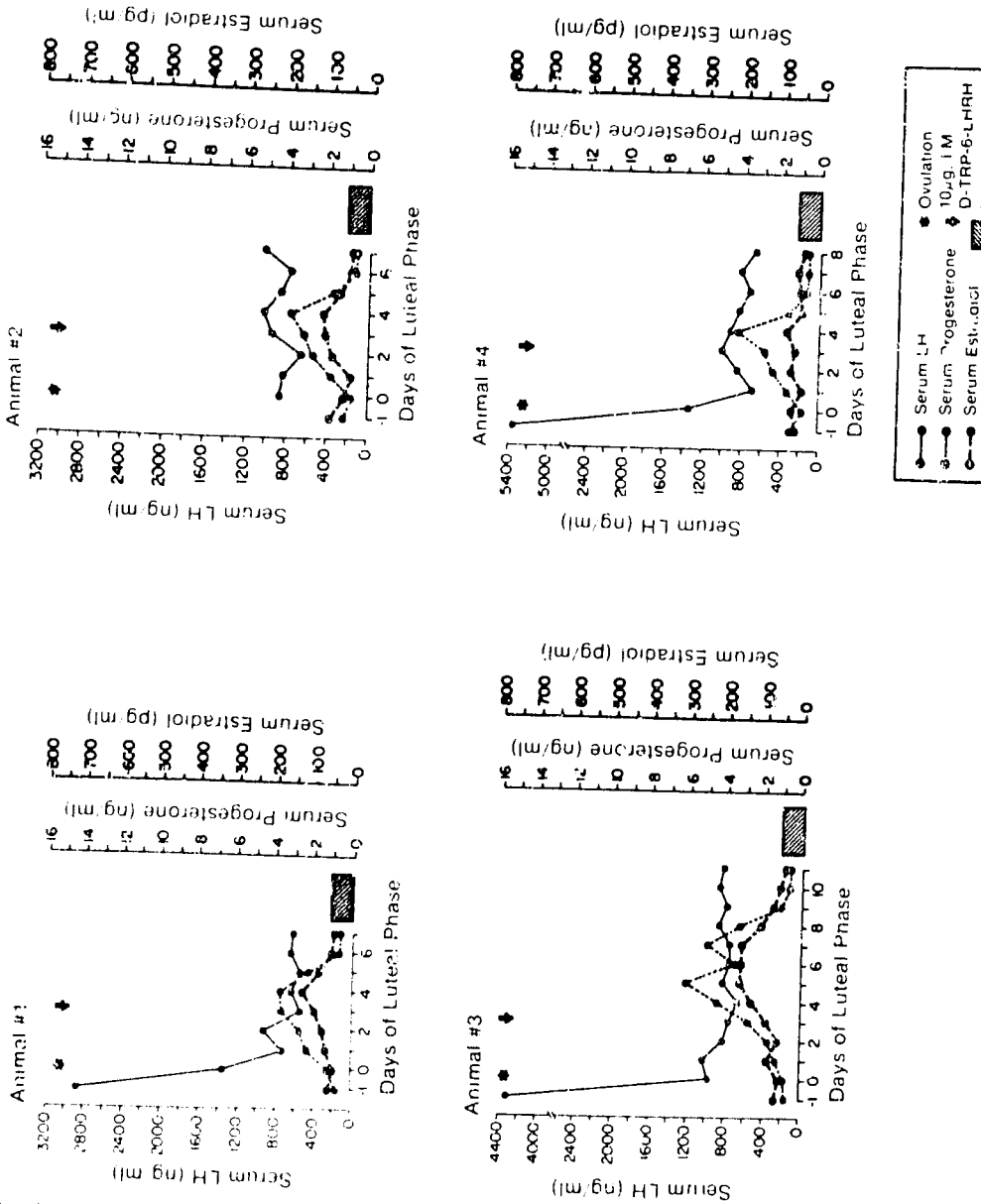


Fig. 8-1. Serum concentrations of LH, progesterone, and estradiol during luteal phase of rhesus monkeys that received 10 μg [D-Trp^6]-LHRH IM on day 3 postovulation. (Figs. 8-1 through 8-5 and 8-7 from Asch RH, Siler-Khodr TM, Smith CG et al: Luteolytic effect of D-Trp^6-luteinizing hormone-releasing hormone in the rhesus monkey (*Macaca mulatta*) J Clin Endocrinol Metab 52:565, 1981)

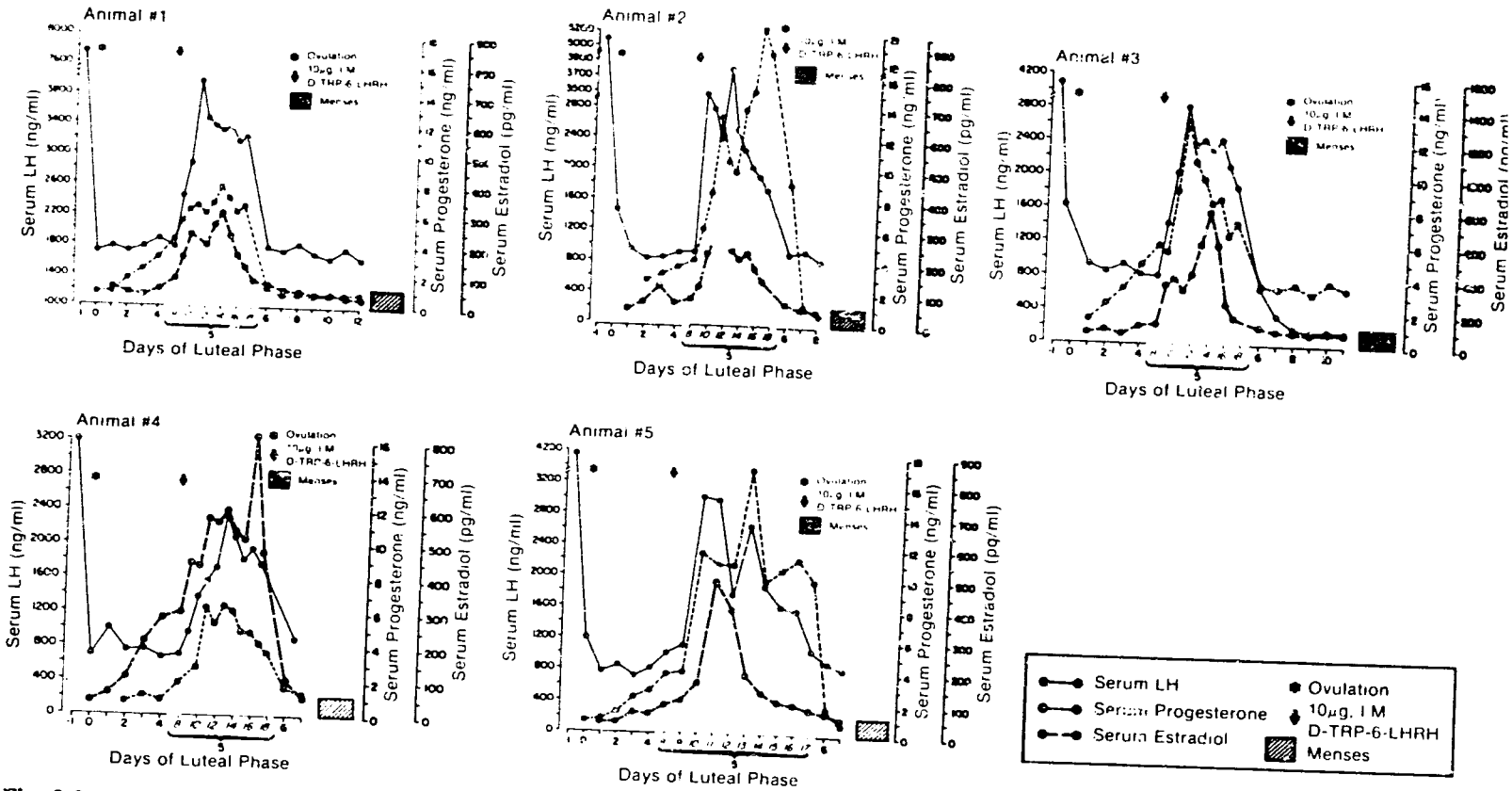


Fig. 8-2. Serum concentrations of LH, progesterone, and estradiol during luteal phase of rhesus monkeys that received 10 μg [D-Trp[6]]-LHRH IM on day 5 postovulation.

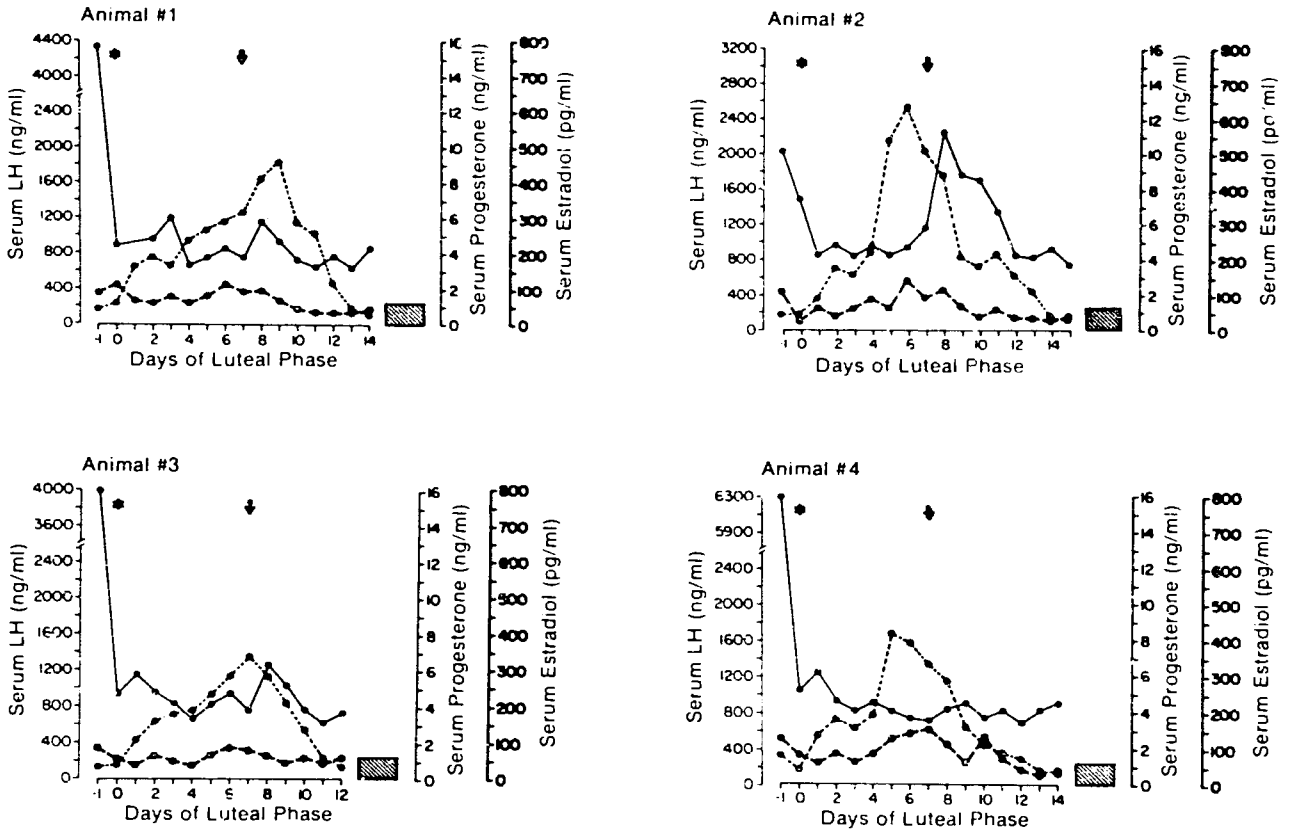


Fig. 8-3. Serum concentrations of LH, progesterone, and estradiol during luteal phase of rhesus monkeys that received 10 μg [D-Trp^6]-LHRH IM on day 7 postovulation.

TABLE 8-1. Effects of [D-Trp^6]-LHRH Administration on the Luteal Phase Length of the Rhesus Monkey

DAY OF ADMINISTRATION (POSTOVULATORY)			LUTEAL PHASE LENGTH (DAYS)	
[D-Trp^6]-LHRH	hCG	n	Individual Animals	X ± S.E.M.
3		4	7-7-11-8	8.2 ± 0.9*
5		5	12-8-11-7-7	9.0 ± 1.0*
7		4	14-15-12-14	13.7 ± 0.6
5	6–10	3	14-14-15	14.3 ± 0.3
6–10	6–10	4	16-15-19-15	16.0 ± 1.0
	6–10	25		15.2 ± 0.8
Untreated Colony		50		15.1 ± 0.6

different concentrations of [D-Trp^6]-LHRH ranging from 1 to 100 ng/ml. After basal levels of progesterone production were measured, hCG (50 ng/ml) was added to each tube to determine if steroid production could be stimulated. Table 8-2 presents the results of this experiment. No significant differences in progesterone production occurred between the control cells and the cells treated with the analog. However, the increase in progesterone production in response to the addition of hCG to the media was virtually the same for each culture tube and was not inhibited by the presence of [D-Trp^6]-LHRH. These results, although obtained *in vitro*, cast doubts on the possibility of a direct action of [D-Trp^6]-LHRH on the corpus luteum cells as an explanation for its luteolytic effect.

LUTEOLYTIC EFFECT OF [D-Trp^6]-LHRH IN HYPOPHYSECTOMIZED RHESUS MONKEYS

The lack of *in vitro* direct effect of the LHRH analog on the corpora luteal cells suggests a different mechanism in action to induce luteolysis. A second possibility is that the luteolytic effect of [D-Trp^6]-LHRH is mediated by the alteration induced by the drug in pituitary gonadotropin secretion. Ferland and co-workers have shown that LHRH is capable of inducing a decrease in ovarian LH receptors in the rat by increasing the concentrations of serum LH.[14]

TABLE 8-2. *In Vitro* Effects of [D-Trp^6]-LHRH on Progesterone Production by Corpora Lutea

		[D-Trp^6]-LHRH (ng/ml)			
	CONTROL	1	25		x ± S.E.M.
Baseline	128	120	150	121	114	126 ± 8
hCG stimulated	215	168	275	255	200	224 ± 24

1. [D-Trp^6]-LHRH does not affect CL steroidogenesis *in vitro*;
2. Its luteolytic site of action is probably not direct at the gonadal level, but probably by hypersecretion of LH resulting in a down regulation of ovarian LH receptors.

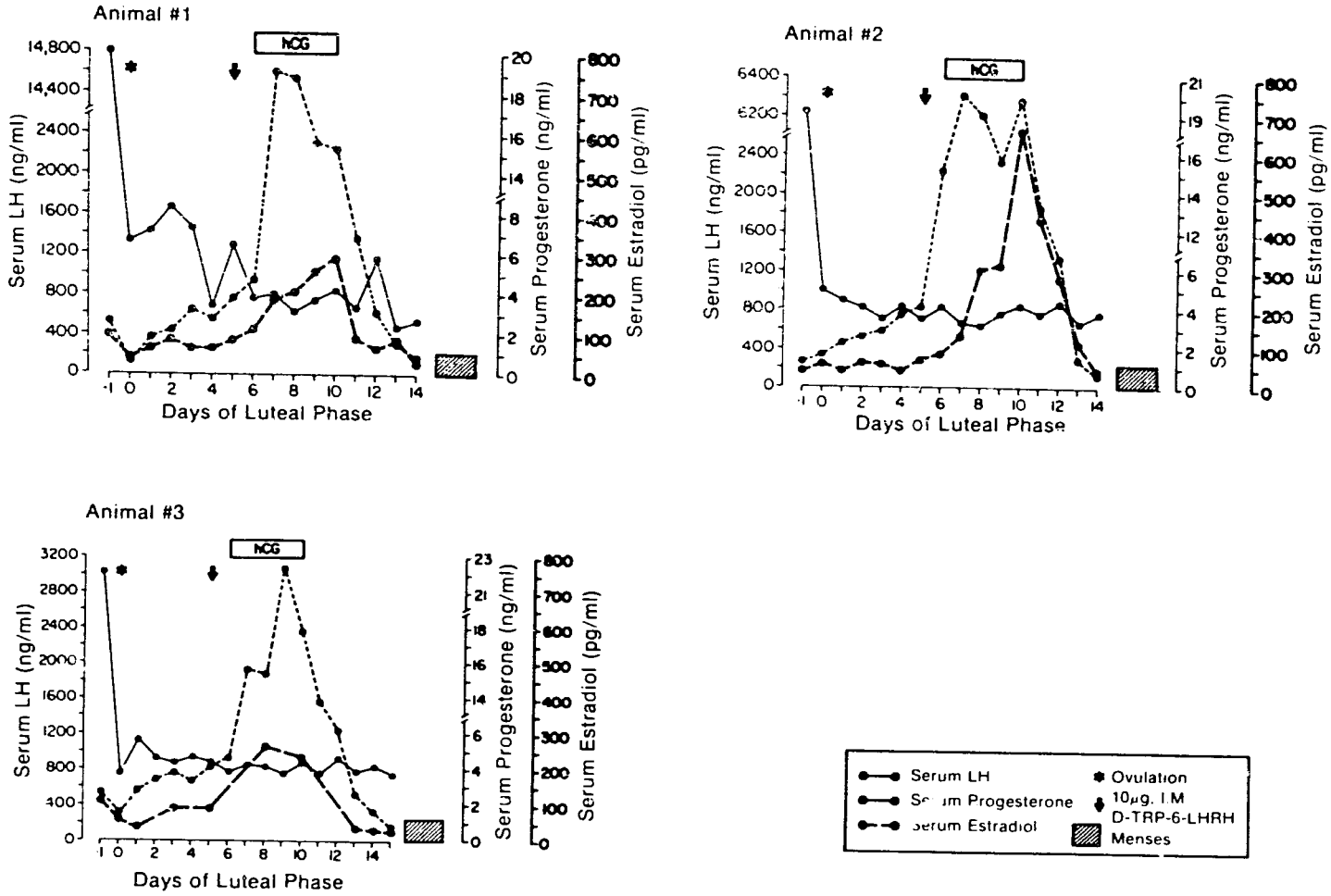


Fig. 8-4. Serum concentrations of LH, progesterone, and estradiol during luteal phase of rhesus monkeys that received 10 μg [D-Trp[6]]-LHRH IM on day 5 postovulation and increased doses of hCG from days 6 through 10 postovulation (30 IU on day 6; 60 IU on day 7; 90 IU on day 8; 180 IU on day 9; 360 IU on day 10).

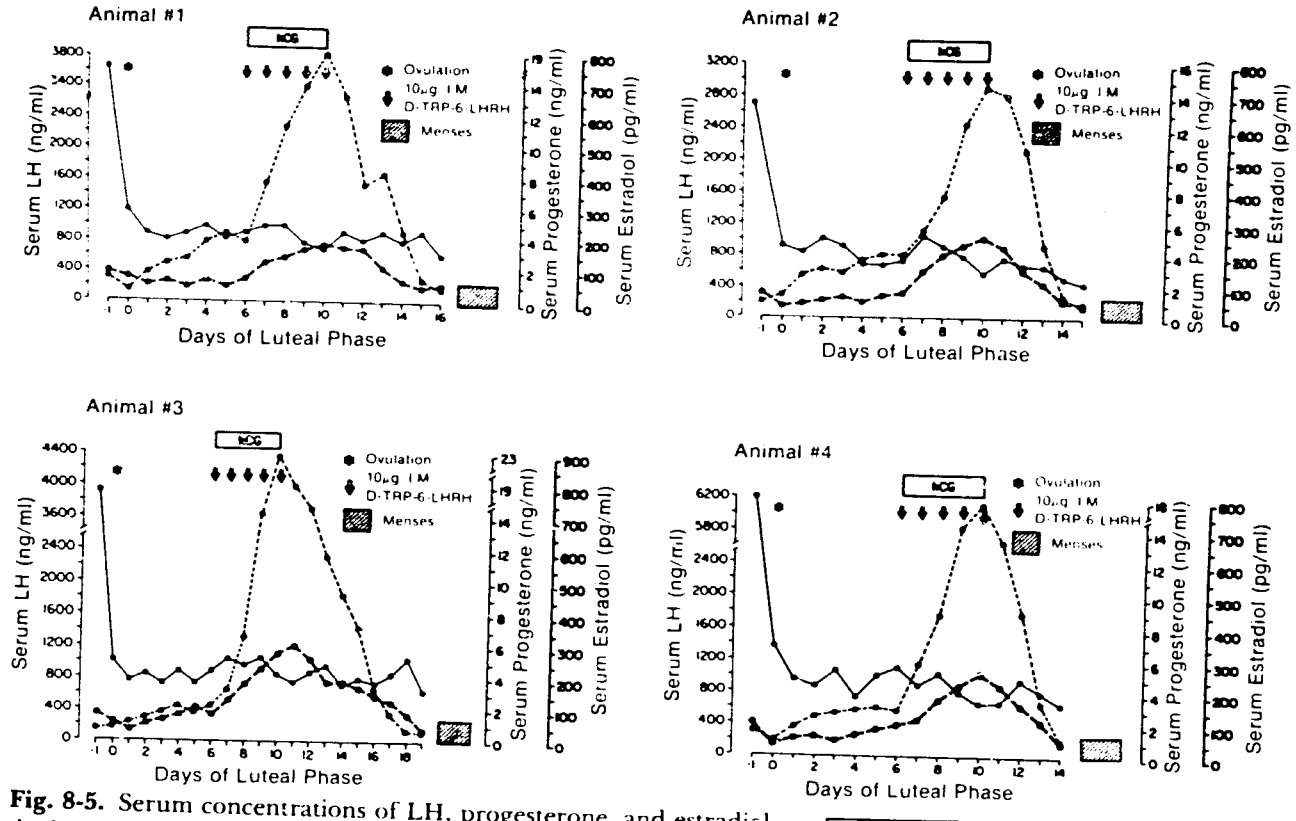


Fig. 8-5. Serum concentrations of LH, progesterone, and estradiol during luteal phase of rhesus monkeys that received daily IM injections of 10 μg [D-Trp^6]-LHRH, and increasing doses of hCG from days 6 through 10 postovulation (30 IU on day 6; 60 IU on day 7; 90 IU on day 8; 180 IU on day 9; 360 IU on day 10).

In order to evaluate the possible role of pituitary gonadotropin in the luteolytic effect of [D-Trp^6]-LHRH, we administered this analog to totally hypophysectomized rhesus monkeys in which ovulation had been induced with exogenous gonadotropins.[2] The study was performed in three animals that were hypophysectomized at least 9 months before the experiments. At the time of their admission into the study, the animals were also receiving a weekly maintenance dose of methylprednisolone acetate 8 mg/kg intramuscularly. All animals were chronically amenorrheic and exhibited an absence of circulating levels of LH (basal and after intravenous administration of 150 μg LHRH) by the mouse interstitial cell testosterone assay for LH–hCG determination.[29] Chronic lack of ovarian stimulation was further revealed by repeated laparoscopic examination. The completeness of hypophysectomy was confirmed histologically in retrospect in a single animal that subsequently died of acute pancreatitis. No pituitary remnants were present in the sella turcica.

Ovulation was induced in two separate treatment cycles in each animal with human menopausal gonadotropins (hMG) and hCG according to the regimen described by DiZerega and Hodgen in rhesus monkeys.[13] Briefly, this technique consists of the daily intramuscular administration of 37 IU of FSH and LH (hMG) until the serum total estrogens reaches a level of 350 pg/ml, at which time 1,000 IU of hCG are given intramuscularly. Under these conditions, ovulation occurred in both cycles of treatment in each animal 24 to 48 hours after the administration of hCG.

Of the total six ovulatory induced cycles, single ovulations were observed in four cases. One animal presented two ovulations, one in each ovary, and in the sixth animal, four corpora lutea were formed, three in the right ovary and one in the left. During the first induced ovulatory cycle of each animal, 10 μg [D-Trp^6]-LHRH was administered intramuscularly in (1 ml) saline solution on days 3 and 5 postovulation. (This dose, as described, is luteolytic in the rhesus monkey.)

On the second induced ovulatory cycle, 1.0 mg of [D-Trp^6]-LHRH was administered by infusion on day 5 postovulation. Utilizing microsurgical techniques, the ascendant branch of the uterine artery was dissected free of its supporting vascular sheath. Previous studies have shown that in the nonpregnant rhesus monkey the ascendant branch of the uterine artery provides more than 90% of the ovarian circulation.[31] A 30-gauge needle fitted to Silastic tubing was placed in the lumen. The selection of the correct artery was confirmed by the initial administration of sufficient methylene-blue dye in saline to stain the corpus luteum. The infusion was performed with a Harvard infusion pump at a rate of approximately 0.17 ml/minute for 1 hour (total volume = 10 ml). Blood flow of the ascendant branch of the uterine artery was not interrupted by the infusion. Blood (2 ml) was collected from the main ovarian vein by direct puncture with a 27-gauge needle and syringe under visual observation through an operating microscope at −30, 0, 10, 20, 30, 40, 50, 60, and 90 minutes relative to initiation of the infusion. Systemic blood was drawn daily from the saphenous or femoral vein. Serum obtained from peripheral and ovarian blood was used for progesterone determination by RIA.

The administration of [D-Trp^6]-LHRH according to a regimen that had

been shown to produce luteolysis in intact, normally cycling rhesus monkeys[4] did not affect the luteal phase of induced ovulatory cycles in chronically hypophysectomized rhesus monkeys (Fig. 8-6). Serum progesterone concentrations and lengths of the luteal phases were not different from those reported previously in untreated animals from our colony (Figs. 8-3, 8-4; and Table 8-1). Circulating progesterone levels reached a peak between days 6

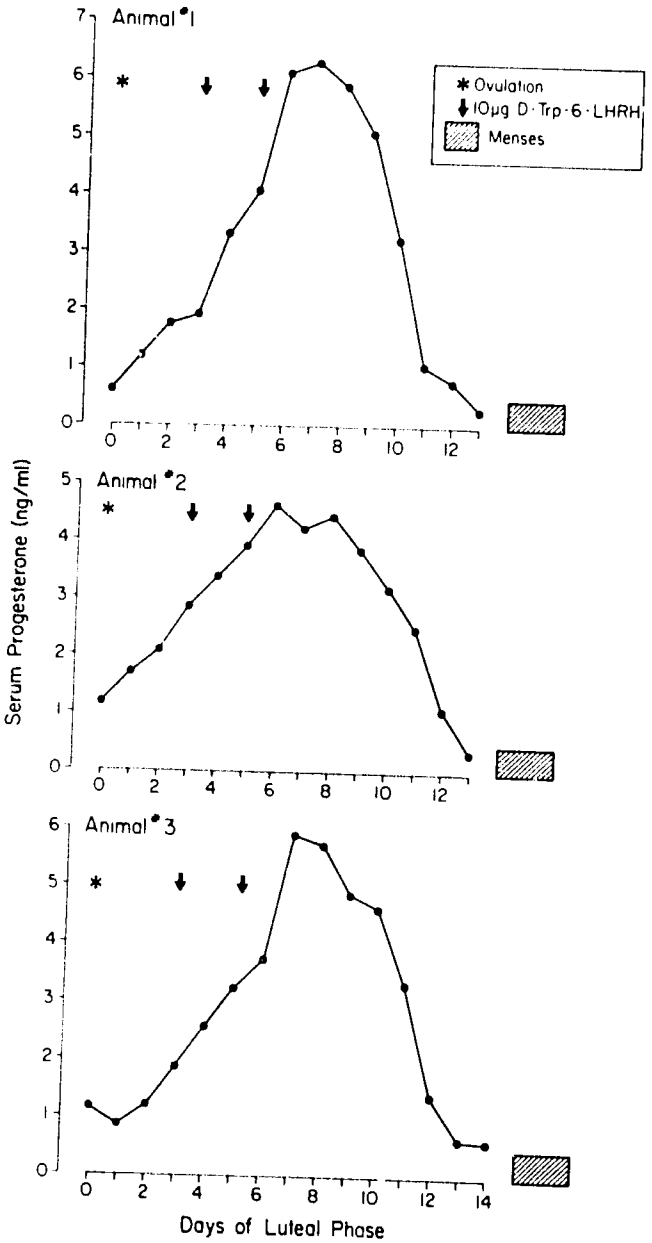


Fig. 8-6. Serum progesterone concentrations and luteal phase lengths of induced ovulatory cycles in hypophysectomized rhesus monkeys treated with 10 μg [D-Trp^6]-LHRH on days 3 and 5 postovulation. (Asch RH, Eddy CA, Schally AV: Lack of luteolytic effect of D-Trp-LH-RH in hypophysectomized rhesus monkeys (*Macaca mulatta*). Biol Reprod [in press])

and 7 postovulation and ranged from 4.6 to 6.3 ng/ml. Administration of 10 μg of [D-Trp^6]-LHRH on days 3 and 5 postovulation did not suppress the normal increase of serum progesterone concentrations that occur during the first week postovulatory. The length of the luteal phases in the three treated animals was 13, 13, and 14 days, respectively. These results were not different from luteal phase duration of untreated animals in our colony, but were statistically longer than that observed in intact animals treated with [D-Trp^6]-LHRH on days 3 and 5 postovulation[4] ($P < 0.01$ and < 0.05, respectively, determined by Student's *t* test).

The ovarian infusions of 1 mg [D-Trp^6]-LHRH likewise did not affect the luteal phase of three rhesus monkeys; peripheral serum progesterone concentrations and luteal phase lengths were normal (12, 13, and 14 days, respectively). In addition, the ovarian infusions of this large amount of the LHRH analog did not produce any marked decrease of progesterone concentrations in the ovarian vein blood during the period of this study (Fig. 8-7).

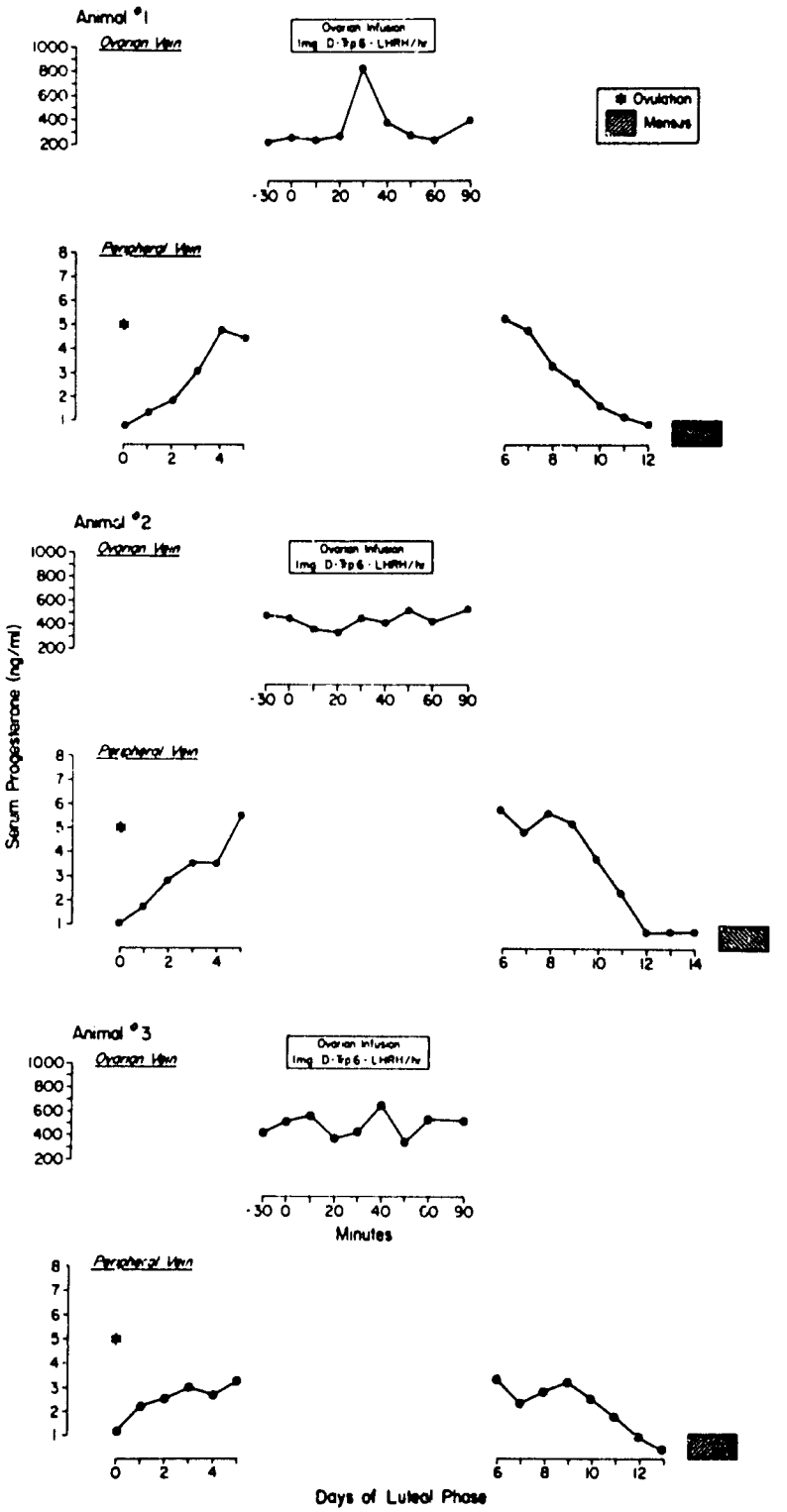
Results of the present studies complement our *in vitro* experiments. The lack of effect of [D-Trp^6]-LHRH in doses shown to be luteolytic in intact animals during the luteal phase of hypophysectomized rhesus monkeys induced to ovulate with hMG/hCG casts doubts upon the theory of a possible gonadal site of action of the LHRH agonists in the induction of luteolysis in primates. It is apparent from our studies that the presence of the pituitary is essential for the luteolytic effect of the LHRH agonists in primates, suggesting that the "desensitization theory" may be an important factor in the inhibition of luteal function.

ABSENCE OF LHRH BINDING SITES IN CORPORA LUTEA

As described, membrane-specific receptors for LHRH have been documented in extrapituitary tissue, including ovary in several mammalian species.[10,15,18,22] Some authors have postulated that LHRH and potent agonists induce luteolysis in small laboratory animals through their effect at the level of such gonadal receptors. However, no information exists about the presence of such receptor sites in primate gonads.

This last experiment was designed to study the presence of LHRH receptors on rhesus monkey corpora lutea.[7] Crude plasma membranes were prepared from adult male rat pituitary and rhesus monkey corpora lutea, lung, kidney, liver, and small bowel. Luteectomy was performed between days 5 and 8 after ovulation using microsurgical techniques. The tissue obtained was used immediately for the study.

Fig. 8-7. Peripheral and ovarian vein progesterone concentrations of induced ovulatory cycles in hypophysectomized rhesus monkeys that received an ovarian infusion of 1 mg [D-Trp^6]-LHRH on day 5 postovulation.


Animal #1
Ovarian Vein
Ovarian Infusion
1mg D-Trp6-LHRH/hr
Ovulation
Menses
Peripheral Vein
Animal #2
Ovarian Vein
Ovarian Infusion
1mg D-Trp6-LHRH/hr
Peripheral Vein
Animal #3
Ovarian Vein
Ovarian Infusion
1mg D-Trp6-LHRH/hr
Minutes
Peripheral Vein
Serum Progesterone (ng/ml)
Days of Luteal Phase

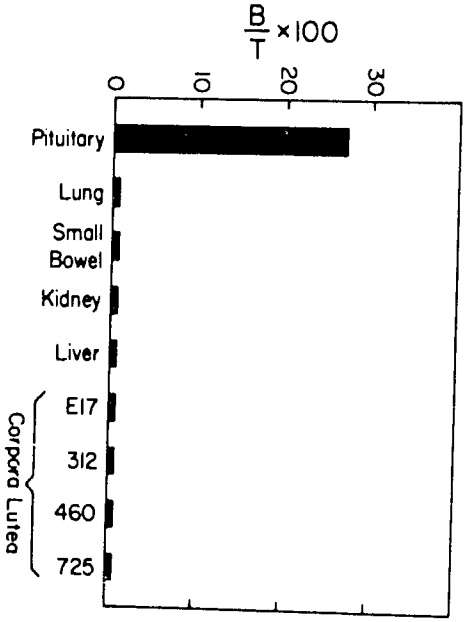


Fig. 8-8. Specific binding of ^{125}I D-Ala^{6}-analog to rat pituitary and corpora lutea, lung, kidney, liver, and small bowel from regularly cycling rhesus monkeys. (Asch RH, Van Sickle M, Rettori V et al: Absence of LH-RH binding sites in corpora lutea from rhesus monkeys (*Macaca mulatta*). J Clin Endocrinol Metab 53:215, 1981)

The D-Ala^{6} analog was radioiodinated according to the lactoperoxidase method initially described by Miyachi and co-workers[23] and modified by Marshall and Odell.[21] Bio Rad Enzymobeads were the source of lactoperoxidase and glucose in the iodination reaction. Labeled and unlabeled analogs were separated using ion exchange chromatography on carboxymethylcellulose (Sigma, fine mesh). Labeled analog was stored at 4°C, *p*H 7.5, in 0.05 M ammonium acetate. The specific activity of the ^{125}I-labeled LHRH analog was measured by self-displacement in the radioreceptor assay (800–1000 μCi/μg).

Luteectomy induced early onset of menses in all the animals. Ovarian vein concentrations of progesterone dropped as early as 10 minutes after luteectomy. Peripheral concentrations of serum progesterone were markedly diminished on the day following surgery.

Specific binding of (^{125}I) D-Ala^{6} analog to plasma membranes of various tissues was tested. The protein concentration per tube used in binding studies was as follows: 150 μg pituitary, 90.4 μg lung, 147.2 μg kidney, 165.6 μg liver, and 97.2 μg small bowel. For rhesus monkey corpora lutea: E 17, 122.8 μg; 321, 128.8 μg; 460, 132 μg; and 725, 138.5 μg.

Nonspecific binding was determined in the presence of 1000-fold excess of unlabeled analog. Rat pituitary plasma membranes specifically bound 27% of the radioiodinated analog (Fig. 8-8). However, no specific binding of the LHRH analog for lung, kidney, liver, small bowel, or corpora lutea from rhesus monkeys was observed.

SUMMARY AND CONCLUSIONS

1. Administration of single injections of [D-Trp^{6}]-LHRH during the early luteal phase (days 3–5 postovulatory) of the rhesus monkey consistently

induces early onset of menses and decreases in serum progesterone concentrations.

2. The luteolytic effect of [D-Trp^6]-LHRH is prevented by simultaneous or successive administration of chorionic gonadotropin in doses similar to those of the peri-implantation period.
3. [D-Trp^6]-LHRH may have potential as a contraceptive if used early in the luteal phase. The rescue of corpus luteum activity when hCG is administered simultaneously or following [D-Trp^6]-LHRH suggests that its effective use during midluteal phases of pregnant cycles is unlikely.
4. [D-Trp^6]-LHRH does not affect progesterone production by dispersed cells from rhesus monkey corpus luteum (*in vitro*).
5. Local infusions of [D-Trp^6]-LHRH into the ovarian circulation do not affect the progesterone concentrations in ovarian vein blood of hypophysectomized rhesus monkeys.
6. No specific LHRH receptors were found in crude plasma membrane preparation of rhesus monkey corpora lutea.

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9

Antifertility Effect of an LHRH Agonist (WY 40972) in the Baboon

V. DANIEL CASTRACANE

JOSEPH W. GOLDZIEHER

The synthesis of LHRH analogs has stimulated great expectations for a new mode of contraceptive regulation.[5] Many studies in rats, nonhuman primates, and humans have indicated a luteolytic action of these potent synthetic agonists. Recently, however, exogenous and endogenous chorionic gonadotropin have been reported to prevent the luteolytic action in both monkeys and women[1,2,13] and the contraceptive potential of these agonists has been proportionately diminished. We have studied the Wyeth LHRH agonist Wy 40972 for its luteolytic action during the luteal phase in nonpregnant baboons and for its abortifacient potential in pregnant baboons at the time of first missed menses. This agonist is a potent luteolytic agent in the rat, and this is the first study with this agonist in the primate.[5] We employed various dosage regimens and routes of administration in order to examine both strength and duration requirements.

MATERIALS AND METHODS

All animals were normally cycling, mature female baboons (*Papio cynocephalus*) housed in individual cages in proximity to and in visual contact with a breeder male. The menstrual cycle of each female had been followed for at least one year by daily observations of the changes in perineal sex skin. Our previous studies had determined that ovulation occurs 1 to 3 days prior to sex skin deturgescence, and therefore the first day of deturgescence has been considered as day 3 postovulation.

Normally cycling female baboons received 1 mg WY 40972 daily for 3 consecutive days in the early luteal phase (days 3, 4, and 5 postovulation) or in the midluteal phase (days 8, 9, and 10 postovulation). Baboons treated in

These studies were supported by Contract PARFR-223, The Program for Applied Research on Fertility Regulation. The agonist WY 40972 was generously supplied by Dr. Alan Corbin of Wyeth Laboratories, Inc. The technical assistance of Cornelio Celaya, Phillip Czar, and Oscar Clayton in animal studies and Daniel Martinez, Edna Wright, and James Kyle in the radioimmunoassay laboratory is greatly appreciated.

the early luteal phase were bled daily from days 3 through 10 and every other day thereafter until day 20 postovulation. Baboons treated in the midluteal phase were bled on days 4, 6, and 8 through 12, and every other day thereafter until day 20 postovulation. Vehicle-treated control animals were bled on a similar schedule. All venipunctures were from the femoral vein under light ketamine anesthesia. Animals were observed daily for changes in perineal sex skin or menses. Plasma was analyzed by radioimmunoassay (RIA) for estradiol and progesterone, as previously described.[4] Estradiol antiserum was generously supplied by Drs. D. C. Collins and K. Wright (Emory University) and progesterone antiserum by Dr. P. N. Rao of our institution.

Female baboons were mated during the periovulatory period on the basis of previous individual sex skin cycle records and of daily observation of subtle changes in sex skin during the mated cycle. Males were removed on the first day of sex skin deturgescence, which was day 3 postovulation. Pregnancy was confirmed by the measurement of plasma estradiol and progesterone on days 16, 18, and 20 postovulation as reported from this laboratory.[12] Confirmed pregnant baboons were treated with WY 40972 starting on day 20 postconception according to the following regimens:

1. A single large dose (10,000 μg) administered IM on day 20
2. Three daily doses (1000 μg) administered IM on days 20, 21 and 22
3. Administration from an osmotic minipump (Alzet), which releases 1 μl/hr for 7 days, equivalent to a daily dose of 2.4 mg WY 40972

Animals were bled on days 20 through 26, 30, and 33. Maintenance of pregnancy was determined by plasma levels of estradiol and progesterone, vaginal bleeding, and any changes in perineal sex skin. Control animals received either saline injections or an empty osmotic minipump. Results from the control animals have been combined into a single group.

RESULTS AND DISCUSSION

Administration of the agonist WY 40972 at a 1-mg daily dose for 3 consecutive days at the early or midluteal phase of the cycle resulted in an inconsistent luteolytic response. Mean luteal phase length (± S.E.) in the control group was 17.7 ± 0.7 days (n = 7). Treatment in the early luteal phase shortened it to 14 and 16 days in two animals; the third had a normal luteal phase length of 20 days. The short luteal phases were accompanied by an early decline in plasma progesterone. Plasma estradiol was transiently elevated in two of three baboons following treatment but in a few days returned to control levels (Fig. 9-1). Treatment in the midluteal phase also shortened it to 15, 15, and 19 days; plasma progesterone declined earlier than in controls in the two baboons with short luteal phases. In all three animals a dramatic and prolonged increase in estradiol was evident following treatment (Fig. 9-2). In all baboons the menstrual cycle immediately following the treated cycle was normal, based on the pattern of perineal sex skin turgescence and deturgescence.

These results demonstrate comparable luteolytic effectiveness upon early

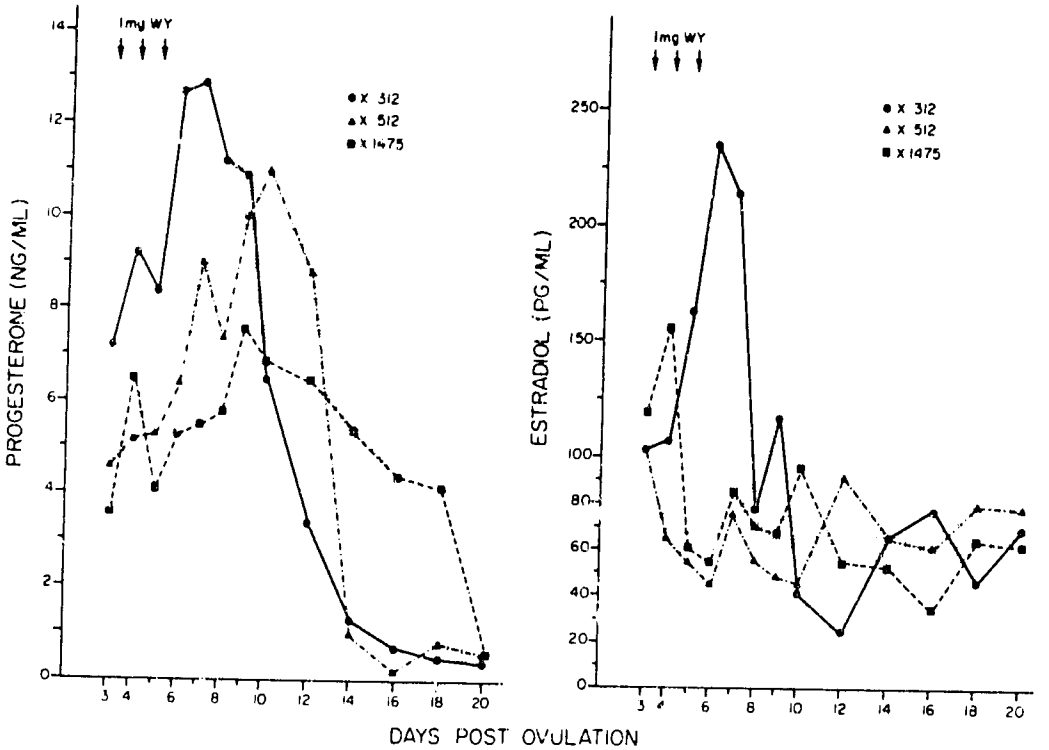


Fig. 9-1. Effect of three 1-mg doses of WY 40972 administered in the early luteal phase (days 3, 4, and 5 postovulation) to normally cycling baboons on plasma progesterone and estradiol levels. Stippled area represents mean levels (±S.E.) of progesterone or estradiol in control baboons. Mean luteal phase length in control baboons was 17.7 ± 0.7 days (n = 7); in the three treated baboons, luteal phase lengths were 14, [illegible] and 20 days.

or midluteal phase treatment in the baboon. Asch and co-workers[1] have demonstrated the luteolytic action of a similar LHRH agonist at the early but not the midluteal phase in the rhesus monkey. Midluteal effectiveness of other agonists has been demonstrated in the human.[3,9]

Luteolytic effects of microgram doses of other LHRH agonists have been reported. Since there is presently no evidence for qualitative differences in various agonists, the minimal luteolytic activity of milligram doses, as used in this study, suggests the possibility of a biphasic effect. The possibility that the milligram quantities administered in this study may have resulted in some such biphasic response has not been investigated. Of additional interest is the observation that these very large doses of WY 40972 produced no perceptible adverse or toxic reactions in any of the animals.

Only limited studies with LHRH agonists have been done in the baboon. Subcutaneous infusion of [D-Trp^6]-LHRH to baboons for 7 days during the early luteal phase had no effect on either luteal phase length or plasma progesterone, although a significant increase in both plasma LH and estrogen

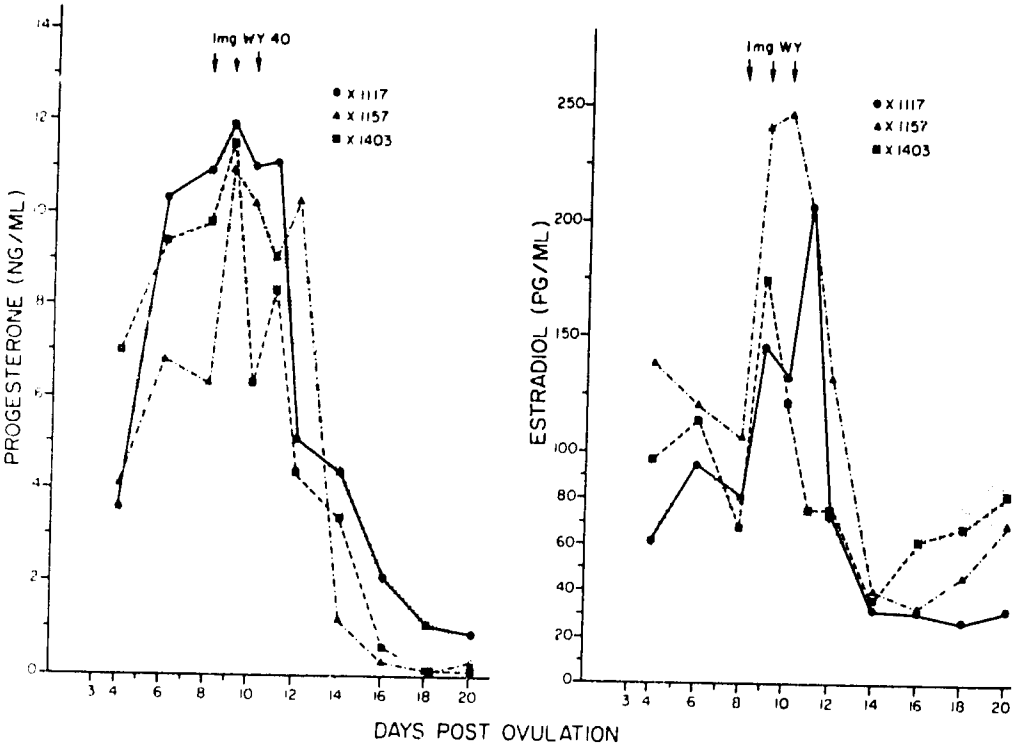


Fig. 9-2. Effect of three 1-mg doses of WY 40972 administered at the midluteal phase (days 8, 9, and 10) to normally cycling baboons on plasma progesterone and estradiol levels. Stippled areas are the same as in Figure 9-1. Mean luteal phase length in control baboons was 17.7 ± 0.7 days (n = 7); in the three treated baboons, luteal phase lengths were 15, 15, and 19 days.

was noted.[6] Subcutaneous implantation of a pelleted formulation of two different LHRH agonists immediately following sex skin deturgescence (early luteal phase) resulted in a shortening of the luteal phase and a decrease in plasma progesterone.[14] Implantation of the agonist pellets in pregnant baboons was associated with a decline in plasma levels of chorionic gonadotropin and progesterone although only one of four treated baboons aborted.[10]

As in the present studies, other reports have demonstrated luteolytic activity of similar LHRH agonists. These were not always expressed by a shortened luteal phase but, in some cases, only by a depression of plasma progesterone levels.[7,8,11]

In our initial study in pregnant baboons, we used a very high single dose (10 mg) of WY 40972 on day 20 postovulation. Three baboons treated in this manner displayed a transient decline in plasma progesterone (Fig. 9-3). Two of the three baboons also showed a large increase in plasma estradiol levels 24 hours after drug administration, with no further effect on estradiol levels thereafter. All three baboons maintained an essentially normal pregnancy except for the transient fluctuations from the normal pattern of plasma estradiol and progesterone (Fig. 9-3). One explanation for the lack of effect of

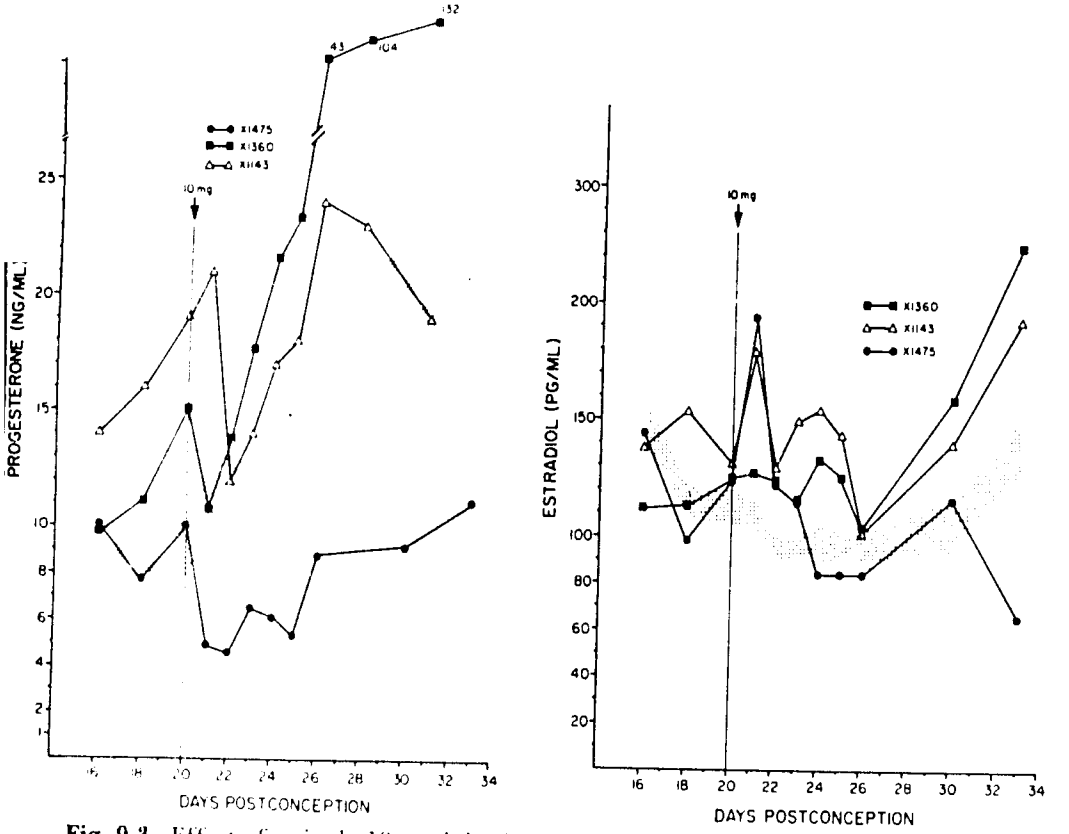
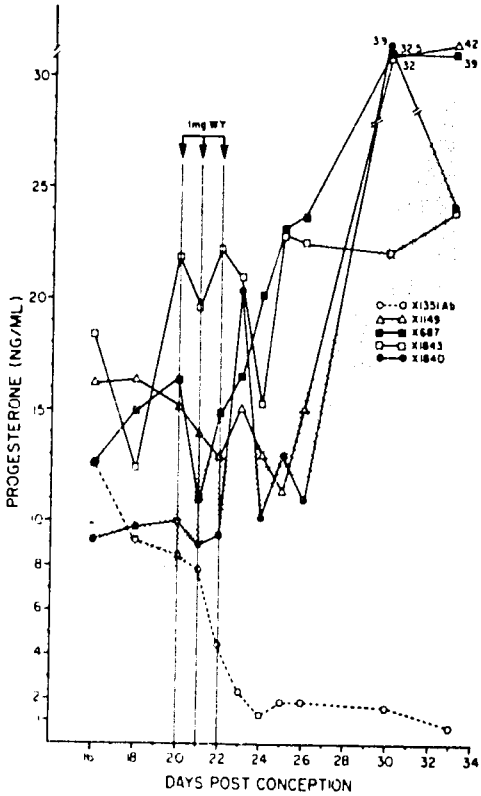


Fig. 9-3. Effect of a single 10-mg injection of WY 40972 on day 20 postconception on plasma progesterone and estradiol levels i . pregnant baboons. Arrow and vertical line indicate day of administration. Stippled areas represent mean levels (± S.E.) of plasma progesterone or estradiol in control pregnant baboons. Individual baboons are represented by different symbols.

this single administration may be the short duration of action of the agonist, and in subsequent trials we expanded the duration of treatment.

In the second study, we used a relatively lower dose, 1 mg, administered on 3 consecutive days, again starting on day 20 postovulation. Of five pregnant baboons treated with this 3-day regimen, one aborted. Progesterone levels in this animal were already declining at the time treatment was initiated, and it is difficult to exclude the possibility of a spontaneous abortion. In the remaining four baboons, there were slight, brief declines in plasma progesterone which soon recovered and followed the normal pattern. In these four animals, estradiol levels were elevated after the first or second administration of the agonist, and only returned to control values at about 10 days after the initiation of treatment (Fig. 9-4).

We next utilized an osmotic minipump (Alzet) which releases 1 μl/hr over a 7-day period. The concentration of the analog was 100 mg/ml, and therefore 100 μg/hr, or 2.4 mg/day, was released over the 7-day period. Of four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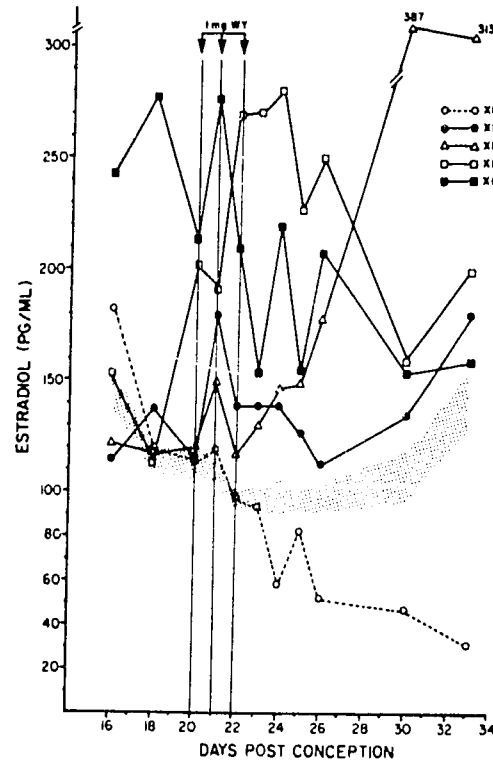
DAYS POST CONCEPTION
1mg WY
X1351Ab
X1149
X687
X1843
X1840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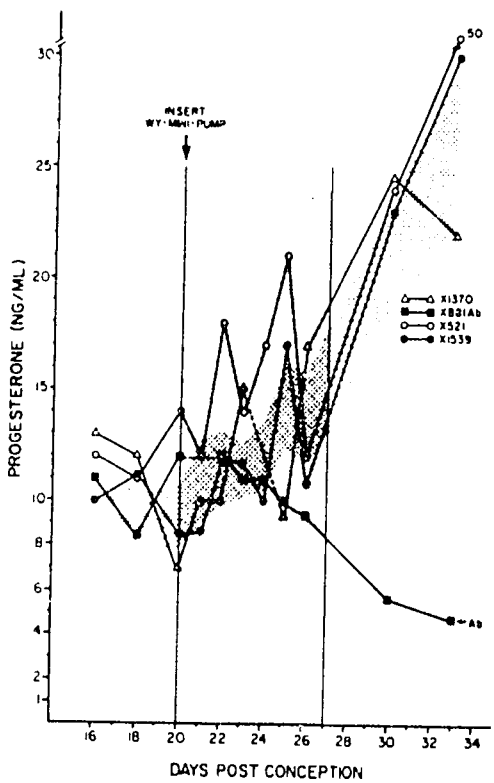
DAYS POST CONCEPTION
1mg WY
387
313
X135
X184
X114
X184
X68


PROGESTERONE (NG/ML)
DAYS POST CONCEPTION
INSERT
WY-MINI-PUMP
50
X1370
X831Ab
X521
X1539
Ab

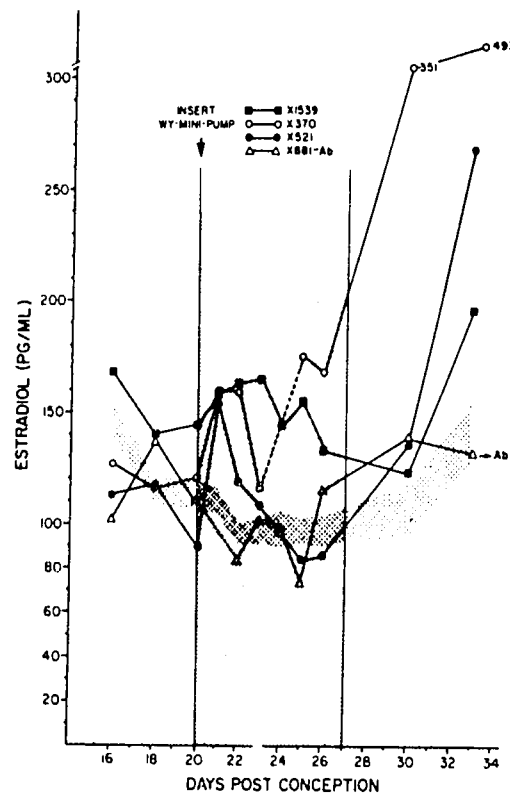

ESTRADIOL (PG/ML)
DAYS POST CONCEPTION
INSERT
WY-MINI-PUMP
X1539
X370
X521
X881-Ab
351
493
Ab

Fig. 9-4. Effect of three 1-mg doses of WY 40972 administered on days 20, 21, and 22 postconception on plasma progesterone and estradiol levels in pregnant baboons. Arrows and vertical lines indicate days of treatment. Stippled area is same as in Figure 9-3.

Fig. 9-5. Effect of WY 40972 administered from an osmotic minipump on plasma progesterone and estradiol levels in pregnant baboons. Release rate (100 μg/hr) was constant over a 7-day period as indicated by the cross-hatched area from days 20 through 26. Stippled area is same as in Figure 9-3.

treated baboons, the one which aborted had never developed the increase in plasma progesterone characteristic of normal pregnancy. Vaginal bleeding was first detected at 48 days postovulation, long after the expiration of the osmotic minipump. In the three baboons that maintained their pregnancies, insertion of the minipumps produced essentially no effect on plasma progesterone levels and a slight increase in estradiol levels after 1 to 3 days, which subsequently subsided to control levels (Fig. 9-5).

These attempts to interrupt pregnancy in the baboon with megadoses of Wy 40972, a potent synthetic LHRH agonist, are discouraging. There were only two abortions in a total of 12 treated animals and at least one of these may have been a spontaneous abortion. There is some suggestion from the osmotic minipump results that a sustained exposure might be more effective. The use of high doses of this agonist for short durations as an early pregnancy abortifacient agent appears unpromising and, indeed, studies from other laboratories with other LHRH analogs in both nonhuman primates and humans appear to be in accord. Results with the "megadoses" of LHRH which we have employed suggest that merely increasing the dose may not be an effective approach.

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10

Female Contraceptive Potential of "Super" Agonists of LHRH as Assessed in Infrahuman Primates

BRIAN H. VICKERY

Over the past six years a steadily increasing number of reports have enumerated actions of LHRH which appeared inappropriate to the physiological function of this hormone. Thus, doses of LHRH that are appreciably higher than those required to stimulate gonadotropin release and to stimulate ovulation have been noted to delay onset of puberty, inhibit ovulation, and even suppress luteal function. These findings have been confirmed with the superactive synthetic analogs. The unexpected and unexplained nature of these effects caused them to be dubbed, generically, paradoxical antifertility effects of LHRH and its agonistic analogs.[14,15,41]

OVULATION INHIBITION

Johnson and associates[5] showed [D-Leu^6]-LHRH ethylamide to delay vaginal opening and inhibit ovarian growth in the immature rat, prevent normal cycling, and cause reversible atrophy of the ovaries and uterus of the mature rat. This ability to prevent cycling has been made the basis of a screening assay for LHRH agonists[38] and has been extended to a variety of species including cattle[24] and even dogs.[45] The rapid reversal of the estrus-suppressant activity has led to a suggestion that LHRH agonists could be of use for cycle synchronization in farm animals.[46]

Guided by initial reports that LHRH and its agonist analogs were poorly effective in the more readily available laboratory primates (such as rhesus[1] or bonnet[35] macaques) but that baboons showed good response,[31] we began investigation of ovulation suppression in baboons. To minimize trauma during chronic drug administration the LHRH agonists ([D-Trp^6]-LHRH ethyl-

I acknowledge the close collaboration of Ms. Georgia I. McRae and Professor Vernon C. Stevens. Excellent technical assistance was provided by Susan Rinne, Patricia Kirchgatter, Christa Tsai, John Rowland, Anne Worden, Richard Seidenberg, Karen Bergstrom, and John E. Powell. Radioimmunoassays were performed by William Briones, Agnes Bajka, and Dorothy Tallentire, and at the laboratories of Dr. Scott Monroe and Professor Vernon Stevens. Some of the studies were supported in part by NIH Contract N01-HD-0-2811 to Syntex Research.

amide and [D-Ala^6]-LHRH ethylamide) were formulated as compacted cholesterol pellets[20] and implanted subcutaneously. When pellets containing a total of 100 μg/kg were implanted on the first or second day of menstruation, ovulation was delayed by about 2 weeks.[47] Other short-term studies were reported showing a similar effect of [D-Ser(TBU)^6]-LHRH ethylamide on ovulation when administered by daily injection to stumptailed macaques.[19-22] Longer-term studies were then mounted in both rhesus[50] and stumptails[21] and have shown that 6 to 7 months' continuous treatment with either [D-Trp^6]-LHRH ethylamide or [D-Ser(TBU)^6]-LHRH ethylamide is consistent with continuous suppression of ovulation (Fig. 10-1). A rapid return of ovulation follows cessation of treatment and is succeeded by a normal luteal phase, as judged by circulating levels of progesterone. Ovulation suppression, as shown in this case with [D-Nal(2)^6]-LHRH,[38] is associated with breakthrough bleeding or, at multiples of the minimal effective dose, with amenorrhea after the first cycle of treatment (Table 10-1).

LH levels are undetectable through the period of dosing (Fig. 10-2), but FSH levels show no consistent change. Preovulatory surges in estradiol are not seen and basal levels are reduced to early follicular phase values. Fertility is markedly suppressed but incidence of mating is unchanged.[50] In the stumptailed macaque, gonadotropin release was not stimulated by an exogenously supplied estrogen surge;[22] in the rhesus, injection of 1000 IU of hCG at midcycle did not induce ovulation.* These results seem to indicate downregulation to be occurring at both pituitary and ovarian levels.

Several trials involving women have had results comparable with those involving infrahuman primates. An early study showed that daily subcutaneous injection of 5 μg/day [D-Ser (TBU)^6]-LHRH ethylamide, administered from menstruation on, prevented ovulation in four women.[39] Similar findings resulted from 100 μg per day of LHRH,[32] and 2.5 μg/day of [D-Trp^6]-LHRH ethylamide.[10] Nasal administration of [D-Ser (TBU)^6]-LHRH ethylamide has established that ovulation is consistently inhibited by daily doses of 400 μg or above.[5,7] Irregular breakthrough bleeding or amenorrhea is observed. Twenty-six women used no other contraceptive, and no pregnancies resulted. Ovulation returns within 4 weeks of discontinuation of up to 6 months of treatment. Mean estradiol levels, particularly in the amenorrheic population, are lowered following either nasal or subcutaneous administration.[2,7]

DESYNCHRONIZATION OF CYCLES

Sheehan and colleagues showed that a single injection of [D-Trp^6]-LHRH ethylamide during the early follicular phase in women caused a delay in ovulation.[42] Three injections of 50 μg [D-Trp^6]-LHRH ethylamide during menstruation in rhesus monkeys caused an insufficient luteal phase and protected from pregnancy.† We have been unable to duplicate this latter finding with three daily injections of 50 μg [D-Nal (2)^6]-LHRH, although delay of ovulation was observed (Fig. 10-3).

(*Text continues on p. 114*)

*Vickery BH, McRae G: Unpublished data.

†Hodgen G: Personal communication, 1981.

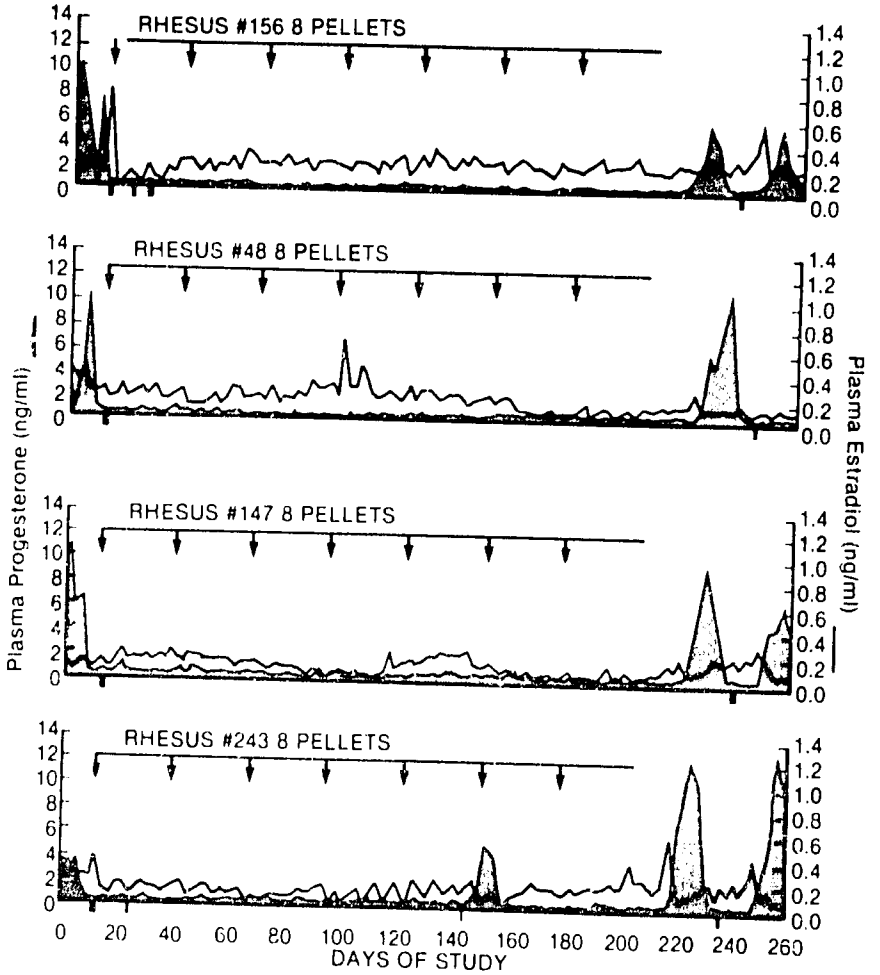

Fig. 10-1. Effect of subcutaneous implantation of 1 mg per animal per month of pelleted [D-Trp^6]-LHRH ethylamide for 7 months on menstrual bleeding and plasma levels of progesterone and estradiol in female rhesus monkeys.

TABLE 10-1. Effect of Daily Injection of an Aqueous Solution of [D-Nal(2)^6]-LHRH on Incidence of Ovulation and Breakthrough Bleeding in Rhesus Monkeys

DOSE µg/day	NO. ANIMALS	OVULATIONS PER CYCLES TREATED*	BTB† ( )‡ DAYS§
—	5	13/14	—
0.31	3	7/9	—
1.25	3	1/9	13(2)
5.00	3	0/9	10(1)
20.00	2	0/6	2(1)‖
80.00	3	0/9	3(1)‖

*Duration of treatment was 90 consecutive days.
†BTB = Breakthrough bleeding.
‡In anovulatory cycles only.
§Number of animals showing BTB.
‖Bleeding associated with first cycle of treatment only.

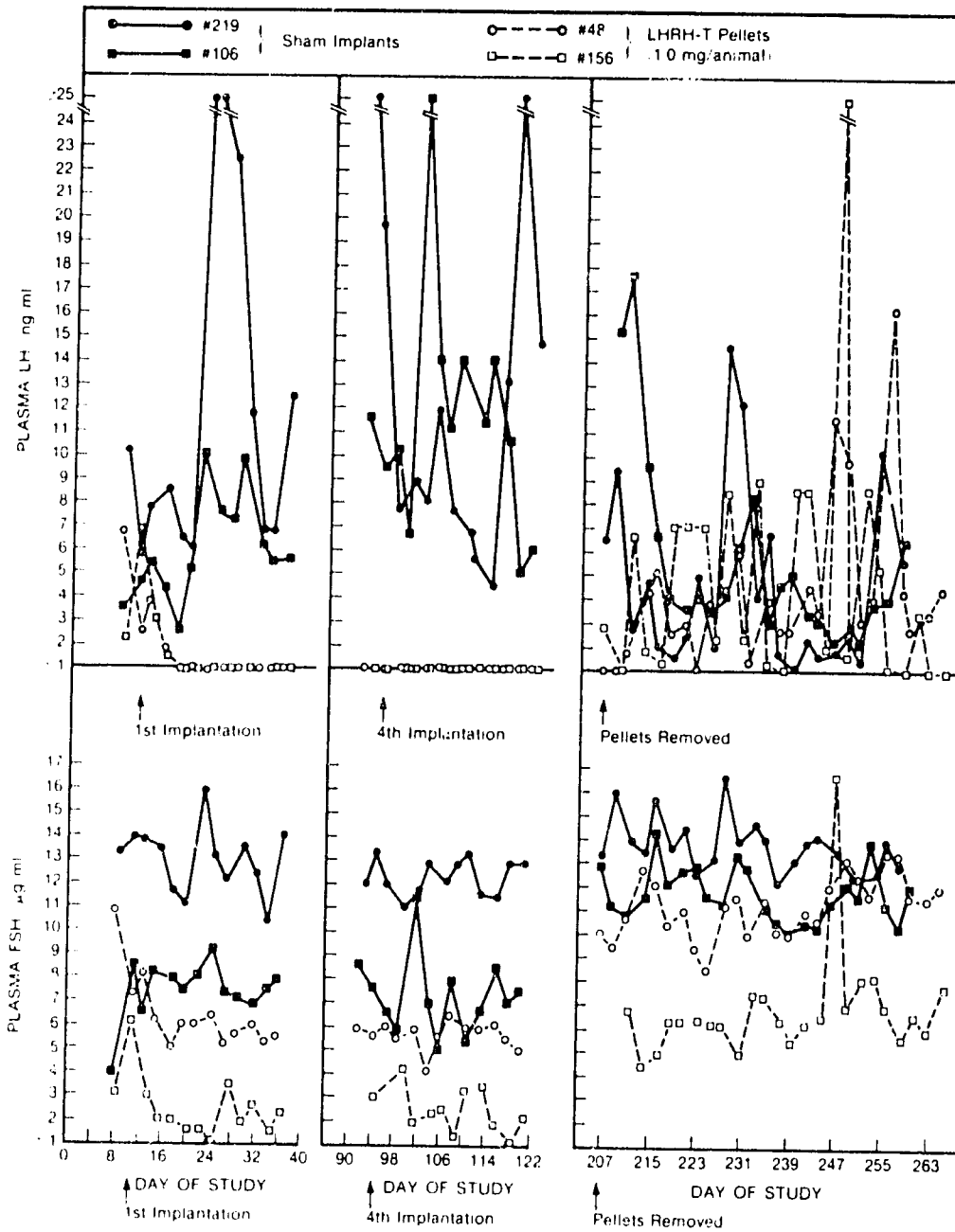

Fig. 10-2. Effect of 7 months' chronic treatment with 1 mg per animal per month of pelleted [D-Trp^6]-LHRH ethylamide on plasma levels of LH and FSH in female rhesus monkeys. Left panel: first month of treatment; middle panel: fourth month; right panel: month following cessation of treatment.

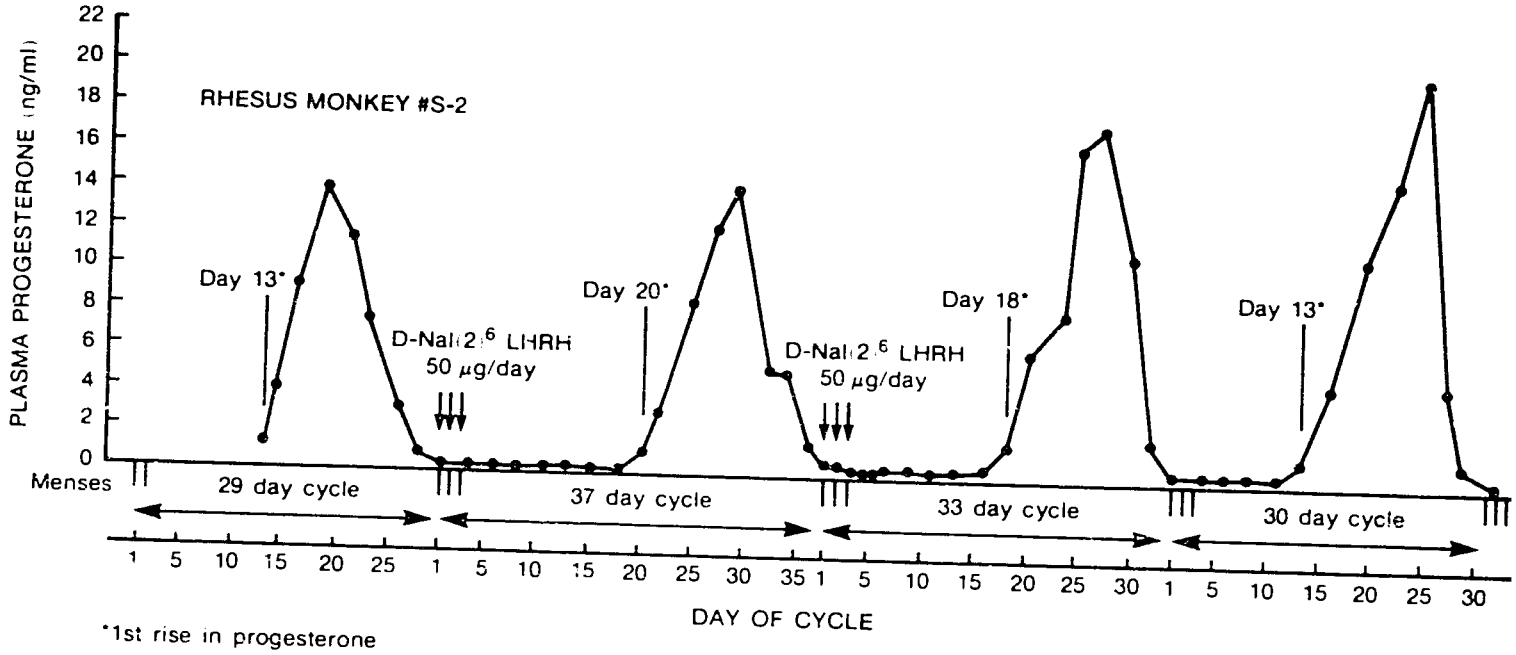

Fig. 10-3. Effect of three daily injections during menstruation (50 μg per animal per day of [D-Nal(2)6]-LHRH in two consecutive cycles) on intermenstrual interval and plasma levels of progesterone in a female rhesus monkey.

LUTEAL SUPPRESSION AND PREGNANCY INTERRUPTION

Studies in rats[16] and rabbits[40] on the postovulatory effects of LHRH agonists led to the suggestion by Corbin and co-workers[14,15] that these agents might be effective interceptive agents acting via luteolysis. Premature demise of the corpus luteum has also been seen in the dog following treatment with one of these agents.[45] The suggestions were rapidly applied to investigation in women. Preliminary promising results were obtained with injection of LHRH itself;[30,33] [D-Trp^6]-LHRH ethylamide[12]; or nasal administration of [D-Ser (TBU)^6]-LHRH ethylamide.[34] The number of doses ranged from 2 to 5; all studies, which were conducted in the luteal phase of nonpregnant women, demonstrated luteolysis. However, more recently, attempts to suppress luteal function in the face of exogenous hCG administration[8,11] or during early pregnancy[11] have fared poorly. The studies, which deal with the ability of a course (10 days) of treatment with hCG to counteract the effect of two injections of LHRH agonist, seem a somewhat limited basis for the poor prognosis for these agents during early pregnancy.

Our own studies, using two injections of up to 50 μg/day [D-Nal(2)^6]-LHRH approximately 9 and 8 days before expected menstruation in the baboon, have shown no change in luteal phase length or in circulating levels of progesterone. Our previous studies with pelleted [D-Trp^6]-LHRH ethylamide or [D-Ala^6]-LHRH ethylamide implanted approximately 4 days after ovulation, on the other hand, showed a clear-cut luteal suppression and premature menstruation.[47] Accordingly, a further trial in baboons was performed using ten daily injections of 2, 10, or 50 μg/day [D-Nal(2)^6]-LHRH starting on the third day after ovulation.[49] A significant, dose-related suppressive effect on luteal phase progesterone was observed (Table 10-2). At the highest dose level integrated values of progesterone for the luteal phase were 54% of contemporary control values and 58% of values observed for previous cycles in the same animals. Luteal phase duration was shortened by treatment but not in a dose–response-related manner.

Similar data have been reported for women administered [D-Ser (TBU)^6]-LHRH ethylamide, 600 μg/day intranasally starting 0 to 2 days after ovulation and continuing until onset of menstruation.[9] Strangely, daily injection of 10 μg [D-Ser (TBU)^6]-LHRH ethylamide in women starting 5 to 7 days after

TABLE 10-2. Effect of Ten Daily Injections of [D-Nal(2)^6]-LHRH Starting 3 Days after Ovulation upon the Integrated Values of Plasma Progesterone during the Luteal Phase

TREATMENT μg/DAY	NO. ANIMALS	INTEGRATED VALUE* FOR PLASMA PROGESTERONE ± S.E.	
		Cycle Prior to Treatment	Treatment Cycle
Placebo	3	74 ± 25	84 ± 11
2	3	86 ± 20	74 ± 5
10	3	72 ± 10	52 ± 19
50	3	77 ± 24	45 ± 5

*Arbitrary units by planimetry.

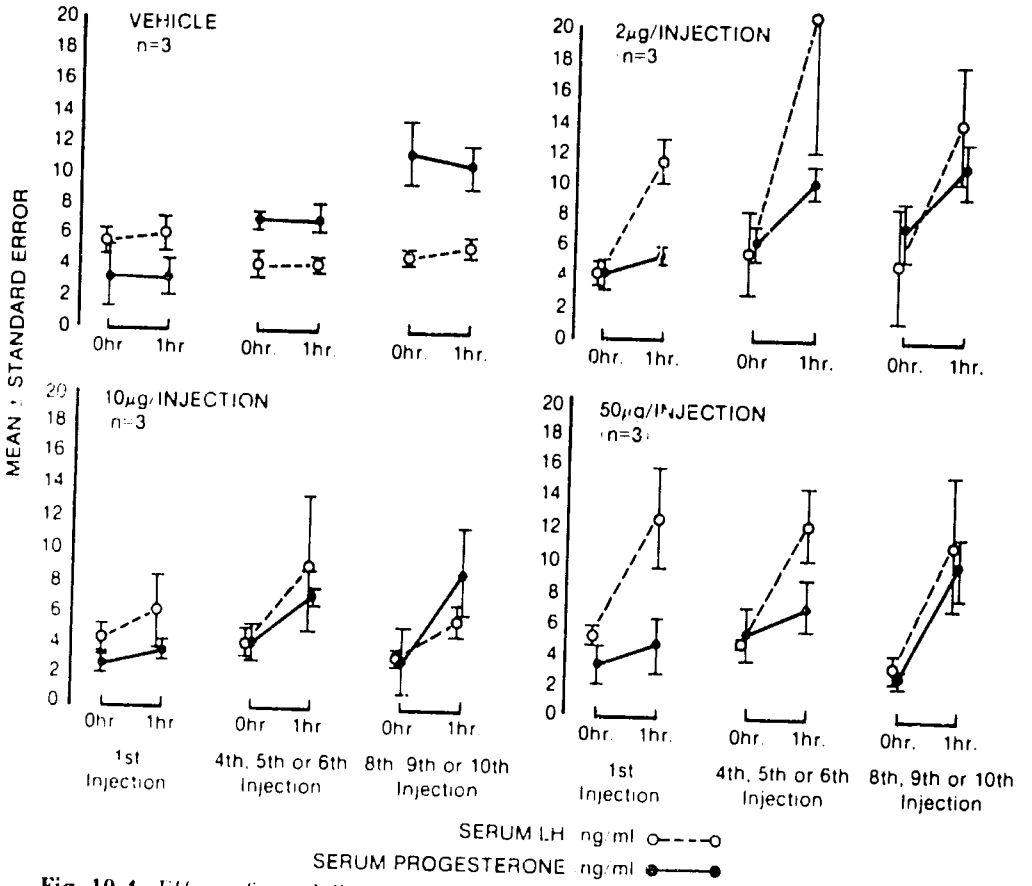

Fig. 10-4. Effect of ten daily injections of [D-Nal(2)^6]-LHRH starting 3 days after ovulation upon acute responses in serum LH and progesterone levels in female baboons.

ovulation and continuing until onset of menstruation had no effect on luteal phase progesterone and appeared to extend luteal phase duration.[9]

It was concluded that the timing of onset of treatment was critical. No data were given on the bioequivalence or blood levels resulting from the different doses and routes used. The mechanism of action of this luteal suppressive effect remains obscure, particularly in light of the continued acute responsivity in the baboon of both pituitary and corpus luteum (Fig. 10-4). A recent study has shown plasma estrone (but not estradiol) levels to rise following [D-Leu^6]-LHRH ethylamide treatment in baboons (Fig. 10-5)*. In view of the suggested luteolytic effect of estrogen in the primate, these estrogen effects may play a part in the luteolytic mechanism.

Presumably the combination of continuous dosing (pellets) and the timing of start of administration (~4 days after ovulation) with [D-Trp^6]-LHRH

*Stevens VC: Personal communication, 1980. Also Figures 10-7, 10-10, 10-11.

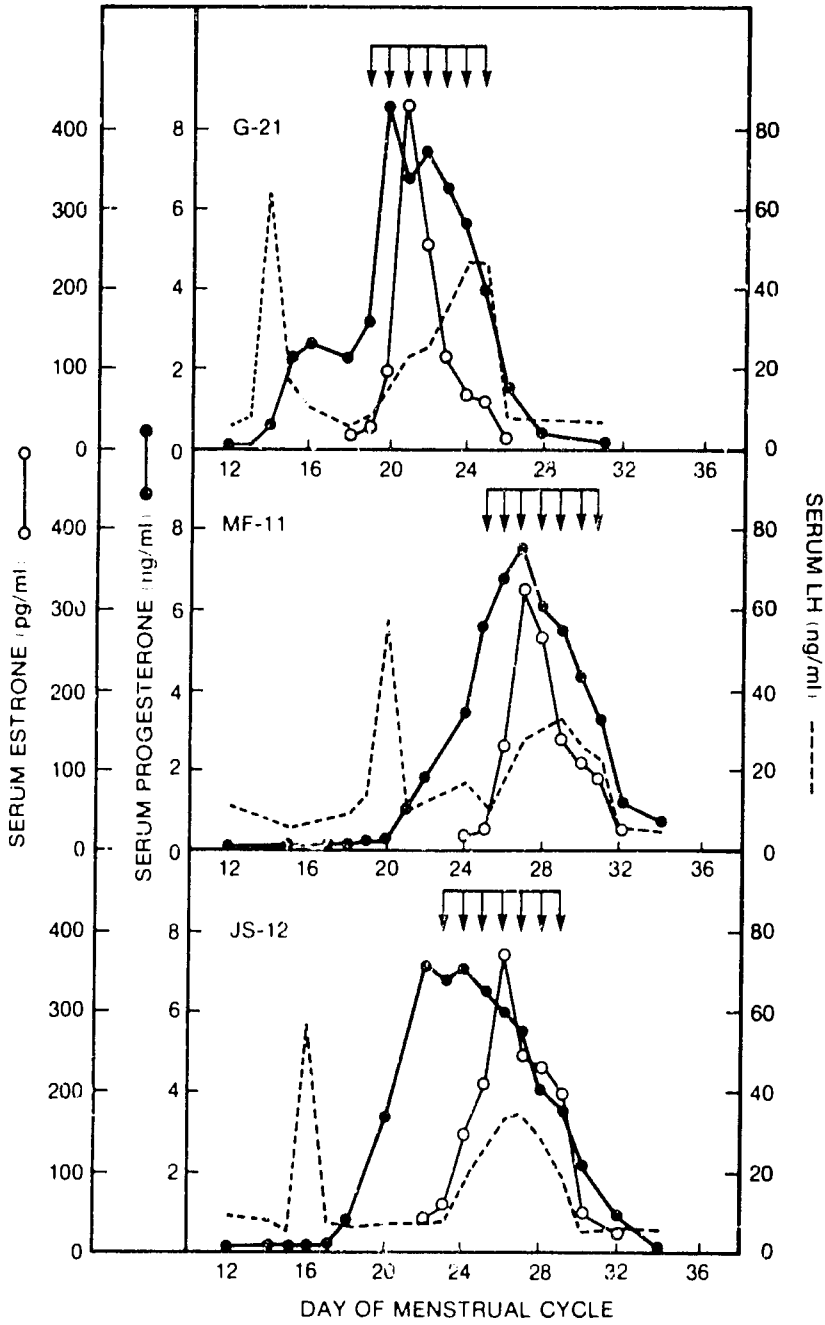

Fig. 10-5. Effect of subcutaneous injection of 1.5 mg [D-Leu^6]-LHRH ethylamide daily for 5 days during the luteal phase on serum levels of progesterone, estrone, and LH in three baboons.

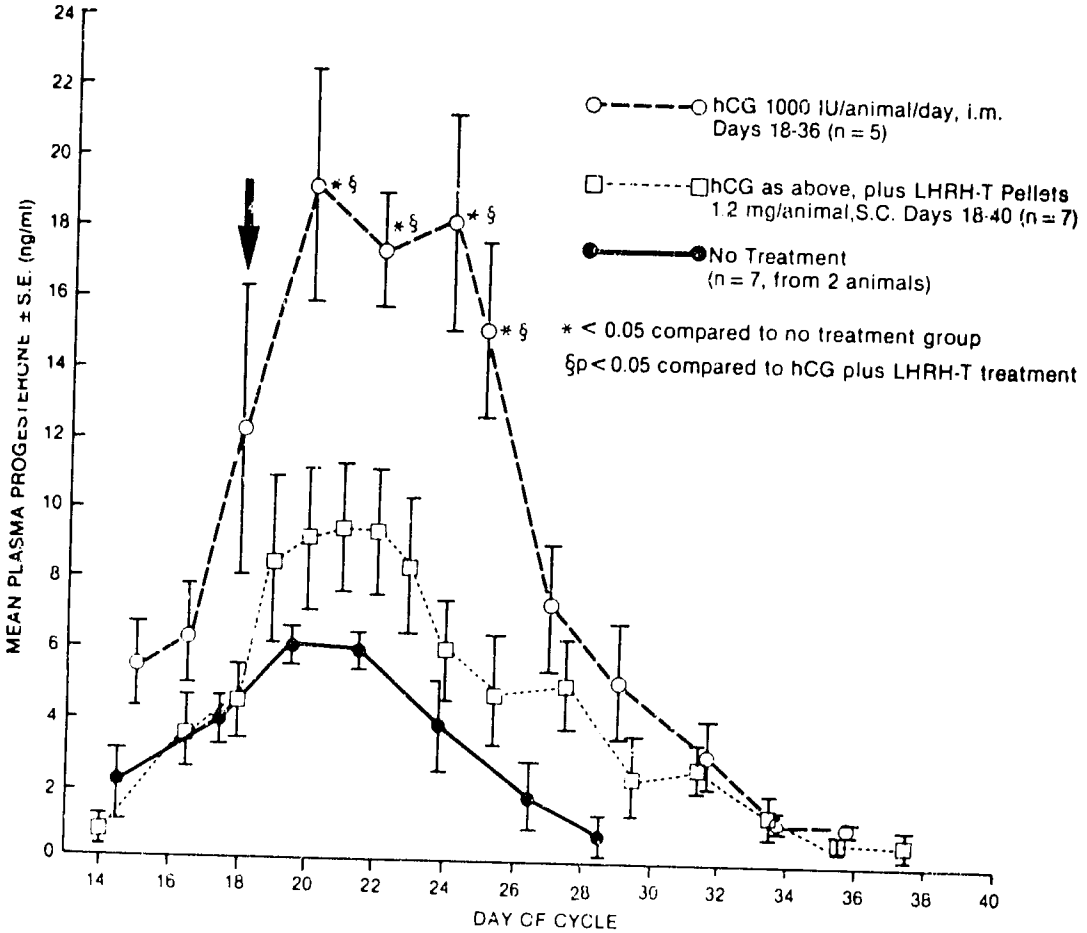

Fig. 10-6. Effect of concurrent treatment with pellets of [D-Trp^6]-LHRH ethylamide plasma (LHRH-T) and hCG on luteal phase duration and plasma levels of progesterone in rhesus monkeys; 1 mg of LHRH-T was subcutaneously implanted per animal from days 18 through 40 of the cycle; 1000 IU hCG per animal was injected IM daily from days 18 through 36 of the cycle. (Asterisk indicates $P < 0.05$.)

ethylamide can account for our finding[48] that this agent antagonizes the effects of exogenous hCG, both on luteal phase duration and on progesterone levels in rhesus monkeys (Fig. 10-6). Daily subcutaneous injection of 1.5 mg [D-Leu^6]-LHRH ethylamide has recently been shown to antagonize the steroidogenic effect of 1000 or 2000 IU hCG given daily for 3 days to female baboons (Fig. 10-7). Of course the possibility that rhesus monkeys and baboons are more sensitive than women to the paradoxical effects of these agents cannot be ruled out; however, it seems unlikely in light of reports of decreased sensitivity to gonadotropin-releasing effects.[1]

The results of our studies in pregnant baboons[36] also contrast with the

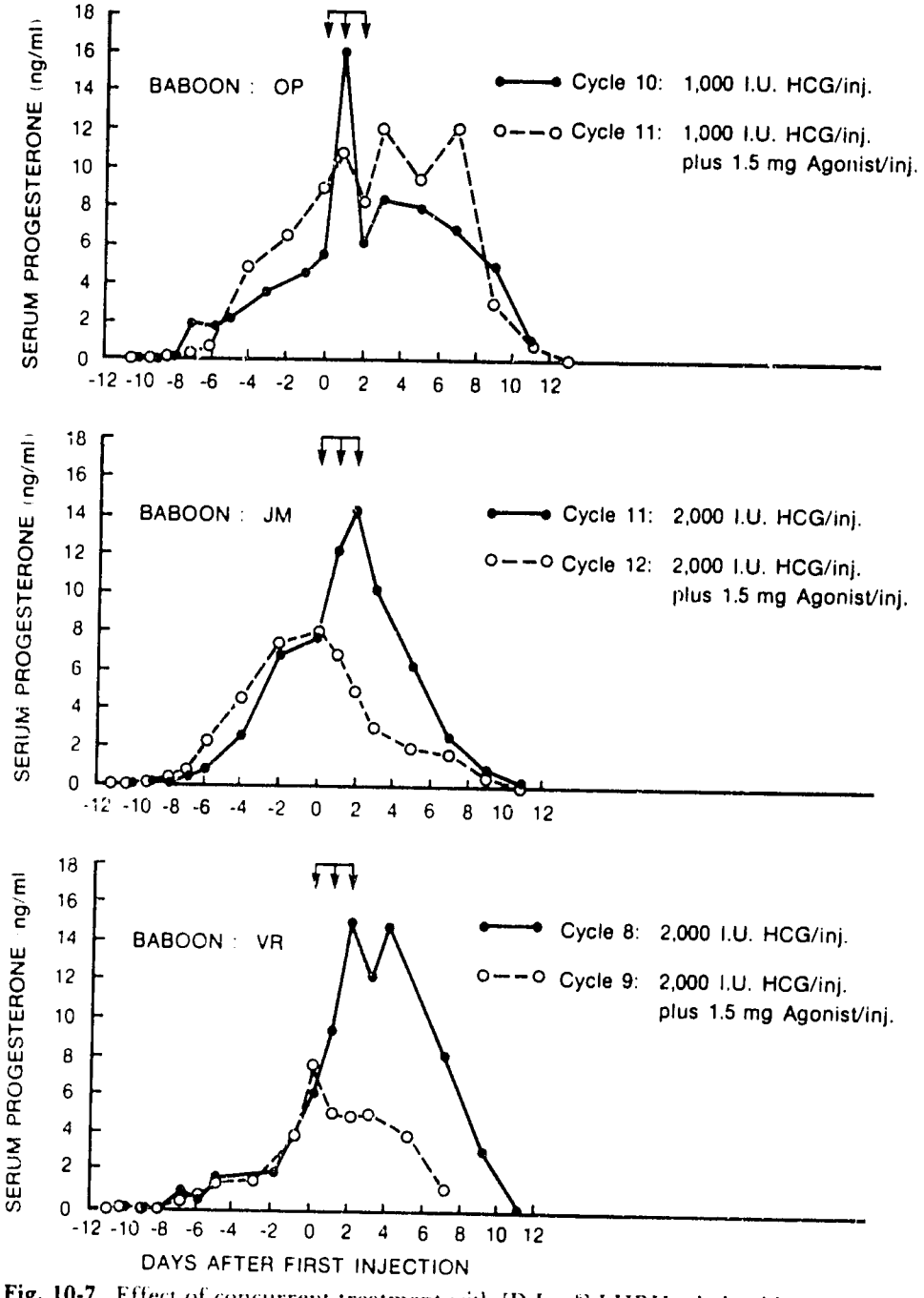

Fig. 10-7. Effect of concurrent treatment with [D-Leu^6]-LHRH ethylamide (TAP-144; A43818) and hCG on serum levels of progesterone in the luteal phase of baboons (*P. papio*). Arrows indicate injection of either hCG (1000 IU in one animal, 2000 IU in two animals) or concurrent injection of hCG (1000 IU in one animal, 2000 IU in two animals) and 1.5 mg [D-Leu^6]-LHRH ethylamide. Blood samples were withdrawn 1 hour after injection of peptide hormones.

reported lack of effect of LHRH agonists in pregnant women.[10] Administration of 125 μg/kg [D-Nal(2)[6]]-LHRH (n = 2) or [D-Trp[6]]-LHRH ethylamide (n = 4) by pellet implantation beginning on the 14th to 21st day of gestation resulted in termination of pregnancy in two of six treated baboons. In all treated animals circulating progesterone levels, presumably luteal in origin, failed to rise in response to rising levels of chorionic gonadotropin as they did in controls (Fig. 10-8). This result, and the rhesus monkey experiment showing antagonism of the steroidogenic effect of exogenous hCG,[50] may be

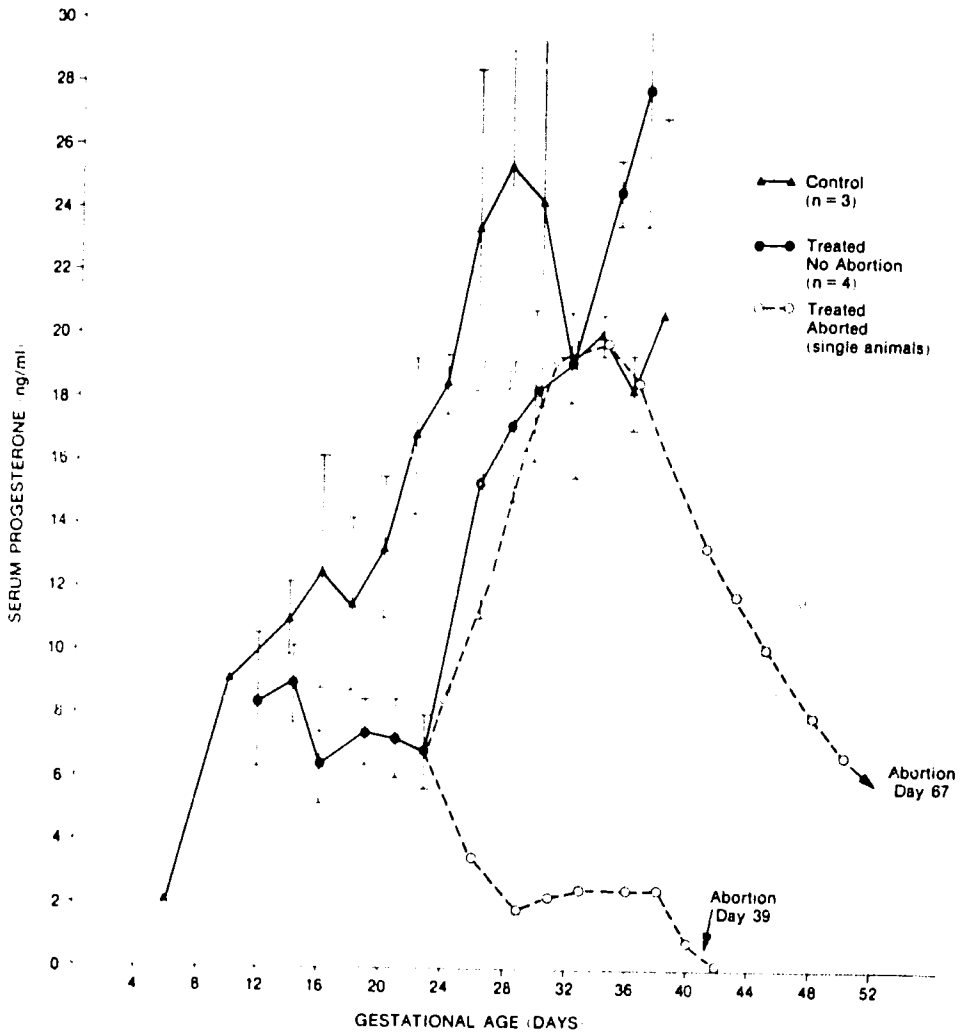

Fig. 10-8. Effect of subcutaneous implantation of 125 μg/kg [D-Trp[6]]-LHRH ethylamide (n = 4) or [D-Nal(2)[6]]-LHRH (n = 2) on serum levels of progesterone starting on day 14 through day 21 of gestation in pregnant baboons.

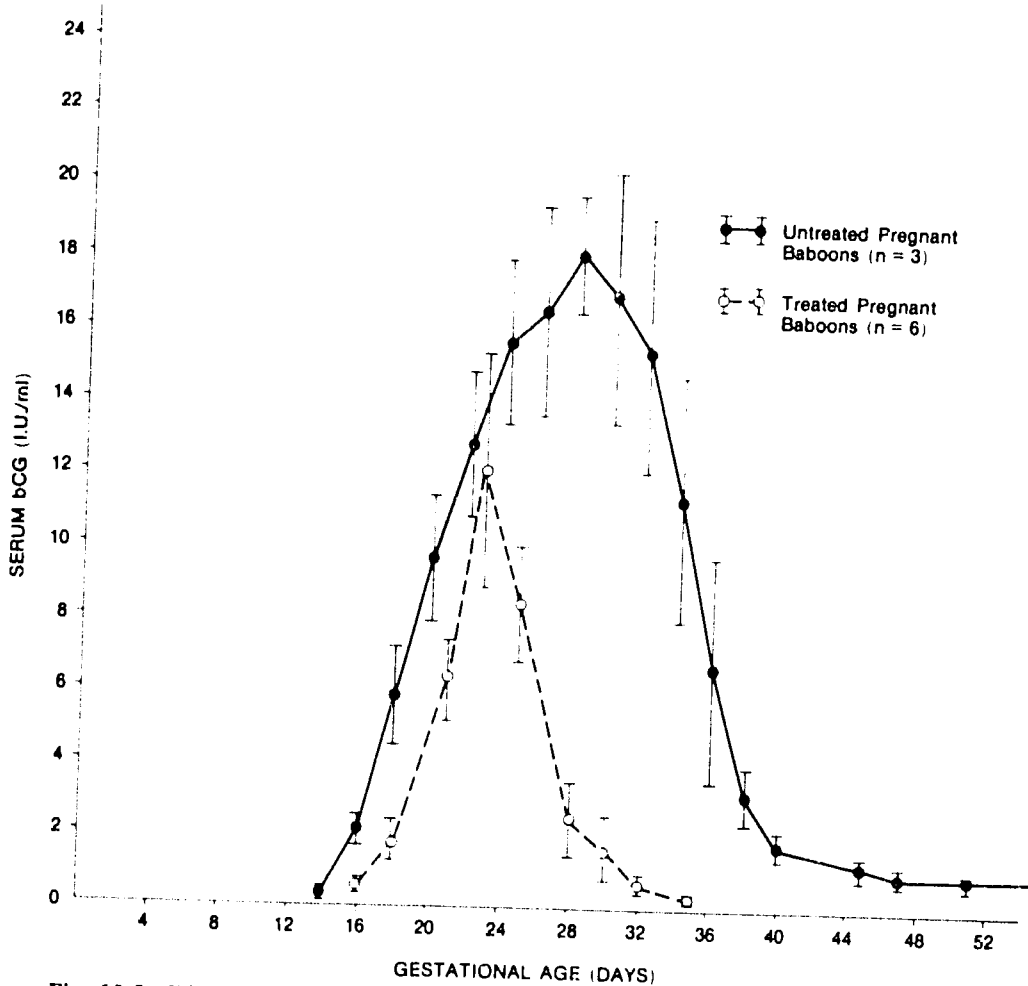

Fig. 10-9. Effect of subcutaneous implantation of [D-Trp^6]-LHRH ethylamide or [D-Nal(2)^6]-LHRH on serum levels of chorionic gonadotropin starting on day 14 through day 21 of gestation in pregnant baboons.

likened to *in vitro* studies that have demonstrated an agonist of LHRH to directly inhibit the response to hCG in dispersed rat luteal cells.[13] This direct activity appeared to be mediated via specific binding of the agonist [D-Ser(TBU)^6]-LHRH ethylamide. More recently it has been suggested that the effect is due to a functional uncoupling of the LH/hCG receptor complex from adenylate cyclase.[3] After the 23rd day of gestation progesterone levels did rise markedly (Fig. 10-7), but this was thought to reflect a placental contribution in keeping with recent findings in rhesus monkey.[23] Interestingly, the profile of circulating chorionic gonadotropin was truncated in all treated animals (Fig. 10-9). Suppressive effects on circulating progesterone levels in pregnant baboons have also been observed following daily injection of

LHRH* and [D-Leu^6]-LHRH ethylamide (Fig. 10-10). Again, suppressive effects on chorionic gonadotropin secretion were indicated (Fig. 10-11). In view of the recent findings of presence of and putative gonadotropin-releasing function of an LHRH-like molecule in the placenta,[18,27-29,37,43,44] this may represent a down-regulation of placental receptors, similar to that noted for the pituitary.[4,17]

*Edgren RA, Stevens VC: Personal communication, 1980.

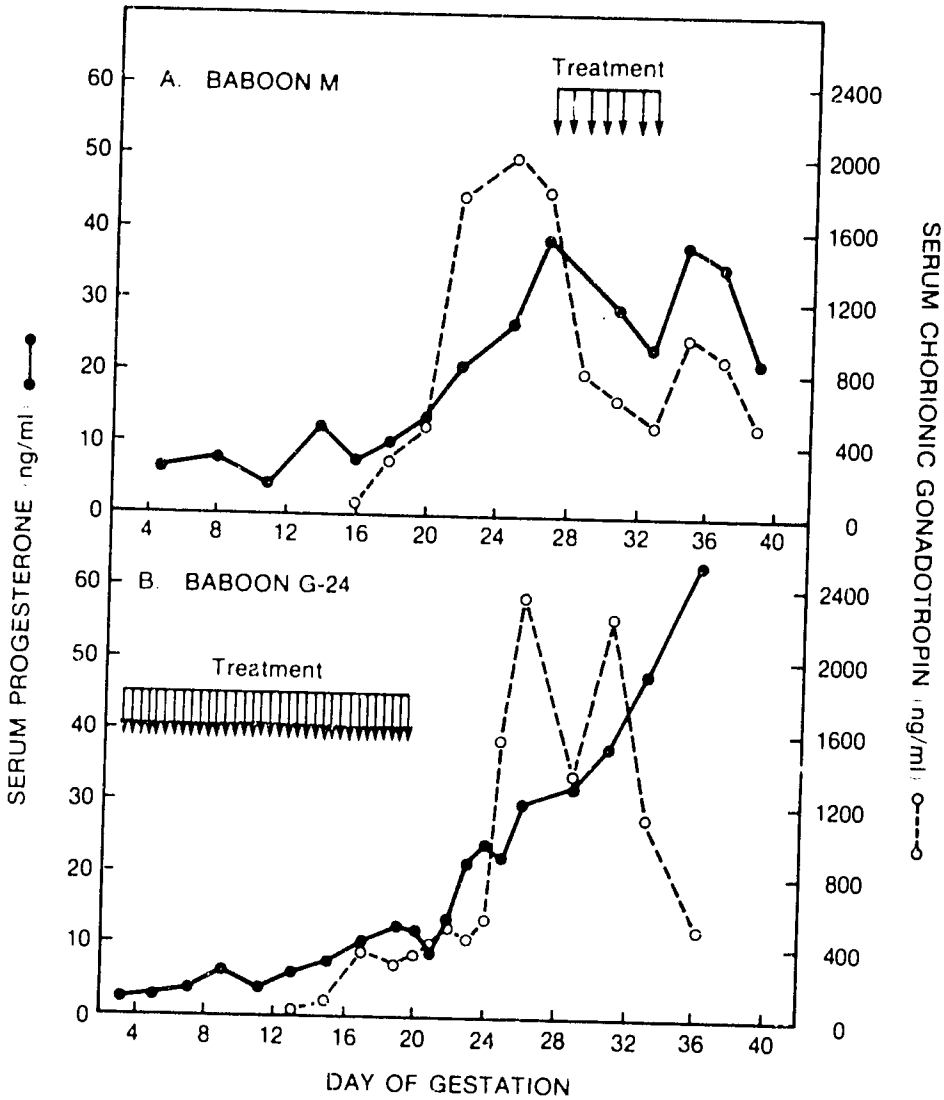

Fig. 10-10. Effect of subcutaneous injection of [D-Leu^6]-LHRH ethylamide upon serum levels of progesterone and chorionic gonadotropin in pregnant baboons. Panel A: effect of seven consecutive daily injections of 2.0 mg per injection starting on day 27 of gestation. Panel B: effect of 2.0 mg per injection twice daily starting on day 3 and continuing until day 19 of gestation.

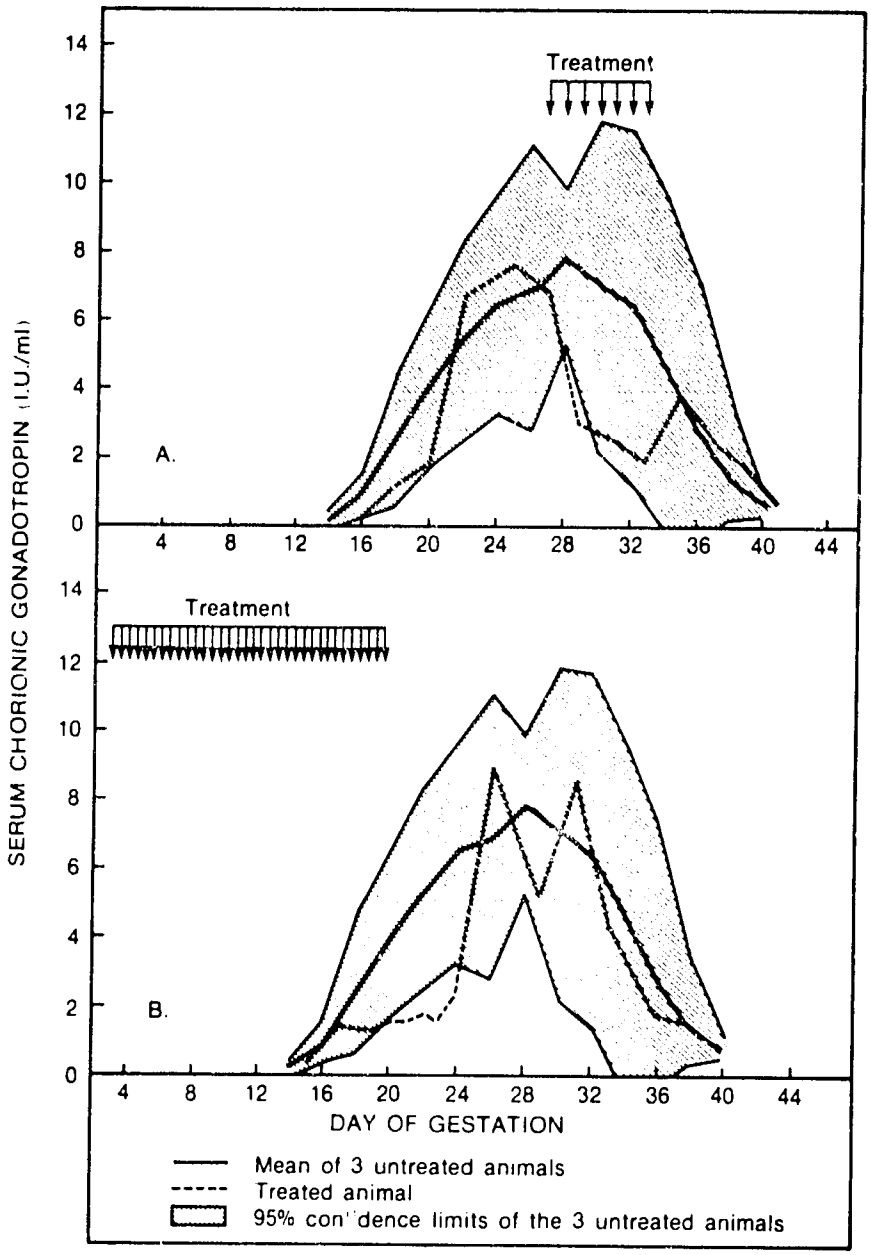

Fig. 10-11. Comparison between serum chorionic gonadotropin levels in untreated pregnant baboons (n = 3) and individual animals treated with [D-Leu^6]-LHRH ethylamide. Dosing is as described in Figure 10-10. Shaded area indicates 95% confidence limits of untreated animal values.

CONTRACEPTIVE POTENTIAL

From the experience with the LHRH agonists so far, the prognosis for their use as ovulation-inhibitors is good. The only drawbacks so far voiced are the questions of desirability of the associated amenorrhea; possibility of either a menopausal-like state from too little estrogen production, or the dangers of "unopposed" estrogen; and lack of oral activity in presently available agents.

Amenorrhea may be preferable to irregular bleeding episodes and may have to be a consequence at present. Data so far have not indicated either a hypo- or a hyperestrogenic state to result from even long-term treatment.[7] It is fortunate that the extreme potency of these "super" agonists has allowed nasal administration to be used in these early studies. Whether this two-log order increase in dose requirement for nasal over injectable quantity will be economically feasible is not clear. However, work is proceeding on alternative routes and formulations (*e.g.*, vaginal depots and depot injectables). Clearly, should the lack of toxicity noted for these agents continue, we can anticipate an addition to our contraceptive pharmacopeia.

The uses of LHRH analogs for desynchronization, follicular luteinization in the absence of ovulation, or induction of inadequate luteal phase are in the very early stages of investigation but do not appear promising.

Finally, the interceptive/menstrual-induction ability of these agents is controversial. Studies of very limited duration of dosing and minimal dose level manipulation with LHRH agonists in women have shown little promise. On the other hand, multiple-dose or chronic administration via pellets in infrahuman primates indicates a higher probability of success particularly at the higher dose levels. It is anticipated that when optimum conditions have been elucidated in the infrahuman primate, a reexamination in women will permit a final judgment.

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11

Evaluation of Antifertility Effects of LHRH Analogs in the Guinea Pig

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ARPAD I. CSAPO

An attractive option for reversible unisex contraception is the use of LHRH and its agonistic analogs to "down-regulate" ovarian and testicular functions. Since LHRH has a short half-life, analogs with increased biological activity have been synthesized.[13,20] However, a "bottleneck" in the preclinical development of these compounds is the species differences that limit the translation of animal studies to man. This impediment might well be exposed by identifying a species with "human-like" regulatory characteristics. In this effort, reproductive biologists repeatedly considered the guinea pig as a suitable model for preclinical studies.[7]

The guinea pig has been considered human-like for the following reasons. Early ovariectomy terminates pregnancy; abortion occurs through the expulsion of the uterine contents rather than through absorption of the conceptuses; the onset of labor is initiated at high progesterone levels; and parturition cannot be delayed by progesterone treatment.

Our interest in the guinea pig as a "model" species for pharmacological studies was substantiated by our experience with PGE_2-analog of exceptional clinical efficacy in postconceptional therapy.[8] This intramuscularly active compound, Sulproston, although relatively inactive in the rat, predictably terminated pregnancy in the guinea pig.[11] Similarly, the progesterone synthesis inhibitor Isoxazol (Win 17,625), which had been 100% effective in the rats,[10] was ineffective in the guinea pig and human. Encouraged by the human-like qualities of the guinea pig and these correlative drug responses, we examined the possible luteolytic and abortifacient effects of two LHRH analogs, [D-Trp^6-Pro^9-NEt]-LHRH (Salk) and [D-6-(2-Naphthyl-alanine]-LHRH (Syntex) in this species during different phases of the gestation.

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MATERIALS AND METHODS

PEPTIDES

The LHRH analogs, [D-Trp^6-Pro^9-NEt]-LHRH and [D-6-(2-Naphthyl-alanine]-LHRH were provided by J. Rivier of the Salk Institute of Biological Sciences and by B. Vickery of the Syntex Research Laboratories, respectively.

ANIMALS

Line breed guinea pigs were purchased from Camm Research Laboratories, N.J. Within 12 hours after spontaneous delivery at term, the females were cohabited with fertile males and the time when the vaginal plug was first observed was designated as day 0 of pregnancy. Each animal was then housed in an individual cage with 14 hours of photoperiod and *ad lib* purina chow. In animals where treatment was started on or after day 10, pregnancy was confirmed either by laparotomy or by palpation, under Fluothane anesthesia.

EXPERIMENTAL DESIGN

To study the contributions of ovaries and the placentae to progesterone biosynthesis, pregnant guinea pigs were sacrificed on days 10 (n = 5), 23 (n = 5), 30 (n = 5), and 43 (n = 13) of gestation. Both the ovaries and the placentae were removed, rinsed in saline, blotted dry, and stored below −20°C. The ovaries of seven nonpregnant guinea pigs were removed 5 days postpartum.

The effect of the Salk LHRH analog was studied in 96 guinea pigs at three different gestational ages: preimplantation, days 0 and 1; postimplantation, days 10 and 16, when the corpora lutea are indispensable; and gestational day 42, when the corpora lutea are already dispensable for maintenance of pregnancy. Of these 96 animals, 52 (experimental) received different doses of the peptide in corn oil by subcutaneous injection (s.c.) twice daily for 5 to 8 days. The remaining 44 control animals received 0.5 ml of the vehicle alone. The grouping of these animals and their treatment schedules are summarized in Table 11-1.

TABLE 11-1. Grouping of Experimental Animals and Their Treatment with [D-Trp^6-Pro^9-NEt]-LHRH

GROUP SYMBOL	NO. ANIMALS	GESTATION DAY	TREATMENT DAYS	TOTAL DOSE/ANIMAL μg
A	12	0*	8	2400 (2 × 150/day)
B	12	1	7	2100 (2 × 150/day)
C	12	10	5	750 (1 × 150/day)
D	5	10	7	2100 (2 × 150/day)
E	7	16	5	500 (2 × 50/day)
F	4	42	5	500 (2 × 100/day)

*Sperm-positive day (vaginal plug).

All animals that started receiving LHRH analog between day 0 and day 16 (Groups A–E) were sacrificed on day 21 of pregnancy, whereas those whose treatment was started on day 42 (Group F) were sacrificed on day 47 of pregnancy. Syntex LHRH-analog (2 × 50 μg/day) was given to three cycling and six pregnant guinea pigs, twice daily for 5 days.

COLLECTION OF SAMPLES

At autopsy, the weights of the fetuses and placentae were measured. Heart and uterine vein blood samples from all the experimental animals treated with Salk LHRH analog and their controls were collected into heparinized disposable syringes. Plasma was immediately separated, snap-frozen, and stored below −20°C. The ovaries and uterine and placental tissues were also stored below −20°C. In our study with the Syntex compound, 2-ml samples of ocular vein blood were taken immediately before, during, and after treatment.

HORMONE ASSAYS

Progesterone concentration was measured by radioimmunoassay using a specific antiserum raised in the rabbit against progesterone (11α-hemisuccinate)-BSA. Since the lipid-rich samples of the guinea pigs increased the within- and between-assay variability, a dual chromatography system was routinely used to isolate progesterone from both plasma and tissue samples prior to assay.[7] The levels of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) were measured following chromatographic separation of PGFM from PGF on silica gel (silicic acid) columns.[7]

STATISTICAL ANALYSIS

The differences in the hormone levels in the groups of control and experimental animals were analyzed by Student's *t* test, and the Chi-square test was used to determine the statistical difference in the percent normal pregnancies in the different groups.

RESULTS

The timing of the luteoplacental shift of progesterone biosynthesis for the maintenance of pregnancy in the guinea-pig is described in Figure 11-1. Before day 23 of pregnancy, the ovarian progesterone content gradually increased and far exceeded the placental content. After day 30, the ovarian progesterone content started to decrease and the placental progesterone content sharply increased, indicating the shift in progesterone biosynthesis from ovaries to the placentae for pregnancy maintenance. The functional significance of the corpora lutea was shown by our earlier finding that ovariectomy in the guinea pig before day 25 of gestation tips the endocrine balance and the animal aborts. However, if the ovariectomy was performed on day 30 of gestation, after the luteoplacental progesterone shift, the animals do not

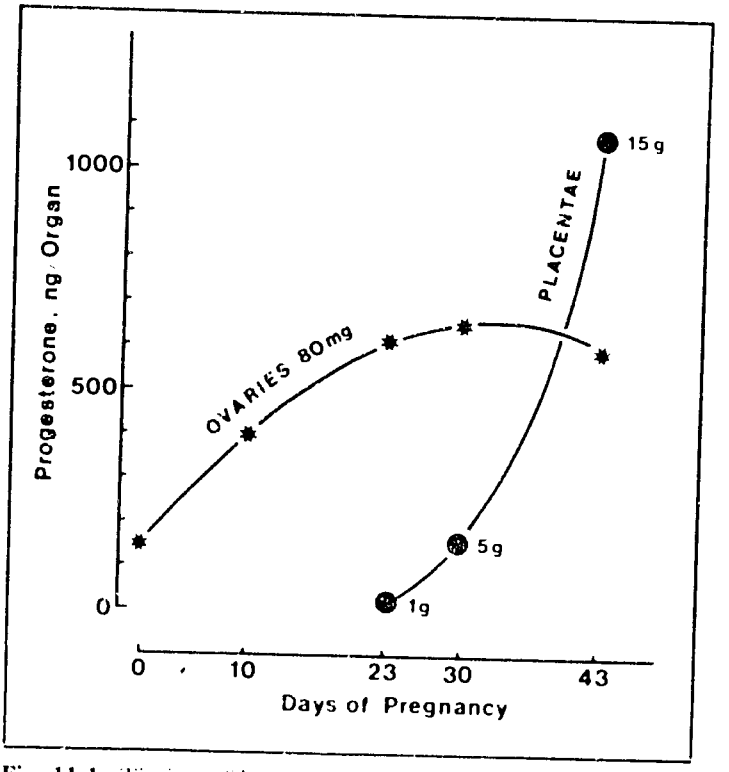

Fig. 11-1. Timing of luteoplacental shift in progesterone biosynthesis. Note that although weight of the two ovaries was constant—78.9 ± 5.1 mg (n = 35)—total placental weight of litter sharply increased from 1.0 ± 0.2 g (day 23) to 5.1 ± 0.5 g (day 30), and to 15.4 ± 1.8 g on day 43.

abort.[9] These studies determined the days on which the treatment could be started to examine the possible luteolytic and abortifacient action of LHRH analog in the guinea-pig model.

Table 11-2 illustrates that between days 0 and 42 of pregnancy, Salk LHRH agonist had no predictable action on pregnancy, despite repeated administration during 5 to 8 days of doses totaling 500 to 2400 μg in oil. The weights

TABLE 11-2. Failure of [D-Trp^{6}-Pro^{9}-NEt]-LHRH to Interrupt Pregnancy in Guinea Pigs*

GROUP SYMBOL	FETAL WEIGHT (G)		PLACENTAL WEIGHT (G)		% PREGNANT	
	Control	*Experimental*	*Control*	*Experimental*	*Control*	*Experimental*
A	0.17 ± 0.02	0.16 ± 0.02	0.99 ± 0.04	1.06 ± 0.05	58	67
B	0.16 ± 0.01	0.16 ± 0.02	0.91 ± 0.03	0.95 ± 0.04	66	75
C	0.11 ± 0.01	0.16 ± 0.02	0.77 ± 0.04	0.82 ± 0.06	100	92
D	0.09 ± 0.05	0.11 ± 0.02	0.60 ± 0.08	0.71 ± 0.04	100	100
E	0.11 ± 0.01	0.09 ± 0.01	0.77 ± 0.04	0.77 ± 0.03	100	100
F	15.30 ± 2.60	17.20 ± 3.10	3.70 ± 0.60	4.14 ± 0.70	100	100

*Autopsy on day 21, except in Group F, where the animals were sacrificed on day 47 of pregnancy.

TABLE 11-3. Progesterone Levels (ng/ml or g), after [D-Trp^6-Pro^9-NEt]-LHRH Treatment (2 × 50 μg/day) from Day 16 to 20 of Pregnancy*

HEART PLASMA	UTERINE VEIN PLASMA	UTERINE TISSUE	PLACENTAL TISSUE	OVARIAN TISSUE
		Vehicle Control (6)		
93 ± 15	121 ± 18	25 ± 5	18 ± 3	7518 ± 1210
		Experimental (7)		
117 ± 18	141 ± 14	22 ± 6	16 ± 5	8650 ± 980

*All values are mean ± S.E.M. Animals were sacrificed at day 21.

of fetuses and placentae in the control and experimental animals were similar. This indicated that during tubal migration of the fertilized ovum and after implantation, both before and after the luteoplacental shift in progesterone biosynthesis, this compound has little therapeutic promise.

Table 11-3 shows no significant difference in progesterone levels in heart and uterine vein plasma, uterus, placenta, and ovaries of control and experimental animals treated from day 16 to 20 of pregnancy (Group E). Similarly, in the other groups of animals autopsied either on day 21 or 47 of gestation, progesterone levels were identical in the control and LHRH-treated groups. The levels of PGF and PGFM in the uterine vein plasma of the experimental animals were unchanged from the values in the normal pregnant control animals (Table 11-4).

Figure 11-2 shows the progesterone levels in guinea pigs treated with Syntex LHRH analog from day 3 to 7 of ovulation and during days 7 to 11 of pregnancy. During the normal estrus cycle (Panel A, Normal) the plasma progesterone levels gradually increased until day 10 postovulation before the levels started to decline, whereas in one treated animal decrease in the progesterone levels was observed on day 7 of ovulation. The administration of this analog from day 7 to 11 of pregnancy (Panel B) caused a decrease in progesterone levels in two of the three animals studied. One of these guinea pigs aborted 90 hours after the initiation of the treatment. The administration of Syntex analog in three guinea pigs from day 20 to 24 of gestation had no effect on the levels of progesterone, and the pregnancy was intact in these animals.

DISCUSSION

Our studies indicate that [D-Trp^6-Pro^9-NEt]-LHRH had no impact on pregnancy in the guinea pig model, not even at high and repeated daily doses.

TABLE 11-4. PGF and PGFM Levels after [D-Trp^6-Pro-^{9}-NEt]-LHRH Treatment (2 × 50 μg/day) from Day 16 to 20 of Pregnancy*

	UTERINE VEIN PLASMA, NG/ML	
	PGF	PGFM
Control (6)	0.53 ± 0.10	2.41 ± 0.31
Experimental (7)	0.42 ± 0.08	3.17 ± 0.15

*All values are mean ± S.E.M. Animals were sacrificed on day 21.

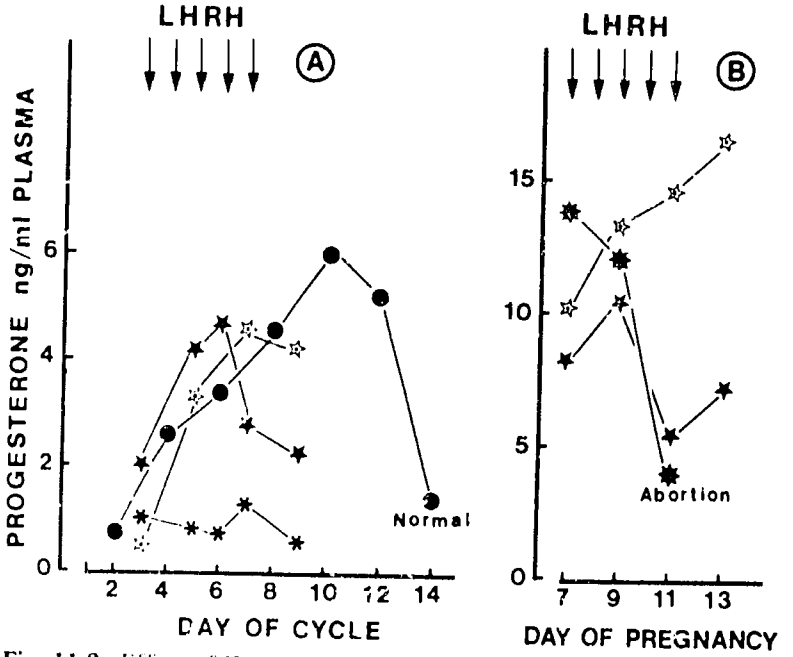

Fig. 11-2. Effect of [D-6-(2 Naphthyl-alanine)]-LHRH on progesterone levels in ocular vein plasma of cycling and pregnant guinea pigs. Panel A indicates mean progesterone levels of five animals during postovulatory period (normal) and effect of LHRH treatment twice daily (2 × 50 μg) from day 3 to 7 of ovulation on three animals. Note decrease in progesterone levels on day 7 in one treated animal. Panel B indicates progesterone levels in guinea pigs treated from day 7 to 11 of pregnancy.

The fetal and placental weights and the levels of progesterone, PGF, and its stable metabolite (PGFM) were almost identical in the control and experimental animals. These observations indicate that this LHRH analog, at the dose levels studied, has little therapeutic promise if given during tubal migration of the fertilized ovum or after its implantation, both before and after the luteoplacental shift in progesterone biosynthesis. On the contrary, the higher (though statistically not significant) ovarian progesterone content observed in the LHRH analog-treated animals (2 × 50 μg/day), having lower litter size (4.6 ± 0.7) than the controls (5.8 ± 0.6), could be interpreted as an indication of the drug's luteotropic action.

Our preliminary observation that [D-6-(2-Naphthyl-alanine]-LHRH lowered peripheral progesterone levels in nonpregnant and early pregnant guinea pigs suggests that this analog might be luteolytic in these animals.

Since the progesterone levels were normal and pregnancy remained intact when three guinea pigs were treated after day 20 of pregnancy, the luteolytic action of Syntex LHRH analog appears limited to only a few days during the cycle and very early pregnancy. However, the results obtained from a pilot study using a small number of animals must be interpreted with caution and needs to be verified in a larger study. The difference in the response obtained with the two different analogs in the guinea pig could be attributed to the

difference in their potencies or the degree to which structural modifications of these analogs augment the secretory response of the endocrine gland.

Studies with LHRH analogs are consistent in showing their luteolytic and abortifacient actions in rats.[14,21,22] However, the effects observed in primates are not always antireproductive in inherence. Synthetic LHRH and its analog [D-Ala, des-Gly^{10}]-LHRH, which increased the serum LH levels in rats, were ineffective in inducing considerable rise of serum LH in the rhesus and bonnet monkeys.[1,17] Similarly, Salk LHRH analog, which is a potent abortifacient in rats,[21] failed to interrupt pregnancy in the human.[6] Since this analog did not affect pregnancy in the guinea pigs, it indicates that the guinea pig and the primates might have similar responses to LHRH treatment.

In the human, LHRH and its analog [des-Gly-NH_2^{10}, proethyl amide]-LHRH had no significant effect on luteal phase length, if given immediately following ovulation.[15,16] However, when given late in the luteal phase, the luteolytic effect was more pronounced.[16] On the other hand, in the monkeys luteolysis was evident when [D-Trp^6]-LHRH was injected on day 3 to 5 of ovulation, and had no effect when the treatment was delayed to day 7 post-ovulation.[2] In the guinea pigs also, Syntex LHRH analog curtailed the corpus luteum function, as indicated by the decrease in peripheral plasma progesterone levels, if given during the estrus cycle or in very early pregnancy, but had no effect when the treatment was delayed until day 20 of gestation.

These observations indicate that LHRH and its agonistic analogs have a luteolytic potential, if given during the "critical" phase of the menstrual cycle or in early pregnancy. The intraspecies variations reported with different LHRH analogs could be due to the different experimental designs used to study the drug response. The interspecies variation in the LHRH response indicates that these peptides might have different site(s) of action. Their antifertility effects could be due to the loss of gonadotropin receptors in the gonads by a sustained release of gonadotropins[12,18,19] or to their extrapituitary effects on the gonads to inhibit steroidogenesis.[3]

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12

Discussion: Physiology and Contraceptive Effects of LHRH and Analogs in Female Animals: Agonists

KAMRAN S. MOGHISSI, MODERATOR

Isolation and synthesis of LHRH in 1971 were soon followed by the development of potent agonist and antagonist peptides in the hope that these compounds might prove to be useful for stimulation or inhibition of gonadotropin release and ovulation.

The preceding presentations have focused on the many effects of agonistic analogs of LHRH in experimental animals. Originally it was believed that LHRH and its agonists might be useful for induction of ovulation and enhancement of fertility. Indeed, these compounds are capable of releasing pituitary LH and FSH and inducing ovulation in a variety of animal models. Early studies, however, established that agonistic peptides were also potent inhibitors of the reproductive process. Depending on the species used, dosage given, and mode and frequency of administration, these peptides have exhibited paradoxical effects such as anovulation, luteal phase deficiency, and termination of established pregnancies.

The antireproductive property of these analogs is directly related to their potency. In general, the greater the agonistic activity, the greater the antifertility property.

Administration of a parent LHRH molecule and a large array of agonists pre- and postimplantation to inseminated rats inhibits pregnancy. Extensive studies utilizing numerous agonists in several other animal models have demonstrated predictable postcoital contraceptive effects.

Supportive evidence for paradoxical antifertility effects of LHRH agonists comes also from other studies which show that this class of peptides has the ability to retard puberty, disrupt estrous cycle, initiate premature ovulation, induce luteolysis, cause ovarian and uterine regression, reduce fertility in inseminated animals, and inhibit the ovarian/uterine stimulation produced by hCG. These effects are reversible, and when treatment has been discontinued or the pregnancy terminated, normal reproductive processes are promptly

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Panelists: Ricardo H. Asch, V. Daniel Castracane, Alan Corbin, Brian H. Vickery. *Discussants:* William F. Crowley, Jr., Horacio B. Croxatto, Fernand Labrie, Hugo Maia, Jr., Ehard F. Nutting, Khairia Omran, Jurgen K. Sandow, Manfred Schmidt-Gollwitzer

restored. Thus, even though these peptides are known to release gonadotropins, to stimulate ovulation, and to promote fertility, their antifertility properties, which have been amply documented, make them attractive candidates as possible contraceptives.

A series of elegant studies in rhesus monkeys by Knobil and his associates[1] have shown that gonadotrophs of the intact pituitary respond to unvarying pulsatile GnRH stimulation originating from the arcuate nucleus of the hypothalamus by releasing pulses of FSH and LH. Immature follicles in the ovary respond to this unvarying gonadotropic stimulation by increasing in size and secreting increasing quantities of estradiol, which in turn causes the discharge of the preovulatory gonadotropin surge, when it exceeds a critical threshold.

Knobil[1] has demonstrated that the administration of an unvarying pulsatile GnRH replacement in rhesus monkeys with a hypothalamic lesion (arcuate nucleus) that have abolished endogenous GnRH production reestablishes normal ovulatory cycle. When intermittent GnRH replacement regimen was changed to a continuous infusion of the peptide, gonadotropin secretion was inhibited. This inhibition was gradually reversed when the pulsatile mode of GnRH administration was reinstituted. Alterations of frequency, or amplitude of pulsatile administration of GnRH in these studies, resulted in various disturbances in the pattern of LH, FSH, and their ratios.

These experiments clearly demonstrate that administration of LHRH and its analogs in a nonpulsatile fashion or in excessive amounts invariably leads to the phenomenon of "down-regulation" and a state of hypogonadotropic hypopituitarism and all of the expected effects of absent or decreased sex steroids on the target organs.

In the female, LHRH agonistic analogs produce large increments in serum LH levels, distorted FSH patterns, inappropriate prolactin secretion, and decreased concentration of gonadotropin and prolactin receptors in the ovary. These changes are associated with luteolysis, impaired steroidogenesis, delayed estrogen secretion, decreased progesterone secretion, and elimination of uterine and/or placental support resulting either in infertility or inhibition and/or termination of pregnancy.

Several investigators have reported also findings suggestive of extrapituitary effects of these analogs. In hypophysectomized pregnant rats, administration of LHRH or agonists leads to pregnancy failure, implying a direct effect on either the ovary, placenta, or both. Other studies have demonstrated that the placenta contains LHRH and is capable of synthesizing this peptide. LHRH agonists can block follicular maturation and ovulation and reduce ovarian FSH/LH receptors induced in hypophysectomized immature rats by exogenous gonadotropins. The aromatization of androgens to estrogens by monolayer cultures of granulosa cells can be inhibited by LHRH and its agonists. Further evidence for the recognition of these peptides by the ovary has been provided by demonstrating that LHRH and several agonists can induce ovulation in hypophysectomized proestrous rats in a dose-related manner.

Questions were raised about ovulation occurring following LHRH analog administration in hypophysectomized rats. In particular it was pointed out that in many instances pars post-tuberalis of the pituitary, which is supradiaphragmatic, remains intact after hypophysectomy. Experiments have also

been carried out in which the growth curve of male rats has been observed following hypophysectomy. Initially, the growth of the treated animals was arrested. Six weeks after hypophysectomy immunoreactive LH and FSH were recovered from hypothalamic fragments using a method that is usually used for the extraction of LHRH. This suggests that with large doses of agonists it is possible to mobilize the LH from the remnant of the pars tuberalis of the pituitary. An approach to determine whether incomplete hypophysectomy may be responsible for ovulation occurring in hypophysectomized animals treated with agonists is to measure the LH shortly (1 hour) after the administration of an analog.

In response to these critiques it was indicated that in hypophysectomy experiments LH assays were performed at frequent intervals for 3 hours. Thus incomplete hypophysectomy could not explain ovulation induction with agonistic peptides in these animals. Admittedly the major mechanism by which LHRH agonists exert their effect is through the pituitary–gonadal axis. However, there is considerable evidence that ovulation in hypophysectomized animals is not due to mobilization of LH, but is the result of an extrapituitary LH release. Receptor sites for LHRH are present in other tissues and it is possible that pharmacologic doses of analogs may elicit the release of LH from some of these sites. It is not known whether endogenous LHRH is released in sufficient amount into general circulation (as opposed to the portal system) to stimulate the release of LH from extrapituitary sites under normal conditions.

Administration of LHRH agonists to rhesus monkeys 3 or 5 days postovulation was found to cause a significant shortening of the luteal phase and decrease in serum progesterone values. These effects were not observed when the peptide was administered on day 7 postovulation. Administration of hCG prevented the shortening of the luteal phase in animals treated with the analog. When the analog and hCG were injected simultaneously, no alteration of the luteal phase was observed.

In vitro experiments using corpora lutea tissue have shown that LHRH analogs ([D-Trp-]-LHRH) are incapable of stimulating progesterone synthesis. These findings cast doubts on the possibility of a direct action of the analogs on the corpus luteum cells for its luteolytic activity. The luteolytic effect of the analogs may be mediated by drug induced-alterations in pituitary gonadotropic secretion. However, local infusion of the analog into the ovarian circulation of hypophysectomized monkeys in whom ovulation was induced with gonadotropin did not affect the length of the luteal phase or the level of serum progesterone concentration. Finally, attempts to identify LHRH receptors in the crude plasma membrane preparation of rhesus monkey corpora lutea have not been fruitful.

In one study, administration of the agonist (Wy 40972) to baboons at the early or midluteal phase of the cycle resulted in an inconsistent luteolytic response, most animals showing a shortening of the luteal phase and/or decline in plasma progesterone concentration. Attempts to interrupt pregnancy in the baboon with the analogs were discouraging. Merely increasing the dosage of analogs to achieve abortifacient action was not effective.

In another study, a reasonably good abortifacient effect was observed. The termination of pregnancy in this study was associated with a marked decline

in blood progesterone levels. Examination of abortoses resulting from successful termination of pregnancy in baboons has not revealed any teratogenic effect. However, because of the small number of animals in these studies, further evaluation is required. There are also some data available on the effect of suboptimal doses of LHRH analogs given to baboons for ovulation inhibition which resulted in escaped pregnancies. Here again no teratogenic effect was observed in the offspring.

Subcutaneous implantation of compacted cholesterol pellets containing an LHRH agonist in baboons on the first or second day of the menstrual cycle resulted in delay of ovulation by about 2 weeks. Similar findings have been reported in stumptailed macaques and in women. Longer-term studies in both rhesus and stumptailed macaques have shown that 6 to 7 months' continuous treatment bring about suppression of ovulation. A rapid return of ovulation follows cessation of treatment and is succeeded by a normal luteal phase. The ovulation and fertility suppression is associated with amenorrhea or breakthrough bleeding. Endocrine studies indicate undetectable levels of LH, inconsistent changes in FSH and suppression of pre-ovulatory surge of estradiol. These results are suggestive of down-regulation of both pituitary and ovarian function. LHRH receptors in the ovaries of the baboon have not been identified as yet, but studies for their identification are in progress.

In women, results comparable with those in subhuman primates have been observed. Administration of LHRH analogs orally, parenterally, or intranasally, and in different dosages, consistently inhibited the ovulation. Irregular breakthrough bleeding or amenorrhea has been commonly observed. Ovulation usually returns within 4 weeks

In the baboon, analogs given in two injections of up to 50 μg/day 8 to 9 days before the expected onset of menses showed no change in luteal phase length or circulating levels of progesterone. However, studies using implanted pellets 4 days after ovulation show a clear cut luteal suppression and premature menstruation. When baboons were given ten daily injections of different doses of the analog, a significant dose-related suppressive effect on luteal phase progesterone was observed.

Very similar data have been reported for women who received the agonist [D-Ser(TBU)]-LHRH ethylamide intranasally 0 to 2 days after ovulation and continuing until onset of menses, whereas injections starting 5 to 7 days after ovulation had no effect on luteal phase progesterone and appeared to extend luteal phase duration. This indicates that the timing of onset of treatment was critical. The rise of plasma estrone in treated baboons shown in some studies may be responsible for luteolysis, since the luteolytic effect of estrogen in the primates has been documented.

Considered together, the data indicate that the prognosis for LHRH agonists as ovulation suppressants is good. The potential drawbacks consist of resulting amenorrhea, irregular bleeding, possibility of menopause-like state or, alternatively, the danger of unopposed estrogen. Another disadvantage is the lack of biologic activity when these peptides are administered orally. Fortunately, the extreme potency of "super" agonists allows nasal administration. It may be also feasible to adminster these peptides by vaginal routes or in the form of implants. Data on the development of hypo- or hyperestrogen state are scanty.

Another concern with chronic use of agonistic peptides is the development of endometrial abnormalities resulting from unopposed effect of estrogen in prolonged anovulatory state, as demonstrated in a later presentation in this workshop. Endometrial pathology is more likely to occur when the absence of progestational effect (anovulation) is associated with hyperestrogen state.

The use of LHRH analogs for follicular luteinization without ovulation or for induction of luteal phase effects does not appear promising.

Finally, the interceptive (menstrual-induction ability and termination of early pregnancy) properties of agonistic peptides, though promising in some experimental animals, remains an area of controversy since preliminary studies in humans show little promise.

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PHYSIOLOGY AND CONTRACEPTIVE EFFECTS OF LHRH AND ANALOGS IN FEMALE ANIMALS: ANTAGONISTS

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13

GnRH Antagonists: Physiologic and Contraceptive Applications in the Female Rat

CATHERINE RIVIER

JEAN RIVIER

WYLIE W. VALE

GnRH is of physiologic significance in regulating reproductive functions. Experiments have shown that active or passive immunization to GnRH results in infertility and gonadal atrophy in both male and female animals.[1,2,7,8,15,20,22] Another approach to investigating some of the functions of the endogenous GnRH has used the administration of GnRH antagonists during various phases of the reproductive cycle.

A large number of GnRH antagonists have been synthesized by several groups in recent years[27,35] Though many of the early modifications of the decapeptide GnRH yielded antagonists that could only block ovulation in acute experiments, the first peptide that was potent enough to be used in long-term *in vivo* studies was [D-pGlu[1],D-Phe[2],D-Trp[3,6]]-GnRH, which was shown to inhibit pregnancy in the rat.[10] Subsequently, much more potent and long-acting antagonists have been developed and used to inhibit reproductive functions in both male[29,30] and female[31,32] rats.

At this time, GnRH antagonists have been administered before and during puberty, during the estrous cycle, and at various times of pregnancy. The following sections present our studies of the acute and chronic administration of GnRH antagonists to the female rat.

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MATERIALS AND METHODS

Two GnRH antagonists were used:

Antagonist I: Ac-Δ^3-Pro^1-pCl-D-Phe-D-Trp-Ser-Tyr-D-Trp-N$^\alpha$Me-Leu-Arg-Pro-Gly-NH_2

Antagonist II: Ac-Δ^3-Pro^1-pF-D-Phe-D-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2

Sprague-Dawley female rats (180–200 g) were maintained under a 14-hour light, 10-hour dark regimen and fed Purina Chow and water *ad libitum*. When required, ovariectomy was performed under ether anesthesia 3 weeks before the experiment. Studies of the estrous cycle used only animals which had exhibited at least three consecutive 4-day cycles. For fertility experiments, intact animals were allowed to adjust to the environment for one week and were then caged individually with a male of proven fertility. Mating was assessed in the morning by the presence of a vaginal plug and of sperm in the vaginal lavage, this time being designated day one of pregnancy. Blood samples were obtained periodically under light ether anesthesia. The outcome of pregnancy was monitored by the length of gestation and the number of delivered pups.

Plasma-prolactin (PRL) levels were measured with the NIAMDD kit and expressed in terms of rat-PRL-RP1 standard. Plasma progesterone (P_4) levels were measured as described by Rivier and Vale.[32]

Both antagonists, synthesized as described by Rivier and others,[27] were dissolved in corn oil by extensive vortexing and injected subcutaneously (sc) in a volume of 0.2 ml. Unless otherwise mentioned, Antagonist I was administered at a 5-mg/kg biweekly (BW) dose and Antagonist II at a 1-mg/kg BW dose.

TIME–COURSE OF ACTION OF GnRH ANTAGONISTS *IN VIVO*

We have used two approaches to gain an appreciation of the duration of action of the antagonists.

DURATION OF ACTION OF ANTAGONIST II IN OVARIECTOMIZED FEMALE RATS

The time-course of action of Antagonist II on LH secretion was evaluated in gonadectomized rats. A catheter was placed in the jugular vein of the animals in order to allow blood sampling in freely moving animals. Corn oil or 200 μg Antagonist II was injected sc and blood samples were obtained over a 24-hour period. As illustrated in Figure 13-1, this antagonist significantly inhibited LH production for at least 24 hours. Similar results were obtained with a larger dose of Antagonist I (not shown). These data provided evidence that LH secretion could be consistently suppressed by a single daily administration of the peptides.

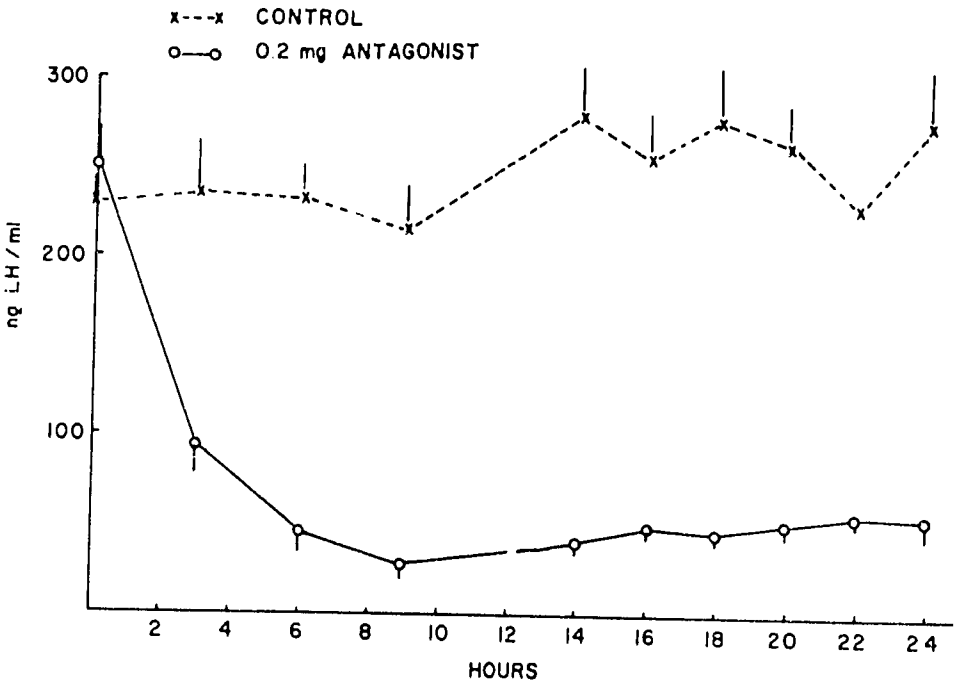

Fig. 13-1. Duration of Antagonist II action on LH secretion in ovariectomized female rats. Cannulated ovariectomized female rats received corn oil (x---x) or 200 μg (Antagonist II) (o---o). LH levels were measured over a 24-hour period. Each point represents the mean ± S.E.M. of six animals.

ABILITY TO BLOCK OVULATION WHEN ADMINISTERED 36 HOURS BEFORE OVULATION

Another test consists of the administration of the antagonists on the morning of diestrus-II. A blockade of ovulation was interpreted to indicate that the peptides inhibited the proestrus LH surge normally occurring 36 hours after their administration. Such an experiment is illustrated in Table 13-1. In this case, comparison of the effect of the antagonist injected either on proestrus or on diestrus-II indicates that while 20 μg Antagonist I totally blocked ovulation when given on proestrus, approximately 1 mg antagonist was necessary to block ovulation when administered on diestrus-II. Since we had observed that 1 mg of the same peptide would interfere with LH secretion for at least 24 hours, it is possible that either the effect of this dose of peptide did not last up to 36 hours, and/or that enough circulating LH was present for ovulation to take place.

ACUTE EFFECT OF GnRH ANTAGONISTS ON OVULATION

The ability of GnRH antagonists to interfere with the events leading to ovulation was tested in several ways.

TABLE 13-1. Effect of [Ac-Δ^3-Pro^1,pCl-D-Phe^2,D-Trp^{3,6},N$^{\alpha}$Me-Leu^7]-GnRH (Antagonist I) on Ovulation

TREATMENT	TIME OF INJECTION	NO. RATS OVULATING
Control	Proestrus	10/10
10 μg Antagonist I	Proestrus	4/10
20 μg Antagonist I	Proestrus	0/10
Control	Diestrus-II	8/8
0.2 mg Antagonist I	Diestrus-II	2/6
1.0 mg Antagonist I	Diestrus-II	0/7

ABILITY OF THE ANTAGONISTS TO BLOCK OVULATION

The test used most widely consists in administering the peptides to regularly cycling adult female rats at noon on the day of proestrus. On the following morning the ovaries are dissected free of fat, the oviducts are flushed and the presence of ova is checked under a microscope.

Table 13-2 illustrates data obtained in this test with five potent antagonists. With the exception of [Ac-Δ^3-Pro^1,pCl-D-Phe^2,D-Trp^{3,6},N$^{\alpha}$Me-Leu^7]-GnRH, the other four peptides completely blocked ovulation at a 10-μg dose.

ABILITY OF THE ANTAGONISTS TO INHIBIT THE PROESTRUS LH SURGE

Normally cycling adult female rats exhibit a surge of LH secretion occurring between 4:00 and 8:00 p.m. on proestrus, and this surge is believed to induce ovulation in the rat. We therefore tested the effect of corn oil or two doses of Antagonist I injected on noon at proestrus, on plasma LH levels measured 4.5 hours later. The 10-μg dose was chosen because it had been shown to block ovulation in only part of the animals, and could therefore allow comparison between inhibition of LH release and inhibition of ovulation. As illustrated in Table 13-3, 10 μg Antagonist I inhibited ovulation in 12 of 26 rats. Though both groups of animals had plasma LH levels that were significantly lower than those of controls, the antagonist-treated rats that did ovulate had higher LH values than those that did not. All rats receiving 20 μg Antagonist I failed to ovulate and showed a marked inhibition of LH secretion.

TABLE 13-2. Effect of GnRH Antagonists on Ovulation

GnRH ANTAGONISTS	NO. RATS OVULATING	
	5 μg at Proestrus	*10 μg at Proestrus*
[Ac-Δ^3-Pro^1,pCl-D-Phe^2,D-Trp^{3,6},N$^{\alpha}$Me-Leu^7]-GnRH	4/8	4/8
[Ac-Δ^3-Pro^1,pCl-D-Phe^2,D-Trp^{3,6}]-GnRH	2/11	0/10
[Ac-Δ^3-Pro^1,pF-D-Phe^2,D-Trp^{3,6}]-GnRH	0/10; 3/10; 4/8	0/10
[Ac-Δ^3-Pro^1,pNO_2-D-Phe^2,D-Trp^{3,6}]-GnRH	3/9	0/10
[Ac-Δ^3-Pro^1,3,4-Cl_2-D-Phe^2,D-Trp^{3,6}]-GnRH	4/7	0/10

TABLE 13-3. Effect of [Ac-Δ^3-Pro^1,pCl-D-Phe^2,D-Trp^{3,6},N$^{\alpha}$Me-Leu^7]-GnRH (Antagonist I) on the Proestrus LH Surge

TREATMENT	NO.	PLASMA LH LEVELS (NG/ML)
Control	8	375 ± 67
10 μg Antagonist I (ovulating)	14	162 ± 36
10 μg Antagonist I (nonovulating)	12	90 ± 11
20 μg Antagonist I (nonovulating)	8	72 ± 6

SHORT AND LONG-TERM EFFECT OF GnRH ANTAGONISTS ON THE ESTROUS CYCLE

In a first series of experiments, a single 200-μg dose of Antagonist II was administered to regularly cycling adult female rats starting on diestrus II. Subsequent vaginal smears were recorded for the next 16 days. This treatment interfered with cyclicity in 50% of the animals for the next 10 days; within 12 days all rats had resumed normal cycles (Fig. 13-2A). When the same amount of peptide was injected every fourth day (also starting on diestrus II), cyclicity was totally blocked 2 days after the second injection in all animals and did not resume over the next 3 weeks of treatment (Fig. 13-2B). The vaginal smears of these animals showed aberrant alternate patterns of leukocytic and cornified cells, with several periods of prolonged estrus. Since prolonged estrus is characterized by the failure of follicles to rupture, it can be inferred that the antagonist treatment was accompanied by elevated numbers of preovulatory follicles for extended periods of time. Whether this is at least in part due to inhibition of FSH secretion remains to be determined.

In an attempt to determine the amount of the antagonist necessary to completely block the estrous cycle, a first dose of 50 μg Antagonist I was injected daily for 8 days; as illustrated in Table 13-4, this treatment interfered with 40% of the cycles. When the dose was increased to 200 μg daily in the same animals, all cycles were disrupted. These data seem to indicate that the dose of the antagonist required to interfere with the estrous cycle on a long-term basis is larger than that capable of acutely blocking ovulation and that inhibiting LH release in short-term experiments. It can be postulated that

TABLE 13-4. Effect of Daily Treatment with 50 μg or 200 μg [Ac-Δ^3-Pro^1,pCl-D-Phe^2,D-Trp^{3,6}, N$^{\alpha}$Me-Leu^7]-GnRH (Antagonist I)

TREATMENT	NO. REGULARLY CYCLING RATS
Control days 1–20	10/10
50 μg Antagonist I, days 1–8*	6/10
200 μg Antagonist I, days 9–20	0/10

*Antagonist I was first given at a 50-μg daily dose for days 1 through 8 then at a 200-μg daily dose for days 9 through 20.

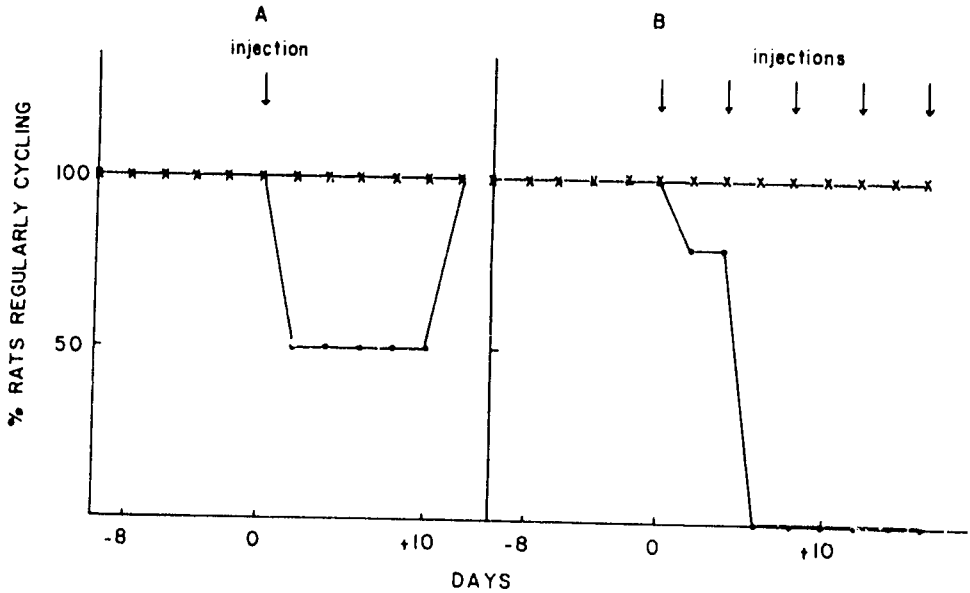

Fig. 13-2. Short- and long-term effects of Antagonist II on estrous cycle. (*A*) Corn oil or one 200-μg dose of Antagonist II was administered on diestrus-II to regularly cycling rats. Daily vaginal smears were obtained for three cycles before as well as for 12 days after the injection. Each point represents ten animals (X = control; ● = antagonist). (*B*) Multiple injections of corn oil or 200 μg Antagonist II.

the blockade of ovulation only requires that the LH surge occurring in the afternoon of proestrus be abolished, while a long-term effect of the antagonist necessitates a 24-hour complete inhibition of gonadotropin secretion.

EFFECT OF GnRH ANTAGONISTS ON THE OCCURRENCE OF PUBERTY

An enhanced capability of the hypothalamus to release GnRH and an increase in responsiveness of the ovaries to gonadotropin, as well as a progressive elevation in PRL secretion, are believed to actively participate in the maturational process that leads to puberty.[33] However, the interrelated events responsible for the onset of puberty are still poorly understood.

In an effort to get additional information on the hormonal requirement of puberty, we injected prepubertal (26-day old at the beginning of the experiment) female rats with corn oil or 0.5 mg Antagonist I for 20 days. Blood

TABLE 13-5. Effect of Daily [Ac-Δ^3-Pro^1,pCl-D-Phe^2,D-Trp^{3,6},N$^{\alpha}$Me-Leu^7]-GnRH (Antagonist I) on the Onset of Puberty

TREATMENT	NO.	DAY OF VAGINAL OPENING
Control	10	34.8 (31–40)
0.5 mg Antagonist I	5	35.8 (31–42)
0.5 mg Antagonist I	5	> 46

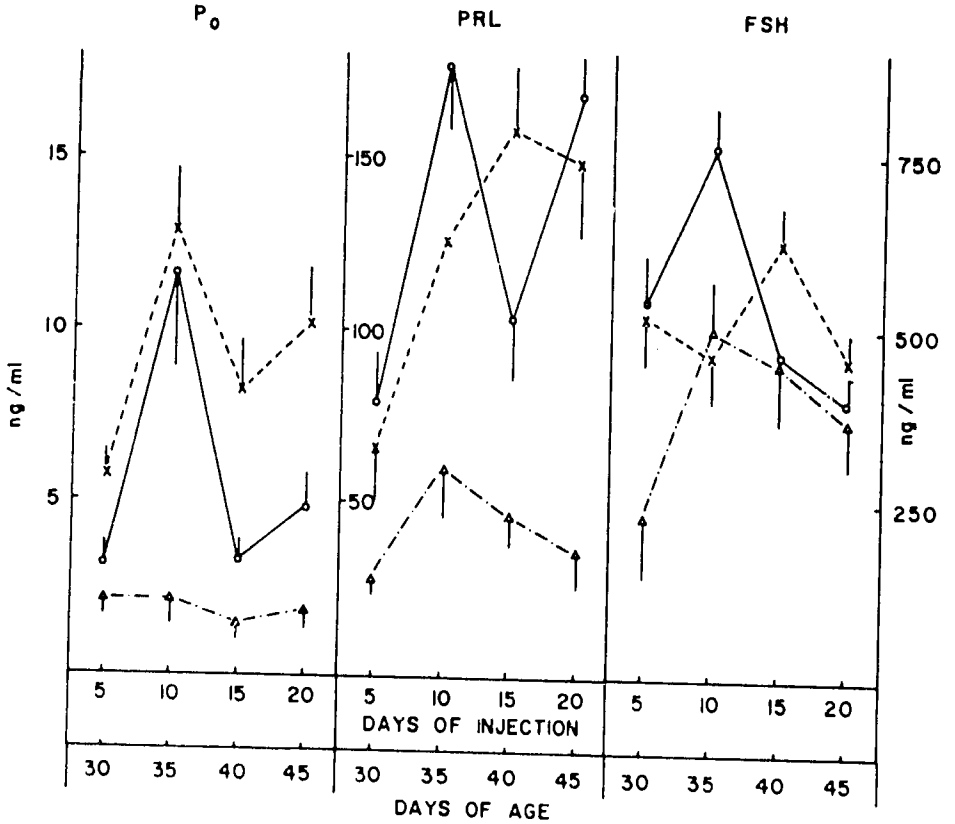

Fig. 13-3. Effect of Antagonist I on onset of puberty. Corn oil or Antagonist II was injected daily to immature female rats, starting at day 26 of age. (x---x = Control; o---o = antagonist-treated rats showing no delay of vaginal opening; Δ---Δ = antagonist-treated rats showing delay of vaginal opening while being treated.)

samples were obtained periodically and vaginal opening was checked daily. As illustrated in Table 13-5, under this treatment regimen, 50% of the treated rats had an onset of puberty comparable to that of control animals, while the other 50% showed no vaginal opening at the end of the experiment (day 46). In those rats in which vaginal opening was delayed, both PRL and P_0 secretion was inhibited, while plasma FSH levels were only slightly lowered (Fig. 13-3). In the absence of E_2 measurements, we could not determine the relative importance of this hormone, but our data support the importance of PRL in the maturational process—a role which is reported to be linked to an enhancement of ovarian responsiveness to gonadotropins.

EFFECT OF GnRH ANTAGONISTS ON CONCEPTION

As shown, intermittent treatment with GnRH antagonists can disrupt the estrous cycle. Is this event accompanied by infertility? In order to study this question, six adult female rats received 200 μg Antagonist II every fourth

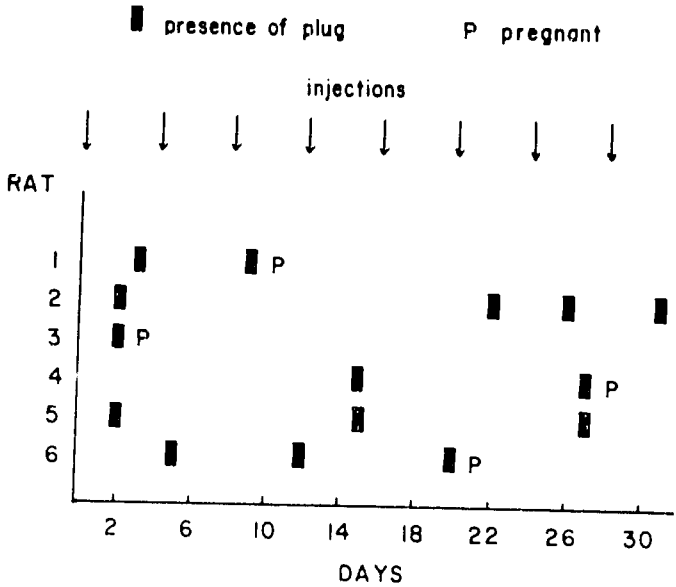

Fig. 13-4. Effect of intermittent administration of Antagonist II on mating behavior and fertility. Six regularly cycling adult Sprague-Dawley rats (180–200 g) received 200 μg Antagonist II every fourth day for 4 weeks. They were then caged individually with males of proven fertility while treatment was continued. The presence of a plug in the bedding was recorded, and mating was confirmed by sperm in the vaginal lavage. Treatment was continued and males were kept with females until pregnancy was established by palpation. Arrows indicate times of injection.

day for 4 weeks while being caged alone. They were then caged with a male each while the treatment was continued. The presence of a plug in the bedding was checked daily, and if present, the occurrence of mating was confirmed by the presence of sperm in the vaginal lavage. The females were removed from the male only after pregnancy could be established by palpation. As illustrated in Figure 13-4, all six females mated, though on an irregular basis. Pregnancy (as monitored by the delivery of healthy pups) occurred in four of six animals over a 30-day period. These data indicate that the intermittent administration of an antagonist, while compatible with active mating behavior, can disrupt the cycle and is accompanied by a certain degree of infertility. By contrast, daily administration of Antagonist I, which completely stopped cyclicity (see Table 13-4), resulted in the total absence of mating behavior (not shown).

EFFECT OF GnRH ANTAGONISTS ON MAINTENANCE OF PREGNANCY

As previously reported,[31,32] administration of GnRH antagonists interfere with pregnancy. The analogs appear to be more effective during the later days (8–12) than earlier days (1–7) of gestation. This is illustrated in Figure

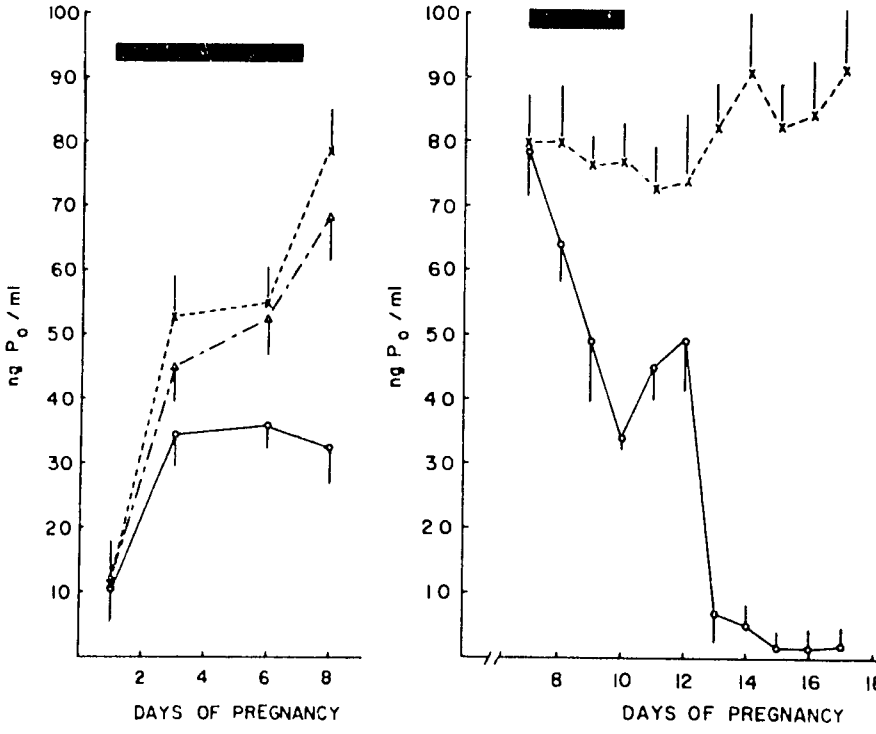

Fig. 13-5. Effect of Antagonist II on plasma progesterone P_o levels during pregnancy. The experiment was divided in two halves. (*Left*) Corn oil or Antagonist II was injected once daily from day 1 through 7 of pregnancy (black bar indicates period of treatment). Blood samples were obtained under ether anesthesia before each injection on days 1, 3, 6, and 8. Length of pregnancy and number of pups were recorded. (x---x = Corn oil; Δ---Δ = antagonist-treated animals who had a normal pregnancy; ○---○ = antagonist-treated animals who aborted.) Each point represents the mean ± S.E.M. of six (x) or three (Δ,○) rats. (*Right*) Corn oil or Antagonist II was administered from days 7 through 10 of pregnancy. The animals were bled as described. (x---x = Corn oil; ○---○ = antagonist-treated animals; all six aborted.) Each point represents mean ± S.E.M. of six rats.

13-5. Daily injections of Antagonist II during the first 7 days after mating result in a 50% inhibition of pregnancy. While antagonist-treated rats who did not abort had plasma P_o levels comparable to those of control rats, termination of gestation was accompanied by a drop in P_o secretion. Administration of Antagonist II from day 8 through 12 after mating was accompanied by a 100% rate of fetal resorption and a marked drop in P_o production.

Measurements of plasma PRL levels have indicated that they were not significantly lowered in antagonist-treated rats who received the peptide during the first week of pregnancy. Since PRL represents the major luteotropic stimulus in early gestation,[9,18,21,33] the relative inefficacy of the peptide during this time could be at least partially explained on this basis. Additionally, GnRH antagonists do not significantly lower estrogen production in early gestation,[32] a hormone required for implantation.[25] By contrast, the sharp

drop in plasma P_4 and LH observed when Antagonist II was administered on individual days encompassing the day 8 through day 12 period,[32] is paralleled by resorption of developing fetuses. Such data are in good agreement with previously reported studies showing that administration of antibodies against GnRH, LH, or P_4 around day 10 of pregnancy is deleterious to the fetuses[6,12,17,19,22,25] and support the recognized importance of these hormones as luteotropic hormones during this time of pregnancy.

In conclusion, results obtained in pregnancy experiments emphasize the temporal relationship between the efficacy of GnRH antagonists to terminate pregnancy and the antagonist's induced changes in hormonal secretion. In particular, their midgestation abortifacient action appears to be directly linked to their ability to lower progesterone secretion. The exact site of action of GnRH antagonists, however, remains to be determined. There is considerable evidence that GnRH agonists can act at the level of the gonads and uterus to inhibit reproductive processes.[3-5,10,14,16,24,26,34] Additionally, GnRH antagonists have been shown to compete with radiolabeled GnRH agonists for binding to gonadal membrane fractions[33] and to antagonize the effect of GnRH on luteal cells in culture.[11] We therefore cannot rule out the possibility that some of the consequences of antagonist administration were in part mediated through nonpituitary receptors. However, whatever their mechanisms of action, GnRH antagonists appear to be promising future regulators of fertility.

SUMMARY

These data are consistent with a physiologic role of endogenous GnRH in controlling most of the events of the reproductive cycle in the female rat. Although the exact mechanisms through which GnRH interferes with reproductive functions need further clarification, it is obvious that their ability to inhibit gonadotropin, and consequently steroid secretion, closely parallels their deleterious effects.

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14

Activities of LHRH Antagonists in Rhesus Monkeys During the Periovulatory Interval of the Menstrual Cycle and Early Pregnancy

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CLARK W. SMITH

The ultimate goal in designing and synthesizing of antagonists of LHRH has been their application for the predictable inhibition of ovulation in women. To date, however, the biological evaluation of LHRH antagonists has been largely restricted to infraprimate species.

The antagonistic activity of a few LHRH analogs has been evaluated in nonhuman primates under experimental conditions where gonadotropin secretion was provoked by exogenous estradiol, LHRH administration, or electrical stimulation of the hypothalamus.[5,6,18] While those studies are of interest, the ability of LHRH antagonists to inhibit spontaneous preovulatory discharge of gonadotropins during normal menstrual cycles would provide a more meaningful estimate of potential utility for the control of human fertility.

This chapter describes experiments with [D-Phe^2,Pro^3,D-Phe^6]-LHRH and [(<Glu-Pro)^1,D-Phe^2,D-Trp^{3,6}]-LHRH administration to rhesus monkeys during the periovulatory interval of the menstrual cycle. The results indicate that LHRH antagonists can prevent ovulation in the monkey and modify spontaneous preovulatory endocrine events; however, more potent antagonists and/or improved regimens of peptide administration are required to uniformly inhibit gonadotropin surges.

Several studies have implicated a role for LHRH in the endocrine control of human pregnancy. The human placenta contains large quantities of LHRH throughout gestation,[19] and *in vitro* studies have shown that placental tissue can synthesize LHRH.[3] LHRH increased the *in vitro* release of human chorionic gonadotropin (hCG) from placental tissue without altering the release of chorionic somatomammotropin.[12] Furthermore, the administration

of LHRH to pregnant monkeys was reported to increase serum chorionic gonadotropin concentrations.[20] Preliminary experiments described here show that the LHRH antagonists [D-Phe^2,Pro^3,D-Phe^6]-LHRH and [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]-LHRH do not alter serum concentrations of chorionic gonadotropin in rhesus monkeys during early pregnancy.

MATERIALS AND METHODS

ANIMALS

Adult rhesus monkeys with a history of menstrual cycles of normal duration were selected for study. The animal care and husbandry procedures for this colony of monkeys has previously been described in detail.[8]

STUDIES DURING THE PERIOVULATORY PHASE OF THE MENSTRUAL CYCLE

Treatments with LHRH analogs were initiated 24 to 48 hours prior to the anticipated midcycle gonadotropin surges, or after preovulatory gonadotropin surges had already commenced. The estimated times for spontaneous midcycle gonadotropin surges were based on published descriptions of the duration of elevated serum estrogen concentrations required to elicit gonadotropin discharges in the rhesus monkey.[11]

Briefly, serum estrogen values must exceed a threshold between 100 and 200 pg/ml for 42 hours to stimulate LH and FSH discharges, although estrogen concentrations in excess of 200 pg/ml are effective in 36 hours. Midcycle gonadotropin peaks in rhesus monkeys occur between days 9 and 13 of the menstrual cycle with day 11 being the mean value.[20] Administration of LHRH antagonists 24 to 48 hours prior to spontaneous surges of LH and FSH required serum estradiol concentrations between 100 and 200 pg/ml for two consecutive days after day 7 of the menstrual cycle. Administration of an LHRH antagonist when midcycle gonadotropin discharges were already in progress required serum estradiol values to exceed 100 pg/ml for two or more days after day 9 of the menstrual cycle, with an increase of more than 50 pg/ml in the previous 24 hours.

Antagonists were administered subcutaneously in 1 ml of corn oil every eight hours (0700, 1500 and 2300 hours) for a total of six injections; the dose of antagonist was 50 mg/injection for a total dose of 300 mg. The number of monkeys treated with each peptide was dictated by the available supply of peptide. Blood samples (5 ml) were obtained once daily beginning on day 1 of the menstrual cycle and continued until the onset of the next menses; blood samples were also obtained every eight hours during administration of the antagonist. Serum was harvested from each blood sample and assayed in duplicate for FSH, LH, estradiol-17β and progesterone using previously described radioimmunoassays.[8,25] The ovaries in each monkey were viewed by laparoscopy in the second half of the treatment menstrual cycle to ascertain the presence or absence of a corpus luteum.

STUDIES DURING EARLY PREGNANCY

Female rhesus monkeys were caged with fertile males on days 11 through 15 of the menstrual cycle. Blood samples were collected prior to treatment and pregnancy was verified by the qualitative determination of monkey chorionic gonadotropin (mCG) in peripheral serum using a specific radioimmunoassay.[7,23] LHRH antagonists were administered subcutaneously in 1 ml of corn oil at the doses and times indicated for each experiment. Serum concentrations of mCG, estradiol-17β and progesterone during the experimental interval were determined in duplicate using the radioimmunoassay methods cited.

LHRH ANTAGONISTS

Three LHRH antagonists were studied in the rhesus monkey. [D-Phe^2,Pro^3,D-Phe^6]-LHRH inhibits ovulation in 100% of treated rats when administered at a dose of 750 μg at noon on proestrus.[10] Ovulation is inhibited in all rats by 250 μg of [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]-LHRH.[17] The most potent antagonist used in these studies, [(<Glu-Pro)^1,D-Phe^2,D-Trp^{3,6}]-LHRH, is totally effective in inhibiting ovulation in rats at a dose of 200 μg.[21]

RESULTS

CONTROL STUDIES

Figure 14-1 shows serum concentrations of FSH, LH, estradiol, and progesterone for two control monkeys receiving injections of corn oil during midcycle gonadotropin surges. Serum hormone concentrations and secretion patterns were normal in every respect.[26] Of particular importance are the smooth, continuous patterns of serum LH and FSH concentrations during the gonadotropin surges. The mean serum hormone concentrations for three control monkeys injected with corn oil 24 to 48 hours prior to the anticipated midcycle discharges of LH and FSH are shown in Figure 14-2. The time of preovulatory gonadotropin secretion was correctly predicted, and serum concentrations and patterns of the gonadotropins and steroid hormones were normal. Corpora lutea with ovulatory stigmas were observed at the time of laparoscopy in all control monkeys. Additional information on control monkeys has previously been published in detail.[21,25]

ADMINISTRATION OF [D-Phe^2,Pro^3,D-Phe^6]-LHRH DURING GONADOTROPIN SURGES

Figure 14-3 presents the serum hormone values for three monkeys in which treatment with [D-Phe^2,Pro^3,D-Phe^6]-LHRH was initiated after the midcycle discharges of gonadotropin had already commenced. Serum gonadotropin values declined to near basal values in Monkey 23B (Figure 14-3A) after the first antagonist injection, and remained near basal values for the next two

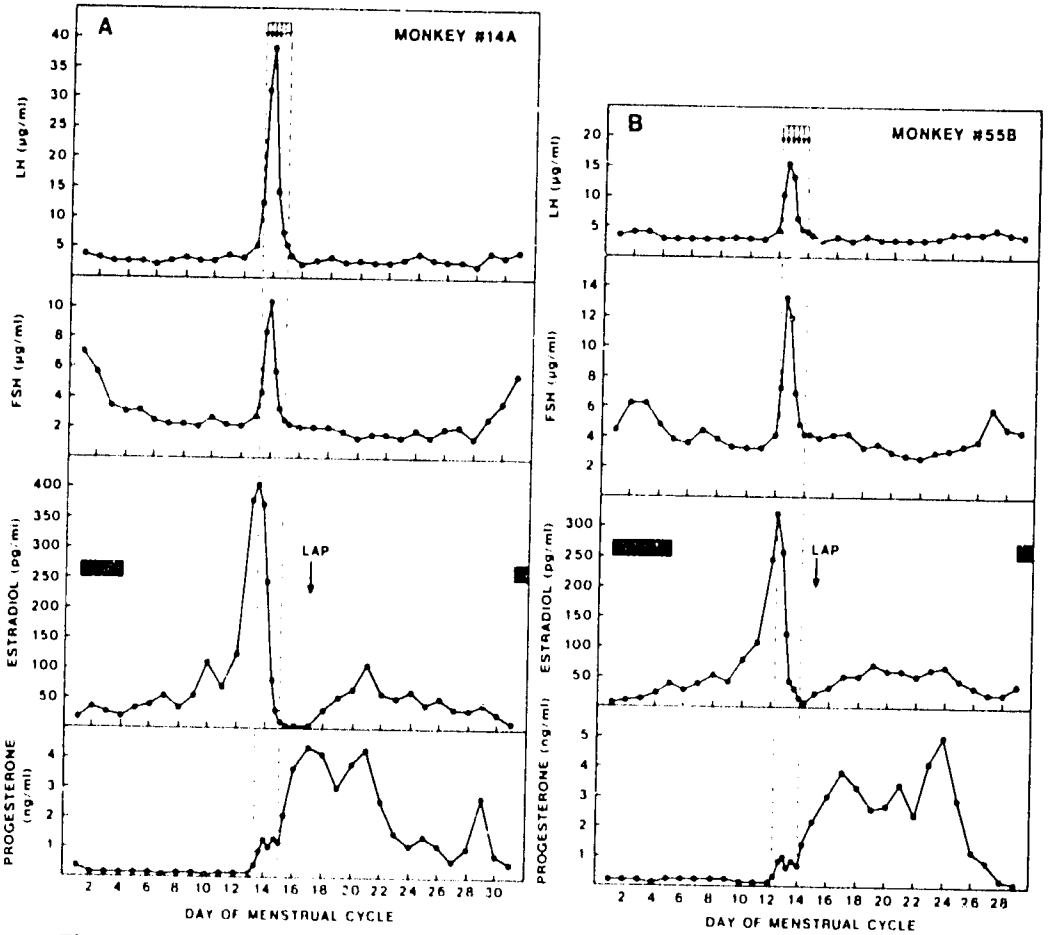


Fig. 14-1. Serum concentrations of LH, FSH, estradiol, and progesterone in two control monkeys injected with corn oil during midcycle gonadotropin surges. Vertical dashed lines enclose treatment interval; dark horizontal bars indicate menstrual bleeding; arrow indicates day of laparoscopic examination of ovaries. (Wilks JW, Folkers K, Humphries J *et al:* Effect of [D-Phe^2,Pro^3,D-Phe^6]-luteinizing hormone releasing hormone, an antagonist, on preovulatory gonadotropin secretion in the rhesus monkey. Biol Reprod 23:1, 1980)

weeks. Although the pattern of serum progesterone concentrations indicated the presence of functional luteal tissue, laparoscopic visualization of the ovaries in this monkey did not reveal any prominent ovarian structures.

A modest 2.5-fold elevation in serum LH values was observed in a second monkey treated with [D-Phe^2,Pro^3,D-Phe^6]-LHRH during the gonadotropin surges (Monkey 30B, Figure 14-3B). Serum FSH concentrations in this monkey clearly showed a midcycle discharge; however, the secretion was discontinuous and therefore abnormal. Laparoscopic examination of the ovaries in this animal also failed to reveal the presence of a corpus luteum, yet the elevated serum progesterone values indicated the presence of functional luteal tissue.

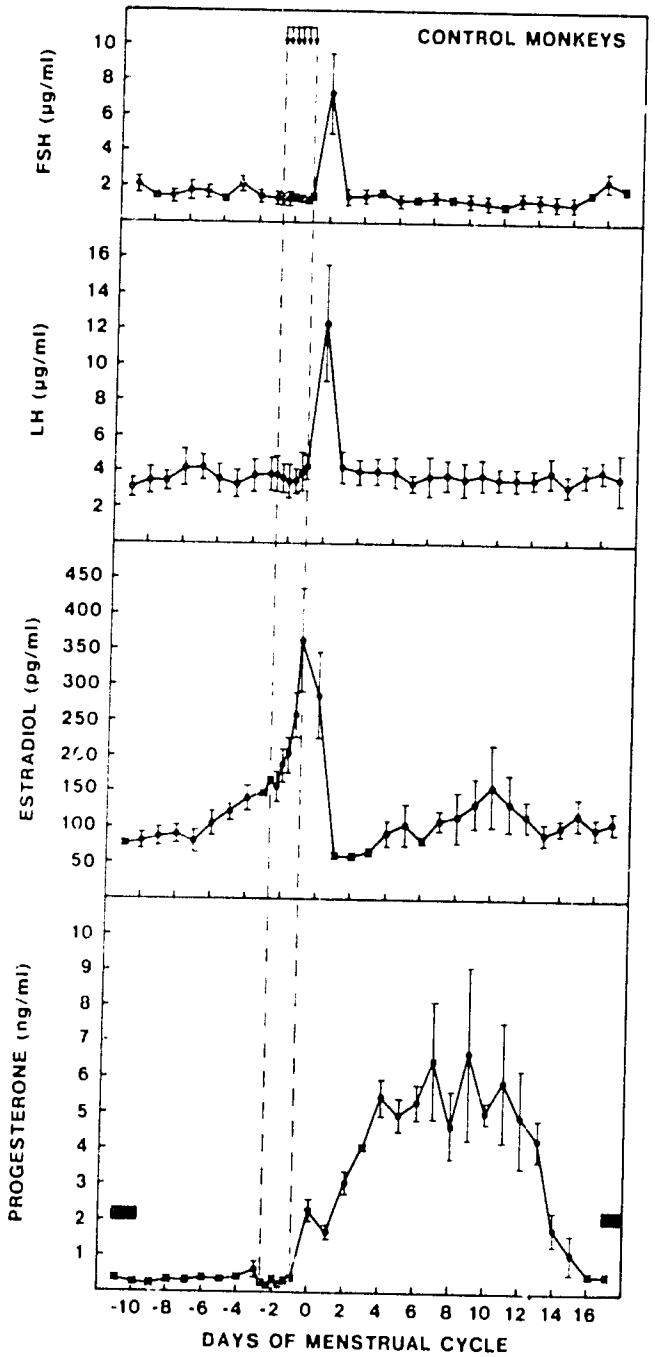
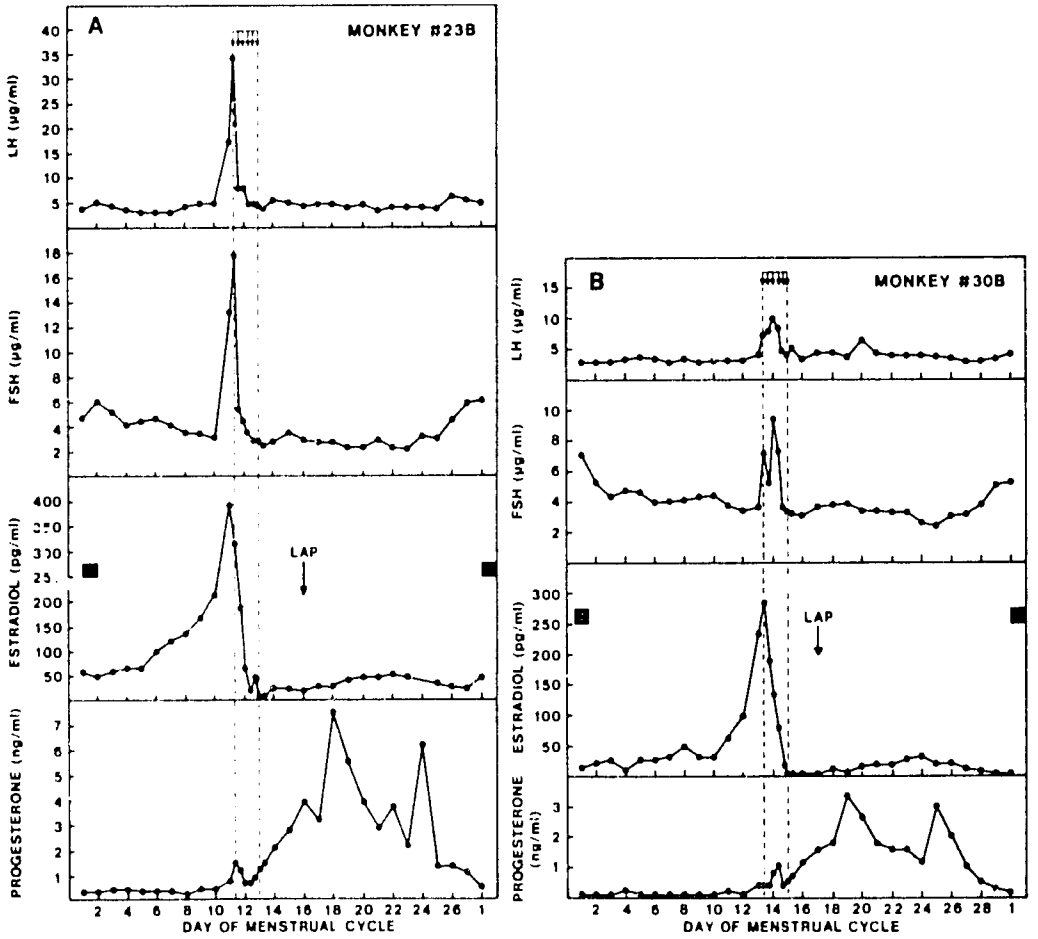


Fig. 14-2. Mean (± S.E.) serum concentrations of LH, FSH, estradiol, and progesterone in three control monkeys given corn oil injections 24 to 48 hours prior to midcycle gonadotropin surges. Hormone values were aligned according to time of LH peak (day 0). Vertical dashed lines enclose injection interval, which on the average commenced 3 days prior to LH and FSH peaks. (Wilks JW, Folkers K, Bowers CY *et al:* Inhibition of preovulatory gonadotropin secretion in the rhesus monkey by [(<Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH. Contraception 22:313, 1980)



Hormone values for the last monkey treated with the LHRH antagonist during midcycle gonadotropin secretion are shown in Figure 14-3C (Monkey 332). Serum LH concentrations fell after the first injection of [D-Phe^2,Pro^3,D-Phe^6]-LHRH, but then rebounded in the second half of the treatment interval; the secretion pattern of LH was discontinuous and the surge was of an unusually long duration (nearly 5 days). A preovulatory surge of FSH was not observed in this monkey. Serum estradiol and progesterone values showed a normal pattern throughout the menstrual cycle. Laparoscopic examination of the ovaries on day 17 of the menstrual cycle revealed the presence of a corpus luteum with an ovulatory stigma.

ADMINISTRATION OF [D-Phe^2,Pro^3,D-Phe^6]-LHRH 24 TO 48 HOURS PRIOR TO GONADOTROPIN SURGES

Serum gonadotropin and steroid hormone concentrations for two monkeys treated with [D-Phe^2,Pro^3,D-Phe^6]-LHRH beginning 24 to 48 hours prior to an expected spontaneous gonadotropin surge are shown in Figure 14-4.

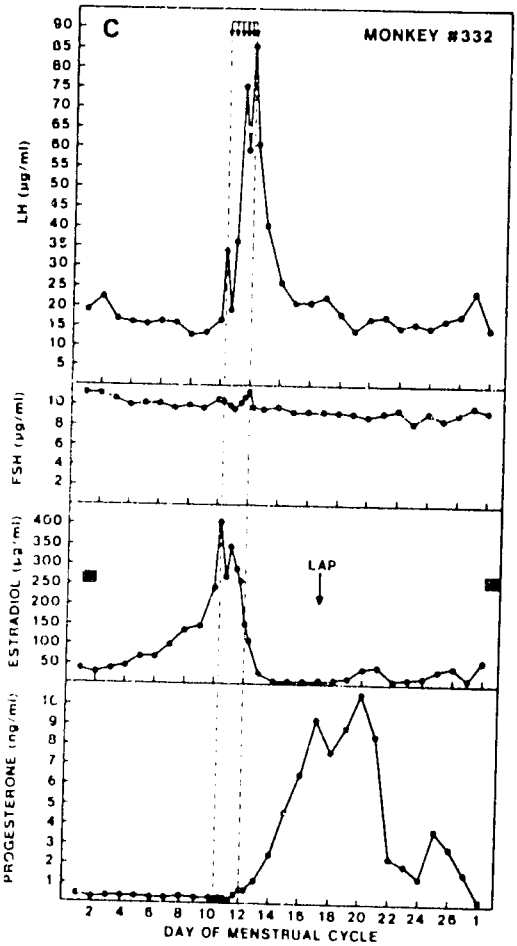


Fig. 14-3. Serum concentrations of LH, FSH, estradiol, and progesterone in monkeys treated with [D-Phe^2,Pro^3,D-Phe^6]-LHRH during midcycle gonadotropin surges. Vertical dashed lines enclose treatment interval; dark horizontal bars indicate menstrual bleeding; arrow indicates day of laparoscopic examination of ovaries. (Wilks JW, Folkers K, Humphries J *et al:* Effect of [D-Phe^2,Pro^3,D-Phe^6]-luteinizing hormone releasing hormone, an antagonist, on preovulatory gonadotropin secretion in the rhesus monkey. Biol Reprod 23:1, 1980)

Serum estradiol values in Monkey 31C (Figure 14-4A) fell during the first half of the treatment interval with [D-Phe^2,Pro^3,D-Phe^6]-LHRH, but then rebounded despite the continued administration of the antagonist. The midcycle LH rise was small and of less than 16 hours' duration; the midcycle elevations in FSH could not unambiguously be classified as a surge. A small, vascular corpus luteum was observed at the time of laparoscopic examination of the ovaries, but an ovulatory stigma was not present. Elevated serum progesterone concentrations indicated the presence of functional luteal tissue.

Hormone values for a second monkey treated with [D-Phe^2,Pro^3,D-Phe^6]-LHRH prior to the gonadotropin surges are shown in Figure 14-4B. Serum estradiol concentrations fell sharply to 15% of pretreatment values during the first half of the antagonist treatment period, but then rebounded. There was a sharp increase in serum estradiol values 3 days after the last antagonist injection, followed one day later by surges of LH and FSH of normal duration and magnitude. A corpus luteum with an ovulatory stigma was observed at the time of laparoscopy, and serum progesterone concentrations indicated the presence of functional luteal tissue.

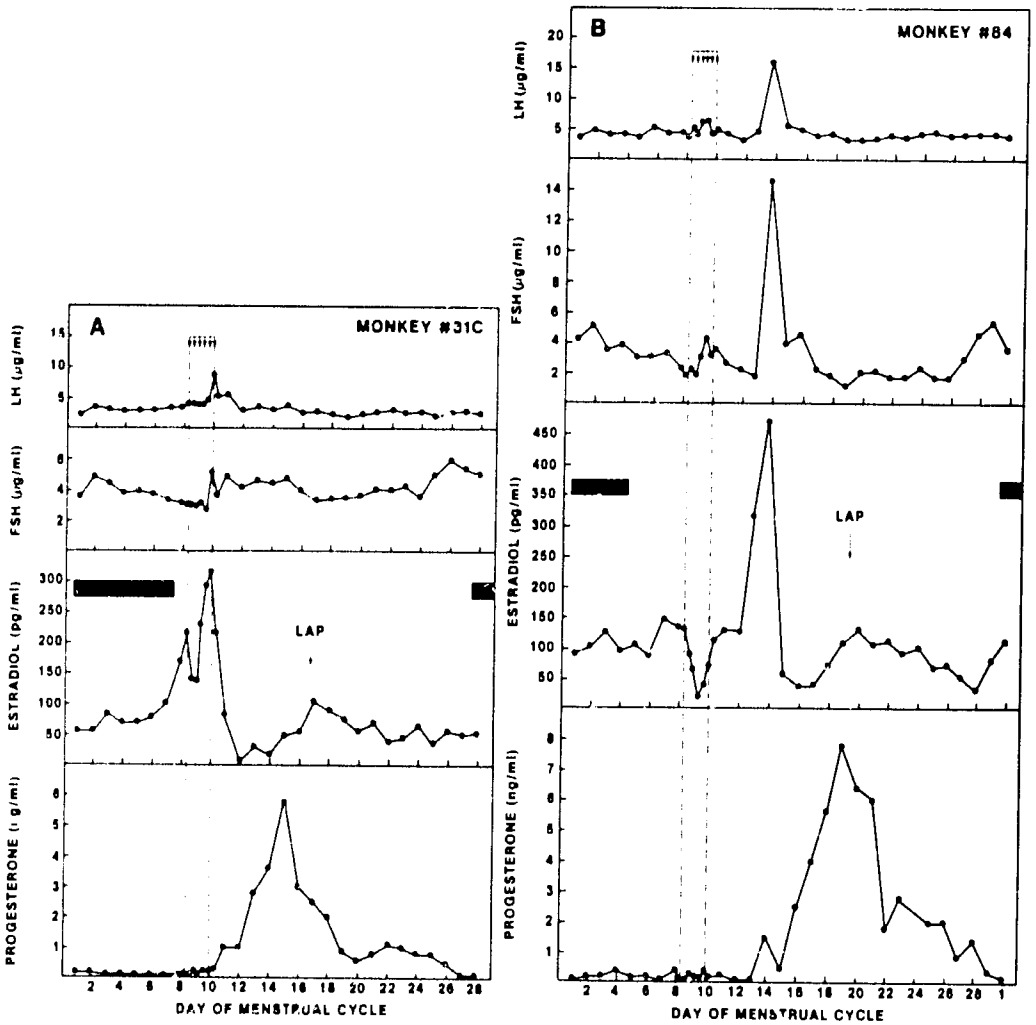


Fig. 14-4. Serum concentrations of LH, FSH, estradiol, and progesterone in monkeys treated with [D-Phe^2,Pro^3,D-Phe^6]-LHRH 24 to 48 hours prior to anticipated midcycle gonadotropin surges. Vertical dashed lines enclose treatment interval; dark horizontal bars indicate menstrual bleeding; arrow indicates day of laparoscopic examination of ovaries. (Wilks JW, Folkers K, Humphries J *et al:* Effect of [D-Phe^2,Pro^3,D-Phe^6]-luteinizing hormone releasing hormone, an antagonist, on preovulatory gonadotropin secretion in the rhesus monkey. Biol Reprod 23:1, 1980)

ADMINISTRATION OF [(<Glu-Pro)^1,D-Phe^2,D-Trp^{3,6}]-LHRH 24 TO 48 HOURS PRIOR TO GONADOTROPIN SURGES

Figure 14-5 illustrates the serum hormone concentrations in a monkey treated with [(<Glu-Pro)^1,D-Phe^2,D-Trp^{3,6}]-LHRH beginning on day 9 of the menstrual cycle. Serum concentrations of estradiol fell during the first 16

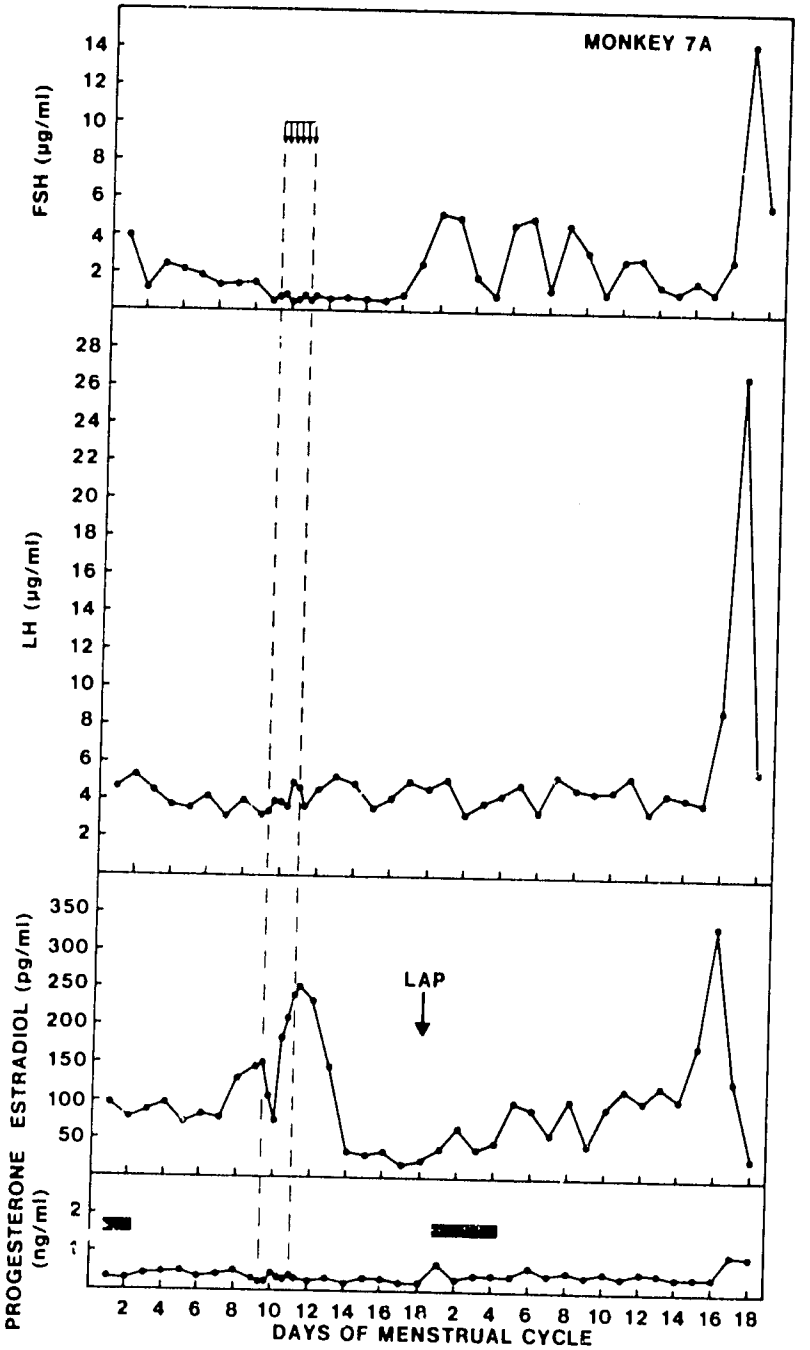


Fig. 14-5. Serum concentrations of LH, FSH, estradiol, and progesterone in a monkey treated with [(<Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH 24 to 48 hours prior to anticipated midcycle gonadotropin surges. Vertical dashed lines enclose treatment interval; dark horizontal bars indicate menstrual bleeding; arrow indicates day of laparoscopic examination of ovaries. (Wilks JW, Folkers K, Bowers CY *et al:* Inhibition of preovulatory gonadotropin secretion in the rhesus monkey by [(<Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH. Contraception 22:313, 1980)

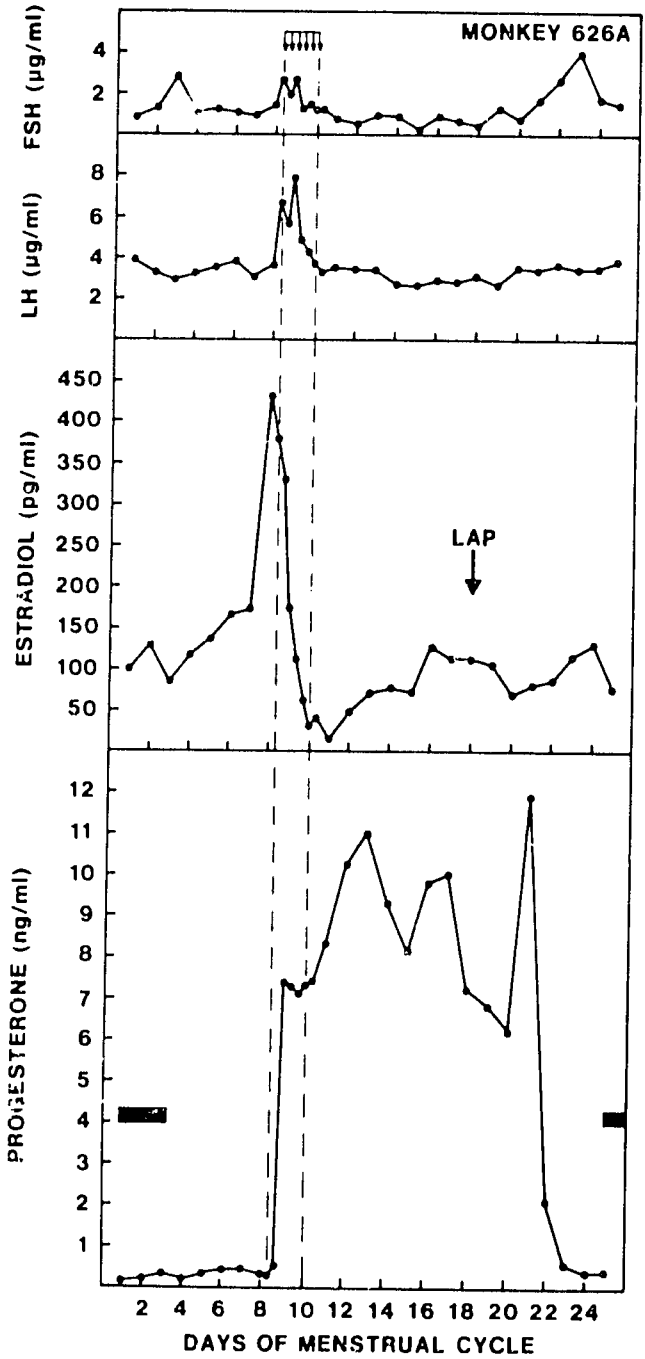


Fig. 14-6. Serum concentrations of LH, FSH, estradiol, and progesterone in a monkey treated with [(<Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH after preovulatory gonadotropin surges had already commenced. Vertical dashed lines enclose treatment interval; dark horizontal bars indicate menstrual bleeding; arrow indicates day of laparoscopic examination of ovaries. (Wilks JW, Folkers K, Bowers CY *et al:* Inhibition of preovulatory gonadotropin secretion in the rhesus monkey by [(<Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH. Contraception 22:313, 1980)

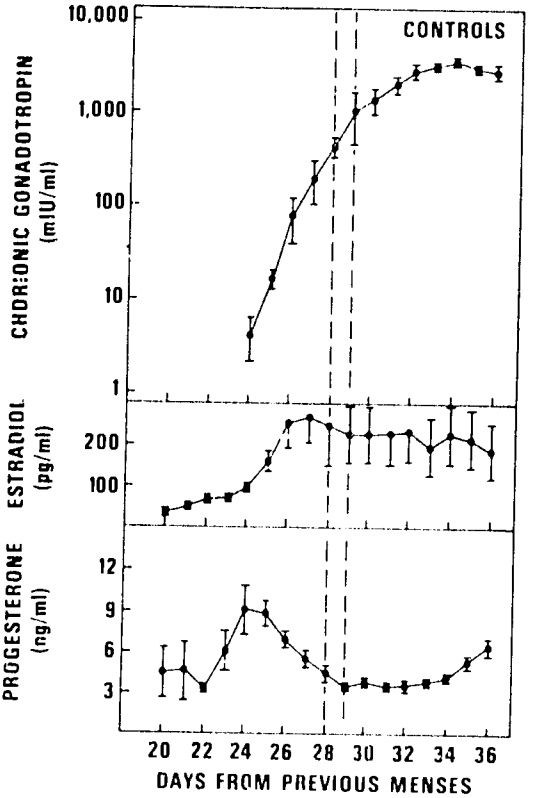


Fig. 14-7. Mean (± S.E.) serum concentrations of chorionic gonadotropin, estradiol, and progesterone in three control monkeys given vehicle injections on days 28 and 29 of fertile menstrual cycles. Vertical dashed lines enclose treatment interval.

hours of antagonist administration, but then rebounded. Despite serum estradiol concentrations greater than 150 pg/ml for 72 continuous hours in this monkey, FSH and LH surges were not observed. Within 3 days after the last peptide injection, serum estradiol values had declined to early follicular phase levels. Low serum progesterone concentrations indicated that ovulation and luteinization did not occur, and only follicles were observed on the ovaries at the time of laparoscopy.

Menstrual bleeding commenced on day 9 of the menstrual cycle, eight days after the last peptide injection. Continuation of daily blood collection in this monkey revealed distinct surges of LH and FSH on day 17 of the subsequent menstrual cycle. Midcycle discharges of LH and FSH were already in progress in another monkey (Figure 14-6) at the time of the first injection of [(<Glu-Pro)^1,D-Phe^2,D-Trp^{3,6}]-LHRH on day 8 of the menstrual cycle. The gonadotropin surges continued during administration of the antagonist although the dual peaks indicated discontinuous secretion. A corpus luteum was observed at laparoscopy, and elevated serum progesterone concentrations indicated that it was functional.

EFFECT OF LHRH ANTAGONISTS ON SUBSEQUENT MENSTRUAL CYCLES

Blood samples were obtained from all monkeys once each week in the menstrual cycles following administration of the LHRH antagonists, or corn oil vehicle. Elevations in serum progesterone consistent with functional luteal

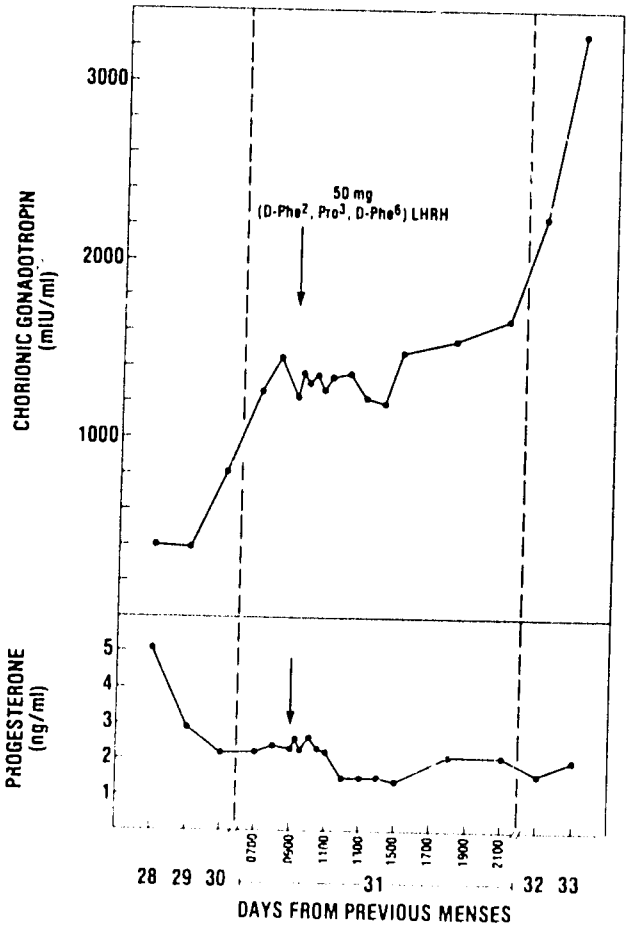


Fig. 14-8. Serum chorionic gonadotropin and progesterone concentrations in a monkey treated with [D-Phe^2,Pro^3,D-Phe^6]-LHRH on day 31 of a fertile menstrual cycle. Samples were collected once daily, except following injection of the LHRH antagonist (when samples were obtained at frequent intervals).

tissue were observed in all monkeys suggesting that subsequent ovarian function was not altered.

CONTROL SERUM HORMONE CONCENTRATIONS IN EARLY PREGNANCY

Figure 14-7 depicts the mean serum concentrations of chorionic gonadotropin (mCG), estradiol, and progesterone for three monkeys receiving control vehicle injections on days 28 and 29 of fertile menstrual cycles. Serum concentrations of mCG were undetectable until late in the luteal phase, but increased rapidly following implantation to reach peak values approximately 34 days from the previous menses. Estradiol values increased following the appearance of chorionic gonadotropin and then remained elevated. Serum progesterone also rose in response to the appearance of mCG, but then slowly declined reaching a nadir at the time of the highest serum mCG values.

ADMINISTRATION OF [D-Phe^2,Pro^3,D-Phe^6]-LHRH DURING EARLY PREGNANCY

Figure 14-8 shows serum mCG and progesterone concentrations for a pregnant monkey studied on days 28 through 33 from the previous menses. Blood samples were collected once daily except that samples were obtained at frequent intervals on day 31 from the previous menses. [D-Phe^2,Pro^3,D-Phe^6]-LHRH was administered subcutaneously in 1 ml of corn oil at a dose of 50 mg at 0900 hours on day 31 from the previous menses. There were no marked changes in serum mCG concentrations following administration of [D-Phe^2,Pro^3,D-Phe^6]-LHRH, and values increased progressively through day 33 in a manner characteristic for this stage of gestation. Serum progesterone values did not change significantly after administration of the antagonist. This monkey aborted her pregnancy 76 days after the administration of [D-Phe^2,Pro^3,D-Phe^6]-LHRH, but it is not possible to relate this event to administration of the antagonist.

ADMINISTRATION OF [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]-LHRH DURING EARLY PREGNANCY

Figure 14-9 shows serum mCG, estradiol, and progesterone values for a monkey receiving subcutaneous injections of [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]-LHRH at 0700, 1500, and 2300 hours on days 28 and 29 of a fertile menstrual cycle. The dose of the LHRH antagonist was 5 mg/injection for a total dose of 30 mg. The patterns and serum values of mCG, estradiol and progesterone were normal following administration of the peptide. Pregnancy progressed uneventfully in this monkey.

DISCUSSION

LHRH antagonists altered and modified periovulatory endocrine events in the rhesus monkey. Despite elevations in serum estradiol concentrations of sufficient duration and intensity to provoke gonadotropin surges at midcycle, elevations in LH or FSH were not observed in a monkey treated with [(<Glu-Pro)^1,D-Phe^2,D-Trp^{3,6}]-LHRH (Figure 14-5). The LHRH antagonists did not uniformly or completely inhibit gonadotropin secretion or prevent luteinization in the remaining monkeys. However, the antagonistic activity of the peptides was evidenced by one or more of the following observations in these monkeys.

1. An immediate cessation of the LH and FSH surges
2. Abnormal, discontinuous secretion of LH and/or FSH
3. Unusually short or prolonged gonadotropin surges
4. LH or FSH surges of diminished magnitude compared with those of control monkeys
5. Absence of concomitant FSH and LH peaks.

Corpora lutea were not observed at the time of laparoscopy in two monkeys treated with [D-Phe^2,Pro^3,D-Phe^6]-LHRH, and in another monkey the corpus luteum lacked an ovulatory stigma. However, serum progesterone concen-

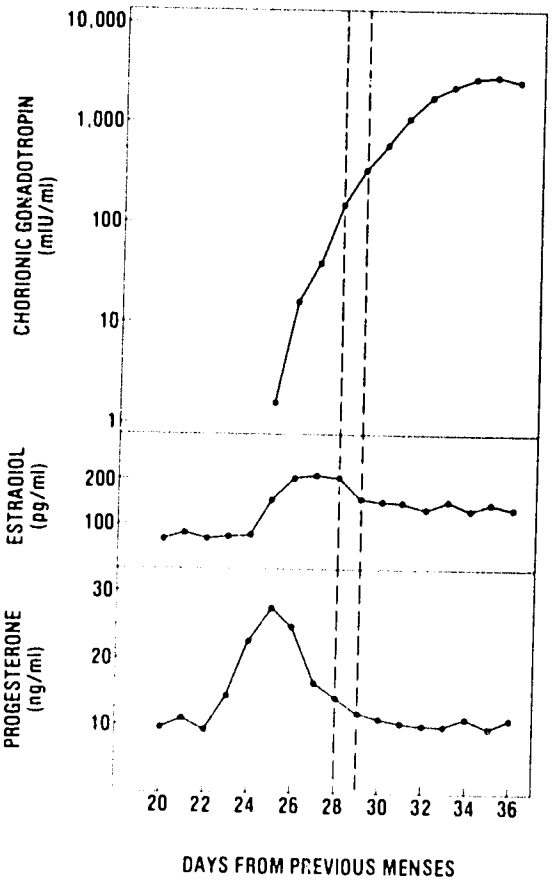


Fig. 14-9. Serum chorionic gonadotropin, estradiol, and progesterone concentrations in a pregnant monkey treated with [D-<Glu[1],D-Phe[2],D-Trp[3,6]]-LHRH on days 28 and 29 from previous menses.

trations in all three monkeys indicated that functional luteal tissue with a normal steroidogenic capacity and life span was present. These observations suggest that follicular rupture did not occur following administration of the LHRH antagonist, although the midcycle discharges of LH and FSH were sufficient to cause luteinization of embedded preovulatory follicles. These presumptive ovulatory failures in monkeys treated with LHRH antagonists require confirmation. An LHRH antagonist which predictably prevents follicular rupture and discharge of the ovum without altering luteinization and luteal cell function could provide a new approach to contraception.

An interesting observation during the course of these studies was the effect of LHRH antagonists on serum estradiol concentrations in monkeys when administered 24 to 48 hours prior to an expected midcycle gonadotropin surge. Both [D-Phe[2],Pro[3],D-Phe[6]]-LHRH and [(<Glu-Pro)[1],D-Phe[2],D-Trp[3,6]]-LHRH reduced serum estradiol concentrations during the first half of the treatment interval; however, serum estradiol rebounded despite the continued administration of the peptide. These changes in serum estradiol were

seen without obvious changes in the secretion patterns of LH or FSH, and were observed whether or not the LHRH antagonist blocked or modified the midcycle gonadotropin surges.

The cross-reaction of the monkey serum LH-like substance in the LH radioimmunoassay used for these studies may have masked important changes in basal LH secretion during treatment with the LHRH antagonists.[14,15] However, other workers have reported that LHRH antagonists do not alter basal serum gonadotropin values in men and chimpanzees.[4,5] Another possibility is that the LHRH antagonists had a direct inhibitory effect upon the preovulatory follicle. This proposal is in contradistinction to the observation that [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]-LHRH did not blunt the *in vitro* stimulation by FSH of estradiol and progesterone synthesis in rat granulosa cells.[9]

Much of our knowledge regarding the physiologic role of LHRH in the primate menstrual cycle has been elucidated by Knobil and colleagues.[13] Their studies indicate that the primary action of LHRH in the rhesus monkey is a permissive one to maintain the functional integrity of the gonadotropin-secreting cells, while estrogens actually regulate gonadotropin secretion through a direct action upon the anterior pituitary. Placement of radiofrequency lesions in the arcuate nucleus of the rhesus monkey abrogates endogenous LHRH secretion; however, ovarian cyclicity can be maintained by the intermittent, hourly infusion of LHRH.[13] Interestingly, estrogen elicits gonadotropin discharges in these monkeys as long as 48 hours after the discontinuation of intermittent LHRH infusions.[22] Furthermore, the follicle destined to ovulate in the rhesus monkey has already been selected by the sixth day of the menstrual cycle.[2] Future studies should evaluate the importance of initiating treatment with LHRH antagonists early in the follicular phase of the menstrual cycle.

Neither [D-Phe^2,Pro^3,D-Phe^6]-LHRH or [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]-LHRH altered serum concentrations of chorionic gonadotropin in the rhesus monkey during early pregnancy. These observations indicate that if LHRH does play a regulatory role in the secretion of chorionic gonadotropin, this function is not inhibited by LHRH antagonists. Additionally, the LHRH antagonists did not alter serum concentrations of progesterone during early gestation; this indicates that like the LHRH agonists, these peptides are incapable of inhibiting luteal function during pregnancy.[1] However, the doses of antagonists used may have been insufficient to elicit an antifertility response. Low doses of [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]-LHRH had no effect on gestation in the rat, whereas high doses (5 mg/day) completely inhibited pregnancy.[16]

CONCLUSION

These preliminary studies showing an alteration of midcycle endocrine events in the rhesus monkey offer the promise that LHRH antagonists will be found to predictably inhibit ovulation in women. Fruitful areas for expanded investigation include:

1. The evaluation of LHRH antagonists with increased potencies and longer biological half-lives

2. The evaluation of treatment regimens emphasizing administration of LHRH antagonists early in the follicular phase of the menstrual cycle
3. Investigations to determine whether the events of follicular rupture and discharge of the ovum following treatment with LHRH antagonists can be dissociated from luteinization
4. Studies of the direct effects of LHRH antagonists upon the ovarian follicle.

Initial attempts to alter early pregnancy in the monkey with LHRH antagonists have not indicated any antifertility activities; however, additional data are required for definitive conclusions.

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15

Antigonadotropic Analysis of GnRH Antagonists in Higher Primates in Relation to Ovulation Inhibition

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Potent GnRH antagonists effectively block ovulation in rodents at low doses. Specifically, [Ac-dehydro-Pro^1,pCl-D-Phe^1,D-Trp^{3,6},N$^\alpha$Me-Leu^7]-GnRH (AI) and (Ac-dehydro-Pro^1,pCl-D-Phe^2,D-Trp^{3,6})-GnRH (AII) were demonstrated to have an effective dose 90 of 20 μg/rat and 10 μg/rat, respectively. Rats weighed 180 to 200 g when injected subcutaneously in corn oil.[14]

A study was undertaken in the chimpanzee (*Pan troglodytes*), a species that closely resembles man in many aspects of its reproductive endocrinology,[7] to determine if GnRH antagonists such as AI and AII have antigonadotropic and antiovulatory activity in higher primates. Differences in endocrinology between species prevent the assumption that data from rodents can be extrapolated directly to man, particularly in view of equivocal results previously obtained in two monkey species with other antagonists active in rodents.[2,16]

MATERIALS AND METHODS

ANIMALS

Ten adult females were selected on the basis of good health, and regular and normal cycles. In nine instances they were successfully trained to submit to alert venipuncture; one animal was bled under ketamine hydrochloride anesthesia (10 mg/kg).

The animals were examined daily and incidence of menses and size of

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sexual swelling recorded, as described by Graham.[6] The animals were fed on Purina monkey chow, supplemented with fruit and water *ad lib.* Ages ranged from 10.8 to 22.3 years and weights from 35 to 58 kg.

DRUG PREPARATION

Analogs were received as a fluffy white powder, and were prepared for subcutaneous injection in corn oil and for intravenous injection in sterile distilled water. Preparations in oil were made by placing an appropriate amount of analog and 15 ml oil into a Thomas teflon pestle homogenizer. After homogenizing for 10 minutes, the mixture was placed in a storage vessel at 5°C. Vortexing before each use produced a pale-gold, opalescent, fine, even suspension. The antagonists were dissolved in distilled water (1 mg/ml) for IV administration. GnRH obtained from the National Pituitary Agency was dissolved in sterile saline at the required concentration.

HORMONE ASSAYS

Estradiol and progesterone were measured by radioimmunoassay, using rabbit antisera against the 6-(0-carboxymethyl)-oxime-bovine serum albumin derivatives of estradiol-17β and progesterone respectively.[12] Cross-reactivities of these antisera with other steroid hormones have previously been established.[12]

For both assays, aliquots of serum samples (250 μl for progesterone, 400 μl for estradiol) and low-, middle-, and high-serum pools were extracted in screw cap tubes with ten volumes of solvent (hexane for progesterone, diethyl ether for estradiol) by vortexing for 15 seconds and then placing the tubes in a horizontal position in a mechanical shaker for 30 minutes. Solvent and serum layers were separated by centrifugation and stored overnight at −20°C. The solvent layer was poured off from the frozen serum pellet and dried down in a vortex-evaporator at 20°C (progesterone) or 10°C (estradiol). One milliliter of Gel-PBS (0.1% solution of Knox unflavored gelatin in phosphate buffered saline; 0.14M NaCl, 0.01M $NaPo_4$, 1:10000 thimerosal, *p*H = 7.6) was added to the tubes to redissolve the extract and the tubes were vortexed for 5 seconds, allowed to stand for 15 minutes and vortexed again for 5 seconds. Duplicate aliquots of 0.4 ml were transferred to assay tubes.

A series of standards (6.25–800 pg/100 μl EtOH for estradiol; 25–1600 pg/100 μl Benzene—EtOH, 9:1 for progesterone) were prepared for each assay by aliquotting directly into assay tubes and drying down. The dried-down standards were redissolved in 0.4 ml Gel-PBS in the manner just described.

From 60 to 70 pg of [2,4,6,7-^{3}H(N)]-estradiol, 101.7 Ci/mmol or 1,2,6,7,-^{3}H(N)]-progesterone, 96.5 Ci/mmol were added to each assay tube in 200 μl of Gel-PBS followed by 200 μl of the corresponding antiserum diluted with sufficient Gel-PBS to bind 40 to 60% of the trace. (Approximate dilutions were 1:5000 for progesterone and 1:1000 for estradiol.) The mixture was shaken at room temperature for 2 hours, whereupon 100 μl immunobead reagent (goat anti-rabbit immunoglobulin, heavy and light chain, Bio-Rad Laboratories) was added and the tubes incubated overnight in a shaking water

bath at 37°. After centrifugation at 400 × g, the supernatant was poured off into scintillation vials containing 3 ml of a PPO/POPOP/Bio-Solv (Beckman)/Toluene liquid scintillation cocktail and counted for 2 minutes in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Assay reliability was assessed by the quality control system of Rodbard and associates.[13] Standard curves were established from a logit log plot of the percent binding versus picograms of steroid standard.[13] Estradiol and progesterone content of each sample were calculated. All values represent the mean of duplicate determinations.

Radioimmunoassays for chimpanzee FSH and LH were performed as previously described.[10,12] The human pituitary preparation LER 907 (20 IU FSH, 48 IU LH 2nd IRP/mg) was used as a standard for both chimpanzee LH and FSH. Chimpanzee gonadotropins cross-react with antibodies to human gonadotropins in such a manner that dilutions of chimpanzee sera are parallel to the human pituitary standard. Assay reliability was assessed by the quality control system of Rodbard and associates.[13] All values represent the mean of duplicate determinations.

EXPERIMENTAL DESIGN

Menstrual Cycle Assay: The primary objective of this study was to test the effect of 0.02, 0.1, and 0.5 mg/kg of AI on the menstrual cycle in order to identify a dose level with evidence of antiovulatory activity. Three or four animals were tested at each dose level. Each subject underwent a vehicle control cycle before drug administration. In control and treatment cycles, venous blood (10 ml) was obtained from the alert subjects on the second day of maximal sexual swelling, then daily from day 6 until the end of maximal swelling (midlate follicular phase). They were also bled on days 1, 3, 6, 9, and 12 after sexual swelling regression (luteal phase). Vehicle or antagonist administration began on day 5 of maximal sexual swelling and was performed immediately after venipuncture, between the hours of 8:00 and 10:00 a.m. Vehicle or analog administration ceased either after the first day of sexual swelling regression (first day of luteal phase), or was terminated when sexual swelling persisted for more than 4 days in excess of the control cycle. (This latter criterion was employed because inhibition of ovulation might result in prolonged sexual swelling and it was necessary to limit drug usage.) After a drug cycle, each animal was rested for one cycle before being reused. One group of animals received 0.1 mg/kg antagonist three times per day at 8-hour intervals.

Duration of Action of Antagonist: In order to investigate the duration of action of AI, 25 μg GnRH challenges were given IV to two alert chimpanzees at 3-hour intervals for 21 hours on the fifth day of maximal sexual swelling; 0.1 mg/kg AI was administered subcutaneously after the first GnRH challenge. Serum was obtained at the time of each GnRH injection and 30 minutes later. An identical protocol, except for administration of oil alone instead of AI in oil, was conducted 2 days earlier. Additional subjects were not studied because of aversion to repeated alert venipuncture.

GnRH Inhibition Assay: Such assays were conducted in four to five subjects and controls. Chimpanzees were anesthetized with 10 to 20 mg ketamine hydrochloride/kg and supplements given IM as needed. Three pre-GnRH venous samples were obtained at 15 minute intervals and antagonist and GnRH were immediately administered IV. Repeated samples were obtained at 15-minute intervals. AI, 1 mg, per subject was administered on day 2, 3, or 4 of menses, immediately followed by 100 μg GnRH. AII, 1 mg, was administered on day 4, 5, or 6 of maximal sexual swelling, immediately followed by 10 μg GnRH: a second dose of GnRH and AII was administered 90 minutes later. In a separate experiment, 1 mg AII was administered twice at a 90-minute interval, but 10 μg GnRH was administered only once, immediately after the second AII injection.

RESULTS

Menstrual Cycle Assay: Results are summarized in Tables 15-1 and 15-2. Control cycles (n = 13; Tables 15-1 and 15-2 and Figures 15-1 and 15-2) showed the anticipated pattern of sexual swelling, gonadotropin and steroid hormone secretion[8,12] characterized by a mean (±S.E.) duration of maximal sexual swelling of 12.5 (±0.76) days, associated with estradiol–17β (E_2) levels rising up to 400 pg/ml in some instances, although other cycles did not show large estradiol peaks. Subsequently, simultaneous LH and FSH peaks of 188.0 (±24.3) and 52.4 (±5.9), respectively, occurred. Sexual swelling regression then followed, and progesterone levels rose to 8 to 18 ng/ml. The mean (±S.E.) interval between the last day of maximal sexual swelling (nominal luteal interval) and first day of menses was 10.7 (±0.7) and mean (±S.E.) duration of menses was 2.8 (±0.3). One control animal had low E_2 values, a very small LH rise, a premature biphasic, low progesterone rise, and an 8-day luteal interval.

At 0.02 mg/kg antagonist, maximal sexual swelling was prolonged by 6 to 9 days compared with controls (Fig. 15-1, Table 15-1). LH and FSH surges were correspondingly delayed, but occurred in most instances shortly before withdrawal of antagonist. Sexual swelling regression was followed by a normal luteal interval and progesterone values within the normal range, suggesting that the antagonist induced only a temporary delay of ovulation. This effect was not seen at the other dose levels (Fig. 15-2).

In all dose groups, sporadic endocrine abnormalities occurred, including double or disjunctive LH peaks in six of 13 instances, no FSH peak in one instance, and neither FSH nor LH peak on one instance. In ten of 13 treated cycles, the LH peak height was less than in controls (Table 15-2) and in nine cycles the mean FSH peak height was significantly reduced compared with controls ($P<0.05$: paired t-test). Other than as noted, the endocrine profiles of the treated cycles showed the expected pattern, with no evidence of altered luteal function resulting from the attenuation of gonadotropin peaks.

Duration of Action of Antagonist: In order to examine the duration of antagonist action more closely, repeated GnRH challenges were given at 3-hour

(*Text continues on p. 174*)

TABLE 15-1. Mean Menstrual Cycle Parameters (Days) in Chimpanzees Treated with [Ac-dehydro-Pro[1], pCl-D-Phe[2], D-Trp[3,6], N^{α}Me-Leu[7]]-GnRH

DOSE (MG/KG)	N	DURATION OF MAXIMAL SEX SWELLING	LUTEAL DURATION	DURATION OF MENSES
Vehicle	4	10.5	9.5	2.0
0.02	4	17.5	8.8	2.8
Vehicle	3	12.7	9.0	3.6
0.1	3	13.7	12.0	3.3
Vehicle	3	13.0	11.3	3.0
0.5	3	14.0	10.0	2.3
Vehicle	3	14.3	12.0	2.7
0.1 · 3 day	3	16.3	11.5	2.7
$\bar{X}$ (± S.E.) Vehicle		12.5 (±0.76)	10.7 (±0.7†	2.8 (±0.3)‡
$\bar{X}$ (± S.E.) Drug		14.7 (±1.2)*	10.4 (±0.8)†	2.8 (±0.4)†

*0.02 mg/kg excluded; n = 9.

†n = 12.

‡n = 11.

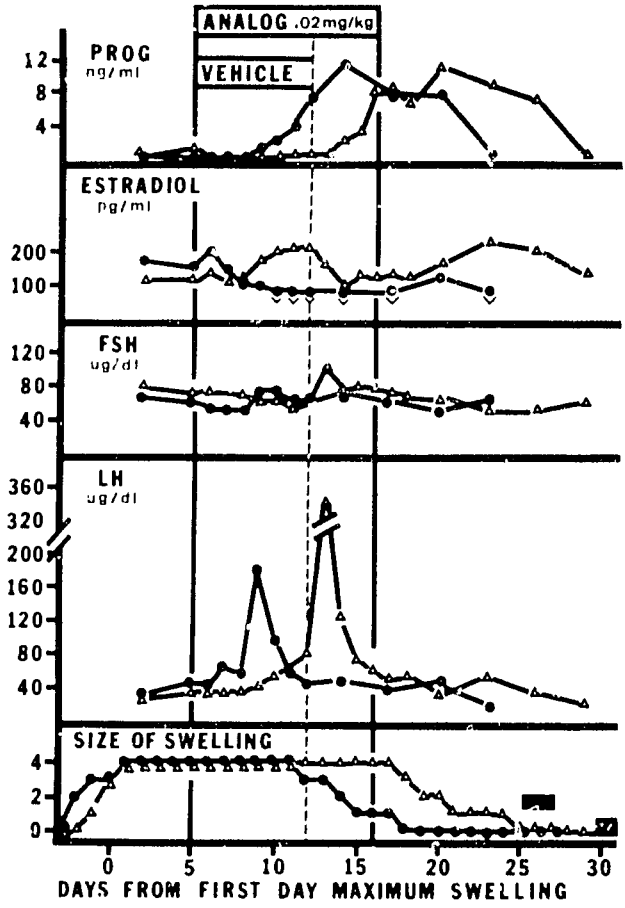

Fig. 15-1. Endocrine and sexual swelling changes during two menstrual cycles of a chimpanzee treated with vehicle (closed circles) and GnRH antagonist A1 (open circles), respectively. Horizontal bars indicate menses. Drug-associated delay in sexual swelling regression and related endocrine events was evident at the 0.2-mg/kg dose level.

TABLE 15-2. Mean Characteristics of Gonadotropin Peaks in Chimpanzees Treated with [Ac-dehydro-Pro[1],pCl-D-Phe[2],D-Trp[3,6], N$^{\alpha}$Me-Leu[7]]-GnRH

DOSE (MG/KG)	N	DAY* OF LH PEAK	DAY* OF FSH PEAK	HEIGHT† OF LH PEAK	HEIGHT† OF FSH PEAK
Vehicle	4	9.0	9.5	123.8	42.1
0.02	4	15.0	15.5	160.8	53.2
Vehicle	3	9.7	9.7	239.9	58.9
0.1	3	11.7‡	13.3‡	220.1	40.8
Vehicle	3	10.3	10.3	250.7	71.4
0.5	3	11.6	13.0	108.7	26.2
Vehicle	3	11.0	11.7	160.0	40.7
0.1 × 3/day	3	10.5	12.0§	84.5	18.2
			$\bar{X}$ (± S.E.) Vehicle	188.0 (± 24.3)	52.4 (± 5.9)
			$\bar{X}$ (± S.E.) Drug	144.8 (± 31.3)	36.0 (± 5.4)

*Day 1 = first day of maximal sexual swelling.
†μg/dl.
‡Delay attributable to 1 subject.
§n = 2.

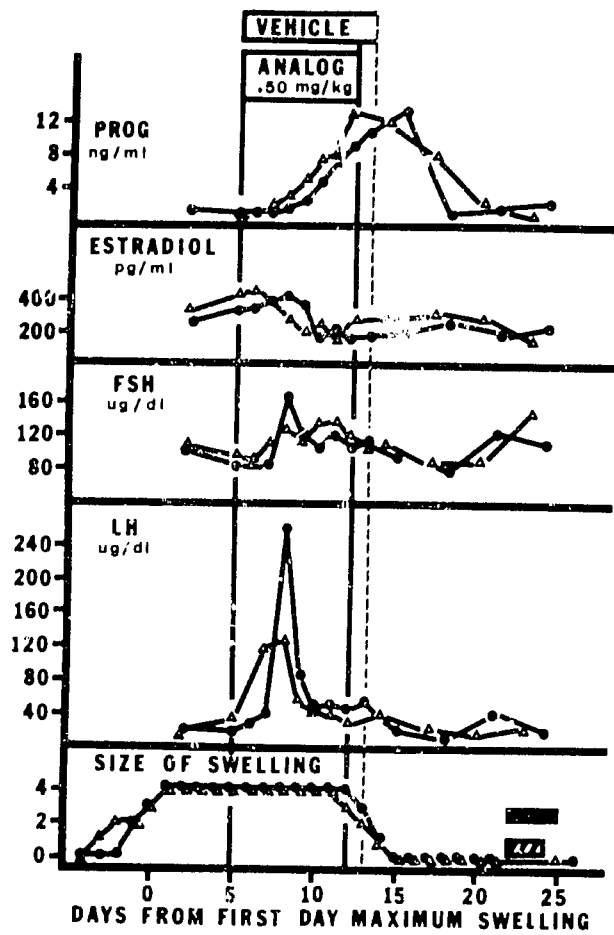

Fig. 15-2. Endocrine and sexual swelling changes during two menstrual cycles of a chimpanzee treated with vehicle (closed circles) and GnRH antagonist AI (open circles), respectively. No effect of the 0.5-mg dose on temporal relationships of menstrual cycle was evident, but reduced LH peak and broad elevation of FSH were frequent findings.

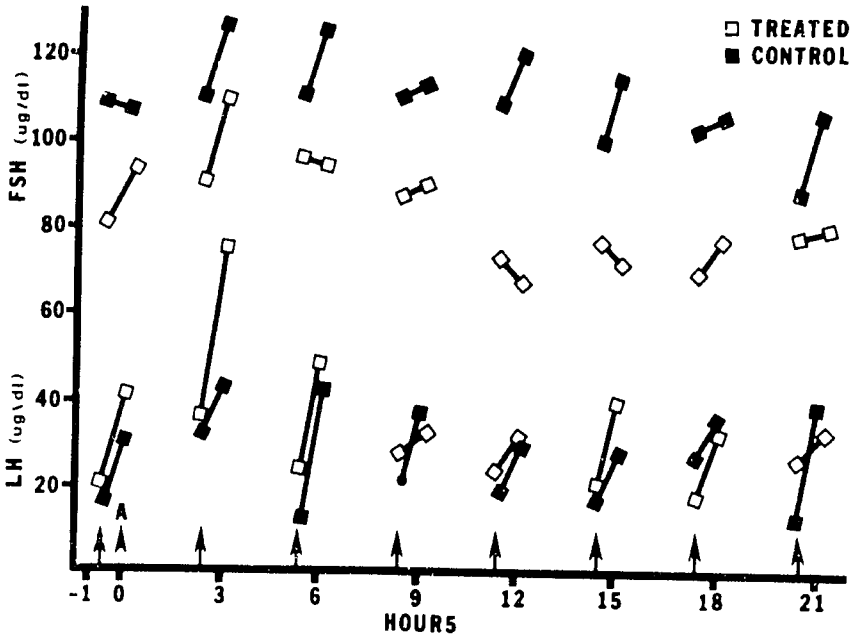

Fig. 15-3. Gonadotropin responses to serial GnRH challenges (arrow) before and after administration of AI (A) to an adult female chimpanzee.

intervals. These did not reveal any consistent effect of antagonist on baseline or GnRH-stimulated LH or FSH levels in two subjects studied after antagonist administration (Fig. 15-3). In Table 15-3, the mean increments in LH and FSH levels after GnRH injection in the control experiment are very similar to the increments after administration of 0.1 mg/kg AI subcutaneously, except that both pre- and post-antagonist baselines and FSH increments were depressed in one animal, an effect evidently independent of antagonist administration. No trend with time in gonadotropin baseline or response to GnRH was observed.

Despite the availability of data from only two subjects, owing to aversion to repeated alert venipuncture, the data provided a reasonable presumption that no consistent inhibition of gonadotropin release, if any, occurs under the test conditions. This conclusion led us to examine the effect of intravenously administered antagonist on GnRH responses, since we questioned the efficacy of the antagonist when delivered subcutaneously in oil.

TABLE 15-3. Mean (± S.E.) Increments in Gonadotropin Levels (μg/dl) after [Ac-dehydro-Pro[1],pCl-D-Phe[2],D-Trp[3,6],N[α]Me-Leu[7]]-GnRH (Successive 3 Hourly GnRH Challenges)

		SUBJECT A	SUBJECT B
LH	Control	17.2 (±3.0)	9.7 (±3.0)
	Analog	16.4 (±4.0)	9.6 (±2.3)
FSH	Control	10.8 (±2.8)	5.6 (±3.0)
	Analog	3.9 (±2.8)	7.5 (±1.8)

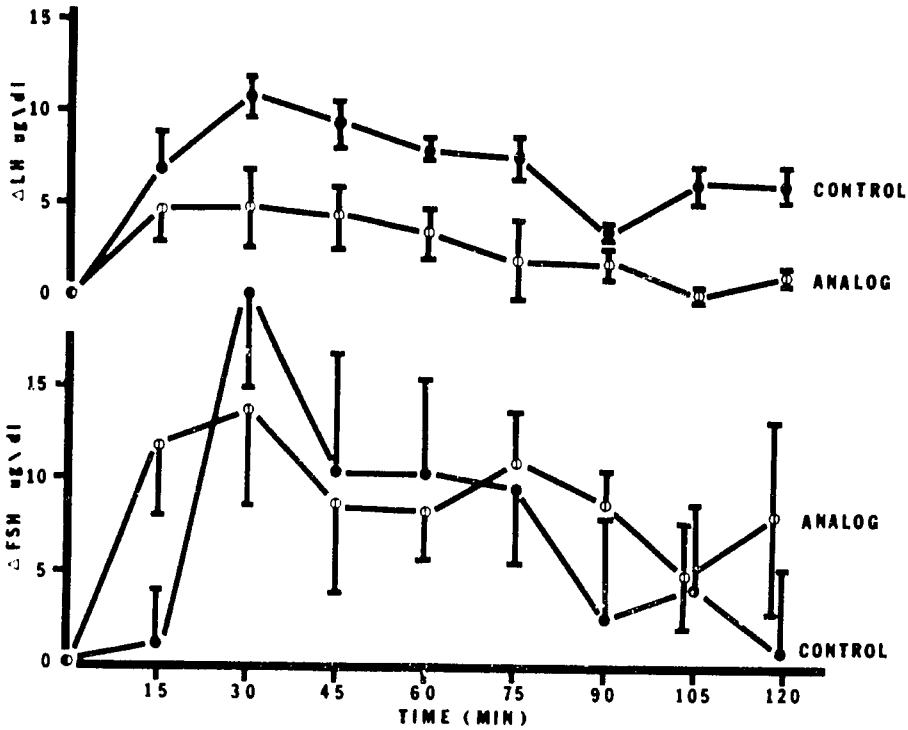

Fig. 15-4. Mean (±S.E.) serum gonadotropin increase over baseline before and after GnRH- and AI- or vehicle-administered IV.

GnRH Inhibition Assay: AI was evaluated in menstruating chimpanzees because, under these conditions, 100 μg GnRH elicits both an LH and FSH response, thus providing two endpoints for evaluation of the antigonadotropic effect of the antagonist.[9]

Interpretation of the data was complicated by the fact that both preanalog baseline and postanalog response was considerably lower in the treated animals (n = 4) than in the controls (n = 4). This effect is apparently attributable to individual variability only, since all samples were run in the same assay. When the data were expressed as increment over preanalog baseline (Fig. 15-4) the area under the LH curve was inhibited 61% by antagonist, when compared with the control curve (one-tailed, unpaired *t*-test: $P<0.025$).

TABLE 15-4. Effect of [Ac-dehydro-Pro[1],pCl-D-Phe[2],D-Trp[3,6]]-GnRH on LH Response

	AREA UNDER THE CURVE; ARBITRARY UNITS		
	1st LHRH Response (Mean area)	*2nd LHRH Response (Mean area)*	*P*
Control	233.8	634.0	<0.05
Treated	143.5	101.75	>0.25
% Inhibition	38.6	84%	
P	>0.15	<0.05	

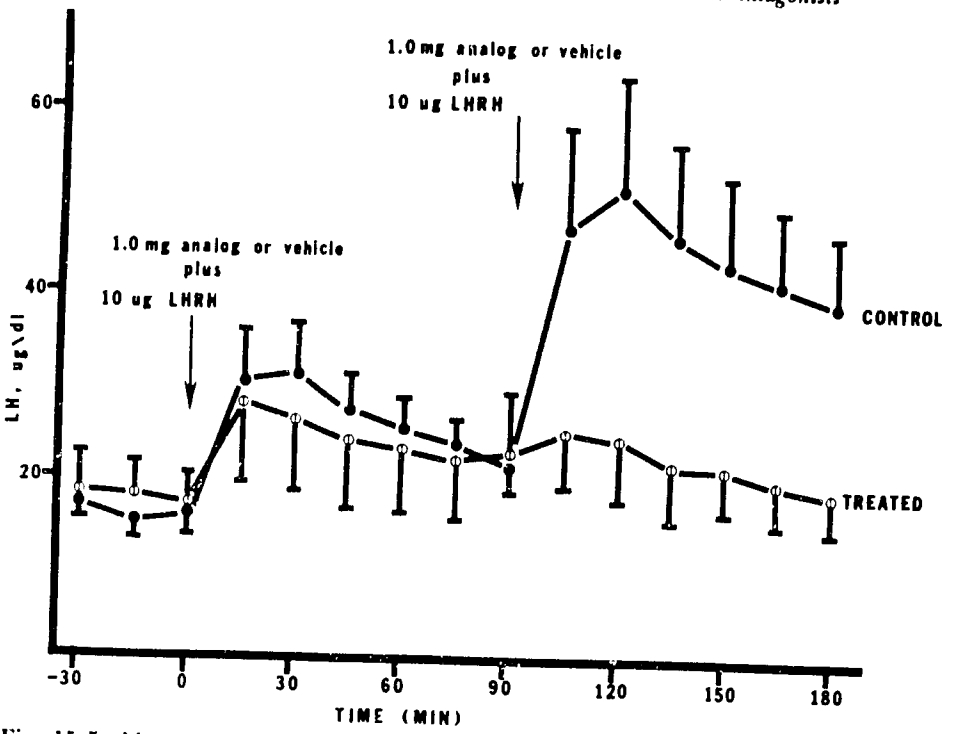

Fig. 15-5. Mean (±S.E.) serum gonadotropin concentration before and after GnRH- and AH-administered IV.

At this point in the study, antagonist AH, twice as potent in the rodent as AI, became available. It was decided to initially evaluate this antagonist in a modified GnRH inhibition assay. The dose of GnRH was reduced to 10 μg to increase the ratio of antagonist. In addition, two doses of GnRH were given at a 90 minute interval, to take advantage of the "self-priming" effect of GnRH[15] and so further enhance the LH response. FSH was not measured, as an FSH response is usually not detectable under these conditions.[9]

Animals receiving GnRH, but no antagonist (n = 5), showed an LH response to each GnRH challenge, the second response being greatly augmented ($P<0.05$; one-tailed t-test) as anticipated (Fig. 15-5). Antagonist administration (n = 5) was associated with a partial (38%) and nonsignificant inhibition of the LH response to the first GnRH challenge (Table 15-4). In contrast, the second GnRH response was almost totally inhibited in the antagonist-treated animals (area under the curve reduced by 81%, compared with controls: $P<0.05$).

In order to explore the reason for lack of inhibition of the first GnRH response, a similar experiment was conducted, but in which only the second injection of AH was associated with a GnRH challenge. Of four control animals with normal baselines, three showed at least fourfold mean LH increments after GnRH, and one a small response (<twofold). Among four analog treated animals, one showed a fourfold increment, one no substantial change, and two a decrement. When areas under the curve were calculated, analog

treatment was associated with approximately a 75% decrement in area under the curve. (Mean responses significantly different in the Mann Whitney Sum Rank Test: $P<0.05$.)

DISCUSSION

Competitive antagonism of GnRH action, and hence suppression of the preovulatory gonadotropin surge, has seemed a logical approach to inhibition of ovulation and/or luteinization for contraception. A series of antagonistic analogs of GnRH are effective antiovulatory agents in rodents, but have not yet been proven in primates.

In this study, AI failed to ablate preovulatory gonadotropin peaks when administered at a dose range of 0.02 to 0.5 mg/kg: there was a trend towards reduced gonadotropin peak height, and occasional perturbation of the normal gonadotropin peak pattern, suggestive of a limited antigonadotropic effect of the antagonist. These effects, although encouraging, were evidently insufficient to inhibit ovulation or luteinization under the conditions tested, since luteal duration and progesterone levels remained normal.

Corbin[2] and Wilks[16,17] likewise noted only mild, incomplete or inconsistent antigonadotropic and antiovulatory effects of the antagonists [D-Phe^2, D-Ala^6]-GnRH, [pyr<Glu-Pro)^1,D-Phe^2,D-Trp^{3,6}]-GnRH, and [D-Phe^2,D-Trp^3, D-Phe^6]-GnRH in *Macaca fascicularis* and *M. mulatta*, respectively, when administered subcutaneously in corn oil in substantial doses before and during the midcycle gonadotropin surge.

This series of studies in which only weak, insufficient, or occasional antigonadotropic effect was evident demand a reassessment of the approach to selection of drug dosage, regimen, and route of administration.

Our initial concern that daily drug administration was insufficiently frequent was not satisifed when thrice daily administration during the menstrual cycle provided no further evidence of antigonadotropic activity or when a repeated GnRH challenge assay with 0.1 mg antagonist/kg injected subcutaneously revealed no immediate or delayed antigonadotropic activity. In this context it is notable that Casper and associates[1] found that 10 mg [D-pGlu^1, D-Phe^2,D-Trp^{3,6}]-GnRH subcutaneously did not affect gonadotropin levels in two postmenopausal women.

These observations led to the question whether the class of antagonists under discussion are capable of significant antigonadotropic action in higher primates, or whether the method of administration was inappropriate. For this reason we turned to examination of the antigonadotropic action of GnRH antagonists administered IV in an acute GnRH challenge assay. These experiments showed that two different analogs (AI, AII) are capable of inhibition of GnRH-induced gonadotropin release when injected intravenously. AI was tested in menstruating chimpanzees at a dose level of 1 mg: 100 μg GnRH. The mean GnRH elevation of LH was reduced 61% by analog administration, but no significant effect on FSH was observed. We felt that 61% inhibition under these conditions was not encouraging for contraceptive potential, especially considering the route of administration. A promising antiovulatory agent should provide closer to 100% inhibition of gonadotropin release in an acute experimental design.

When a more potent analog (AII) in the rat became available, we redesigned the GnRH assay to increase its sensitivity. Hence, data from the two experiments are not directly comparable. AII, 1 mg, was administered to midfollicular chimpanzees, immediately followed by 10 μg GnRH. (Conditions calculated to optimize the LH response and antagonist/GnRH ratio). In addition, a second dose of antagonist and GnRH was given 90 minutes after the first, because an enhanced gonadotropin response is seen under these conditions in the absence of antagonist.[4,15] Surprisingly, only slight inhibition (38%) of the first GnRH-induced LH response was noted, in contrast to that induced by AI. Considering the greater analog/GnRH ratio and greater potency in rodents of AII, this result was totally unexpected. A large LH response to the second GnRH challenge occurred, probably reflecting the so-called self-priming effect of GnRH.[15] The antagonist substantially (81%) blocked this second GnRH response. When AII was given 90 minutes before, and concurrently with a single GnRH challenge, a substantial inhibition occurred, in contrast to the poor inhibition when the two drugs were given in quick succession initially.

AII requires a finite duration of pre-exposure to block a GnRH challenge. The design of GnRH challenge experiments can greatly influence the perception of antagonist potency. Preadministration of antagonist should probably be a standard condition of GnRH-inhibition assays in primates. In light of these facts, it seems possible that AI would have shown evidence of greater potency had it been administered 90 minutes before GnRH challenge.

A further key point is that AI and AII are capable of moderate to substantial acute antigonadotropic action in chimpanzees, provided they are administered IV. The failure to detect a profound effect when AI was administered subcutaneously in corn oil might be a function of metabolism, or of rate of absorption from the vehicle. The choice of vehicle may be the critical factor since two less potent antagonists, [D-Phe^2,D-Trp^3,D-Phe^6]-GnRH and [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH, were moderately effective in inhibiting LH responses to two successive GnRH challenges when administered subcutaneously to chimpanzees in propylene glycol/saline in a similar experimental design to the inhibition assay employed here.[4,5] A vehicle such as corn oil, though likely to provide the desired slow release needed for chronic gonadotropin suppression, might not achieve adequate blood levels of antagonist to block endogenous GnRH action effectively, unless much larger doses are administered.

To develop an effective protocol for primate antiovulatory trials, a more systematic approach to selection of antagonist, vehicle, route of administration, and dose is required.

A GnRH assay similar to that used with AII is suitable for initial evaluation of drug potency. Having identified a drug with adequate potency, a vehicle and route of administration must be selected which are suitable for repeated administration and prolonged drug action. The GnRH challenge assay can again be used to assess these factors.

The next question to answer is how to maintain continuous and adequate suppression of gonadotropin secretion. Given an adequate antagonist, this would appear to be a matter of dose and frequency of administration. These administration parameters can be evaluated in the chimpanzee, either by a GnRH challenge at various levels after antagonist administration, or perhaps

in the male chimpanzee where absence of the cyclic variable can speed up analysis.

Using these approaches to the manipulation and evaluation of drug administration parameters, it should be possible to establish a protocol that permits continuous gonadotropin suppression. One approach to dosing that could be considered in light of our previous results is an initial IV injection of antagonist to obtain high circulating levels, followed by subcutaneous or IM doses to chronically sustain the circulating concentration of antagonist.

A systematic approach to antagonist and dose regimen selection for ovulation suppression in primates is extremely important. In the absence of such an approach, negative results in a menstrual cycle assay are impossible to interpret. It is uncertain whether the particular antagonist tested is inherently ineffective in the primate model, if one of many possible changes in administration protocol might produce the desired effect, or if the principle underlying this approach to contraception is invalid.

The principle on which this approach to ovulation control is based has, in fact, been called into question. Evidence has been presented that endocrinologically normal menstrual cycles can be reproduced in rhesus monkeys deprived of an endogenous source of GnRH and continuously administered small exogenous pulses.[11] This implies that, at least in the rhesus monkey, there is no obligate requirement for a preovulatory GnRH surge. (The gonadotropin surge may result instead from an estrogen-mediated postive feedback sensitization of the gonadotrophs to a relatively constant level of GnRH.)

Support for or against this hypothesis can be recruited from a systematically designed GnRH administration protocol which has been demonstrated to provide adequate chronic suppression of the gonadotropin response to exogenous GnRH challenge. Failure to block midcycle gonadotropin elevation and related events with such a protocol in man or a closely related species such as the chimpanzee would evoke the presumption that there is indeed no requirement for a preovulatory GnRH surge in these species and that GnRH antagonist administration is not a viable approach to ovulation inhibition.

However, there is every reason to keep an open mind on this issue until conclusive experiments can be conducted. Wilks and colleagues[16,17] have reported limited evidence of an antiovulatory effect of GnRH antagonists in rhesus monkeys: replication of their data on a larger scale is necessary. Second, rhesus monkeys differ from chimpanzees in their GnRH responses,[9] and it is quite possible that in higher primates an acute preovulatory GnRH surge is present and necessary for ovulation, whatever the condition in rhesus monkeys.

In view of these uncertainties, it appears premature to proceed directly to attempt to inhibit ovulation in women before validating the ability of GnRH antagonists to block ovulation in animal models closely related to man, such as the chimpanzee.

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16

Discussion: Physiology and Contraceptive Effects of LHRH and Analogs in Female Animals: Antagonists

ROBERT T. CHATTERTON, MODERATOR

In the studies of antagonists of LHRH on ovulation suppression in rats, rhesus monkeys, and chimpanzees reported in this session, all compounds were studied or previously tested for their ability to inhibit ovulation in rats, and ranged in potency for ED_{90} of from 10 to 250 μg as a single dose. Studies in rats showed that the effect of the LHRH antagonists was on LH secretion. Prolactin levels were unchanged, and progesterone administration concomitant with the antagonist prevented its abortifacient action.

Compared with LHRH agonists which are abortifacient before day 6 in the rat, the antagonists are effective only between days 6 through 10, a time in pregnancy when the ovary is LH-dependent as shown by other criteria. Preliminary evidence in primates suggests that antagonists with greater potency in rats are also more potent in primates. However, with the dose regimens employed, ovulation was not consistently suppressed in primates. Some evidence that more frequent administration may be effective was presented, probably more than three times a day, even with the most long-acting antagonists. Escape from suppression in the form of ovulation that was delayed until after LHRH antagonist administration was withdrawn was also observed. Occasional selective suppression of FSH appeared to occur, but this was not associated with anovulation in the treatment cycle nor in the subsequent cycle.

Occasional inhibition of ovulation with follicular luteinization was also observed in monkeys; the possibility of designing a dose-regimen that would consistently produce this effect has been considered a worthwhile objective, but more work must be done to ascertain its feasibility.

Since the action of the antagonists is clearly limited to suppression of LH and FSH secretion by the pituitary (no suppression of CG production has been found), the application of this technology is limited to methods for

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Panelists: Charles E. Graham, Catherine Rivier, John W. Wilks. *Discussants:* William F. Crowley, Jr., Joseph W. Goldzieher, Charles E. Graham, Hugo Maia, Jr.

suppression of ovulation. Usefulness of the LHRH antagonists will require methods of administration that will allow more continuous exposure of the female to the compound or preparations that have an even slower rate of inactivation in the body.

LHRH-ANALOG AGONISTS AND ANTAGONISTS AS ANTIFERTILITY AGENTS IN THE HUMAN FEMALE

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17

Induction of Luteolysis by Postovulatory Intranasal Administration of [D-Ser (TBU)^6-des-Gly-NH$_2$ ^{10}]-LHRH Ethylamide (Buserelin): A Postcoital Contraceptive Approach

ANDRE LEMAY

NACIA FAURE

FERNAND LABRIE

The isolation and synthesis of LHRH,[33,34] the hypothalamic decapeptide that stimulates pituitary LH and FSH secretion, and the availability of potent LHRH analogs have permitted a large number of studies on the control of reproductive functions. Synthetic releasing hormones can induce ovulation in the experimental animal,[45] and in some cases humans,[38,50] thus providing a potential new therapeutic approach for the treatment of human infertility, but only limited success has been obtained.

The difficulties in achieving therapeutical success in treating human infertility are most likely related to the now well-recognized paradoxical antifertility effects of these neuropeptides in both female[5,14,21,24,25,41] and male[3,4,39] experimental animals. Thus, the administration of LHRH or a potent agonist on diestrus I or II in normal female rats has been shown to delay the expected day of mating and vaginal cornification.[5,6,17,25] Gonadotropin-releasing peptides have also potent postcoital contraceptive activity in female rats[14,21,24] and rabbits.[18]

Although the precise mechanisms responsible for the antifertility effects of LHRH and its agonists are not yet fully understood, at least part of these effects are probably mediated by the loss of gonadal receptors and desensitization secondary to induced release of endogenous gonadotropins, thus providing the best evidence for the mechanisms involved in the antifertility effects of LHRH and its agonists.[3,4,21,25] There is also a direct inhibitory effect

These studies were supported by NIH Contract and MRC Grant (MA-6558). The authors gratefully acknowledge the supply of Buserelin by Hoechst Pharmaceuticals.

of LHRH agonists at the gonadal level[9,19,20,27,32,41,46,50] although the relative importance of the pituitary and gonadal sites of action remains to be assessed. Another site of the antifertility effects of LHRH peptides is the adenohypophysis where decreased gonadotropin responsiveness to LHRH is observed after pharmacologic or repetitive doses of LHRH agonists.[16,43,47]

LUTEOLYSIS INDUCED BY INJECTION OF LHRH OR LHRH AGONISTS

Following the observation of such potent antifertility effects of LHRH and its agonistic analogs in the animal, we studied the possibility of a luteolytic effect of treatment with LHRH during the luteal phase in normal women. In 1978, we first observed that repeated subcutaneous injections of 250-μg doses of LHRH led to early luteolysis in 4 women (28). As illustrated in Figure 17-1, the administration of five subcutaneous doses of LHRH at 4-hour intervals on the seventh day after the LH peak in a representative subject causes a second peak of LH and FSH. On the day of LHRH treatment, estradiol and progesterone are increased. During the following 4 days, serum progesterone and estradiol decreased precociously and menstruation appeared 3 days earlier than in the control cycle.

This initial study was next extended to 17 treatment cycles.[40] When administered between days 1 to 9 after the LH peak, LHRH shortened the luteal phase from 1 to 4 days in 16 of 17 treatment cycles. Of the three women who had a luteal phase shortened by only 1 day, two had unusual spotting 3 and 5 days before the beginning of their menses. It was then noticed that the treatment with LHRH seemed to be more efficient when applied on two successive days as compared with a single day (four women).

There was also a better efficiency of treatment when LHRH was administered in the middle rather than in the early luteal phase. Figure 17-2 illustrates the shortening of the luteal phase and the percent inhibition of serum progesterone level according to day of treatment after the LH peak. In fact, in all cases where treatment was administered between days 1 and 5, the shortening of the luteal phase was 1.4 ± 0.2 day and mean progesterone inhibition was $28.7 \pm 6.0\%$. When treatment was administered on days 6 to 9, shortening of luteal phases was 3.3 ± 0.2 days and mean progesterone inhibition was $54.8 \pm 6.7\%$.

A similar luteolytic effect has been observed by other investigators using subcutaneous injection of potent LHRH analogs. Koyama and associates[26] reported that five daily injections of 100 μg of [des-Gly-NH_2^{10} Pro-ethylamide^9]-LHRH early in the luteal phase did not shorten the luteal phase but led to a significant decrease of plasma progesterone levels. Casper and Yen[12] showed that one or two 50-μg doses of [D-Trp^6,Pro^{9}NEt]-LHRH administered during the middle of the luteal phase induced an early and concomitant decline in circulating levels of estradiol and progesterone, as well as a short luteal phase and premature menstruation. Raynaud and others[40] have shown an inhibition of progesterone secretion during the luteal phase by subcutaneous injections of two LHRH agonists in the monkey (*Macaca fascicularis*). More recently, Asch and colleagues[1] demonstrated a luteolytic effect of a

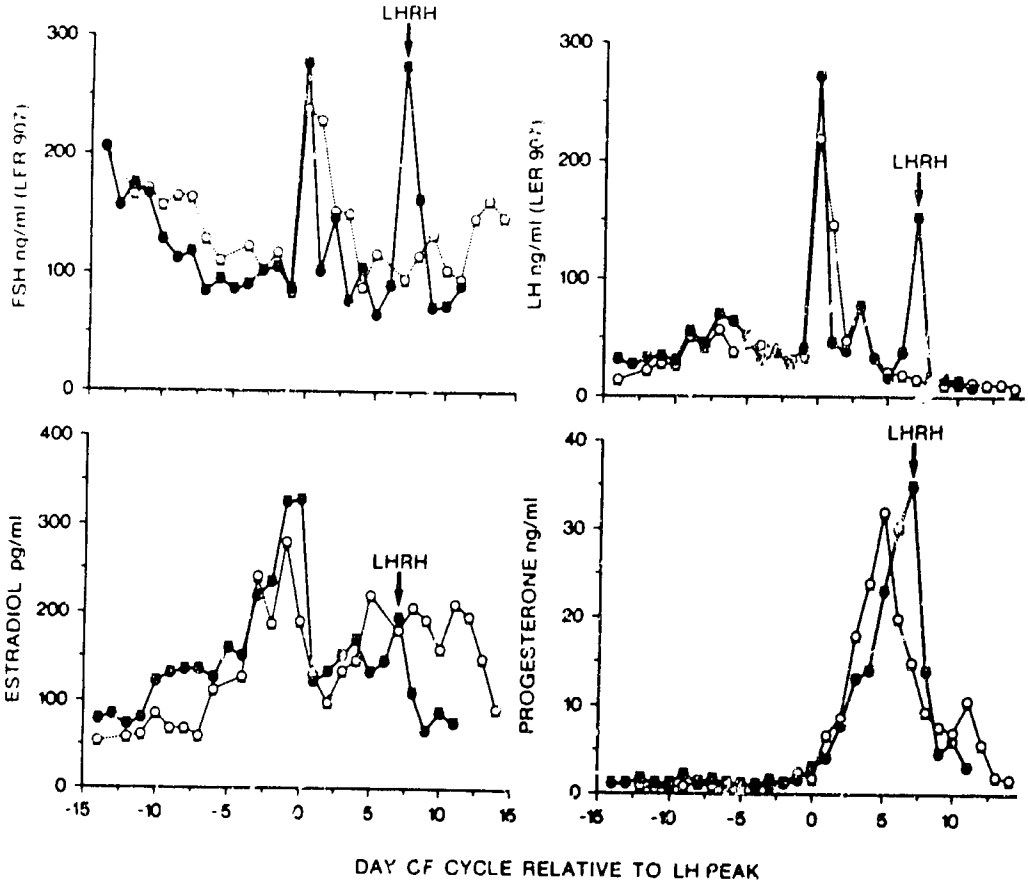


Fig. 17-1. Daily LH, FSH, estradiol, and progesterone levels in a woman during pretreatment control (○-----○) and LHRH (●—●) treatment cycles. On day 7 after LH peak, five 250-μg doses were injected subcutaneously every 4 hours.

single IM injection of [D-Trp^6]-LHRH in the rhesus monkey (*Macaca mulatta*). Luteolysis was consistently induced when the analog was injected on days 3 or 5 postovulation. However, no effect was observed when treatment was given on day 7 postovulation.

LUTEOLYSIS INDUCED BY INTRANASAL ADMINISTRATION OF BUSERELIN

Since the potent LHRH agonist Buserelin [D-Ser(TBU)^6-des-Gly-$NH_2{}^{10}$]-LHRH ethylamide[44] is active by the intranasal route,[49] we then studied the effect of this LHRH agonist administered intranasally during two successive menstrual cycles on a single day between days 4 and 9 after the LH peak in normal women. Figure 17-3 illustrates the typical pattern of serum LH and progesterone levels in one woman during six consecutive menstrual cycles:

two control pretreatment, two treatment, and two control posttreatment cycles. When 500 μg of the analog was administered twice at 0800 and 1700 hours on day 5 or 6 after the LH peak during the third and fourth cycles, respectively, the luteal phase was shortened by 4 days. Since a serum sample was taken before the second administration of the LHRH analog, LH level had almost returned to basal, whereas progesterone concentration reached the highest level of the luteal phase. Thereafter, progesterone concentration declined rapidly during the next 4 days leading to premature menstruations. In the two posttreatment cycles, the length of the luteal phase as well as the progesterone profile were similar to those observed during pretreatment cycles.

The effects of intranasal administration of the LHRH analog on luteal phase length and serum progesterone concentration in six normal women are summarized in Figure 17-4. When the 12 pretreatment, treatment, and posttreatment cycles were considered, the luteal phase was shortened from 13.6±0.3 to 10.9±0.3 days after LHRH analog treatment ($P<0.01$); length of the luteal phase was 12.8 ±0.3 days in the posttreatment cycles. When the surface areas under the serum progesterone curves were calculated starting from the day of LHRH analog treatment, the progesterone was reduced to 61.3±9.2% of control and returned to 97.3±13.3% of control in the posttreatment cycles.

This first study with Buserelin clearly indicated that luteolysis can be induced after intranasal administration of two 500-μg doses of the neuropep-

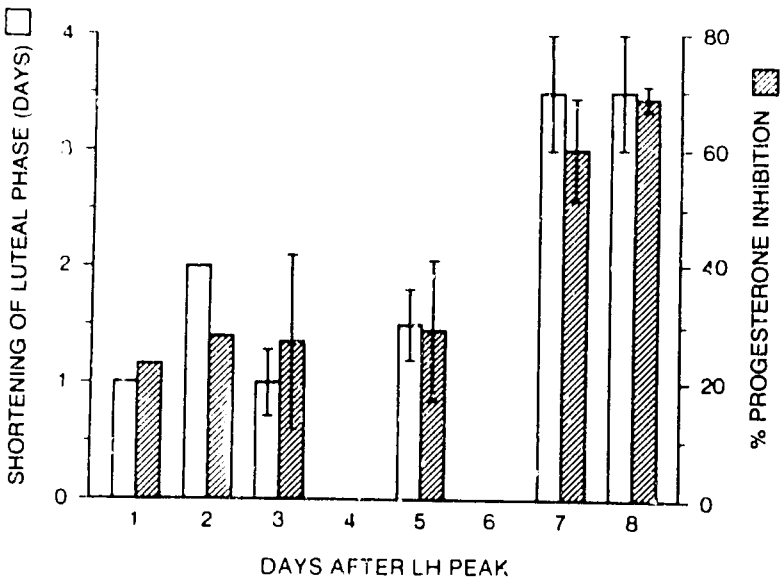


Fig. 17-2. Relationship between day of LHRH treatment after LH peak, shortening of luteal phase, and percentage of progesterone inhibition. Five 250-μg doses of LHRH were injected subcutaneously every 4 hours during a single day. Inhibition of progesterone was evaluated by calculating surface under progesterone curve starting on day of LHRH treatment.

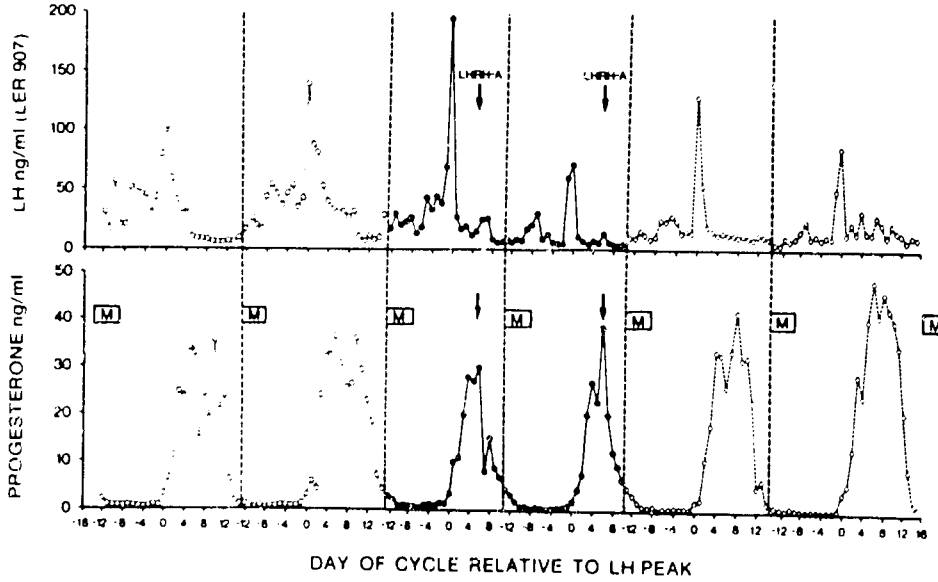


Fig. 17-3. LH and progesterone profiles in a woman during six consecutive cycles. On days 5 and 6 of the third and fourth cycle, 500 μg Buserelin was administered intranasally at 0800 and 1700 hours. On day of treatment, hormone levels reported were those observed at the time before the second administration of the LHRH analog. Luteal phase was shortened by 4 days in the two treatment cycles. (M = menstruation)

tide during a single day at midluteal phase. No side-effect was observed and apparently normal cycles occurred immediately after treatment.

SENSITIVITY OF GONADOTROPIN RESPONSE AND CORPUS LUTEUM FUNCTION TO SINGLE INTRANASAL ADMINISTRATION OF BUSERELIN

Although the mechanism of the antifertility action of LHRH and its agonistic analogs remains to be fully understood, the down-regulation of gonadal receptors and functions clearly demonstrated in the animal[8,23,24] provides a probable explanation for the acute luteolytic effect observed in the human. In fact, the antifertility effects of the LHRH peptides observed in male and female rats are parallel to their gonadotropin-releasing activity.[15] Moreover, exogenous LH and hCG have also been shown to be luteolytic in pregnant sheep[36] and pregnant and pseudopregnant rabbits and rats.[31,42] Such treatments with exogenous gonadotropins have been shown to lead to desensitization of ovarian and testicular gonadotropin receptors and function.[4,29]

It has been observed in both male and female rats that the desensitizing activity of LHRH and its agonists depends not only on the maximal plasma LH levels reached but also on the duration of this elevation.[13,15] Consequently, it was important to use a dose of the LHRH analog which leads to a maximal rise of plasma gonadotropin levels and also has a long duration of action.

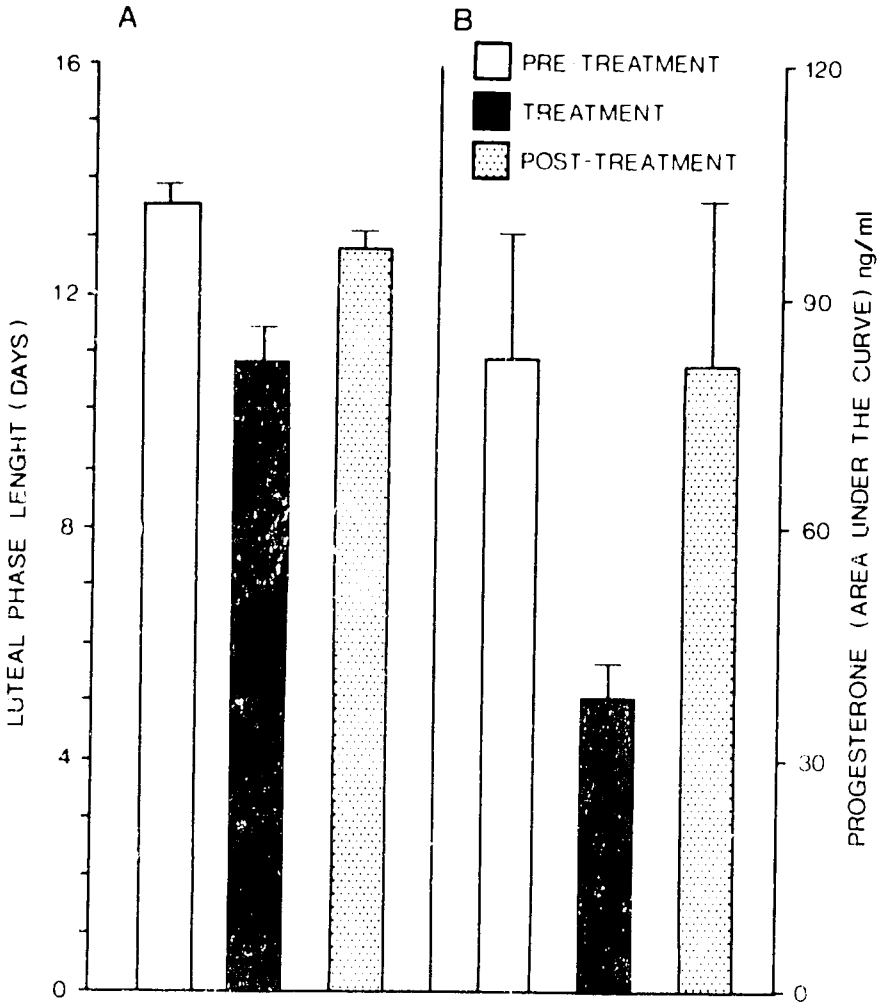


Fig. 17-4. (*A*) Average luteal phase length and (*B*) area under progesterone curves of 12 pretreatment, treatment, and posttreatment cycles. The day of spontaneous LH peak was taken as day 0. Progesterone values were calculated in each cycle starting from day of LHRH analog treatment.

The pituitary LH and FSH responses to LHRH peptides are known to vary according to the different stages of the menstrual cycle.[37,48] Using a 5-μg subcutaneous dose of Buserelin, an intermediate response was found at midluteal phase.[16] Our previous experiments in normal women with LHRH suggested a better efficiency for luteolysis when the peptide was administered between days 6 and 9 after the LH peak.[30] In order to optimize the induction of luteolysis by intranasal insufflation of Buserelin, it appeared important to study the gonadotropin response and the corpus luteum function after single increasing doses of the analog administered at midluteal phase.

Blood samples were obtained every other day relative to the time of Buserelin administration for days 1 to 10 and daily for the remainder of the cycle

in 24 normally cycling women who were studied during at least four menstrual cycles corresponding to two pretreatment, one treatment, and one posttreatment cycles. In most of the subjects, the study was continued for two more cycles corresponding to a second treatment cycle followed by another posttreatment cycle. Single doses of 50, 100, 200, 500, 1000 or 1500 μg Buserelin were insufflated into the nose at 0900 hours on a single day ranging from day 6 to 12 after the LH peak. On the day of LHRH analog treatment, blood samples were taken at −½, −¼, 0, ½, 2, 4, 6, 8, 11, 14 and 24 hours, in order to evaluate the acute hormonal responses.

Figure 17-5 illustrates the serum LH response to increasing doses of Buserelin administered by nasal spray. At the 50- and 100-μg doses, there is already a significant elevation of serum LH concentration at 30 minutes, while maximal levels are reached at 2 hours and 4 hours, respectively. With increasing doses of LHRH analog, the response occurs earlier and a maximum is reached at 4 hours. At the 1000- and 1500-μg doses a 10- to 15-fold stimulation is obtained and serum LH levels are still above basal values at 14 hours.

The effect of increasing doses of Buserelin is better illustrated when serum LH is expressed as areas under the 24-hour response curves (Figure 17-6). An approximately 50% maximal response is already observed at the dose of 100 μg of the neuropeptide, and a near-maximal stimulation is obtained at the 200-μg dose. The present data indicate that an intranasal dose of 200 to 500 μg of Buserelin gives a near maximal stimulation of gonadotropin secretion in women at the midluteal phase.

The luteolytic effect of single doses of Buserelin is shown in Figure 17-7, which illustrates the hormonal profiles in a woman studied during six consecutive cycles. In the first two control cycles the luteal phase lasted 15 and 16 days after the LH peak. On days 10 and 6 after the LH peak of during the third and fifth cycles, 500 μg and 1500 μg of LHRH analog were insufflated. Four hours after treatment, there was an important stimulation of serum LH and FSH levels, while estradiol and progesterone levels were also increased. During the following days, serum progesterone and estradiol concentration decreased progressively and premature menstruation appeared 13 days after the LH peak. In the posttreatment control cycles (fourth and sixth cycles), luteal phase length returned to normal (15 and 16 days) and the hormonal profile was normal.

Figure 17-8 compares the effect of increasing doses of Buserelin on serum progesterone levels in all pretreatment, treatment, and posttreatment cycles. Up to 200 μg, there is no apparent change in serum levels of the steroid. At the 500-μg dose, there is a tendency for decreased progesterone concentration during the treatment cycle. At doses of 1000 μg and 1500 μg, progesterone levels in the treatment cycles are clearly inhibited. The progesterone curves also show the full recovery of the corpus luteum function in posttreatment cycles. Similar curves have been obtained for estradiol (data not shown).

The percent inhibition of progesterone and estradiol concentrations has been measured by calculating the areas under the curves in individual cases starting from the day of LHRH analog treatment. As shown in Figure 17-9, inhibition of the level of the two sex steroids is observed at the 500-μg dose, a maximal inhibition being obtained at 1000 μg and 1500 μg of Buserelin.

(*Text continues on p. 194*)

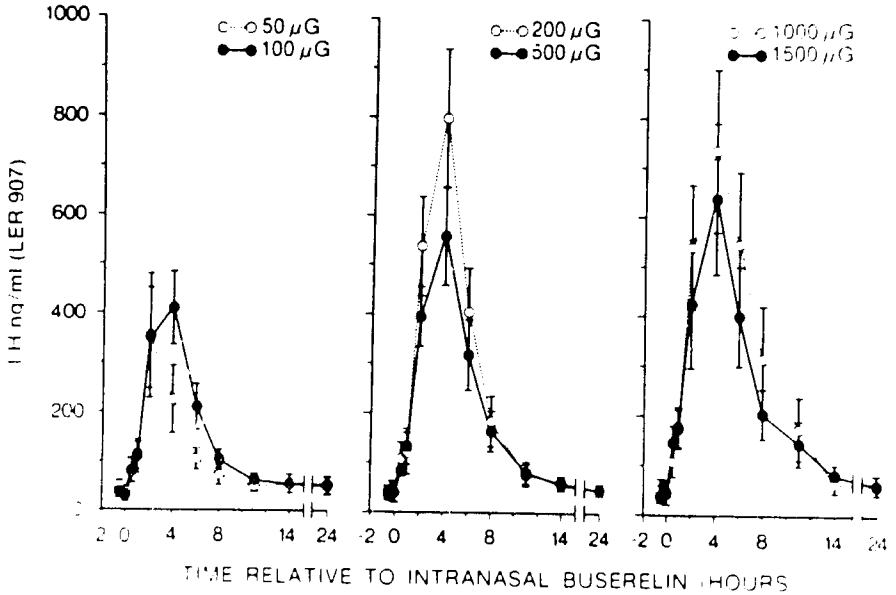


Fig. 17-5. Acute serum LH response to single intranasal insufflation of increasing doses of Buserelin.

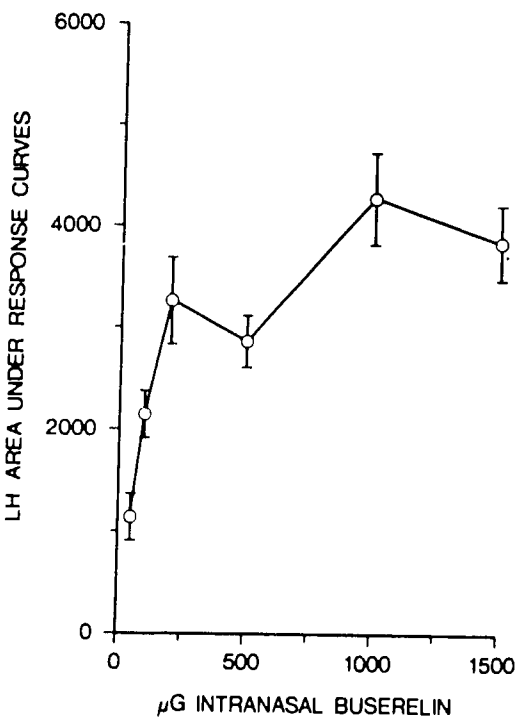
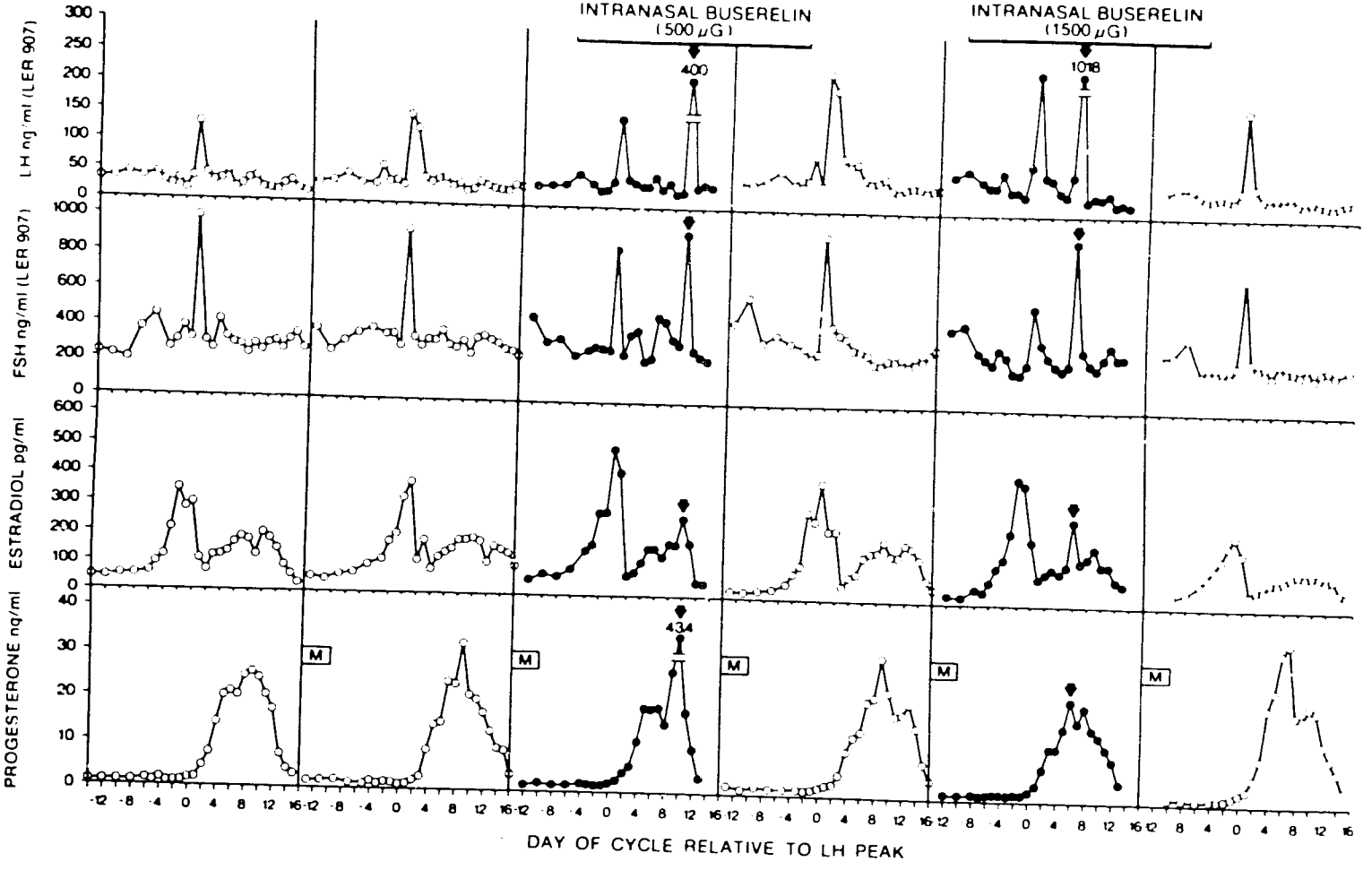


Fig. 17-6. LH surface areas under response curves following single intranasal insufflation of increasing doses of Buserelin. Values are expressed as the mean ±S.E.M. the cumulated 24-hour serum LH levels in individual women.


INTRANASAL BUSERELIN
(500 μG)
INTRANASAL BUSERELIN
(1500 μG)
400
1018
434
M
LH ng/ml (LER 907)
FSH ng/ml (LER 907)
ESTRADIOL pg/ml
PROGESTERONE ng/ml
DAY OF CYCLE RELATIVE TO LH PEAK

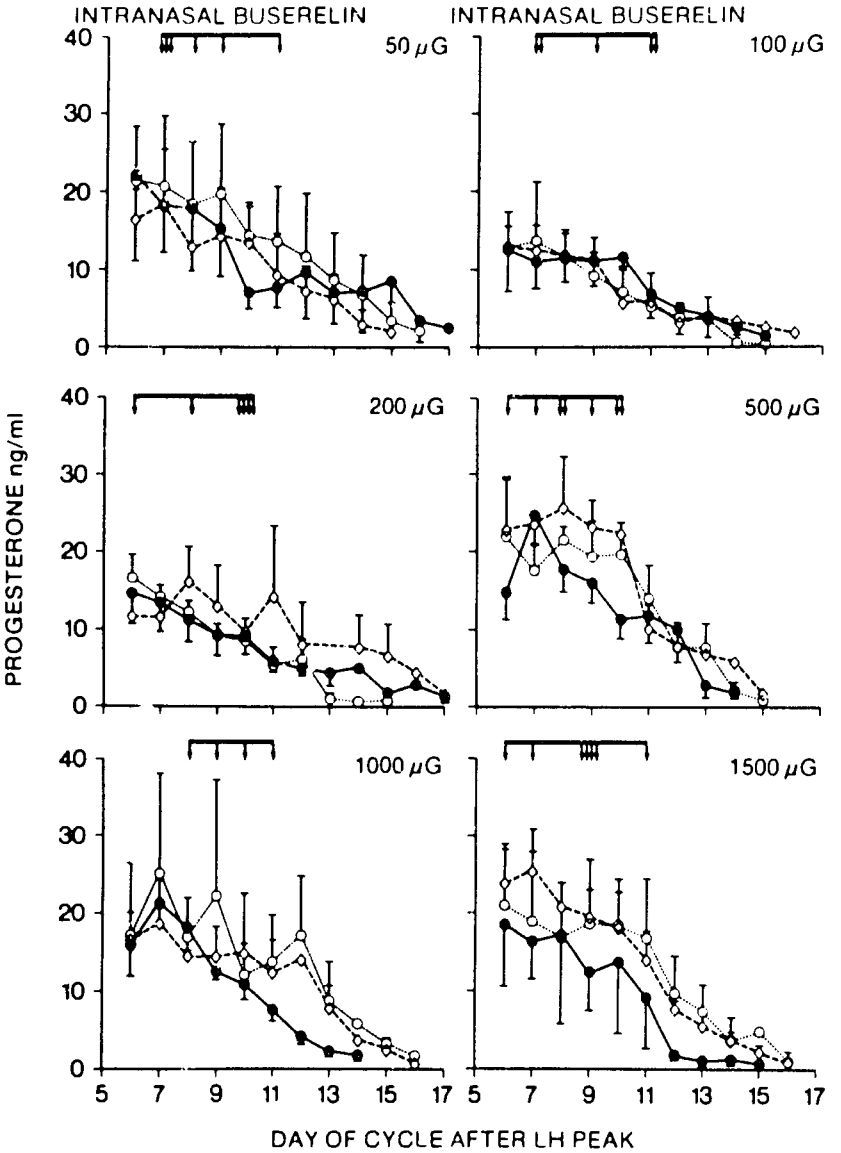


Fig. 17-8. Effect of single increasing intranasal doses of Buserelin on progesterone levels. Progesterone values are means ±S.E.M. of individual levels in each group in pretreatment (○-----○) treatment (●—●), and posttreatment (◇-----◇) cycles. Arrows indicate on which day each woman was treated.

Fig. 17-7. Serum LH, FSH, estradiol, and progesterone levels in a woman during six cycles. A single dose of 500 μg and 1500 μg Buserelin was insufflated into the nose on days 10 and 6 of the third and fifth cycle, respectively. On day of treatment, hormone levels reported were those observed at 4 hours after administration of the LHRH analog. Luteal phase was shortened by 2 days in the two treatment cycles. (M = menstruation)

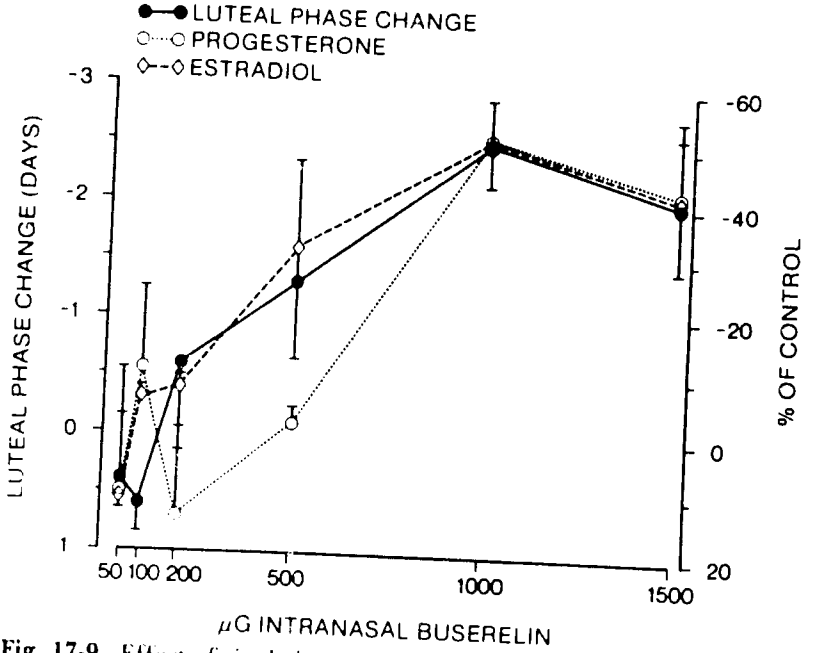


Fig. 17-9. Effect of single increasing intranasal doses of Buserelin on luteal phase length (●—●), serum progesterone (○-----○), and estradiol (◇-----◇). Results are expressed as the mean ±S.E.M. of individual values in each group as compared with control pretreatment cycles. The percent change in progesterone and estradiol was obtained by calculating the area under hormone curves in individual cases starting from day of LHRH analog administration.

The observed inhibition of the corpus luteum function correlates well with the shortening of the luteal phase which is found at doses of Buserelin higher than 200 μg.

The present results clearly indicate that the luteolytic effect of a single dose of intranasal Buserelin administered during midluteal phase is dose-related and is proportional to the amplitude and duration of the gonadotropin response. In subsequent studies on induction of luteolysis, optimal efficiency is likely to be obtained by using single or repeated administration of optimal doses of the agonist.

INTRANASAL BUSERELIN AS A POSTCOITAL CONTRACEPTIVE APPROACH

Because progesterone secretion by the corpus luteum is essential for implantation and maintenance of early pregnancy and luteal phase insufficiency is associated with infertility, the luteolysis induced by intranasal LHRH analog treatment appears as an attractive and practical postcoital contraceptive approach in regularly ovulating women. This approach is near-physiological,

since it is based on the regression of corpus luteum function, an event which normally occurs at the end of each normal menstrual cycle. The aim of the treatment with Buserelin is to control in a precise manner the time of decrease of the corpus luteum function and consecutive occurrence of menses.

In order to use intranasal LHRH analog as a postcoital contraceptive, the obvious important question to be answered is the effectiveness of the treatment at the time of possible onset of a pregnancy. Since hCG is secreted very early after implantation and stimulates corpus luteum function, it is mandatory to demonstrate a definitive luteolytic effect in the presence of rising concentrations of hCG. Bergquist and colleagues[7] and Casper and colleagues[11] have already presented preliminary observations reporting that luteolysis induced by LHRH agonist treatment is prevented by exogenous hCG. In these studies, fixed and relatively high doses (1500 or 5000 IU) of hCG were administered for 7 or 10 days. In fact, using such treatment progesterone concentration was found to be 100% more elevated than during the control cycle. Moreover, levels of hCG reached were not reported; more importantly, the effect of LHRH agonist treatment in the presence of hCG was not compared to the effect of hCG treatment alone.

Casper and associates[11] also reported that subcutaneous administration of high doses of an LHRH agonist on two successive days was ineffective in inducing luteolysis when treatment was started on day 37 to 54 after the expected day of menstruation in pregnant women. In three women the levels of hCG were above 2000 mIU/ml; in only one woman, 50 μg of LHRH analog were administered on days 43 and 44 of the cycle, corresponding to a 2-day period before detection of hCG in the circulation.

Asch and others[1] reported that administration of increasing amounts of hCG from days 6 to 10 after ovulation prevented luteolysis induced by LHRH agonist treatment in the monkey. In this study, the analog was administered either as a single dose on day 5 or daily concomitantly to hCG treatment. When administration of the neuropeptide was repeated from days 6 to 10, acute LH response was not reported. However, there is no apparent increase or decrease in serum LH when measured daily. As mentioned previously, decreased pituitary responsiveness has been observed after repetitive doses of LHRH agonists.[16,43,47] hCG control cycles were not performed in the same animals, but luteal phase length was reported to be longer in another study with an identical hCG treatment.[2] It is also clear that levels of progesterone on day 14 after combined hCG-LHRH analog treatment are much lower than those observed in the hCG cycle reported in the other study.[2]

There was three- to sixfold increase in estradiol and progesterone levels in response to the hCG regimen used. Such an important increase in corpus luteum function is probably not observed at the very beginning of a pregnancy in the monkey. Moreover, in patients followed from the time of ovulation and conception, basal FSH levels fall very rapidly and there is nearly complete blunting of the LH-FSH response to LHRH at a time when estradiol and progesterone levels are still at levels seen during the luteal phase.[23] In spite of the important hCG stimulation of the corpus luteum, it is possible that a luteolytic effect could have been observed if hCG control cycles had been included in the study and that other schedules of LHRH analog administration may be effective.

In order to validate the potential use of intranasal LHRH analog treatment for postcoital contraception in the human, careful and detailed studies should be performed at the very onset of pregnancy. Optimal increasing amounts of hCG could be administered in order to mimick the hCG rise and corpus luteum function usually observed at the beginning of pregnancy. It has been shown that before the fourth week after the last expected menstrual period, hCG levels are usually below 100 mUI/ml.[10,35] Thus, it will be important to study the effect of schedule of administration of an optimal dose of the analog at different levels of hCG. Though our previous studies have shown the optimal doses of Buserelin required to induce luteolysis when the peptide is administered by nasal spray at midluteal phase, the efficiency of such treatment remains to be assessed at other stages of the menstrual cycle. Moreover, in possible schedules of treatment using repeated administration of LHRH analog, the acute pituitary and corpus luteum responses should be noted.

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18

The Contraceptive Use of Buserelin, A Potent LHRH Agonist: Clinical and Hormonal Findings

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WOLFGANG HARDT

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MARIANNE VON DER OHE

Analogs of LHRH with widely differing biological activity have been prepared by deleting or substituting certain amino acids of the decapeptide. The influence of one of the most potent LHRH agonists, [D-Ser(TBU)[6]]-LHRH-(1-9)-nonapeptide ethylamide (Buserelin, Hoe 766), on pituitary and gonadal function has been studied intensively in different species.[13] This LHRH agonist has been estimated to exceed the effectiveness of LHRH in normally cyclic women by a factor of 40.[4] Although the LHRH analog provides a new therapeutic approach for treatment of infertility, only limited success has been reported in humans.[13,14] Chronic administration produces an initial stimulation and subsequent suppression of gonadal function.[14] This "paradoxical" antifertility effect of LHRH agonists is now well documented in both female and male experimental animals.[7,13] Moreover, recent studies indicate that these oligopeptides can also lead to inhibition of reproductive function in humans.[2,4,9,11] As a result, new approaches to fertility control based on LHRH agonists are currently under investigation.

Chronic treatment by intranasal administration of Buserelin inhibited ovulation in all but two of 89 treatment cycles in women.[2,3] Amenorrhea combined with reduced estrogen secretion was often noted. Endometrial biopsies in a very limited number of cases showed mild proliferation in menstruating women and inactive endometrium in amenorrheal women. Our results from continuous treatment with daily doses of 400 μg Buserelin (156 treatment months) were quite different. The morphologic alterations of the endometrium indicated that unopposed estrogen stimulation of target organs may be the main problem of long-term contraception with the dose schedule used.[15]

Extensive investigations with LHRH agonists are necessary for appraisal of this new approach to fertility control. Here we report our findings on chronic

The authors thank Hoechst AG, Frankfurt, F.R.G., who generously supplied Buserelin, and to the nursing staff of the outpatient clinic of the Universitaetsfrauenklinik Charlottenburg for their assistance in the care of our volunteers.

intranasal administration of different doses of Buserelin in 70 women over a total of 411 treatment months.

SUBJECTS AND METHODS

Seventy female volunteers, aged 17 to 42, were selected for study. The women had ovulation proven by biphasic basal body temperature; serum progesterone levels were normal in the control cycle. No volunteer had used oral contraceptives for at least three months prior to the study. Ten of the women had a history of oligomenorrhea or episodes of amenorrhea.

Daily intranasal administration of Buserelin* was started on the first day of menstrual bleeding following the control cycle and was continued normally for 6 months. Once each day, 28 women (Group I) were given 400 μg Buserelin and 28 volunteers (Group II) were treated with 200 μg Buserelin. (Seven women received 200 μg once daily and 21 women, 100 μg twice a day.) Fourteen women (Group III) received 100 μg of the LHRH agonist. Because preliminary results had indicated insufficient inhibition of ovulation by daily administration of 100 μg Buserelin, all women of the latter group used an additional means of contraception (intrauterine copper device).

The application of Buserelin was monitored by clinical examinations and frequent blood samples (two to three times a week, at least 6 hours after intranasal spray application) for determination of FSH, LH, estradiol, and progesterone by radioimmunoassay.[16] In selected cases, blood samples were taken after cessation of treatment until the next menstrual period and the mentioned hormones assayed. Endometrial biopsies (n = 109) were performed on volunteers during different periods of medication. The morphologic findings of the endometrium were based on a 28-day cycle according to Noyes and colleagues.[12] All volunteers had given written informed consent prior to the clinical trial.

RESULTS

A total of 411 treatment months with different doses of Buserelin were recorded. Only the 56 women of Group I (400 μg) and II (200 μg) did not use an additional contraceptive method. Thus, only 337 treatment months can be considered in terms of contraceptive effectiveness. One pregnancy occurred. This volunteer reduced the daily dose from 200 μg to 100 μg Buserelin on her own initiative.

Ovulation occurred approximately one week after reduction of medication. Pregnancy was confirmed by detection of hCG. This woman had a miscarriage 7 weeks postconception (Fig. 18-1).

INHIBITION OF OVULATION

Table 18-1 shows the distribution of serum progesterone concentrations during the course of treatment with different dose regimens of Buserelin. In 291 of 411 treatment months, progesterone levels were consistently below

*Kindly supplied by Hoechst AG, Frankfurt, F.R.G.

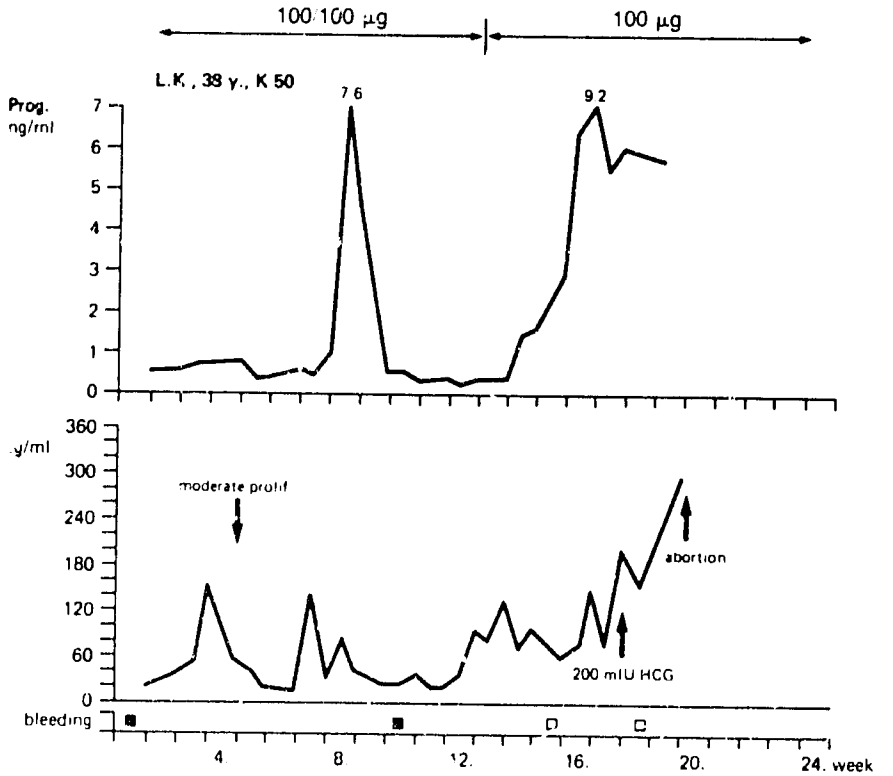


Fig. 18-1. Serum values of estradiol and progesterone in a volunteer (L.K.) during course of Buserelin treatment. Daily dose was reduced from 200 µg to 100 µg; pregnancy occurred.

TABLE 18-1. Distribution of Serum Progesterone Levels of 70 Volunteers During the Course of Treatment with Buserelin.

DOSE REGIMEN HOE 766 (µg) (N)	PROGESTERONE (ng/ml) <1 (%)	1–3 (%)	>3 (%)	MONTHS OF TREATMENT
400 (28)	136 (87)	16 (10.2)	4 (2.8)	156
200 (7)	32 (65)	10 (20)	7 (15)	49*
100/100 (21)	89 (67)	23 (17)	20 (16)	132*
100 (14)	34 (46)	17 (23)	23 (31)	74†
Total	291 (71)	66 (16)	54 (13)	411

Prog.>6 (ng/ml): *5 (2.7 %); †9 (12%)

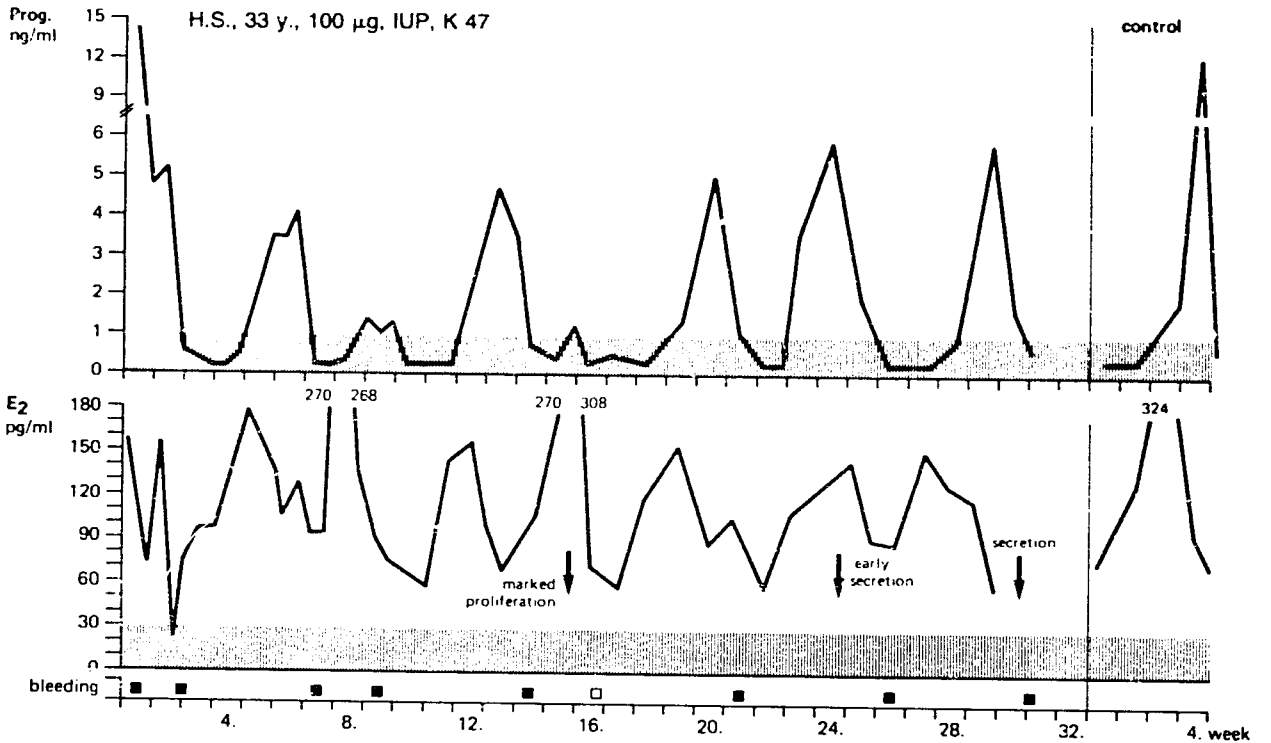


Fig. 18-2. Serum values of estradiol and progesterone in a volunteer (H.S.) during and after course of treatment with 100 µg Buserelin. Arrows indicate day of endometrial biopsy.

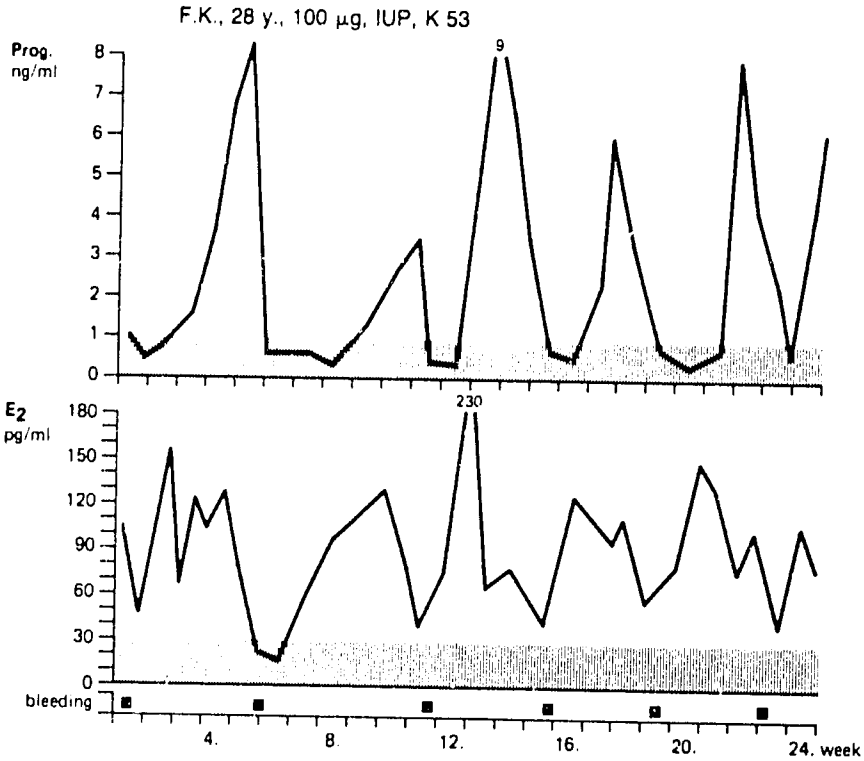


Fig. 18-3. Serum values of estradiol and progesterone in a volunteer (F.K.) during course of treatment with 100 µg Buserelin.

1 ng/ml; anovulation can be assumed. Incidence of anovulation was clearly dose-related and decreased from 87% (400 µg) to 66% (200 µg) and 46% (100 µg Buserelin). Progesterone secretion did not exceed 3 ng/ml during 66 treatment cycles; such findings indicate impaired corpus luteum function or luteinization only. In the remaining 54 treatment months progesterone levels (>3 ng/ml) probably reflected minor suppression of corpus luteum steroidogenesis. In spite of these subnormal progesterone values, no pregnancy occurred during 31 months of treatment in women using no further contraceptive method besides Buserelin (400 or 200 µg).

In women treated with 100 µg Buserelin and using an additional intrauterine device, both subnormal and normal progesterone values and luteal phases of more than 10 days were observed (Figs. 18-2, 18-3). Undisturbed corpus luteum activity can be assumed in these cases.

In summary, anovulation and severe corpus luteum insufficiency were found in 97.2%, 84.5%, and 69.0% of the recorded treatment months in the decreasing dose groups (400, 200, and 100 µg Buserelin). The antifertility effect in women using 200 µg Buserelin once daily or 100 µg twice daily was equal (Table 18-1).

TABLE 18-2. Distribution of Bleeding Pattern of 70 Volunteers During the Course of Treatment with Buserelin.

DOSE REGIMEN HOE 766 (μg)	N	BLEEDING None	Irregular	Regular
400	28(4)*	7	14	7
200	7(2)*	3	—	4
100/100	21(4)*	8	3	10
100	14	1	4	9
Total	70	19 (27%)	21 (30%)	30 (43%)

*Pre-existing cyclic disturbance (amenorrhea)

BLEEDING PATTERNS

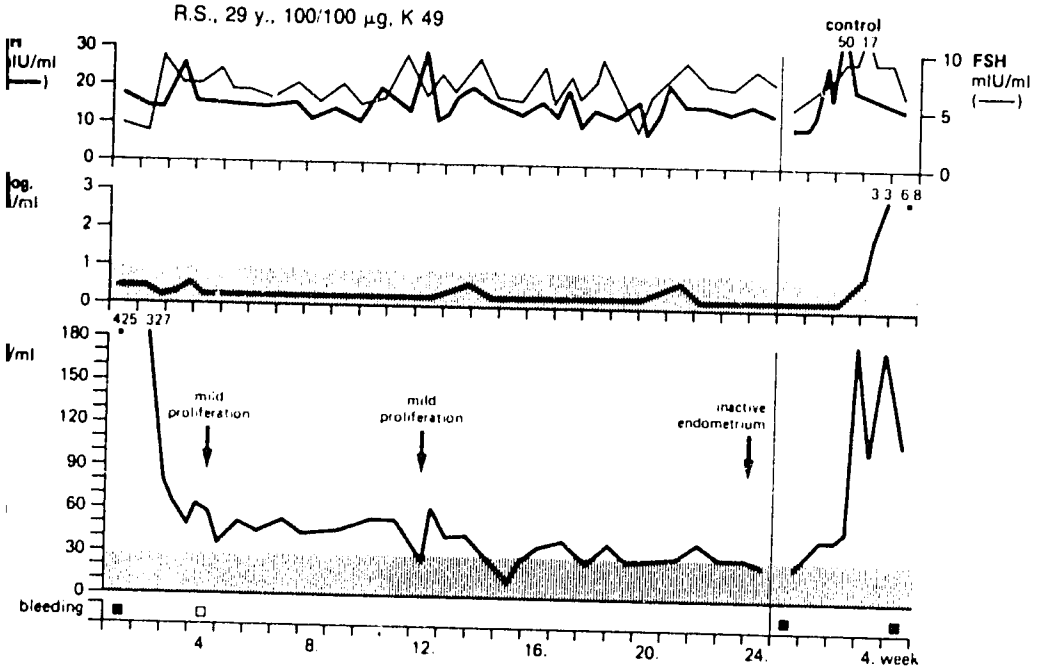
Regardless of the dose regimen, 19 women developed amenorrhea with the onset of medication (Table 18-2, Figs. 18-4, 18-5). It is interesting to note that ten of these volunteers had a pretreatment history of cyclic disturbance. Whereas the incidence of amenorrhea was equal in the higher dose regimens (400 and 200 μg Buserelin), amenorrhea occurred significantly less in Group III (100 μg).

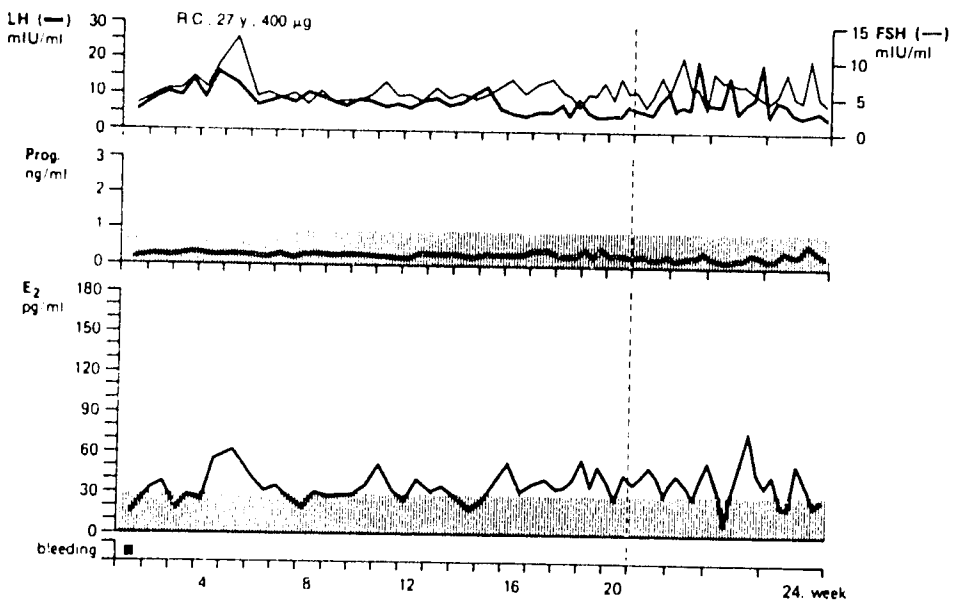
Table 18-2 illustrates the distribution of regular (intervals of 3 to 6 weeks) and irregular (intervals over 6 weeks) bleeding patterns during the course of treatment with different doses of Buserelin. Of the 28 women treated with 400 μg Buserelin, 14 had irregular bleeding intervals or developed amenorrhea during the second half of the observation period of 6 months (8 of 14 volunteers), as shown in Figure 18-6. The occurrence of irregular bleeding intervals was significantly less frequent with the lower dose regimens (Groups II and III). Regular bleeding intervals were found to be dose-related more often in these groups, as shown in Figures 18-2, 18-3, and 18-7.

The total number of irregular and regular bleeding events was similar in Group I (400 μg Buserelin, 79 bleedings during 156 treatment months) and Group II (200 μg, 90 bleedings during 181 treatment months). The highest incidence of bleeding was registered in Group III (100 μg, 54 bleedings during 74 treatment months). Comparing bleeding events with the preceding hormonal pattern revealed that estrogen-dependent bleeding (breakthrough or withdrawal bleeding) was quite common in Group I (400 μg, 59 of 79 bleedings).

Fig. 18-4. Serum values of gonadotropins, estradiol, and progesterone in a volunteer (R.S.) during and after course of treatment with 100 μg Buserelin twice daily. Arrows indicate day of endometrial biopsy.

Fig. 18-5. Serum values of gonadotropins, estradiol, and progesterone in a volunteer (R.C.) during course of treatment with 400 μg Buserelin. Blood samples were obtained daily from week 20 to 24.


R.S., 29 y., 100/100 µg, K 49
control
50 17
FSH
mIU/ml
3 3 6 8
425 327
mild proliferation
mild proliferation
inactive endometrium
bleeding
4. week


LH (—)
mIU/ml
R.C., 27 y., 400 µg
FSH (—)
mIU/ml
Prog.
ng/ml
E2
pg/ml
bleeding
24. week

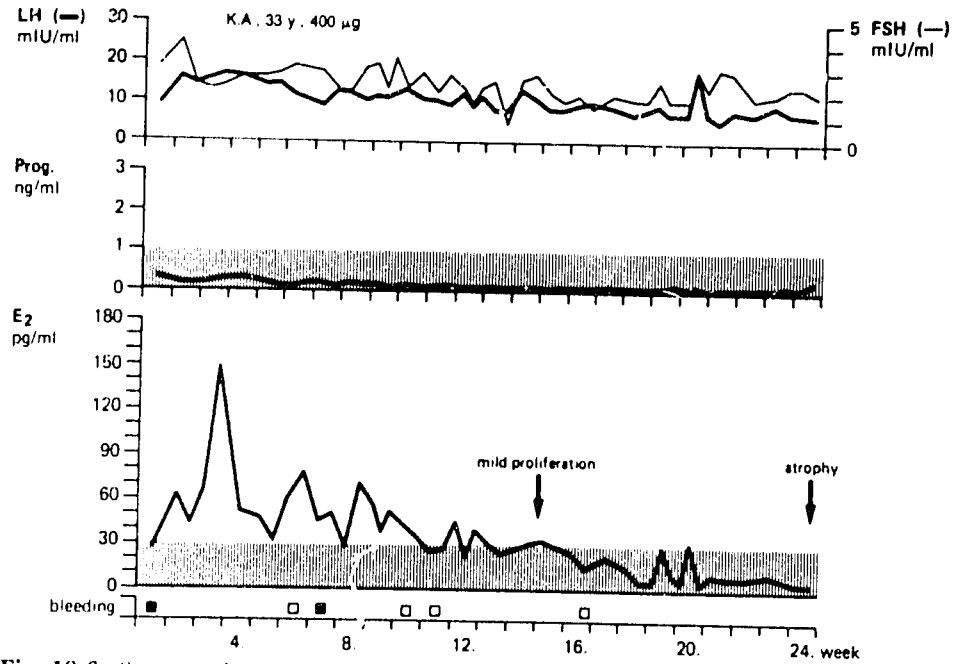


Fig. 18-6. Serum values of gonadotropins, estradiol, and progesterone in a volunteer (K.A.) during course of treatment with 400 µg Buserelin. Arrows indicate day of endometrial biopsy.

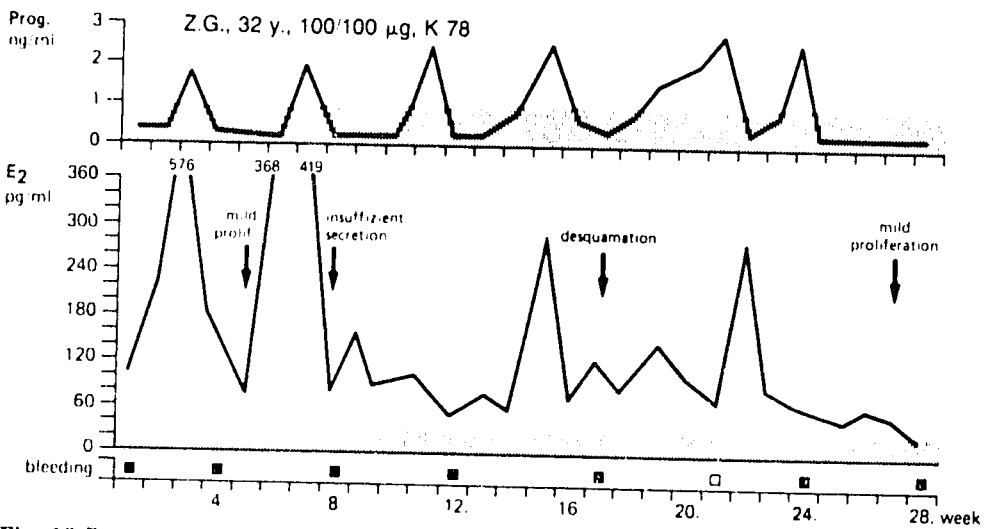


Fig. 18-7. Serum values of estradiol and progesterone in a volunteer (Z.G.) during course of treatment with 100 µg Buserelin twice a day. Arrows indicate day of endometrial biopsy.

Because of the higher incidence of residual cyclic progesterone activity, bleeding in Group II (200 μg) and Group III (100 μg) was mainly induced by progesterone withdrawal (60 of 90 bleeding events and 40 of 54, respectively). Examples are illustrated in Figures 18-2, 18-3, and 18-7. Comparison of the two different dose regimens (400 and 200 μg) in the same volunteer showed the dose-dependent inhibition of progesterone secretion (Fig. 18-8).

HORMONAL PATTERNS

Basal secretion of both gonadotropins remained nearly unaltered during the whole treatment course at all dose levels (Figs. 18-4, 18-5, 18-6, 18-8, 18-10, 18-11, and 18-12). Typical LH peaks following an estradiol surge could not be detected, but could not be definitely excluded on account of sampling intervals (2 to 4 days).

Intranasal administration of Buserelin evokes a pronounced release of LH, FSH, and, subsequently, estradiol during the first day of treatment. This is illustrated in Figure 18-9.

The stimulatory effect of Buserelin on gonadotropin secretion clearly decreased as treatment progressed. One volunteer developed amenorrhea during the course of treatment. However, women with menses-like bleeding during treatment showed only an inconstant reduction of the pituitary activity in terms of LH and FSH release during the progress of treatment (data not shown).

Estradiol secretion showed a tendency to lower values as treatment progressed, especially in group I treated with 400 μg Buserelin.[17] Generally, menstruating women showed higher serum estradiol concentrations and greater cyclic variation of the estradiol pattern than others. In contrast to these general findings, individual estradiol profiles were quite different. Only in women with preexisting endocrine disturbances (n = 10) could the occurrence of low and stable estradiol values combined with amenorrhea be predicted, as shown by way of example in Figures 18-4 and 18-5. In all other instances, progression or inhibition of the follicular maturation process, indicated by the estradiol secretion, could not be predicted.

In Group I (400 μg) 18 women showed marked fluctuations of the estradiol values, at times reaching preovulatory levels (Fig. 18-10). However, in the second half of the treatment period a tendency to lower serum concentrations of estradiol became apparent (Fig. 18-6). In four women, on the other hand, estradiol secretion was not suppressed and marked fluctuation could be observed until the end of treatment.

It is interesting to note that prolonged estradiol secretion caused marked proliferation of the endometrium and was often not interrupted by withdrawal bleeding (Fig. 18-11). At the lower doses (200 and 100 μg), periods of prolonged and elevated estradiol secretion were infrequent because of the higher incidence of progesterone and even estradiol withdrawal bleedings (Figs. 18-2, 18-3, 18-7, and 18-8). This is confirmed by the histologic findings. However, even in these dose regimens cyclic estradiol variations were followed by periods of impaired follicular maturation. Despite unaltered medication, restitution of cyclic variation of estradiol production took place. This is illustrated in Figure 18-12.

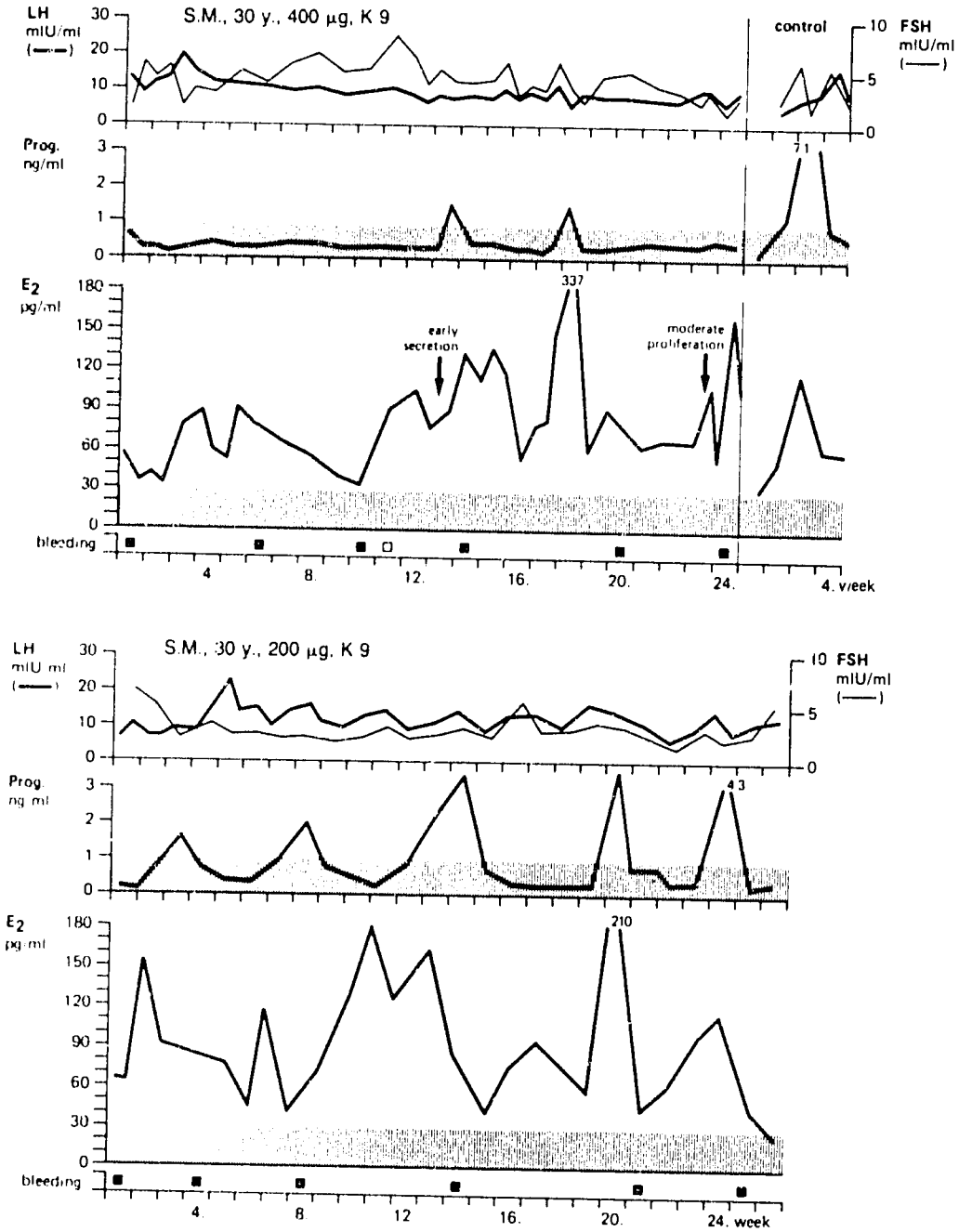


Fig. 18-8. Serum values of gonadotropins, estradiol, and progesterone in a volunteer (S.M.) during course of treatment with 400 and 200 µg Buserelin. Arrows indicate day of endometrial biopsy.

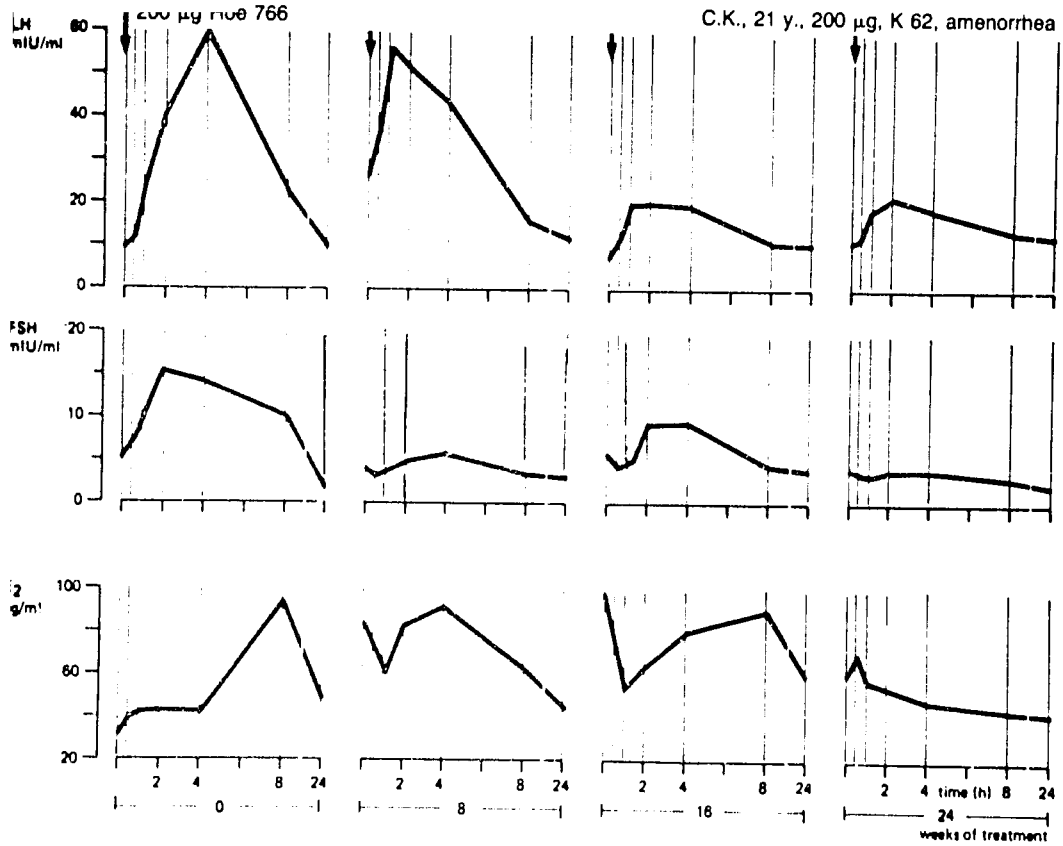


Fig. 18-9. Serum values of gonadotropins and estradiol in a volunteer (C.K.) after stimulation with 200 µg Buserelin during different weeks of treatment with 200 µg Buserelin.

In all treatment groups normal cyclic functions returned after cessation of therapy (Figs. 18-2, 18-4, 18-8, and 18-10). Two women became pregnant during the first cycle after 6 months of administration of Buserelin for contraceptive purposes.

ENDOMETRIAL BIOPSIES

During different periods of treatment, 109 endometrial biopsies were performed on 60 patients. In most cases repeated histologic specimens were obtained. Results for the different dose regimens are given in Table 18-3.

Morphologic findings ranged from inactive endometrium to marked proliferation as well as secretory transformation. The morphologic alterations could be readily correlated with the hormone profiles and bleeding intervals, as shown in Figures 18-2, 18-4, 18-6, 18-7, 18-8, 18-10, and 18-11.

Repeated endometrial biopsies in women with prolonged amenorrhea showed a regression of endometrial proliferation (Table 18-4) for the 200 µg dose regimen and for individual cases in Figures 18-4 and 18-13.

(*Text continues on p. 212*)

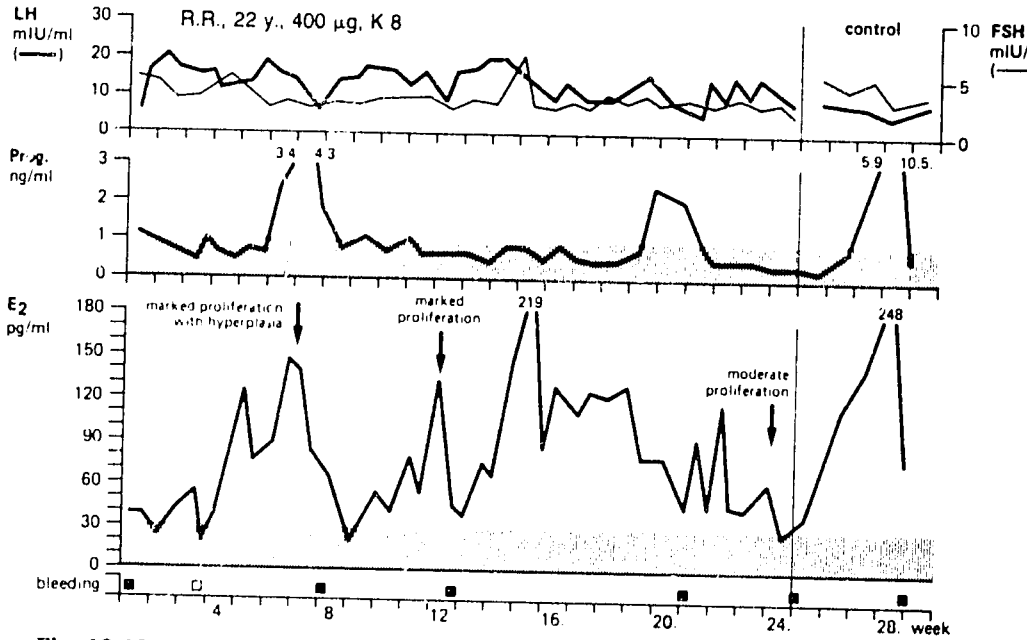


Fig. 18-10. Serum values of gonadotropins, estradiol, and progesterone in a volunteer (R.R.) during course of treatment with 400 µg Buserelin. Arrows indicate day of endometrial biopsy.

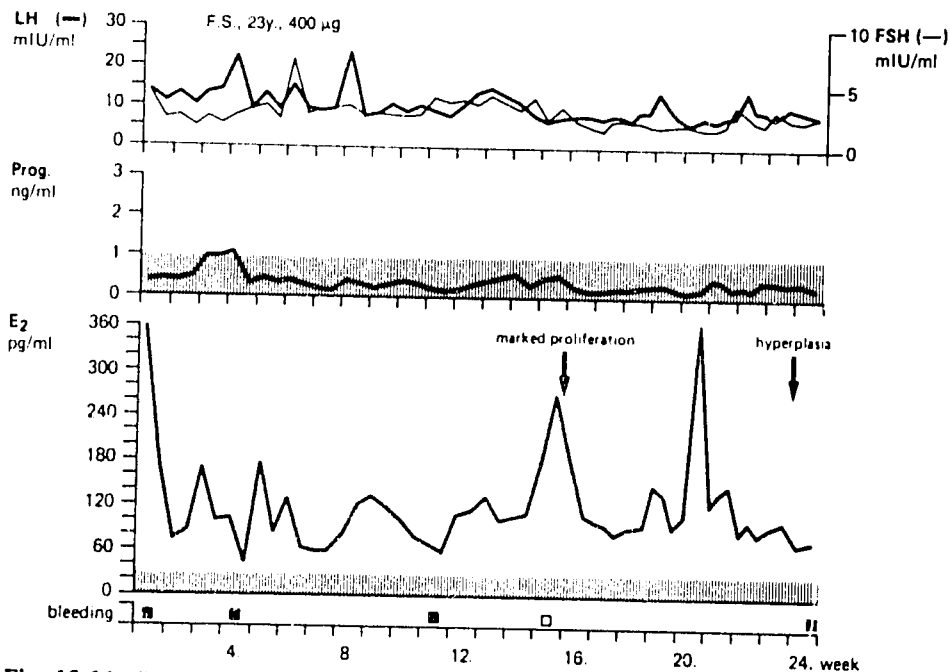


Fig. 18-11. Serum values of gonadotropins, estradiol, and progesterone in a volunteer (F.S.) during course of treatment with 400 µg Buserelin. Arrows indicate day of endometrial biopsy.

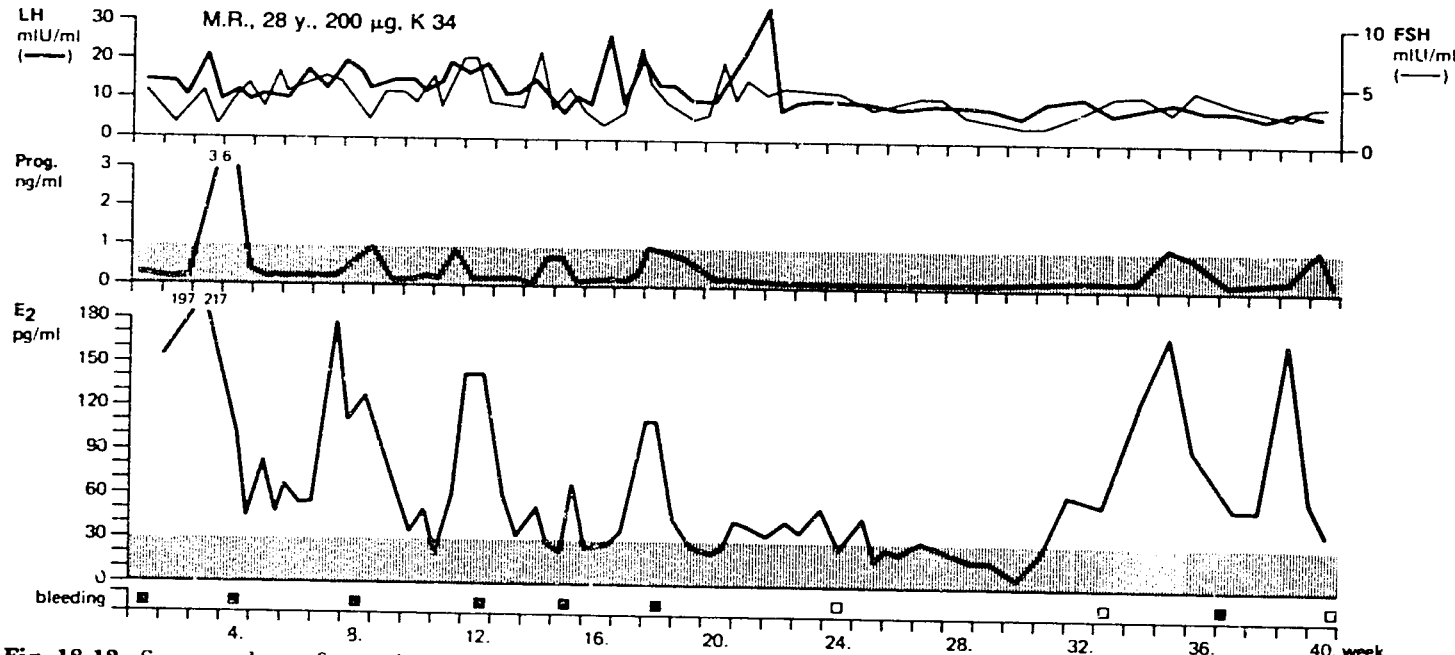


Fig. 18-12. Serum values of gonadotropins, estradiol, and progesterone in a volunteer (M.R.) during course of treatment with 200 µg Buserelin.

TABLE 18-3. Morphologic Findings of the Endometrium of 60 Volunteers During the Course of Treatment with Buserelin.

DOSE REGIMEN HOE 766 (μg)	PROLIFERATION Inactive	Mild	Moderate	Marked	Secretion	N
400	4	4	9	12	2	31
200	3	4	2	1	0	10
100/100	12	14	13	5	7	51
100	1	5	2	6	3	17
Total	20 (18.5%)	27	26	24	12 (11%)	109
		77 (70.5%)				

Regular bleeding: intervals of 3 to 6 weeks; irregular bleeding: more than 6 weeks.

Full secretory transformation of the endometrium was found in Group III only (100 μg Buserelin, Fig. 18-2). Groups I and II showed early transformation with a shortened luteal phase or insufficient transformation similar to the morphologic finding in cycles of infertile women with corpus luteum insufficiency (Figs. 18-7, 18-8).

The incidence of marked proliferation was highest in Group I (400 μg) and could be readily correlated to the prolonged periods of anovulation with unsuppressed or slightly suppressed estradiol secretion (Figs. 18-10, 18-11). These findings were significantly less common in the lower-dose regimen groups (Table 18-5).

SIDE-EFFECTS

Intranasal administration was well tolerated by the volunteers. During the first weeks of treatment only a few women complained of local nasal irritation and short-lived headaches, particularly in the group treated with 400 μg Buserelin. Three of the amenorrheal women receiving 400 μg developed symptoms of estrogen deficiency.

DISCUSSION

The possibility of using potent and long-acting LHRH agonists such as Buserelin via chronic intranasal application for fertility control in women has

TABLE 18-4. Morphologic Findings of the Endometrium of 11 Volunteers (n = 26) during the Course of Treatment with 200 μg Buserelin. Clinical Finding of Amenorrhea.

PROLIFERATION	MONTHS OF TREATMENT 1–2	3–4	5–6	N
Marked	1	—	—	1
Moderate	3	2	2	7
Mild	2	4	—	6
Inactive	1	4	7	12
Total	7	10	9	26

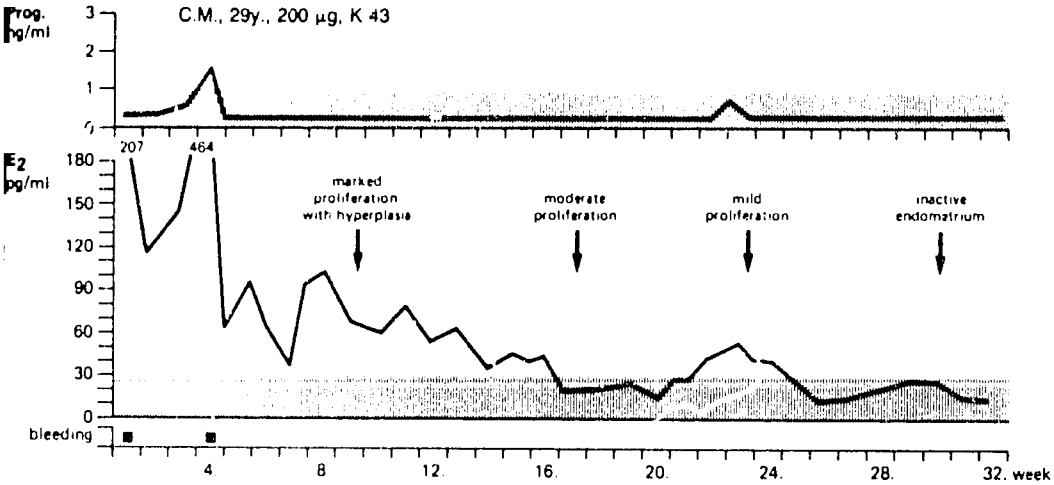


Fig. 18-13. Serum values of estradiol and progesterone in a volunteer (C.M.) during course of treatment with 200 µg Buserelin. Arrows indicate day of endometrial biopsy.

now been well documented by Nillius and others and also by our group, and may offer advantages compared to other hormonal contraceptive-methods.[2,3,11,15] To establish the contraceptive feasibilities of this compound, we extended our recently published study and investigated different dose regimens which are reported in this chapter.

The main difficulty is finding the threshold dose that has a high antifertility effectiveness combined with low undesirable effects on target organs such as breast and endometrium. It is well known that unopposed estrogen stimulation may play a cocarcinogenic role in the development of endometrial cancer.[1,6,10] Extensive investigation is needed before this pharmacologic advance can be used as a safe and effective contraceptive.

Concerning the antifertility effect of Buserelin, daily doses of 400 µg led to a sufficient inhibition of reproductive function in women, mainly as a result

TABLE 18-5. Bleeding Intervals of Volunteers (n = 24) with Marked Proliferation of Endometrium during the Course of Treatment with Buserelin.

DOSE REGIMEN HOE 766 (µg)	N	DAYS M ± SD (range) Last	Next	No
400	12	33 ± 26 (8–84)	8.5 ± 7.5 (2–21)	4*
200	6	20.5 ± 4.8 (16–25)	13.3 ± 3.6 (2–44)	1*
100	6	22.5 ± 5 (11–35)	10.5 ± 3 (9–19)	2*

*Regression of endometrium without bleeding confirmed by endometrial biopsies

of inhibition of ovulation.[3] However, inhibition of follicular maturation, implied by consistently low estradiol secretion, could not be observed in all volunteers. Marked fluctuations of estradiol secretion with values corresponding to those of the preovulatory phase of fertile women are more common. As shown by the hormonal pattern and endometrial biopsies, even periods of prolonged unopposed estrogen stimulation of endometrium, as well as phases of estrogen deficiency, can occur.

A more regular bleeding pattern may minimize the risk of unopposed estrogens on the endometrium. The use of lower dose levels (as shown in this study) leads more often to regular bleeding intervals. These bleedings are mainly caused by progesterone withdrawal. However, the critical threshold dose seems to be between 200 and 100 μg Buserelin. The dose level of 100 μg is not sufficient to inhibit (in some cases) normal follicular maturation, ovulation, and subsequently normal corpus luteum function.

On the other hand, at a daily dose of 200 μg Buserelin, even when ovulation occurs and development of corpus luteum is induced, continuous treatment with the LHRH agonist may cause luteolysis and subsequently impair implantation of a fertilized egg. Recently the antiovulatory and luteolytic effect of intermittent treatment with LHRH agonists have also been reported for monkeys as well as humans.[5,6,8] However, the influence on follicular maturation may also play an important role, which remains to be assessed.

It is known that corpus luteum insufficiency of infertile women is best treated by early stimulation of follicular maturation with human postmenopausal gonadotropins or clomiphene.[17] Yet our data provide evidence that in women developing amenorrhea, a sufficient basal estradiol secretion is maintained but stimulation of the endometrium is only slight. Furthermore, women with prolonged cycles without bleeding showed a decrease of estradiol secretion combined with regression of endometrial proliferation. These findings indicate that a prolonged use of LHRH agonist for contraception may be possible. However, the fear of unsuspected pregnancy in nonbleeding women must be considered.

In summary, our data indicate that 200 μg Buserelin are sufficient for contraceptive effectiveness of this compound. However, the unpredictable behavior of follicular maturation and thus estradiol secretion seems to be the main problem for appraisal of this new approach to fertility control. We are far from a "safe and effective" new contraceptive that can be used for easy routine application by the general population. Extensive investigations are necessary. One suggested mode of administration may be the combination of LHRH agonists with intermittent use of gestagens; another promising way may be the postovulatory induction of luteolysis.[7]

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19

Effect of LHRH on Ovulation and Corpus Luteum Formation in Women

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HUGO MAIA, SR.

ERNESTO TIRONI

ELSIMAR METZKER COUTINHO

The mechanism that controls follicular development, ovulation, corpus luteum formation, and luteolysis in women is not completely understood. The formation of a corpus luteum is a sequel to ovulation, which is initiated by LH in spontaneous cycle and by LH or a surrogate, hCG, in cycles induced by exogenous gonadotropins, clomiphene, or epimestrol.[3,8,9,14]

Vande Wiele and co-workers[15] have shown that normal corpus luteum function is dependent upon continued stimulation by LH. Progesterone secretion by the corpus luteum of the cycles in the rhesus monkey and in women can be stimulated and prolonged by administration of hCG.[6,13] hCG is produced by the time of implantation by the developing trophoblast, and it can be detected in peripheral circulation as early as 8 days following ovulation.[10] hCG is important physiologically; it maintains steroidogenesis in the corpus luteum by its luteotrophic effect. Once the corpus luteum is rescued by the increasing amounts of hCG produced by the trophoblast, it becomes more resistant to the effect of luteolytic agents. Synthetic properties can suppress progesterone production by the corpus luteum, but its effect is readily overcome by hCG.[5]

Synthetic estrogens are also luteolytic. Gore and others have reported that diethylstilbestrol (DES), initiated early in the luteal phase, caused a marked reduction in progesterone production in women.[4] Corpus luteum insufficiency also occurs spontaneously in women, and it is usually associated with unimpaired fertility. Clinical estimates of the incidence of inadequate corpus luteum function vary from 5 to 20%, depending in part upon the criteria used for diagnosis and in part upon the population of patients under consideration. Among women who ovulate spontaneously but seek treatment for infertility or repeated early abortions, inadequate corpus luteum function is less common than among women with anovulation who fail to conceive or

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sustain pregnancy following any induced ovulations.* These observations indicate that proper follicular maturation is a prerequisite for normal corpus luteum development.

Follicular development in women depends on proper LH/FSH secretion ratio. Rising levels of FSH beginning late in the luteal phase provide the gonadotropic and steroid support required for the growth in the preantral follicle, one of which will ovulate in the succeeding cycle. Disturbance in the secretion of FSH and LH early in the cycle affects the growth of the follicle, resulting in either anovulation or in the development of an insufficiently functioning corpus luteum. A prolonged follicular ripening with low serum estradiol and a modest increase of estradiol in the preovulatory phase results in the development of an insufficient corpus luteum.[12] Estradiol preovulatory rise and fall is a prerequisite for the ovulatory surge of LH at midcycle in humans.[2,10] On the other hand, LH induces ovulation only if follicular development is completed, as signaled by the high levels of estradiol. Biochemical studies in animal species have shown that the number of LH receptors increases in the follicle during its development.[2]

Recently, with the synthesis of the hypothalamic hormone (LHRH) that releases both LH and FSH from the pituitary gland, it became possible to investigate the role of LH and FSH on follicular development and corpus luteum function in women. The present chapter describes the effect of LHRH on ovulation in normal menstruating women as well as in some patients with anovulation and sterility. All patients enrolled for these studies provided written informed consent.

LHRH was used in the initial trials to induce ovulation in patients with polycystic ovarian disease.[9] Initial studies have shown that repeated administration of LHRH alone was not successful in inducing follicular development and ovulation in patients with secondary amenorrhea. This failure was in part explained by the abnormal LH/FSH ratio in response to LHRH. Since an excess of LH early in follicular development was deleterious to follicular development, repeated administration of LHRH did not seem to be of therapeutic use in cases of anovulatory sterility. However, in patients with anovulation, in whom follicular maturation was stimulated by epimestrol or clomiphene, administration of LHRH was followed by ovulation when there was evidence that follicular growth was complete.[9] This was usually inferred by estradiol serum levels above 250 pg/ml. In patients treated with epimestrol, in whom the follicle was not mature, administration of LHRH for several days was not successful in inducing ovulation. These studies suggest that LHRH is effective only when growth of the follicle is complete. Administration of LHRH before the follicle is fully developed does not result in ovulation and normal corpus luteum function.

LHRH in experimental animals was shown to exert antifertility capabilities when administered chronically. In women, chronic administration of LHRH potent analogs inhibited ovulation.[11] These studies suggested that LHRH or its analogs could be used as a contraceptive, and they explained the previous failed attempts to induce ovulation in women with LHRH alone. However,

*Mishell DR, Jr. Unpublished data.

daily administration of LHRH is not practical from a clinical point of view. Another regimen of administration of LHRH was then tested. In the last 12 months, 20 patients in our hospital were treated with LHRH in order to block ovulation or induce insufficient corpus luteum. In all these patients, LHRH was administered for only 5 days during different phases of the menstrual cycle.

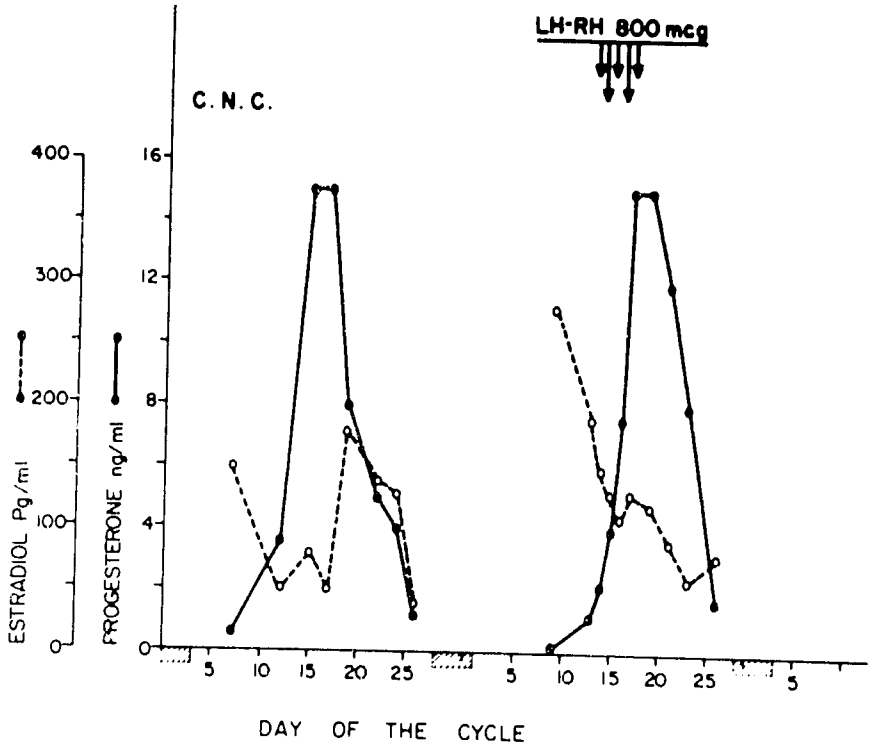
All patients were studied for one cycle before they were enrolled for this study. Blood was drawn for measurement of estradiol, progesterone, and LH levels by radioimmunoassay. All patients were multiparous and had requested tubal sterilization. Corpus luteum function was normal in all cases, as inferred by progesterone levels above 8 ng/ml 8 days following E2 peak. These patients were treated in the second cycle with LHRH. LHRH was injected in the dose of 400 to 1000 μg subcutaneously for 5 days. In four patients, 1 mg LHRH was administered as an intravenous infusion for 5 hours during two consecutive days. LHRH treatment was initiated either before or after ovulation had occurred. In the first group (n = 6), administration was started 5 days after the estradiol peak, when progesterone levels were above 4 ng/ml. In the second group (n = 14) LHRH was given either before follicular growth was complete or on the day of the preovulatory estradiol peak. In all patients, progesterone levels were low, usually less than 1 ng/ml.

LH responses were monitored in all patients during treatment. Basal levels were below 8 mIU/ml before LHRH treatment was initiated. In only two patients, LHRH treatment was started at the time of spontaneous LH surge. In four patients for whom LHRH administration was initiated 5 days following the estradiol peak, progesterone production by the corpus luteum was not reduced during treatment (Figs. 19-1, 19-2). In the remaining two patients, there was even an increase in progesterone levels following LHRH administration. In all patients, progesterone levels were above 4 ng/ml when the subcutaneous injections were initiated. LH levels rose promptly following LHRH injection to reach a plateau 1 hour later. The response to subcutaneous injections of LHRH did not change significantly during the 5-day treatment (Fig. 19-3).

In the second (n = 14) group of patients, LHRH treatment was initiated before ovulation had occurred, as inferred by progesterone levels below 2 ng/ml. In 11 patients, LHRH was administered subcutaneously in the dose of 400 to 800 μg/ml/day for five consecutive days. In the remaining three patients, LHRH was administered as an intravenous infusion for five hours during two consecutive days. The total dose administered was 1 mg/day. In 12 of these patients, LHRH treatment was started before the preovulatory estradiol surge had occurred. In the remaining two, treatment was started on

Fig. 19-1. Effect of LHRH on progesterone production by human corpus luteum *in vivo*. Note that progesterone levels were not affected when LHRH treatment was initiated 5 days following E2 peak.

Fig. 19-2. Progesterone levels during control and LHRH-treated cycles (n = 4). LHRH was started 5 days after E2 peak, when progesterone levels were above 4 ng/ml. Note that the progesterone production was not affected by LHRH treatment.


LH-RH 800 mcg
C. N. C.
ESTRADIOL Pg/ml
PROGESTERONE ng/ml
400
300
200
100
0
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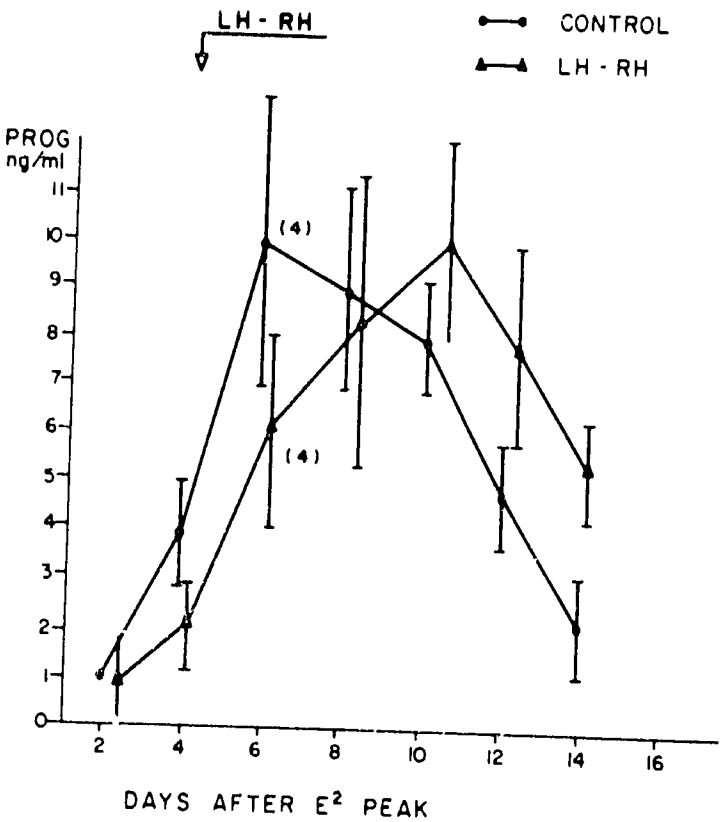
DAY OF THE CYCLE


LH - RH
CONTROL
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PROG
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11
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DAYS AFTER E² PEAK

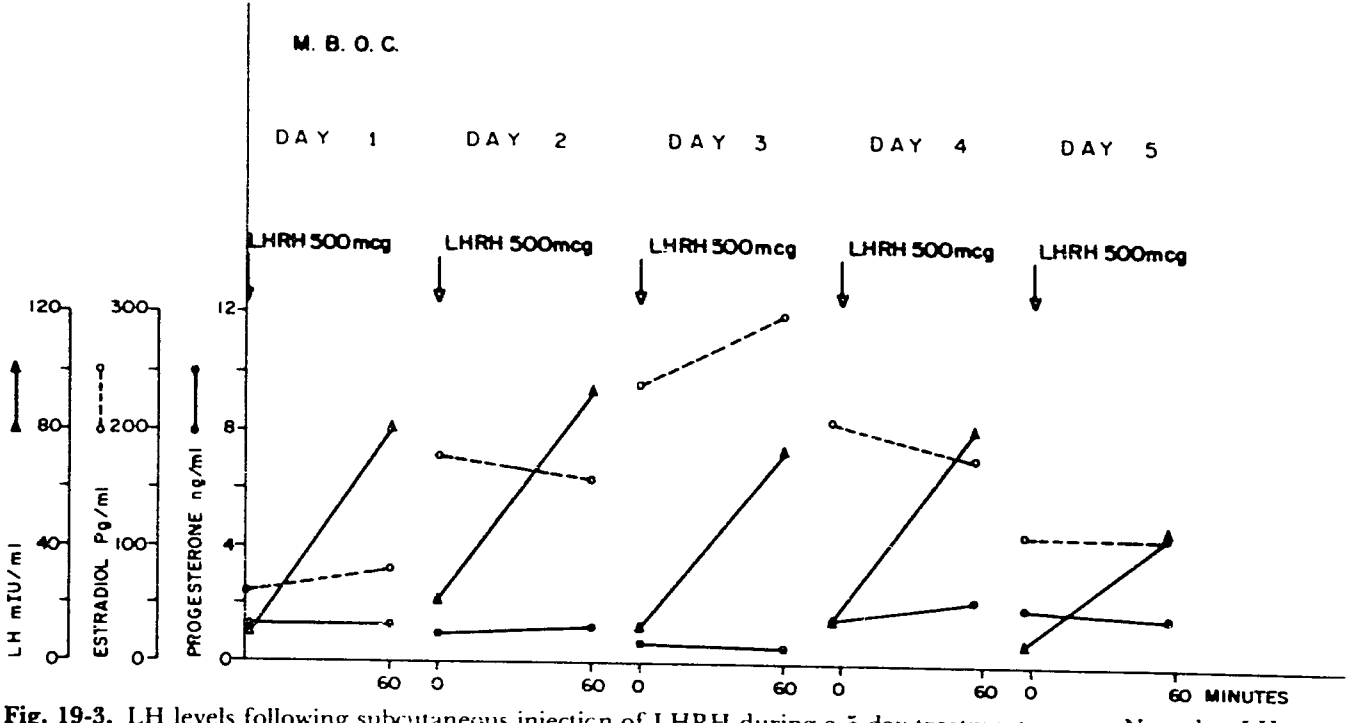

Fig. 19-3. LH levels following subcutaneous injection of LHRH during a 5-day treatment course. Note that LH response did not decrease markedly during treatment.

the day of estradiol surge. Blood was drawn from these patients for measurement of estradiol, progesterone and LH levels by radioimmunoassay. Corpus luteum function was atnormal in all paatients treated with LHRH before the preovulatory estradiol surge (Fig. 19-4). In two patients progesterone levels were below 2 ng/ml following treatment, perhaps suggesting that ovulation had been suppressed (Figs. 19-5, 19-6). In the remaining patients progesterone production was markedly reduced during the treatment cycle (Fig. 19-7). The differences in progesterone levels between the treatment and control cycles were statistically significant. Similar findings were also observed in patients treated with 1 mg. LHRH IV for two days (Fig. 19-8). However, in

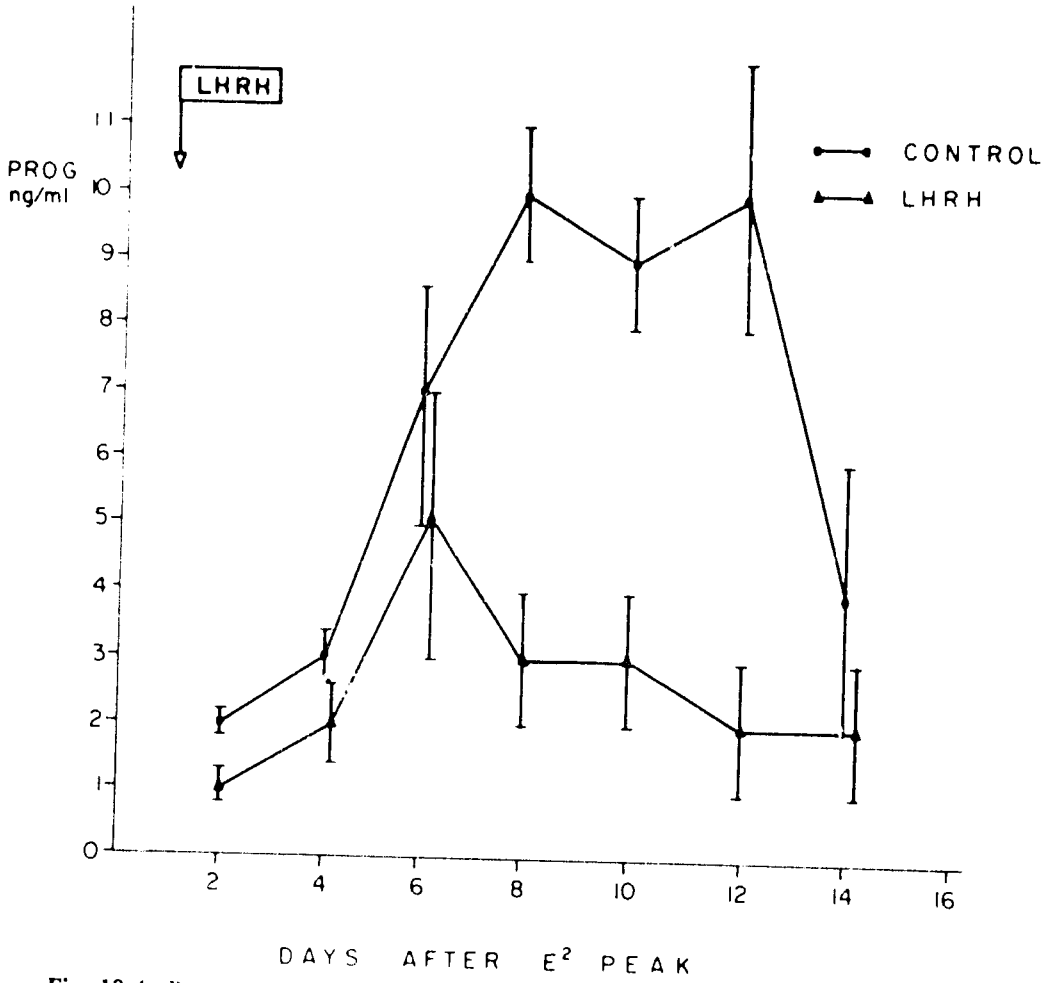

Fig. 19-4. Progesterone levels during control and LHRH-treated cycles (n = 11). LHRH treatment was initiated either before or during preovulatory estradiol peak. LHRH was injected in the dose of 400 to 1000 μg/day during 5 consecutive days. Note that progesterone production was significantly reduced following LHRH administration.

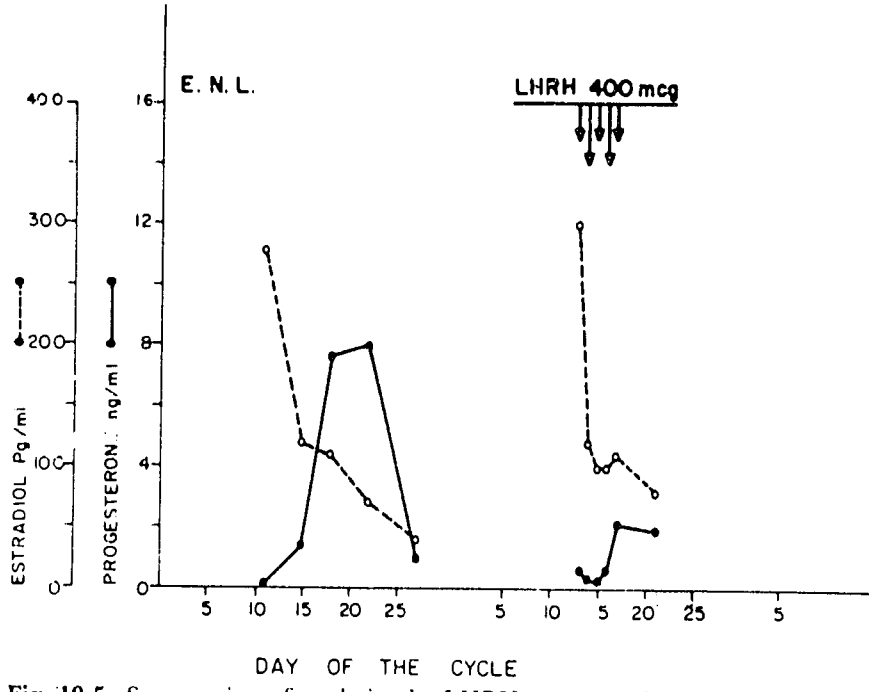

Fig. 19-5. Suppression of ovulation by LHRH treatment in a normally menstruating woman. LHRH subcutaneous injection was initiated at time of preovulatory E2 peak. Note that progesterone levels remained below 2 ng/ml following treatment. Cycle length was shortened in this patient.

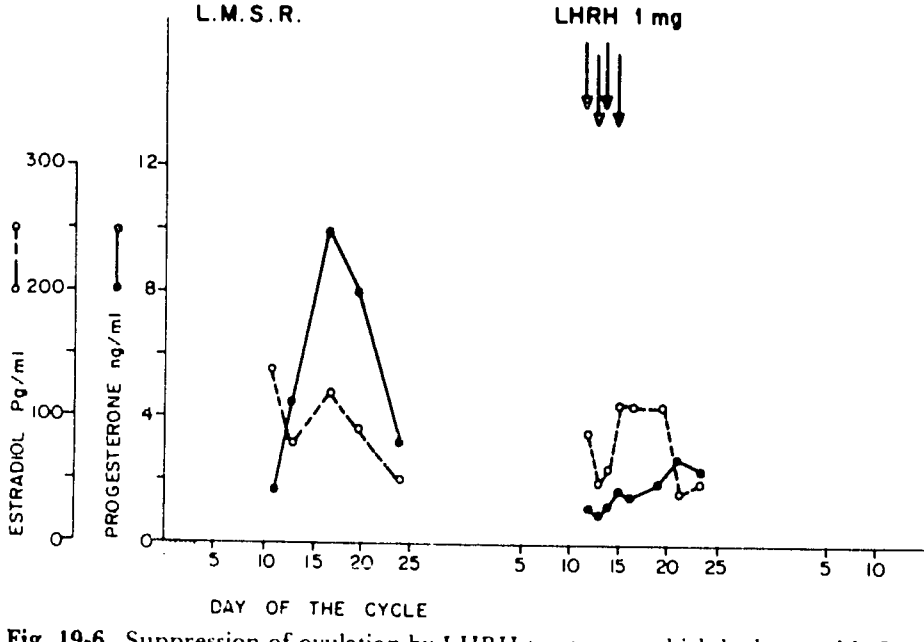

Fig. 19-6. Suppression of ovulation by LHRH treatment, which had started before the E2 preovulatory peak. Note that progesterone levels remained low following treatment.

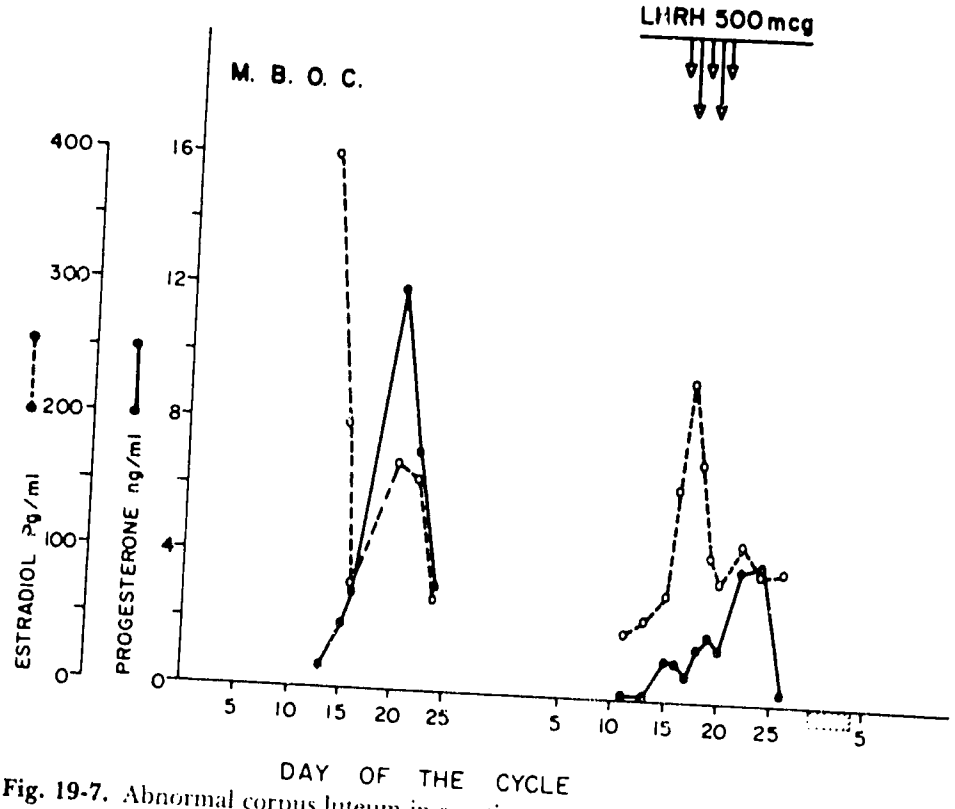

Fig. 19-7. Abnormal corpus luteum in a patient treated with LHRH before ovulation had occurred. Note that LHRH treatment was started before preovulatory estradiol peak.

these patients the response to LHRH on the second day of treatment was markedly reduced, indicating that prolonged stimulation with LHRH has a desensitization effect on the pituitary gland (Fig. 19-9).

These results indicate that the optimal time for administering LHRH should be before ovulation has occurred. The presence of tonic high levels of LH before follicular development is complete, or during the first days following ovulation, will exhaust LH receptors in granulosa cells, inducing an insufficient corpus luteum. In humans, as well as in other species, ovulation follows a surge in LH secretion by a period of 16 to 32 hours when the follicle is mature. While LH stimulates the production of steroid and prostaglandins by the ovarian follicle, the LH peak also incites an important inhibitory action on steroidogenesis and cyclic AMP production during the later half of the preovulatory period.[7] This is accompanied by a decrease in LH and FSH receptors in the granulosa cells. This property of LH to induce the "down-regulation" of its own receptor at ovarian level is one of the mechanisms that prevent an excessive hormone production by the ovary. This property of LH is also shared by other hormones, such as progesterone.

The development of the refractoriness of the follicle to LH stimulation

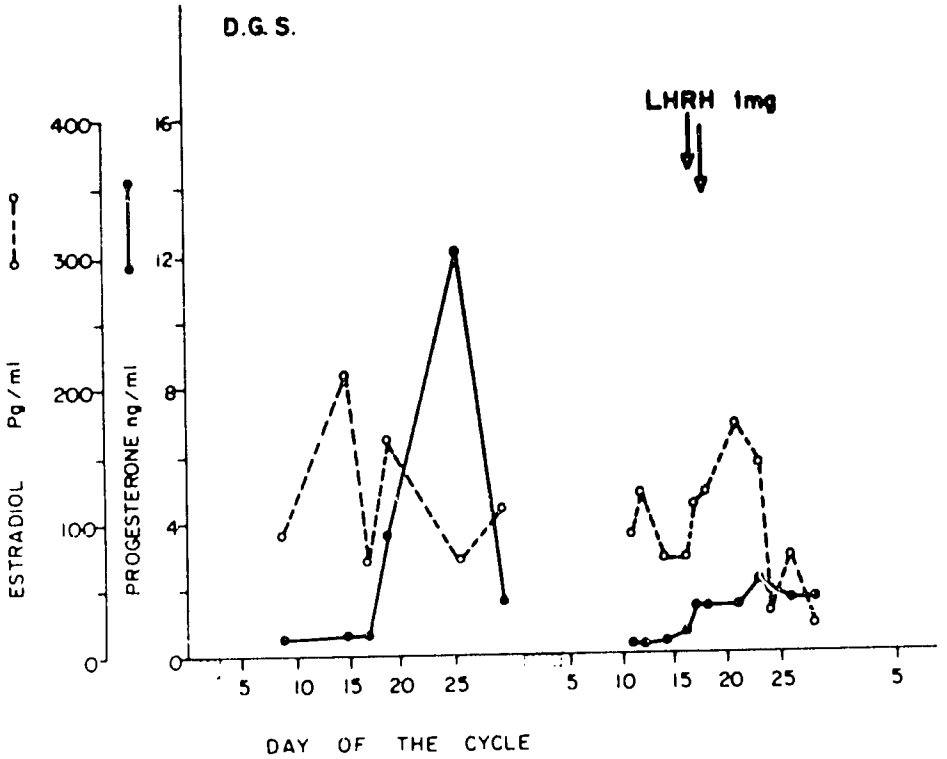

Fig. 19-8. Effect of IV infusion of LHRH on ovulation in women. Note that prolonged infusion of LHRH (5 hr/day), given before follicular maturation is complete, exerted a suppressive effect on ovulation and corpus luteum formation.

after being exposed to this hormone *in vivo* explains why the LH preovulatory peak has to be short-lived in order to have normal corpus luteum function. If tonic high levels of LH are present before follicle maturation is complete or during the first days following ovulation, it will decrease the concentration of its own receptor in the granulosa cells during a critical period of its own development. These results indicate that LHRH can be used to block ovulation or to cause an insufficient corpus luteum in humans, as long as it is administered before ovulation. On the other hand, if LHRH treatment is initiated later in the luteal phase, the effect on progesterone production will be minimal.

These findings are also in agreement with recent observations that LHRH is present in the cytotrophoblast and it can stimulate hCG by the developing embryo. These results indicate that the administration of LHRH after implantation has occurred will probably not affect pregnancy. Thus, there is probably not much future for LHRH as an early abortifacient agent in humans. However, the use of LHRH or its analog during the follicular phase to block ovulation or to induce a defective corpus luteum may have some future as a midcycle contraceptive in women. The development of potent LHRH analogs offers new and exciting possibilities for this field.

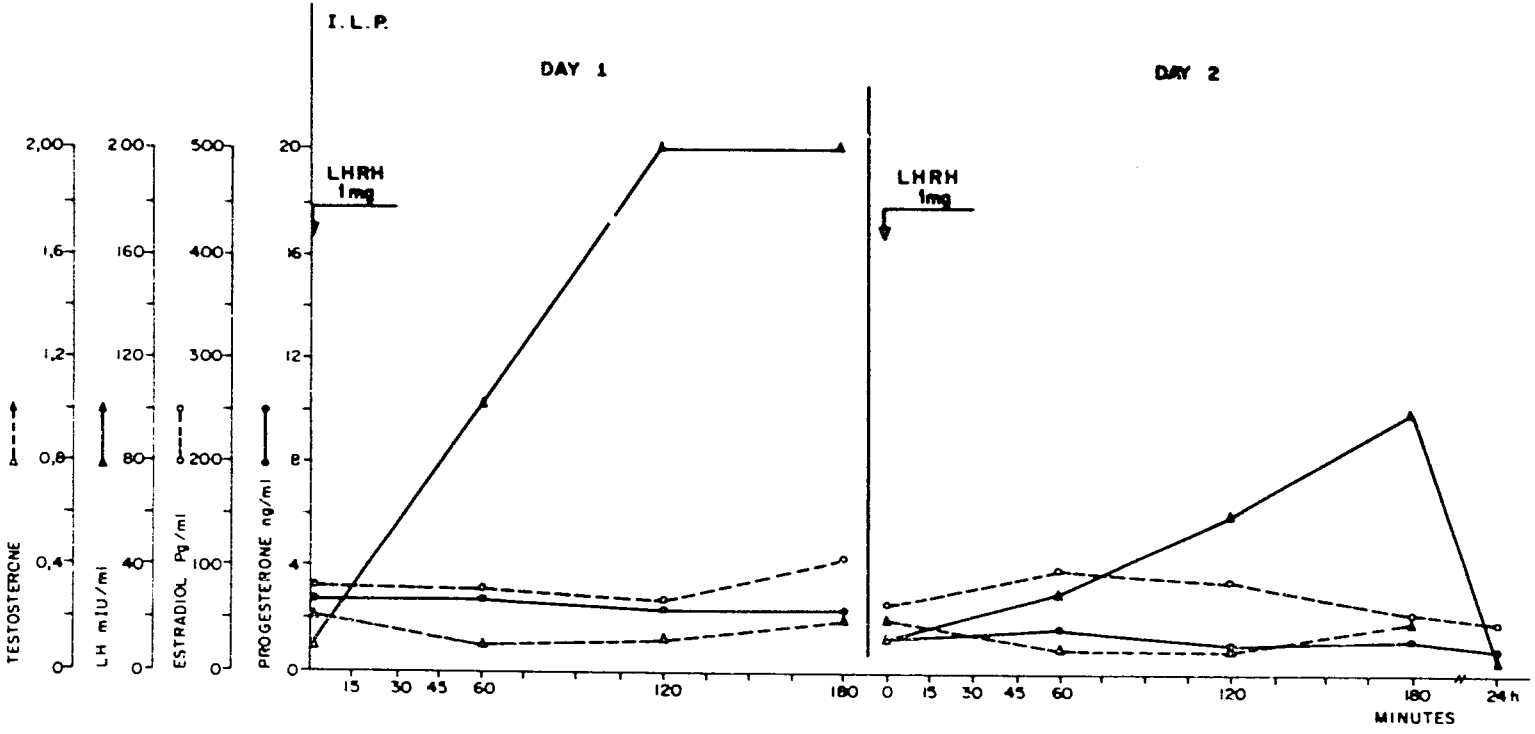

Fig. 19-9. Desensitization effect of prolonged infusions of LHRH on response of pituitary gland. Note that LH release was markedly reduced on the second day of treatment.

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20

The Use of LHRH Agonists and Antagonists as Antifertility Agents in the Human Female

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ANDREW V. SCHALLY

DAVID H. COY

ANA MARIA COUMARU-SCHALLY

The decapeptide LHRH, responsible for synthesis and release of LH and FSH, was isolated and synthesized by Schally in 1971.[9] Later, modifications of the amino acid sequence of this hormone led to the synthesis of analogs with agonistic and antagonistic LHRH activities.[10] Studies indicate that histidine and tryptophan probably exert a functional effect, in addition to providing receptor-binding capacity, since substitutions or deletions in position 2 or 3 greatly decrease LHRH activity.

The primary site of enzymatic degradation of LHRH at the Gly^6-Leu^7 bond, and the introduction of D-isomers of amino acids at position 6, significantly enhance biologic activity. Analogs substituted in position 6 or 10, or both, are ten to one hundred times more potent than LHRH and also possess prolonged activity.

A second site of enzymatic degradation is at the 10 position glycine amide residue. Substitution of an ethylamide at this position leads to enhanced activity. It has been postulated that the greater activity of the analogs, compared with original decapeptide, is the result of resistance to enzymatic degradation and retention at the pituitary tissue. It has also been suggested that LHRH analogs may possess greater affinity than the LHRH for anterior pituitary receptor sites. LHRH agonists stimulate gonadotropin secretion, resulting in elevated levels of both LHRH and FSH. These high circulating levels of LH/FSH may decrease ovarian responsiveness to LH/FSH by decreasing LH/FSH receptor number. This leads secondarily to decreased ovarian steroidogenesis. LHRH analogs may also act directly at the gonadal level to inhibit ovarian hormonogenesis.

STIMULATORY LHRH ANALOGS

The development of highly active stimulatory analogs of LHRH led us to attempt to improve the rate of success in inducing ovulation in anovulatory women who wished to conceive.[5] [D-Ala^6,des-Gly^{10}]-LHRH ethylamide and [D-Leu^6]-LHRH, which systematically raise gonadotropin levels in blood for as long as 24 hours, was administered to infertile women (Figs. 20-1 and 20-2); however, overall percentages of ovulation and pregnancy were low.

In spite of the initially large release of gonadotropins after administration of these stimulatory LHRH analogs, gonadotropin responses rapidly decreased during the prolonged treatment. This resulted in disturbances in gonadotropin secretion with irregular follicular maturation and a low incidence of ovulation (Tables 20-1 and 20-2). The high rate of breakthrough, as well as withdrawal, bleeding indicated improper follicular maturation.

Another potent LHRH analog, [D-Trp^6]-LHRH, was studied in amenorrheic sterile patients. Fifteen women received intramuscular injections of

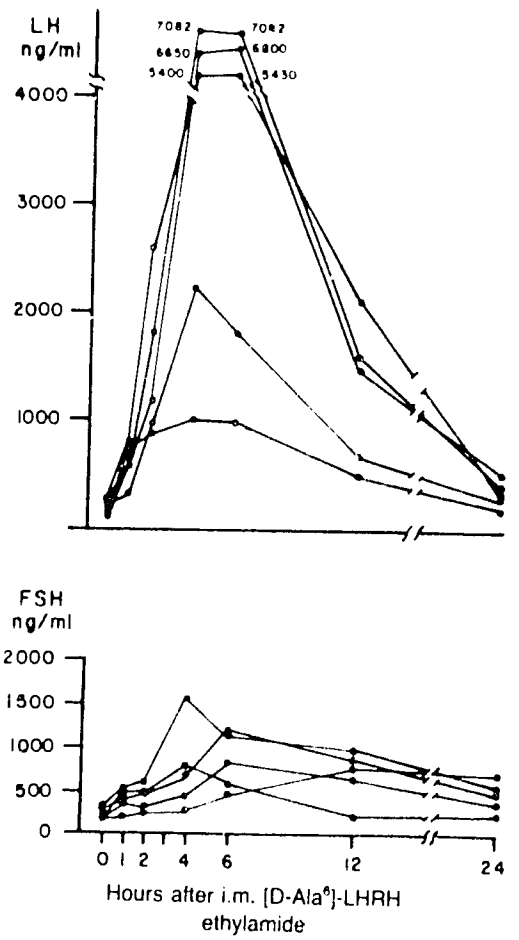

FIG. 20-1. Serum LH and FSH levels following administration of the stimulatory LHRH analog. The effect is potent and gonadotropin release is prolonged.

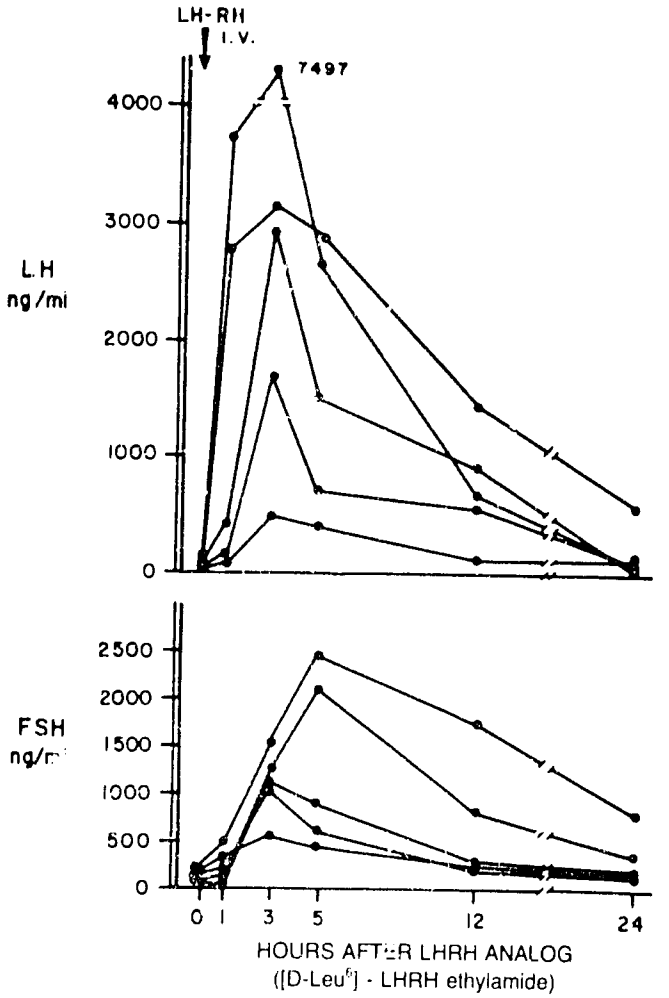

FIG. 20-2. Effect of [D-Leu^6]-LHRH ethylamide on FSH and LH secretion.

TABLE 20-1. Results of Therapy with Daily Injections of [D-Leu^6,des Gly^{10}]-LHRH Ethylamide

CASE NO.	DAILY INJECTION (Frequency)	DURATION OF THERAPY (Days)	DAILY DOSE (mg)	TOTAL DOSE (mg)	TOTAL URINARY ESTROGENS (μg/24 hr)	CLINICAL RESPONSE
1	× 3	13	0.6	7.8	65	BTB*
2	× 3	10	0.6	6.0	75	Ovulation
3	× 3	11	0.6	6.6	84	BTB
4	× 3	12	0.6	7.2	47	WDB,† 5 days later
5	× 3	11	0.6	6.6	21	BTB
6	× 3	10	0.6	6.0	66	Ovulation

*Breakthrough bleeding.
†Witndrawal bleeding.

TABLE 20-2. Attempt of Induction of Ovulation with [D-Ala]-LHRH Ethylamide

CASE NO.	DAILY INJECTION (Frequency)	DURATION OF THERAPY (Days)	DAILY DOSE (mg)	TOTAL DOSE* (mg)	TOTAL URINARY ESTROGENS (μg/24 hr)	CLINICAL RESPONSE
1	× 2	8	0.4	3.2	68	WDB,† 2 days later
2	× 2	8	0.4	3.2	46	No bleeding or ovulation
3	× 2	8	0.4	3.2	87	WDB, 5 days later
4	× 2	8	0.4	3.2	86	WDB, 2 days later

*All patients received an additional dose of 400 μg of [D-Ala]-LHRH ethylamide by an 8-hour infusion on the last days of therapy.

†Withdrawal bleeding.

10 μg of [D-Trp^6]-LHRH three times a day for 10 days. The following day, patients were given an intravenous infusion of 50 μg over a period of 8 hours. In addition, a 50-μg booster of this analog was given intramuscularly. Three of the 15 patients ovulated, but none became pregnant. All women had surges of both LH and FSH after the repeated injections and the infusion of LHRH analog (Fig. 20-3). The FSH surges were lower than LH surges. Daily injections of the analog followed by its infusion produced irregular LH/FSH secretion, which was sufficient to maintain production of estrogens (Fig. 20-4). Each injection of LHRH analog elicited a significant increase in blood levels of gonadotropin, which in turn stimulated the steroid secretion by the ovaries. One woman experienced ovulation, but her progesterone serum levels exhibited a progressive decrement that coincided with early menstrual bleeding. Two additional women showed a similar pattern in progesterone concentrations. These results demonstrated clearly how ovulation followed by irregular progesterone secretion can lead to a short luteal phase in several cases. On the basis of these results, it was suggested that the apparent luteolytic effect of the stimulatory analog, as evidenced by the progesterone serum profile, may contribute to the difficulty in treating anovulatory infertile women.[1]

Our difficulty in inducing ovulation, as well as in dealing with frequent breakthrough bleeding associated with the use of the potent agonist LHRH analogs through six years of experience, led to the consideration of a paradoxical effect explained through a pituitary desensitization induced by repeated administration of LHRH analog. It seemed that treatment could result in the persistent occupancy of available receptor sites, blocking further responsiveness of the gonadotropins. Conversely, when the original decapeptide of LHRH was administered following proper dosage dictated by the estrogen excretion index as a marker of follicular steroidogenesis, follicular maturation and ovulation were achieved in women with polycystic ovarian disease. However, induction of ovulation was not obtained systematically, probably because of the difficulty in establishing the adequate LHRH ther-

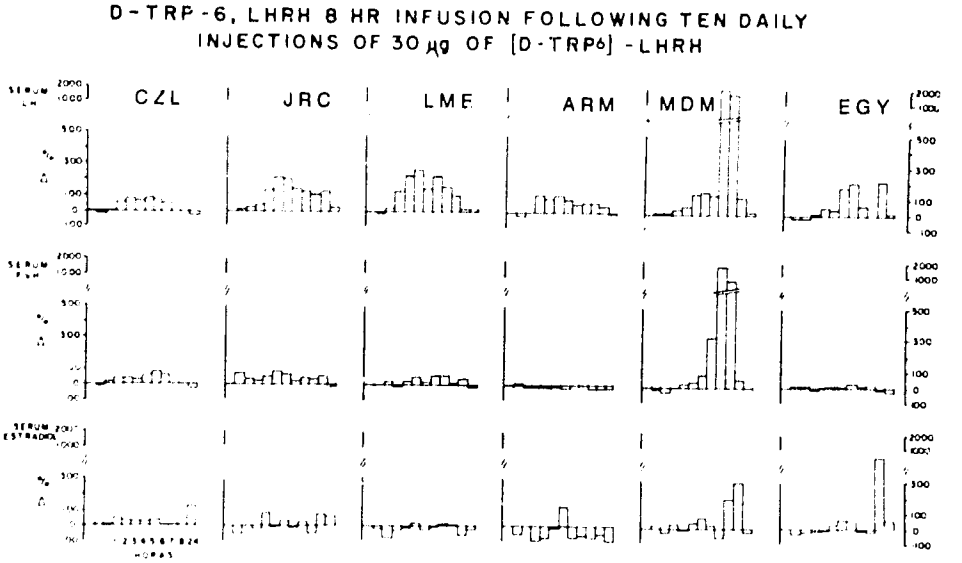

FIG. 20-3. Values of LH, FSH, and estradiol 17β correspond to percent of maximum increment. (Canales ES, Montvelinsky H, Fonseca ME *et al:* The use of a potent stimulatory LHRH analog [D-Trp-6-LHRH] in induction of ovulation. Int J Fertil 25:193–197, 1980)

apeutic regimen. Despite repeated LHRH stimulation, estrogens produced by the ovary declined, and in turn these patients developed the anticipated bleeding.

We had observed that chronic administration of [D-Leu^6]-LHRH ethylamide led to a decreased responsiveness of the pituitary gland (Tables 20-1 and 20-2). Later, it was demonstrated that analogs exert antifertility effects when used in large doses or when administered by repeated doses. It became clear that chronic administration with stimulatory analogs of LHRH could result in a paradoxical effect—inhibition of ovulation in normal women. It has been demonstrated that administration of [D-Ser (^{t}Bu)^6]-LHRH ethylamide, either subcutaneously or intranasally, is able to inhibit ovulation in normal women during the treatment cycle.[2] Similarly, we have attempted to inhibit ovulation in previously eumenorrheic women by IM administration of [D-Trp^6]-LHRH in a dosage of 5 μg once daily to five women for 20 days of the menstrual cycle. Ovulation was inhibited in three of five patients, but menstrual cycles were shortened and breakthrough bleeding occurred frequently. Moreover, some problems derived from this approach; thus inhibition of ovulation by stimulatory analogs of LHRH caused the clinically adverse effect of unopposed estrogen effect, such as irregular uterine bleeding or amenorrhea.

LHRH analogs have also been used for induction of luteolysis.[3] We gave injections of 50 μg [D-Trp^6]-LHRH on each of two consecutive days during the immediate postovulatory phase, producing a short luteal phase and premature menstruation. A premature decline in progesterone levels in seven women suggested that luteolysis took place.

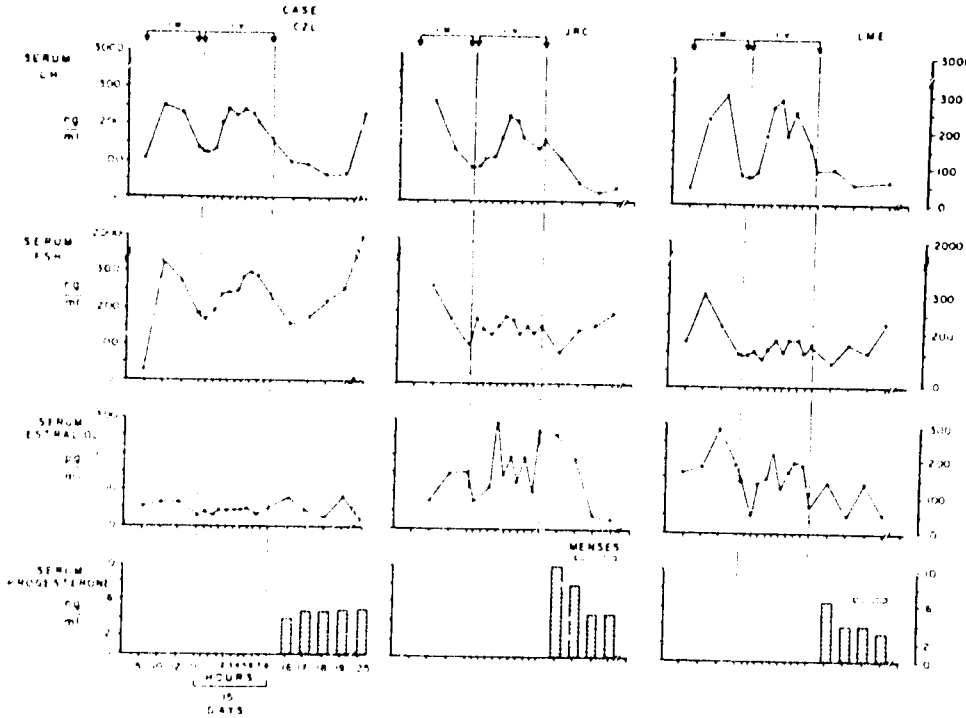

FIG. 20-4. Serum levels of gonadotropins and steroids in three women receiving daily injections of [D-Trp^6]-LHRH over 10 days followed by an additional infusion. (Canales ES, Montvelinsky H, Fonseca ME *et al:* The use of a potent stimulatory LHRH analog [D-Trp-6-LHRH] in induction of ovulation. Int J Fertil 25:193–197, 1980)

Other studies have confirmed the ability of LHRH analogs to induce luteolysis when administered during the midluteal phase.[7] In our experience, the administration of 5 μg [D-Trp^6]-LHRH on days 3 and 4 after ovulation in seven eumenorrheic women resulted in a short luteal phase and premature menstruation, compared with the previous cycle in the same patients. Hormone studies in these patients showed a premature decline of both progesterone and estradiol 17β concentrations in blood, which was proof of the luteolytic effect of LHRH analog. The LHRH agonist was effective as a luteolytic agent when given between 3 and 5 days from the LH surge, in agreement with other studies.[7]

It has been proposed that luteolysis in a previous cycle may interfere with the timing of follicular growth and subsequent corpus luteum formation in the next cycle; however, we did not observe this phenomenon. All patients in our study recovered the normal menstrual cycle when the peptide was withdrawn. From these observations, it is recommended that the appropriate moment to use LHRH should be soon after ovulation, not in the midluteal phase. This will avoid severe corpus luteum insufficiency and subsequent irregular bleeding. It appears that when the analog is given early in the luteal

phase, progesterone levels do not increase, estradiol levels remain more or less constant during this phase, and patients have a fairly regular menstrual cycle. However, we and other investigators have administered LHRH within 2 to 6 days of ovulation, creating inconsistent effects on serum progesterone levels and on the menstrual cycle.[7,8]

In order to induce luteolysis, it is necessary to administer the LHRH during a critical period, which seems to be between 3 and 6 days after LH surge or basal body temperature rise. Concerning the mechanism of LHRH-induced luteolysis, studies in the rat have shown a direct action of LHRH agonists on ovarian steroid production as well as on follicular development. High-affinity binding sites for LHRH have been demonstrated on rat luteal cell membranes. The LHRH agonists can inhibit production by the ovary of hypophysectomized rats and by luteal cells *in vitro*; however, apparently by interfering with binding of LH to its receptor, the agonists also induce a refractory state in which the pituitary *in situ* fails to produce LH after an initial response.[7] The binding of LHRH on ovarian receptors in the absence of the pituitary gland strongly suggests that the LHRH agonists exert a direct action on these tissues, probably through binding with LHRH receptors of the gonads. The site of action and the mechanism of this luteolytic effect of LHRH analogs in the human remain unknown. Therefore, it would be more desirable to attempt LHRH-induced luteolysis rather than inhibition of ovulation, since early regression of the corpus luteum could avoid the inconvenience of breakthrough bleeding and the possibility of chronic stimulation of the endometrium, with the related concern about endometrial hyperplasia.

One problem derives from this approach. Implantation is not prevented when the LHRH analog is used days after the midluteal phase. On the other hand, it is not practical to give the LHRH agonist in the early follicular phase, because it interferes with follicular development.

INHIBITORY ANALOGS OF LHRH

In 1971, Schally proposed that it could be possible to develop inhibiting analogs of LHRH. It was also suggested that replacement or deletion of one or more amino acids in LHRH might result in analogs possessing properties for an effective binding, but lacking those that are necessary for the functional effect. It was also demonstrated that histidine in position 2 and tryptophan in position 3 appeared to exert functional effects; therefore, early inhibitory analogs contained replacements of deletion in these two positions to block the receptor-binding capacity. Later, it was reported that incorporation of D-amino acid in position 6 increased the inhibitory activity of these compounds.[10] Thus, [D-Phe^2-Phe^3-D-Phe^6]-LHRH or [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH blocked LHRH response and ovulation or both in several species of animals. The stimulatory and inhibitory analogs of LHRH seemed to exert their action on the same pituitary receptors as those for LHRH.

These polypeptides possess the features required for binding, but lack those necessary for a functional effect. Thus, by competing with endogenous LHRH for pituitary binding sites, they induce a decrease of both LH and

FSH release. The powerful activity of [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH made possible successful clinical tests in humans, in males as well as in females.[2,6]

When 90 mg of this analog was given intramuscularly to eight normal women on days 12 to 14 of the cycle, gonadotropin response fell to 50 μg of LHRH given 3 and 6 hours later (Fig. 20-5). The injection of 90 mg [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH suppressed the LHRH-induced release of both LH and FSH 6 hours later; however, a rebound effect was noticed 24 hours later. LH secretion in response to LHRH injection 24 hours after the intramuscular injection of [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH was significantly higher than before. FSH release 24 hours later was not significantly different from control.

Figure 20-6 depicts the effect of this LHRH analog in each of the eight cases. These results showed that [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH inhibits the response of LH and FSH to the injection of LHRH during the 6 hours following the administration of this antagonist; however, this effect is lost 24 hours after the injection of the analog (Fig. 20-7). Basal levels of gonadotropins obtained at the time of the repeated injection of LHRH 24 hours after administration of the inhibitory analog were not suppressed in all women, a finding similar to that observed previously in men.[6] A clinical observation was that the menstrual cycle was disrupted in all patients; six presented earlier menstrual bleeding (from 2 to 5 days), whereas two had delayed menses (9 and 15 days after expected). Disruption of the menstrual cycle in all cases

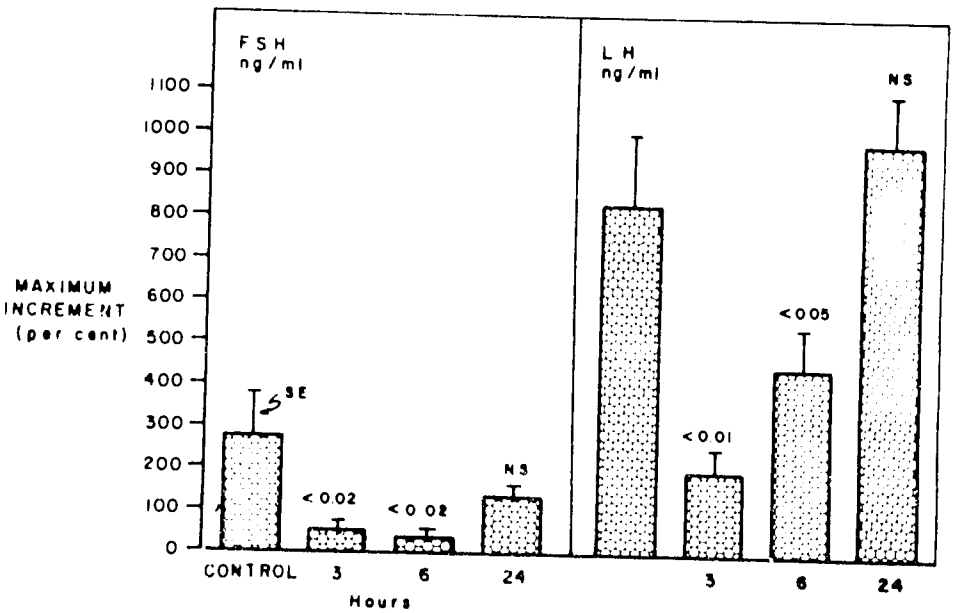

FIG. 20-5. Mean ± (S.E.) responses before and after IM administration of 90 mg [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH. (Canales ES, Montvelinsky H, Zarate A *et al:* Suppressive effect of an inhibitory LHRH analog on the gonadotropin response to LHRH in normal women. Int J Fertil 25:190–192, 1980)

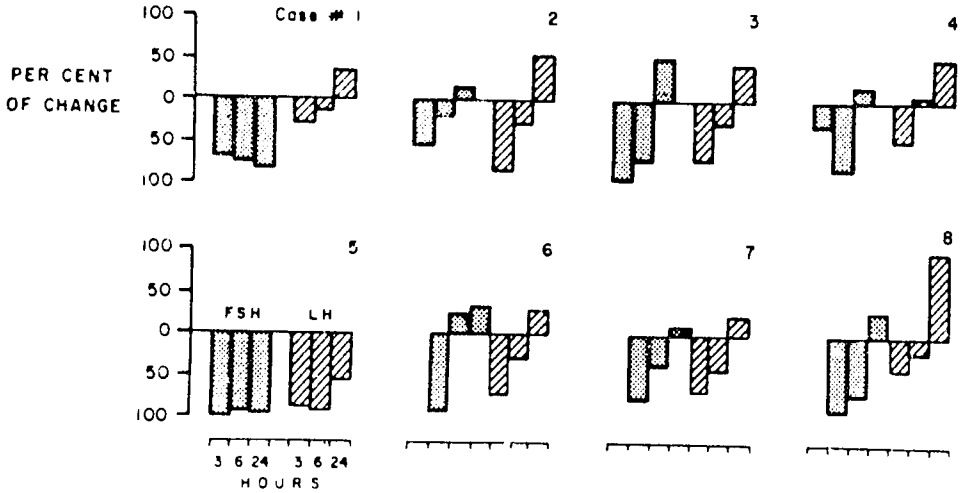

FIG. 20-6. Effect of [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH on FSH and LH serum levels when treated with LHRH decapeptide. (Canales ES, Montvelinsky H, Zárate A *et al:* Suppressive effect of an inhibitory LHRH analog on the gonadotropin response to LHRH in normal women. Int J Fertil 25:190–192, 1980)

demonstrates the anovulatory effect of this potent antagonist. None of these women ovulated.

Other clinical studies have utilized more advanced compounds. [N-Ac-D-Phe^1,D-pCl-Phe^2,D-Trp^{3,6}]-LHRH, administered on day 12 of the cycle in doses of 10 mg, abolished the LH midcycle surge, and ovulation did not occur in eight of 14 normal menstruating women. Six of these women menstruated

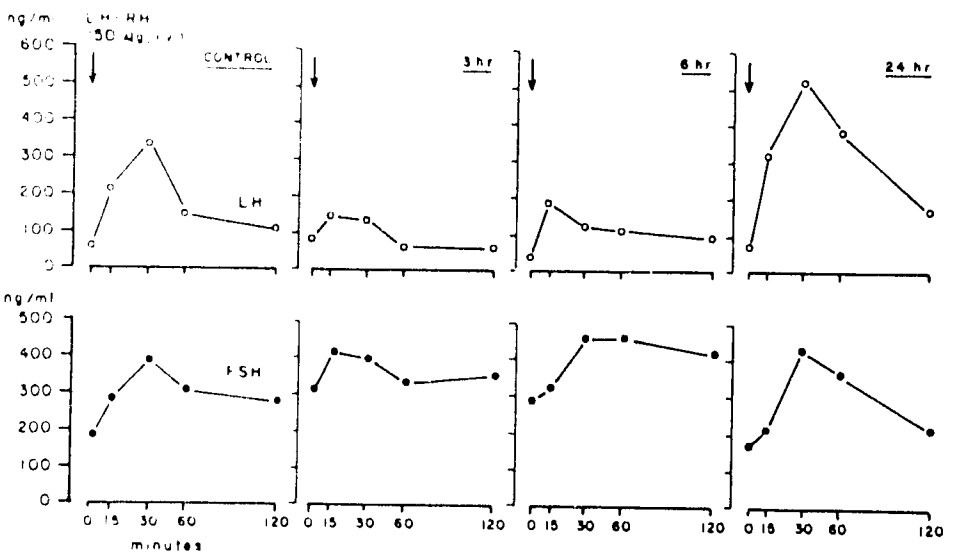

FIG. 20-7. LHRH responses before and after the IM administration of 90 mg [D-Phe^2,D-Trp^3,D-Phe^6]-LHRH in one case. Arrows indicate time of injections.

between 12 and 20 days after LHRH injection, and two patients presented uterine bleeding 4 to 6 days following LHRH administration. The other six women showed some attenuation of both LH and FSH surges, but ovulated. All 14 patients resumed regular menses and ovulation in following cycles, which proves the spontaneously reversible effect. In another study, 5 mg blocked LH midcycle surge in six of nine normal women; however, the menstrual cycle was disrupted since bleeding was delayed up to 20 days. A new analog, [N-Ac-D-pCl-Phe^{1,2},D-Trp^{3},D-Phe^{6},D-Ala^{10}]-LHRH blocked ovulation at 2- to 3-mg doses in five of eight women, but the same problem was observed; menstrual bleeding occurred 20 to 30 days after the single injection of LHRH analog. This analog is very active and has prolonged inhibitory action. The contraceptive value of this analog is currently being evaluated.

In conclusion, we feel that new techniques based on the blockade of the ovulatory surge of LH/FSH could be the basis of birth control methods. Continued research on inhibitory analogs of LHRH may lead to the development of new contraceptives that could be conveniently self-administered and free of untoward side-effects. These analogs could lead to an excellent contraceptive method. LHRH antagonist analogs are not effective when used during the follicular phase of the cycle. Inhibiting progesterone biosynthesis by LHRH analogs does not appear to be an effective contraceptive method.

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21

Clinical Studies with LHRH Agonists

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SAMUEL S. C. YEN

Recently, luteinizing hormone releasing hormone (LHRH) and LHRH agonists have been reported to induce luteolysis in women.[4,7-9] Lemay and others[9] observed that in a small group of subjects the luteolytic effect of LHRH appeared to be less effective in the early part of the luteal phase. However, the question of maximal effectiveness of LHRH agonist on luteolysis remains to be established. The present investigation was undertaken to evaluate the potential clinical usefulness in terms of reproducibility, reversibility, and critical time of administration of LHRH agonist in inducing luteolysis in women.

MATERIALS AND METHODS

Fifteen regularly cycling women (ages 25–39) volunteered for this study, and written informed consents were obtained. A nonhormonal form of contraception was used by all the women. Mean cycle length by history was 28 days (range 27–30 days). In each subject, the menstrual cycle was monitored by recording the basal body temperature (BBT) daily throughout the cycle and by serial determination of serum levels of LH, P, and estradiol (E_2) beginning on day 10 of each cycle.

Following a control cycle in which no treatment was administered, the 15 subjects received a subcutaneous injection of 50 μg of [D-Trp^6,Pro^{9}NEt]-LHRH (LHRH-Ag) on one or two successive days in the luteal phase as previously described.[4] Blood samples were obtained from each subject at approximately the same time each day (usually between 8 and 10 a.m.) and one of the investigators examined the BBT chart. Injections of LHRH-Ag were then begun at various intervals after the biphasic shift in BBT in order to investigate the possible presence of a critical time period in the luteal phase during which the luteolytic action of the peptide might occur. Each of the 15 subjects participated in from one to four treatment cycles for a total of 43 treatment cycles for the group. A subsequent control cycle (recovery cycle)

We thank P. Malcom, J. Beauregard, the nurses of the Clinical Research Center at the University Hospital, M. Reck, J. Wong, J. Boone, J. Aurand, and W. Hopper for their excellent technical assistance; D. Austin, H. Leskovac, and C. Yoza for manuscript assistance; and especially Dr. A. Lem for his advice. Drs. W. Vale and J. Rivier kindly provided the synthetic LHRH agonist used in this study.

was monitored in 14 women to determine the reversibility of any luteolytic effect of LHRH-Ag.

Because normal menses occur approximately 5 days following spontaneous regression of the corpus luteum,[2,5] induction of luteolysis was considered successful if a parallel decline in E_2 and P resulted in premature onset of menses within 5 days of first dose of LHRH-Ag. A failed induction of luteolysis was considered to have occurred if menses began more than 5 days after LHRH-Ag treatment regardless of whether or not the luteal phase length was shortened. In three instances, owing to the inherent inaccuracy of BBT, LHRH-Ag was inadvertently administered within 5 days of the expected onset of menses as determined retrospectively by the hormonal data. These cycles could not be evaluated.

LHRH-Ag was synthesized and provided by J. Rivier and W. Vale at the Salk Institute. *In vitro* studies have demonstrated that this agonist is 144 times more potent than LRF in releasing LH and FSH from rat anterior pituitary cells;[10] this degree of potency has been confirmed by *in vivo* studies of women during the early follicular phase.[3] LHRH-Ag was dissolved in sterile 0.9% NaCl containing 0.9% benzyl alcohol as a bacteriostatic agent (Cutter Laboratories, Berkeley, CA); passed through a millipore filter (0.22 μm, Millipore Corp., Bedford, MA); and used to test sterility. All the LHRH-Ag used in this study was prepared in one batch.

Measurement of LH, E_2, and P levels was made by radioimmunoassay.[1,6,12] Samples from individual studies were run in the same assay. Data analysis was performed by analysis of variance and Student's two-tailed *t*-test.

RESULTS

In 29 of the 43 treatment cycles, "successful" induction of luteolysis occurred. In 11 treatment cycles, LHRH-Ag administration failed to induce luteolysis. Three treatment cycles could not be evaluated.

In Figure 21-1, composite data for the mean (±S.E.) levels of P and E_2 in the luteal phase of the 15 control cycles are compared with the cycles in which induction of luteolysis by LHRH-Ag failed (n = 11) or succeeded (n = 29). Control luteal phase length was 14.4 ± 0.3 days (mean ± S.E.) and did not differ from failed treatment cycle length (13.8 ± 0.4 days); no difference was seen in the E_2 or P levels of these two groups. However, in the 29 successful treatment cycles, luteolysis was confirmed by the premature parallel decline of serum P and E_2 levels and the significant ($P < 0.001$) shortening of the luteal phase (11.1 ± 0.3 days) when compared with the control cycle (14.4 ± 0.3 days).

The distribution of success or failure in the induction of luteolysis, when examined on the basis of the time of administration of the LHRH-Ag during the expanse of the luteal phase, is illustrated in Figure 21-2. When given within 5 days of the LH peak, LHRH-Ag failed to induce luteolysis in ten of 13 cycles (77%). Under the conditions of our study, the early corpus luteum appears to be refractory to the luteolytic action of LHRH-Ag. However, when treatment began on or after day 5, luteolysis occurred in 26 of 27 cycles

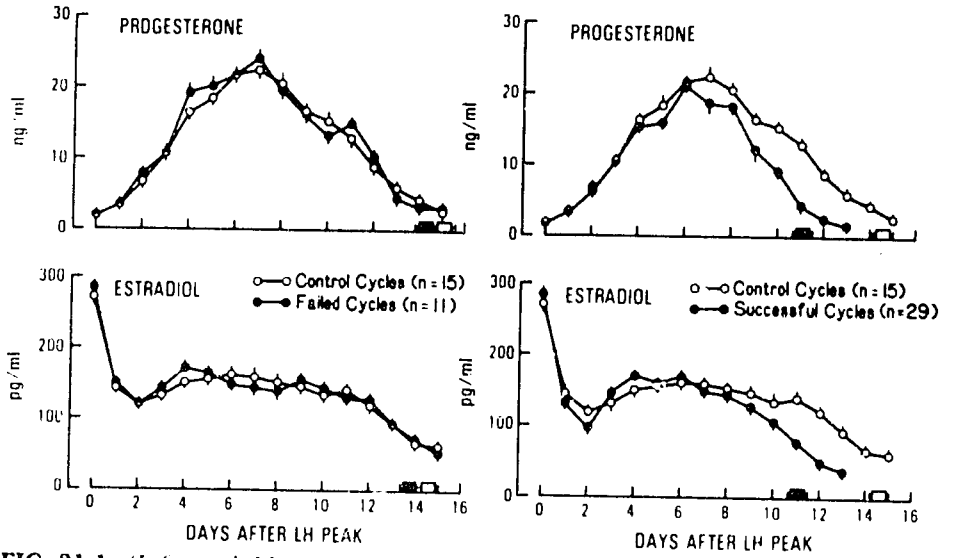

FIG. 21-1. (*Left panel*) Mean (±S.E.) daily serum levels of P and E in 11 failed treatment cycles (●-----●) compared with 15 control cycles (○-----○). Onset of menses (±S.E.) is indicated by boxes (open, control; black, treated). (*Right panel*) Mean (±S.E.) daily serum P and E levels in 29 successful treatment cycles (●-----●) compared with 15 control cycles (○-----○). Onset of menses (±S.E.) is indicated by boxes (open, control; black, treated).

(96%). Reproducibility was thus demonstrated in each subject's sequential cycles as long as the LHRH-Ag was given at the appropriate time.

The reversibility of the luteolytic effect of LHRH agonist was evaluated by monitoring a final nontreatment (or recovery) cycle in 14 of the 15 subjects. In all subjects, the recovery cycle luteal phase length was within 1 day of the control cycle length, and there was no difference in mean luteal phase lengths of control cycles (14.4 ± 0.3 days) and recovery cycles (14.1 ± 0.4 days).

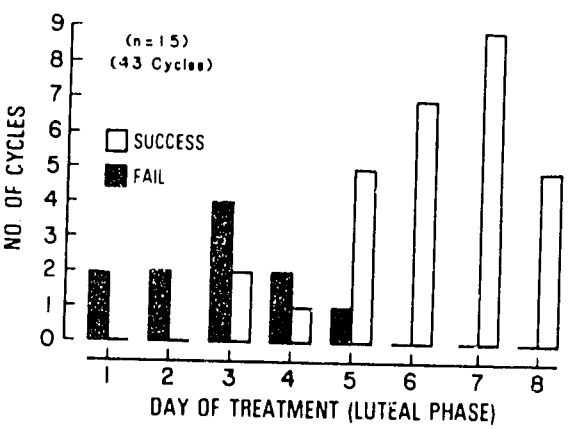

FIG. 21-2. Number of successful and failed treatment cycles according to day of the luteal phase on which treatment with LHRH-Ag was administered.

Neither ovulation nor the adequacy of luteal function seemed disturbed by prior treatment with LHRH-Ag.

DISCUSSION

The present study has demonstrated both reproducibility in individual women and complete reversibility of the luteolytic effect of LHRH-Ag; however, the reproducibility depends on the timing of administration in the luteal phase. When treatment with LHRH-Ag commences 5 to 8 days after the LH peak, premature luteolysis consistently occurs. However, the early corpus luteum appears to be refractory to the luteolytic effect of LHRH-Ag since, under the conditions of our study, administration of the agonist within the first 4 days after the LH peak failed to induce luteolysis. These findings are consistent with the observations of Lemay and associates[9] that multiple injections of LHRH caused premature luteolysis in women when given on days 6 through 9 after the LH surge, whereas administration on days 1 through 5 was far less effective in shortening the luteal phase.

The mechanism of the luteolytic effect of LHRH and LHRH-Ag is unclear. Experimental data suggest three possible sites of action: direct pituitary desensitization, the down-regulation of ovarian LH receptors, and a direct inhibitory action of LHRH on ovarian steroidogenesis. The mechanism of maximal sensitivity in the midluteal phase is also unknown. It is tempting to speculate that an increased delivery of LHRH agonist to the luteal cells is occasioned by the increased vascular supply of the corpus luteum, which becomes maximal at 5 to 8 days after ovulation.[10]

The potential usefulness of LHRH agonist as a contraceptive agent acting by prevention of interception of implantation is obvious. However, extended study is required before long-term application of LHRH agonist-induced luteolysis can be practical. Its use may be restricted by the timing of administration, as evidenced by the inaccurate interpretation of BBT in three of our 43 treatment cycles and by the apparent refractoriness of the early corpus luteum to LHRH agonist's luteolytic action.

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Discussion: LHRH-Analog Agonists and Antagonists as Antifertility Agents in the Human Female

JOSEPH W. GOLDZIEHER, MODERATOR

In order to put various contraceptive and luteolytic regimens with LHRH agonists in perspective, one must keep in mind that the secretory activity of the hypothalamopituitary system produces repeated pulses of LHRH at approximately 90-minute intervals. The secreted peptide has a very short half-life, allowing for a rapid decay of the stimulus. To the extent that various medicinal regimens do not follow this course of events, they are unphysiologic and have a variety of unphysiologic consequences. Frequent or continuous administration simply shuts down pituitary gonadotropin release resulting, in effect, in transient chemical castration that may be observed both in females and males. Between these extremes, administration of various doses of long-acting LHRH analogs, in various regimens, is bound to produce quite a spectrum of events—one which is likely to be further confounded by the inherent variability of response from one individual to another. The early stages of clinical investigation are likely to be associated with confusion—as is indeed the case at the present time.

Animal studies have been helpful, but very large differences have been noted between rodents, nonhuman primates, and humans in terms of dose response. This makes extrapolations little better than educated guesses. Even in experimental animals, conflicting results are apparent. Several investigators have now examined the luteolytic effect of long-acting analogs, beginning at various stages of the postovulatory phase. They have obtained conflicting data as to whether treatment in the early, mid-, or late luteal phase is likely to have a substantial effect on plasma progesterone. A similar inconsistency is seen in clinical studies of the sensitivity of the corpus luteum to this luteolytic effect. Others have questioned whether depressions in plasma progesterone are even meaningful, in terms of the ultimate goal of contraceptive action. It was even suggested, based on clinical observations, that effective luteolytic therapy would have to begin prior to formation of the corpus luteum (*i.e.*, in the mid- or late follicular phase).

The "trapped-ovum" syndrome is considered a somewhat uncommon form of spontaneous infertility, but the condition can be reproduced by hormonal

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Panelists: Andre Lemay, Hugo Maia, Jr., Manfred Schmidt-Gollwitzer, Katharine Sheehan, Arturo Zarate. *Discussants:* Ricardo H. Asch, Horacio B. Croxatto, Wolfgang Hardt, Fernand Labrie, Andre Lemay, Kamran S. Moghissi, Jurgen K. Sandow

manipulations in experimental animals. The question was raised as to whether there was any evidence of this phenomenon as a result of the various clinical manipulations with LHRH agonists, and indeed whether any of the observational techniques used could have identified the phenomenon if it occurred. There appears to be no such information at the present time, but ultrasound scanning might possibly help, and laparoscopic examinations of women undergoing "luteolytic" forms of LHRH agonist therapy would certainly yield valuable information.

Extensive clinical contraceptive studies have been carried out with a variety of regimens using the Hoechst intranasal agonist, Buserelin. As might be anticipated, there is a great deal of individual variation to a given dose of this agent, and it is not entirely clear how much of this is due to the individual and how much to the consistency of the delivery system. In any event, with daily therapy at sufficiently high doses, complete ovarian inhibition and anovulation can be obtained. With progressively lower doses lesser effects are seen, approximating those of the minipill, and eventually doses are given which are subeffective in most individuals.

This spectrum of induced ovarian insufficiency produces the full clinical spectrum of what is termed dysfunctional uterine bleeding by the clinical gynecologist. The symptoms range from total amenorrhea (approximating castration), to infrequent periods, to dysfunctional bleeding, to intermittent and unpredictable ovulation/anovulation. Considering that certain doses in certain individuals produce anovulatory cycles (with an attendant unopposed estrogen effect on the endometrium), the occurrence of endometrial hyperplasia and dysfunctional bleeding is not unexpected.

Although the subjects studied by these clinical investigators did not object to this menstrual anarchy, the side-effects are unlikely to be equally well accepted in real-life situations. Such problems are inherent in any technology that alters estrogen secretion, and more particularly, alters or eliminates the effect of progestational exposure on the endometrium. The problems are entirely analogous to the observations made decades ago during the evolution of the oral contraceptives, where addition of an adequate amount of a potent progestational compound was required to provide an acceptable menstrual pattern. Indeed, the question was raised with regard to the ovulation-inhibiting regimens of LHRH agonists: What is it they do that the oral contraceptives do not do better? One cannot even make an argument about differences in side-effects, since the adverse reactions of greatest concern with contraceptive steroids became apparent only when thousands of individuals used them on a prescription basis. Just as problems with cardiovascular hazards and contact lens misfitting did not turn up during phase I and phase II trials, the relative absence of side-effects of LHRH analogs (except for the menstrual disruption) at this stage should not make one too sanguine. In fact, one of the predictable features of this discussion was the enthusiasm of the investigators engaged in phase II clinical trials, and the distinct pessimism and feeling of *deja vu* among the gynecologists who had lived through the era of steroid contraceptive evolution.

PHYSIOLOGY AND ANTIFERTILITY EFFECTS OF LHRH AND ANALOGS IN MALE ANIMALS

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23

Mechanisms of the Antifertility Effects of LHRH Agonists in the Male Rat

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FLEUR-ANGE LEFEBVRE

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The discovery of LHRH and the availability of the synthetic neurohormone and a large series of its highly potent agonistic analogs has permitted rapid progress in the understanding of the control of pituitary gonadotropin secretion in both experimental animals and humans.[9,23] Although the synthetic releasing hormones provided a potentially new therapeutic approach for the treatment of human infertility,[24,36] only limited success was obtained in the early studies.[19,30]

The difficulties in achieving such therapeutic success in the treatment of human male infertility are most likely related to the now well-documented "paradoxical" antifertility effects in male experimental animals.[2,3,4,6,10,14,20,21,25,28,29,32,34,35] Moreover, treatment with LHRH agonists can also lead to inhibition of reproductive functions in the human, resulting in decreased testicular steroidogenesis in men.[7,8,21,33]

Although the precise mechanisms responsible for the antifertility action of LHRH agonists are not yet fully understood, at least in part, these effects are probably mediated by the loss of gonadal receptors and desensitization secondary to LHRH agonist-induced endogenous LH release.[3,4] The direct inhibitory effect of LHRH agonists at the gonadal level could also play a role, at least at high doses.[1,18,21,37] Another site showing an antifertility effect is the adenohypophysis, where a decreased gonadotropin responsiveness to LHRH follows acute or chronic treatment with LHRH agonists in both men and experimental animals.[28,29,33]

With the potential use of LHRH agonists as a new approach in male contraception and in treating androgen-dependent pathologies (such as cancer

of the prostate) already obvious, it becomes important to investigate the mechanisms responsible for the paradoxical antifertility effects of LHRH agonists. Such knowledge should help to determine effective dosage regimens so that stimulation or inhibition of reproductive functions can be achieved according to clinical needs.

INHIBITION OF TESTICULAR GONADOTROPIN RECEPTORS

In our original description of the inhibitory effects of treatment with an LHRH agonist on testis and secondary sex organ weight in the male rat, the first evidence for the mechanisms involved—namely loss of testicular gonadotropin receptors—was also described.[2] It was then reported that treatment of male rats with the potent LHRH agonist [D-Leu^6]-LHRH-EA, administered as a single injection or as a series of seven relatively large daily doses (1.8 μg), leads to reduced testicular weight, weight of accessory reproductive glands, testicular LH receptor levels, and plasma testosterone concentration. Subsequently, we examined the effects of far lower doses of an LHRH agonist, more nearly approximating the physiologic stimulation with endogenous LHRH.[3] Groups of rats were injected with saline or with 0.008, 0.04, 0.2, 1.0 or 5.0 μg [D-Leu^6]-LHRH-EA once at 8 a.m. or three times a day (8 a.m., 4 p.m., and 12 midnight) for 1 week. The last injection was at 8 a.m. on the seventh day, and the rats were sacrificed 24 hours later.

As illustrated in Figure 23-1, there was a significant loss (approximately 30%) of testicular LH receptors in animals treated with 0.008 μg of the LHRH agonist, whereas a maximal effect (80%) occurred after doses ranging from 0.04 to 0.2 μg. Interestingly, the depletion of LH receptors by the LHRH agonist was more apparent in animals injected one time rather than three times a day. Administration of LHRH agonist had a similar effect on the levels of testicular prolactin receptors, with maximal depletion being observed in animals treated with 0.2 μg of the agonist. Because treatment with 0.04 μg or higher doses of the LHRH agonist leads to a reduction of testicular weight, it is significant that a similar inhibitory pattern was observed when LH and PRL receptor levels were expressed per gram of testis. In addition to reducing testicular weight, LH, and prolactin receptor levels, treatment with doses of 0.04 μg or more decreased plasma testosterone levels and seminal vesicle weight.

INHIBITION OF TESTICULAR STEROIDOGENESIS

These studies have shown that administration of LHRH or its agonistic analogs to adult male rats causes a marked decrease in testicular LH receptors; in weights of the testis, ventral prostate, and seminal vesicles; and in concentration of plasma testosterone.[2,4,20] Studies of Leydig cells from rats treated with hCG have shown that the hCG-induced desensitization is accompanied by a decrease of 17,20-desmolase and 17-hydroxylase activities.[11] Moreover, the extent and nature of the block in the androgen biosynthetic pathway are highly dependent upon the dose of hCG administered.[12]

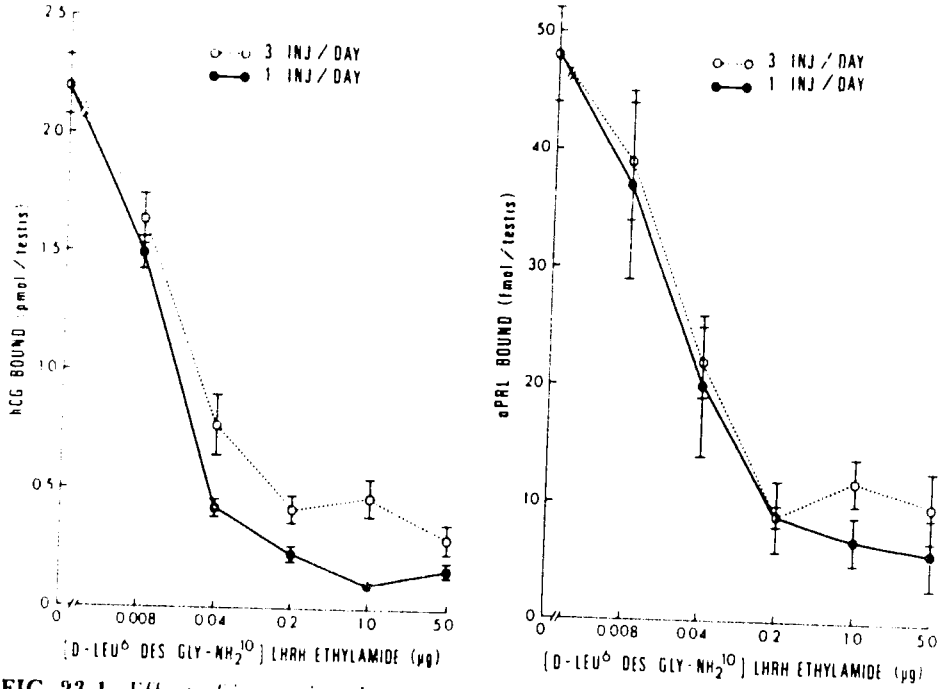

FIG. 23-1. Effect of increasing doses of [D-Leu^6,des-Gly-NH$_2^{10}$]-LHRH ethylamide injected once or three times a day for 7 days on testicular LH/hCG and prolactin receptor levels. (Auclair C, Kelly PA, Coy DH *et al:* Potent inhibitory activity of [D-Leu^6,des-Gly-NH$_2^{10}$]-LHRH ethylamide on LH/hCG and PRL testicular receptor levels in the rat. Endocrinology 101:1890–1893, 1977)

To analyze the steroidogenic pathway during desensitization induced by LHRH agonist treatment, we first examined the time course of changes in testicular and plasma levels of steroid intermediates after daily administration of 1 μg [D-Ala^6]-LHRH-EA. Changes in the levels of testicular LH, prolactin, and FSH receptors were correlated with changes in steroidogenesis. The ratio of 17-OH-progesterone to progesterone decreased rapidly following LHRH agonist treatment, suggesting a rapid inhibition of 17-hydroxylase activity.[6] There was a progressive and dramatic fall in testicular androstenedione and testosterone levels after treatment (Fig. 23-2). A decrease of approximately 25% in the concentrations of these two steroids occurred 24 hours after treatment, with a progressive decrease to 5 to 15% of control level 3 days later. The inhibitory effect on 5α-dihydrotestosterone levels is, however, much less pronounced. The decrease to 30% of control values was observed 1 day after treatment and was followed by a slow increase to approximately 50% of control values at 8 days.

In contrast to the marked changes observed in the androgen biosynthetic pathway, there was very little change in testicular estrogen levels up to 8 days after treatment; however, there was a small increase in the concentration of estrone and 17β-estradiol in the testes 12 hours after the first injection of the peptide. The 20-fold increase of the estrone-to-androstenedione ratio ob-

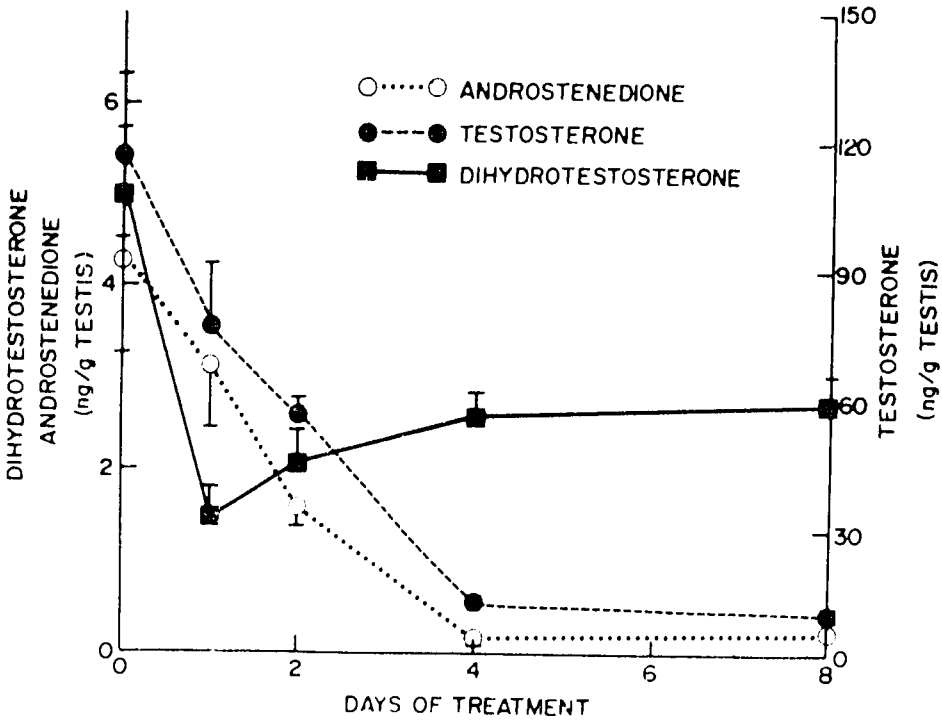

FIG. 23-2. Time course of the effect of daily subcutaneous injection of 1 μg [D-Ala[6],des-Gly-NH$_2$[10]]-LHRH ethylamide on testicular androstenedione (○), testosterone (●), and 5α-dihydrotestosterone (■) in adult rats. (Bélanger A, Auclair C, Ferland L *et al:* Time-course of the effects of treatment with a potent LHRH agonist on the testicular steroidogenesis and gonadotropin receptor levels in the adult rat. Steroid Biochem 13:191–196, 1980)

served between 2 and 4 days after treatment strongly suggests a corresponding increase of testicular aromatase activity.

In Leydig cells, the transformation of pregnenolone into testosterone can occur through two biosynthetic routes, the Δ^4 and the Δ^5 pathways. The relative importance of the two pathways varies according to species, the Δ^4 pathway appearing to be the main biosynthetic route in the adult male rat. As indicated by the changes in the ratio of 17-OH-progesterone to progesterone,[6] 17-hydroxylase activity was already significantly decreased 24 hours after the first injection of the LHRH agonist, that is, at the time when no significant change of LH receptor levels was detected. The apparent maximal inhibitory effect on 17-hydroxylase activity was seen 2 days after treatment, when LHRH receptor levels were decreased to 30% of control values. As indicated by the ratios of androstenedione to 17-OH-progesterone, 17,20-desmolase activity was decreased, also at 24 hours, and a maximal inhibition was seen at 2 days. A similarity of changes of progesterone and testosterone levels in the testes and plasma indicates that the measurement of testicular levels of steroid intermediates provides a valid index of enzymatic activities.

Our data clearly demonstrate that treatment of male rats with the LHRH agonist at doses greater than 10 ng per day causes marked inhibition of basal concentrations of testosterone in the testis; these effects appear to be due to a decrease in the enzymatic activities of 17-hydroxylase and 17,20-desmolase. Interestingly, treatment with low doses of the LHRH agonist can lead to an increased steroidogenic response to oLH. In fact, after daily treatment with 1 or 5 ng of [D-Ala^6]-LHRH-EA for 9 days, the testicular response of progesterone, 17-OH-progesterone, androstenedione, and testosterone increased even though testicular LH receptor levels were unchanged. Treatment with the 10-ng dose of the LHRH agonist—which led to a 50% loss of LH receptors—was associated with a significant increase in testicular androstenedione, testicular testosterone, and plasma testosterone responses to oLH. Zipf and co-workers also observed that relatively low doses of LH caused an increase of the plasma testosterone response to oLH in the presence of decreased testicular LH receptor levels.[37] Such findings raise the possibility that treatment with low doses of LHRH agonists might stimulate Leydig cell function, whereas high doses inhibit testicular functions.[20,25]

INHIBITION OF PITUITARY GONADOTROPIN RESPONSIVENESS

Although a marked reduction in testicular gonadotropin receptors and steroidogenesis has followed treatment with LHRH agonists, the anterior pituitary can also be an important site of the inhibitory action of LHRH agonists on reproductive functions. Impairment of the pituitary gonadotropin responsiveness has been reported after chronic exposure of pituitary cells in culture to LHRH *in vitro*[27] and after repeated administration of gonadotropin-releasing peptides in the rat *in vivo*.[21] Inhibition of the pituitary gonadotropin responsiveness after repeated administration of LHRH or its agonists has also been observed in men and women in different endocrine states.[15,33] These observations raise the possibility that an LHRH-induced loss of the pituitary gonadotropin responsiveness may be in part responsible for the paradoxical antifertility effects observed after LHRH agonist treatment.

Treatment of adult intact male rats with [D-Ala^6]-LHRH-EA for 2 weeks caused a 50% decrease of the LH response to native LHRH (Fig. 23-3). Although basal plasma FSH levels increased twofold after LHRH-EA treatment, the pituitary FSH response, as measured by the area under the plasma FSH curve, remained unchanged (Fig. 23-3B).

A similar inhibition of the pituitary LH response to LHRH after LHRH analog treatment was observed in castrated male rats. Although the pituitary LH response was inhibited by 50%, no effect on the pituitary FSH response was observed (data not shown). Furthermore, the increase in pituitary and plasma LH and FSH levels after castration was significantly suppressed after administration of the analog.

In intact male rats, 1 and 2 days after cessation of chronic treatment with LHRH analog, the pituitary LH responsiveness to native LHRH as measured by the area under the plasma LH response curve was still reduced by 75%.[21] This loss of response was followed by a progressive return toward control

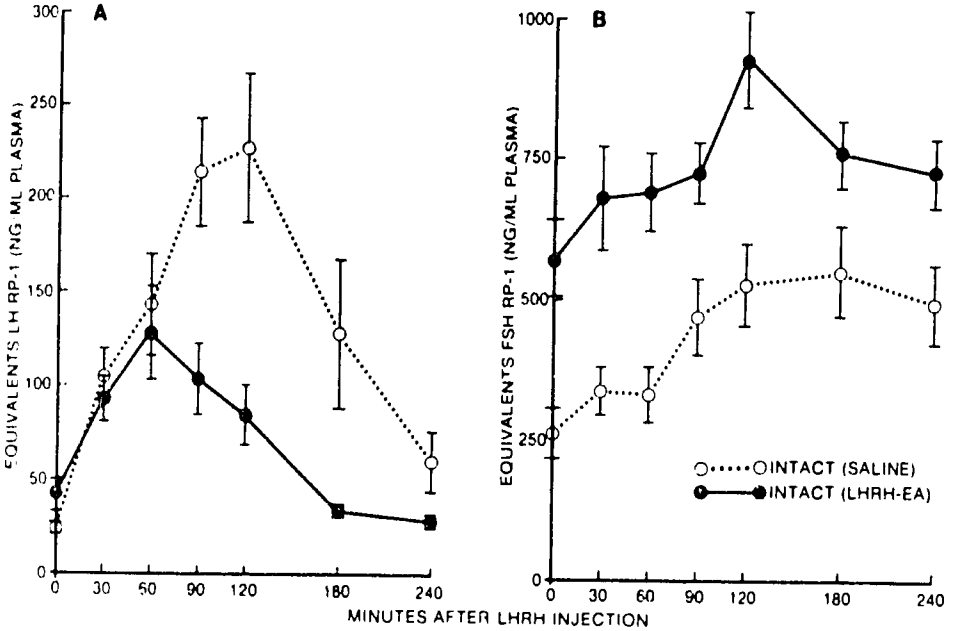

FIG. 23-3. (*A*) Pituitary LH and (*B*) FSH response to native LHRH in intact male rats treated for two weeks with [D-Ala^6,des-Gly-NH$_2^{10}$]-LHRH ethylamide (LHRH-EA). (Labrie F, Bélanger A, Cusan L *et al:* Antifertility effects of LHRH agonists in the male. J Androl 1:209–228, 1980)

levels, with complete recovery 8 days after cessation of LHRH analog administration. Thus, under the experimental conditions used to demonstrate the antifertility effects of LHRH agonists in male rats, the inhibitory effects on gonadotropin receptor levels and steroidogenesis were usually accompanied by a marked decrease of the pituitary LH responsiveness to LHRH.

Inhibition of LH responsiveness to LHRH after LHRH agonist treatment could be due to direct effects of the synthetic peptides on gonadotrophs, to secondary changes of plasma steroid levels,[16] or to both mechanisms. It was therefore of interest to compare the effects of treatment with the gonadotropin-releasing peptide in intact and castrated male rats. The results, which demonstrate that the LHRH agonist exerts a direct inhibitory effect on the anterior pituitary level, are in agreement with the observations of Sandow and colleagues.[28]

INHIBITION OF SPERMATOGENESIS

After determining that short-term treatment with LHRH and its agonists had a dramatic inhibitory effect on testicular gonadotropin receptor levels and steroidogenesis, we studied the effect of long-term treatment with an LHRH agonist on testicular function and spermatogenesis. After 1 week of treatment with [D-Ala^6]-LHRH-EA (100 ng, twice a week), no histologic alterations in

the testes were observed except for some regressive changes in a few seminiferous tubules. However, after 2 to 8 weeks of treatment, marked histologic changes were observed. At 2 weeks, many tubules exhibited degenerative changes and disruption of cellular associations. However, a few tubules appeared relatively normal, and many spermatozoa were found in the epididymis. After 2 weeks of treatment, Sertoli cells in most tubules appeared to contain vacuoles. This abnormality persisted for the entire duration of treatment.

After 4 weeks of treatment, changes in testicular histology were more dramatic. Almost all tubules exhibited signs of regression. In about 25% of the tubules, the process of degeneration was very advanced with almost complete absence of the germ cells and only the Sertoli cells remaining.[25] At this time, only a few spermatozoa could be seen in the epididymis. After 8 weeks of treatment, all the tubules showed advanced degenerative changes. However, no morphologic changes were observed in the Leydig cells at any time interval. Moreover, results of preliminary ultrastructural studies indicate that Leydig cells retain their normal appearance after 1 to 4 weeks of administering the LHRH agonist.

In a second series of experiments, the same LHRH analog was administered using a higher dose, namely 1 μg every second day rather than 100 ng twice a week. Four weeks of this treatment induced degenerative changes in almost all seminiferous tubules, and the process of degeneration appeared more advanced than was observed after treatment with the lower dose. In about 20 to 30% of tubules, both germinal and Sertoli cells were almost completely absent (Fig. 23-4). Again, the Leydig cells did not show any significant morphologic change. Testicular weight was reduced by about 40%.

RELATIVE IMPORTANCE OF THE PITUITARY AND TESTICULAR SITES OF ACTION

Since the antifertility effects of LHRH agonists are parallel to their LH-releasing activity,[14] it has been suggested that endogenous LH release leading to a secondary loss of gonadal receptors and blockage of steroidogenesis is responsible for the antifertility effects observed after such treatment.[3,4,20] However, the early observation by Rippel and Johnson that administration of a potent LHRH agonist to hypophysectomized immature rats prevents the hCG-induced increase in ovarian weight already suggested a direct ovarian site of action of the peptide.[26] More recently, a direct *in vivo* action of LHRH agonists has also been observed by other groups at the testicular level.[1,18]

Valid assessment of the relative importance of the pituitary and gonadal sites of the antifertility action of LHRH agonists can be achieved only under *in vivo* conditions. Although LHRH and some of its agonistic analogs have had direct gonadal effects in hypophysectomized animals, pharmacologic doses of the LH-releasing peptides have been used and no dose-response study has been performed. The present study compares the effects of increasing doses of the LHRH agonist [D-Ser(TBU)^{6}, des-Gly-NH_2^{10}]-LHRH-EA on testicular and ovarian gonadotropin receptors and steroidogenesis in intact and hypophysectomized male rats.

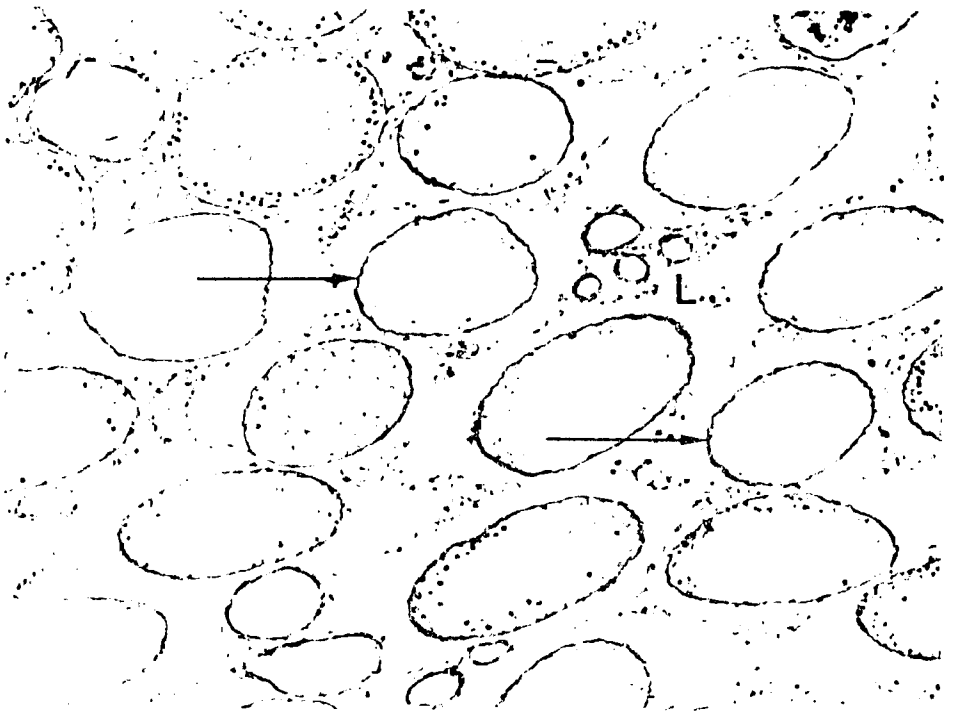

FIG. 23-4. Section through a testis of a rat treated for 4 weeks with the LHRH analog. All tubules present degenerative changes. In some tubules (*arrows*), all germinal and Sertoli cells have completely disappeared. Leydig cells (*L*) have a normal appearance.

As illustrated in Fig. 23-5A, treatment of intact rats with daily doses of 1 or 3 ng [D-Ser(TBU)^6]-LHRH-EA leads to a 35 to 50% stimulation of testicular LH receptor levels; the 10-, 30-, and 100-ng doses decrease LH receptors by 20, 65 and 90%, respectively. By contrast, in hypox animals (Fig. 23-5B), the 1- and 3-ng doses have no effect, whereas the 10-, 30-, and 100-ng doses reduce LH receptor levels by only 20 to 40%. Treatment with the LHRH agonist had no effect on testicular FSH receptors, and only the highest dose (100 ng) had an inhibitory effect on prolactin receptors in intact rats. No effect was seen in the absence of the adenohypophysis.

Because treatment of adult male rats with LHRH agonists leads to blockage of the testicular steroidogenic pathway at the level of 17-hydroxylase and 17,20-desmolase activities,[5] we next investigated a possible effect of similar treatment on the testicular steroidogenic pathway in the absence of the pituitary gland. When the steroidogenic response was examined 2 hours after subcutaneous administration of 10 μg oLH, there was a marked decrease of the testicular levels of 17-OH-progesterone, androst-5-ene, 3β, 17β-diol, and testosterone after treatment with doses of LHRH agonist higher than 10 ng (Fig. 23-6). Though treatment with the 30- and 100-ng doses caused a marked decline in the testicular 17-OH-progesterone levels, the concentration of 17-OH-pregnenolone increased. As indicated by the levels of pregnenolone and

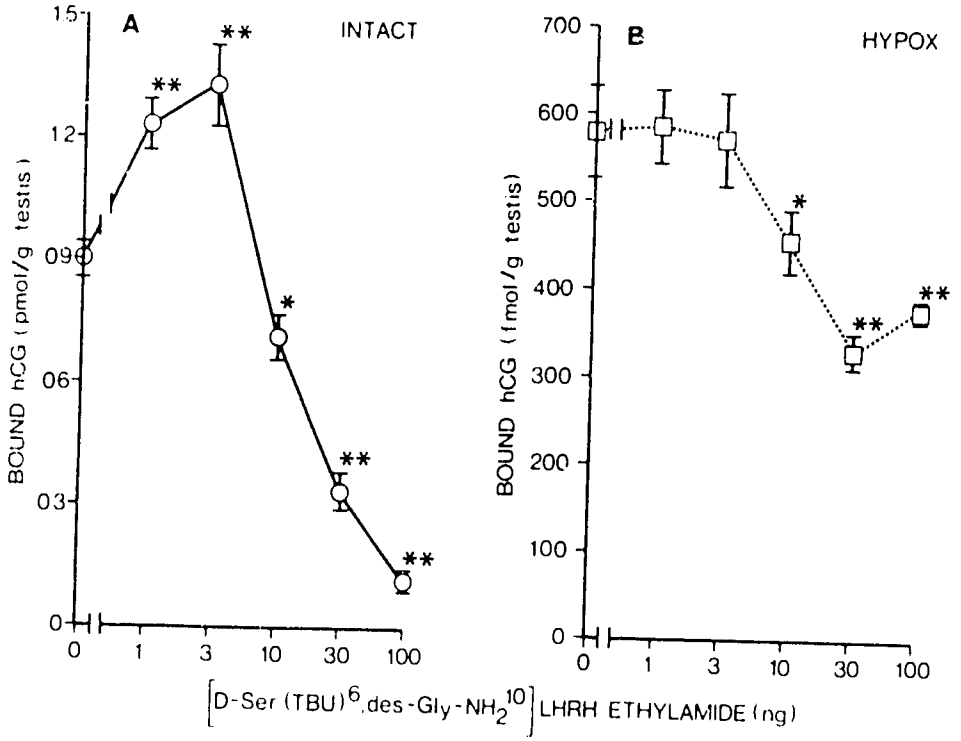

FIG. 23-5. Effect of daily treatment for 9 days on (*A*) intact or (*B*) hypox (3 days earlier) rats with increasing doses of [D-Ser(TBU)^6]-LHRH-EA on testicular LH/hCG receptor levels. The animals were sacrificed 24 hours after the last injection of the LHRH agonist or vehicle alone (1% gelatin–0.9% NaCl). (Séguin C, Bélanger A, Cusan L *et al:* Relative importance of the adenohypophyseal and gonadal sites of inhibitory action of LHRH agonists. Biol Reprod [in press])

progesterone, no sign of blockage at early steps in the steroidogenic pathway was observed after treatment. Low doses (3 and 10 ng) of [D-Ser(TBU)^6]-LHRH-EA stimulated the response of testosterone and androst-5-ene-3β, 17β-diol, but higher doses had a marked inhibitory effect. Basal levels of 5α-dihydrotestosterone were unchanged after treatment with the LHRH agonist, but the response to oLH was slightly increased at the 10- and 30-ng doses. Both basal and oLH-induced androstane-3α, 17β-diol levels increased after treatment with 10 to 100 ng [D-Ser(TBU)^6]-LHRH-EA, thus suggesting increased 5α-reductase activity.

A very different pattern of basal as well as oLH-stimulated testicular steroid levels was found in hypox animals. Although testicular pregnenolone and 17-OH-pregnenolone levels were not detectable in these animals, treatment with any dose (1 to 100 ng) of the LHRH agonist led to a marked inhibition of basal testicular levels of all steroids. Moreover, in contrast with the marked changes of the steroidogenic response to oLH observed in intact animals treated with the LHRH agonist, little or no effect of the same treatment was seen in hypox animals (Fig. 23-7). In fact, only a slight (approximately 25%, $P < 0.05$) inhibition of the testosterone response was seen after treatment

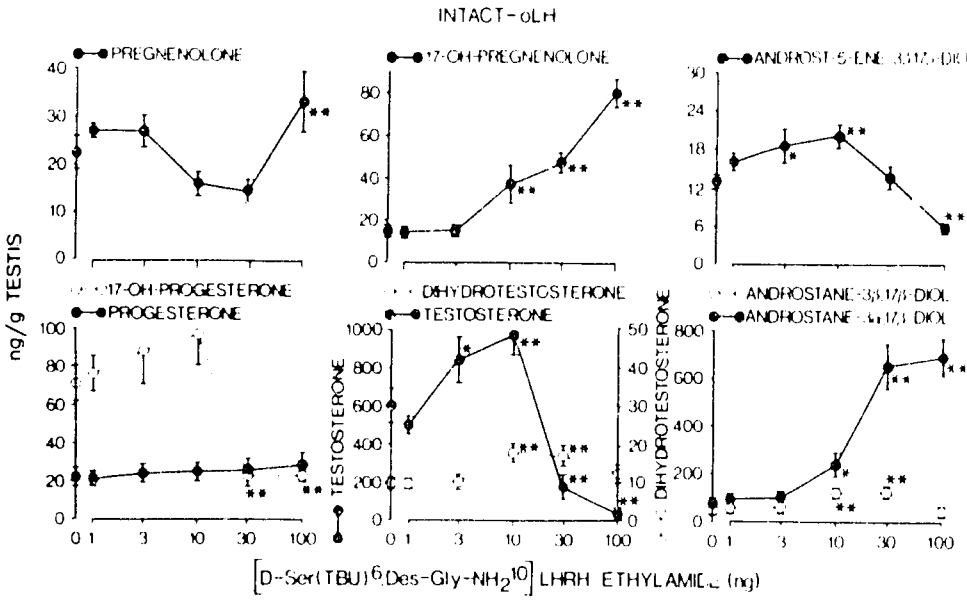

FIG. 23-6. Effect of daily treatment for 9 days of intact adult rats with increasing doses of [D-Ser(TBU)^6]-LHRH-EA on oLH-stimulated testicular levels of pregnenolone, 17-OH-pregnenolone, androst-5-ene-3β, 17β-diol, progesterone, 17-OH-progesterone, testosterone, 5α-dihydrotestosterone, androstane-3α, 17β-diol and androstane-3β, 17β-diol. The animals were sacrificed 24 hours after the last injection of the LHRH agonist and 2 hours after administration of 10 μg oLH. (Séguin C, Bélanger A, Cusan L *et al:* Relative importance of the adenohypophyseal and gonadal sites of inhibitory action of LHRH agonists. Biol Reprod [in press])

with the two highest doses of the LHRH agonist. The 17-OH-progesterone response increased ($P \leq 0.01$) and no significant change was seen in the responses of the seven other steroids measured.

As mentioned, chronic treatment with LHRH agonists can lead to a marked inhibition of spermatogenesis in the rat, significant degenerative changes of the seminiferous tubules being already observed after 2 weeks of treatment.[25] To determine whether the LHRH agonist produces these effects by direct action at the testicular level, hypox rats were injected every second day with 100 ng [D-Ser(TBU)^6]-LHRH-EA for 1 month before histologic examination. Treatment of hypox rats with the LHRH agonist had no effect on the histology of the testis (data not shown).

TESTICULAR LHRH RECEPTORS

The first step in the action of LHRH agonists in the testis is likely to be binding to receptors. We examined the possible occurrence of LHRH receptors in an enriched Leydig cell preparation and studied its binding characteristics using the potent and stable LHRH agonist [^{125}I] [D-Ser(TBU)^6]-LHRH-EA as iodinated tracer.[13] This agonist binds to a single class of high-

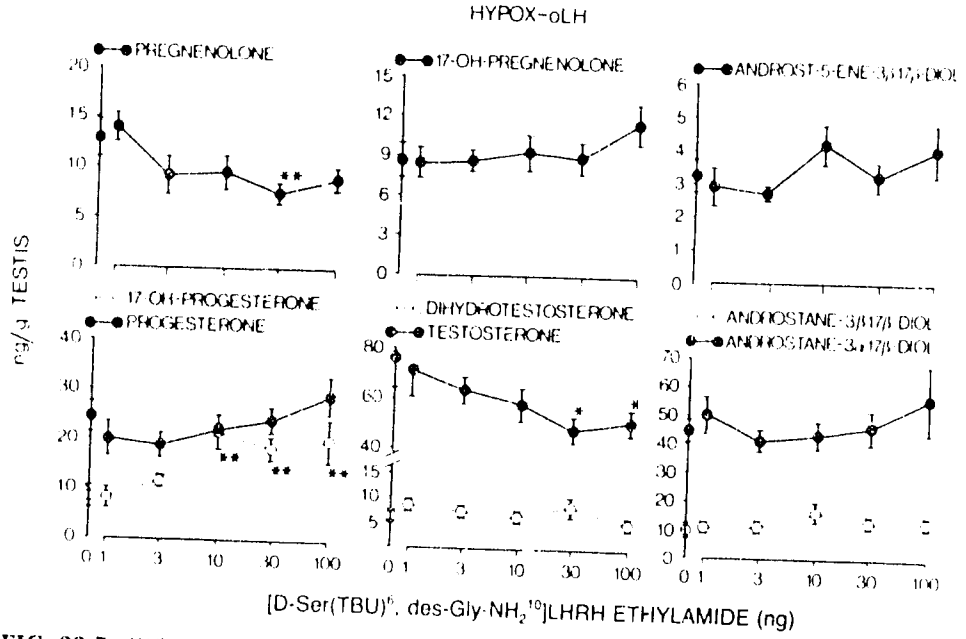

FIG. 23-7. Effect of daily treatment for 9 days of hypox (3 days earlier) adult rats with increasing doses of [D-Ser(TBU)⁶]-LHRH-EA on oLH-stimulated testicular levels of pregnenolone, 17-OH-pregnenolone, androst-5-ene-3β, 17β-diol, progesterone, 17-OH-progesterone, testosterone, 5α-dihydrotestosterone, androstane-3α, 17β-diol and androstane-3β, 17β-diol. The animals were sacrificed 24 hours after the last injection of the LHRH agonist and 2 hours after the administration of 10 μg of H. (Seguin C, Bélanger A, Cusan L *et al:* Relative importance of the adenohypophyseal and gonadal sites of inhibitory action of LHRH agonists. Biol Reprod [in press])

affinity and saturable sites in rat testicular interstitial cells with an apparent dissociation constant of 0.12 nM. Data obtained with increasing concentrations of the homologous unlabeled peptide (Fig. 23-8) show an apparent K_D of 0.25 ± 0.1 nM, thus indicating that [^{125}I] does not alter the affinity of the molecule for the testicular binding sites. No specific binding could be measured in either seminiferous tubules or Sertoli cells. Binding capacity was calculated at approximately 2,500 sites per interstitial cell.

The potency of a representative group of LHRH analogs to displace [^{125}I] [D-Ser(TBU)^6]-LHRH-EA is illustrated in Figure 23-8. The agonistic analogs [D-Ala^6]-LHRH-EA, [D-Ser(TBU)^6]-LHRH-EA, and [Glu^1]-LHRH displaced [^{125}I] [D-Ser(TBU)^6]-LHRH-EA at ED_{50} values of 0.83, 0.97, and 42.8 nM, respectively; LHRH displaced the radiolabeled ligand at an ED_{50} value of 5.64 nM. The LHRH antagonist [D-pGlu^1,D-Phe^2,D-Trp^3,D-Phe^6]-LHRH displaced [^{125}I] [D-Ser(TBU)^6]-LHRH-EA at an ED_{50} value of 3.2 nM. The potency of the LHRH agonists to interact with the LHRH receptor in Leydig cells was closely correlated with the LHRH-releasing activity measured in anterior pituitary cells in primary culture.[22]

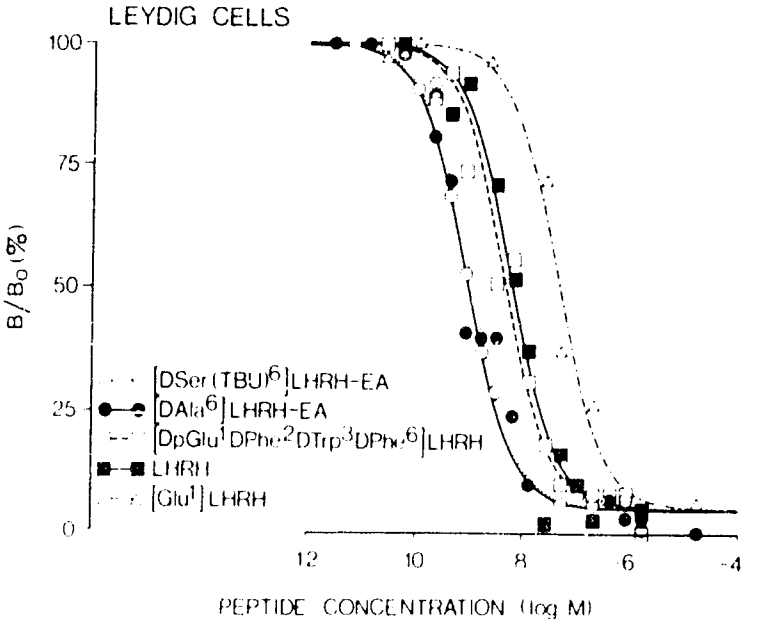

FIG. 23-8. Displacement of [^{125}I] [D-Ser(TBU)^6]-LHRH-EA binding from isolated rat testicular interstitial cells by a representative group of LHRH analogs. (Lefebvre FA, Reeves JJ, Séguin C *et al.* Specific binding of a potent LHRH agonist in rat testis. Mol Cell Endocrinol 20:127–134, 1980)

As mentioned, the close correlation observed between the LH-releasing potency of LHRH analogs and their ability to inhibit testicular functions suggests that endogenous LH release is largely responsible for the antifertility effects of these peptides.[14] In support of the major role of endogenous LH, the inhibitory effects observed after treatment with LHRH agonists (including degeneration of the seminiferous tubules and specific blockage of steroidogenesis) are identical to those obtained after treatment with LH or hCG.[6,12,21]

TESTICULAR LHRH-LIKE MATERIAL

The evidence that LHRH receptors have almost superimposable binding characteristics in the anterior pituitary and testis indicates that the antifertility effects of LHRH and its agonists might be partly mediated by their action through testicular LHRH receptors, at least when high doses of the LHRH agonists are used. LHRH concentration in the portal blood system of the rat has been reported at 150 pg/ml.[17] This observation, combined with the fact that LHRH is diluted 500-fold between the portal circulation and the jugular vein, makes it unlikely that hypothalamic LHRH could have a physiologic action on testicular LHRH receptors. However, it is also unexpected to have specific testicular LHRH receptors that are not linked to a physiologic function. Although the possibility of an action of hypothalamic LHRH at the

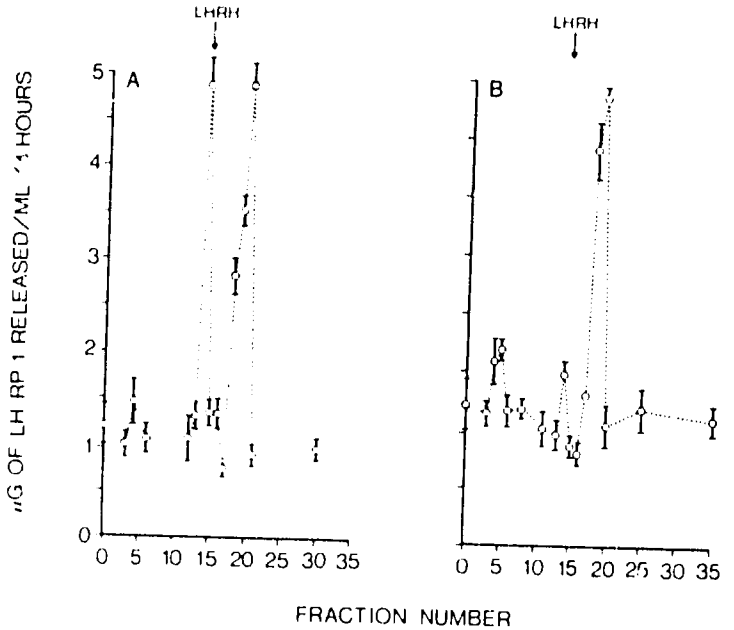

FIG. 23-9. Elution profile of LH-releasing activity from rat testicular acetic acid extract (<50,000M.W.) from (*A*) immature and (*B*) adult rats on an HPLC C-18 column with a gradient of triethylamine-formic acid (0.25M, *p*H 3.0) (TEFA)-acetonitrile (90% to 30% TEFA). LH-releasing activity was measured in rat anterior pituitary cells in culture as described. (Drouin J, Labrie F: Selective effect of androgens on LH and FSH release in anterior pituitary cells in culture. Endocrinology 98:1528–1534, 1976)

gonadal level must be further investigated, the presence of specific testicular LHRH receptors suggests that LHRH secreted locally may be involved in the control of testicular activity, a new area of investigation of great biologic interest.

As illustrated in Figure 23-9, two peaks of LH-releasing activity are seen after separation on HPLC C-18 column of testicular acid extracts from immature (A) and adult (B) rats. The first peak of LH-releasing activity coelutes with LHRH. Studies are in progress to purify and characterize this LHRH-like material further. A similar two-peak elution pattern has also been observed in ovarian tissue.*

SUMMARY

From our findings and surveys of other research, it is evident that treatment with LHRH agonists can cause both gonadal and pituitary desensitization. It is thus likely that the antifertility effects observed after LHRH and LHRH agonist administration result from the simultaneous inhibitory effects of the

*Godbout M, Labrie F: Unpublished data.

synthetic peptides at the gonadal and pituitary levels. However, from the present in vivo data, it appears that gonadal desensitization induced by LHRH agonist-induced endogenous LH release is the main factor involved.

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Antigonadal, Antisteroidal, and Antifertility Effects of LHRH Agonists in Male Animals

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Many analogs of LHRH that have been synthesized are more potent than LHRH in releasing LH and FSH.[25] When administered frequently in large doses, they exhibit antifertility effects in several species.[4,10,18] The mechanisms by which these peptides exert their antifertility actions are complex and are not understood completely at present. They were originally believed to produce all of their effects by way of the pituitary;[11,24] however, subsequent studies have shown that several agonists as well as LHRH have direct inhibitory effects on the gonads, as evidenced by their ability to inhibit *in vitro* steroidogenesis in both testicular and ovarian cells.[9,15] These peptides have shown antisteroidal activity in male and female reproductive tracts.[27]

The various sites of action of LHRH and its agonists are schematically represented in Figure 24-1. One effect of these peptides might be to modify the synthesis and release of LHRH by the hypothalamus, but this has not been extensively investigated.[21] The second and more extensively studied action of LHRH is on the pituitary. Acute administration leads to a release of large amounts of gonadotropin. After long-term treatment, there is a desensitization of the pituitary and impaired gonadotropin release.

A third site of action is at the gonadal level, where the effects are exhibited in two ways:

1. Acute increases in serum gonadotropins produced by LHRH results in down-regulation of gonadotropin receptors and reduced steroidogenic activity
2. The agonists also have direct effects on the gonads to decrease gonadotropin receptor levels and inhibit steroidogenesis.

Finally, LHRH and its agonists inhibit the actions of androgens and estrogens on male and female reproductive tracts.

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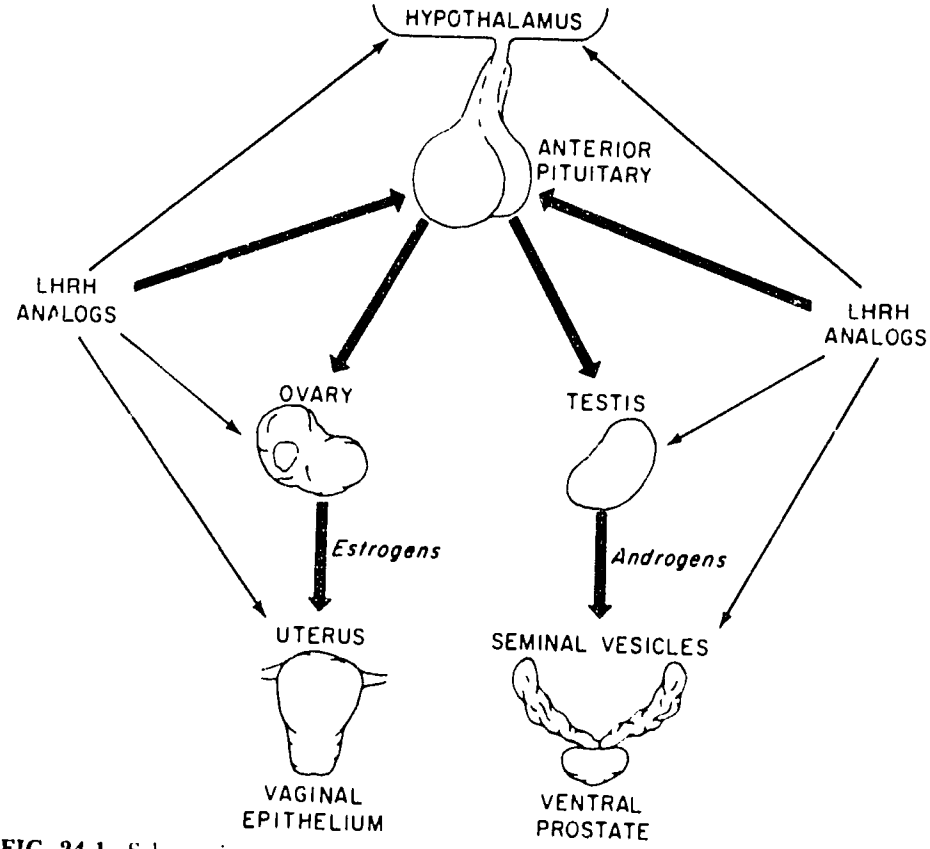

FIG. 24-1. Schematic representation of action sites for LHRH agonists.

This chapter discusses the antitesticular and antifertility effects of LHRH agonists in male animals of several species and focuses primarily on experiments from our own laboratory and related studies in the literature. For these studies, [(im-Bzl)]-D-His^{6},des-Gly^{10}]-LHRH-EA (D-His) and [D-Trp^{6},des-Gly^{10}]-LHRH-EA (provided by Drs. J. Rivier and W. Vale of Salk Institute, California) have biologic activities of 210 and 140 × LHRH, respectively. [D-Trp^{6}]-LHRH (supplied by Dr. A.V. Schally, V.A. Hospital, Tulane University, New Orleans) has a biologic activity of 60 × LHRH.

PITUITARY-MEDIATED ANTITESTICULAR EFFECTS

As noted, it was first proposed that the major antifertility effect of LHRH was mediated via the pituitary. Representative experiments supportive of this contention are illustrated in Figures 24-2 and 24-3. Administration of 10 μg D-His to male rats caused a rapid increase in serum LH levels that reached a peak after 2 hours, then returned to baseline after 8 hours and remained within the normal range over the next 8 days (Fig. 24-2A). The testicular

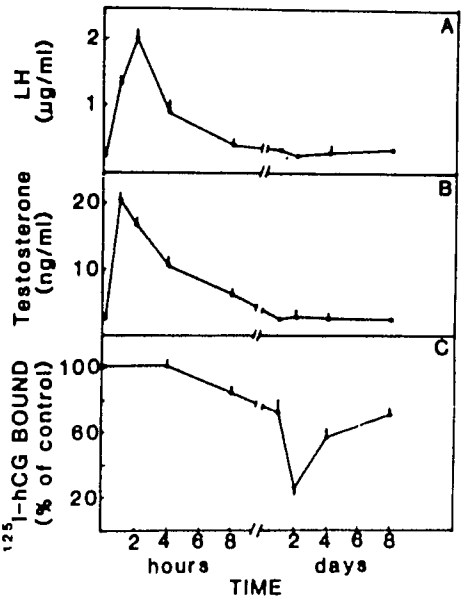

FIG. 24-2. Changes in serum LH, serum testosterone, and testicular LH receptor levels after administration of 10 µg [(im-Bzl)-D-His^{6},des-Gly^{10}]-LHRH-EA(D-His) in adult male rats. (Mean ± S.E. of six rats.)

response to the acute increase in serum LH is shown in Figures 24-2B and 24-2C. Serum testosterone (T) levels also rose rapidly and reached a peak after 1 hour and returned to normal range after 24 hours. The testicular LH receptor levels showed no significant changes over the first 4 hours when the serum LH and testosterone levels had reached their peak. By day 1, there was a considerable loss of LH receptor activity, which reached the lowest level by day 2 before gradually returning towards normal over several days. The

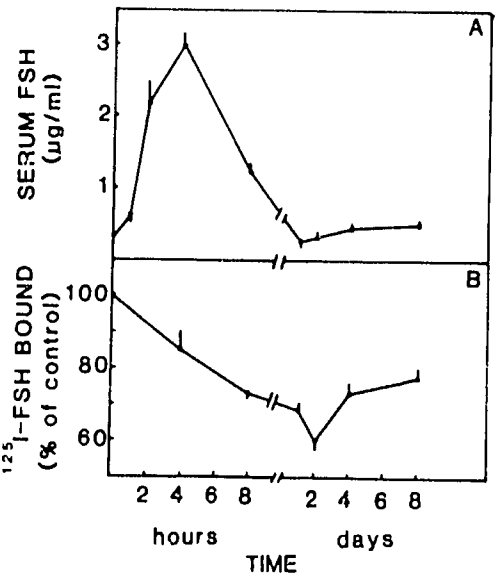

FIG. 24-3. Changes in serum FSH and testicular FSH receptor levels after administration of 10 µg [(im-Bzl)-D-His^{6},des-Gly^{10}]-LHRH-EA(D-His) in adult male rats. (Mean ± S.E. of six rats.)

decrease in testicular LH receptor concentration in response to LHRH or its agonists has been attributed to the releasing hormone-induced increase in serum LH, since similar effects can be produced by the administration of LH or hCG.[2,8,14,26]

D-His also caused an acute increase in serum FSH levels and a protracted decrease in testicular FSH receptor levels (Fig. 24-3). The decrease in FSH receptor concentration started earlier than that of LH and was noticeable 4 hours after administration of D-His. Since administration of LH does not lead to a similar depletion of testicular FSH receptors, the observed decrease after D-His administration could be due to an increase in serum FSH levels.[13] Indeed, administration of FSH to rats has been reported to cause a reduction in testicular FSH receptor levels and a decrease in their *in vitro* testicular adenylyl cyclase response to FSH.[16]

Acute administration of LHRH or its agonists also caused a decrease in testicular prolactin (PRL) receptor concentration.[2,8] Unlike the situation in the case of LH and FSH receptors, a decrease in PRL receptors is not associated with an increase in serum PRL levels.[12] Since administration of oLH or hCG can also cause a reduction of testicular PRL receptor levels, the LHRH-induced decrease in PRL receptors is believed to be due to the acute increase in serum LH.[12]

Although it is generally believed that the antigonadal and antifertility effects of LHRH and its agonists are mediated through the pituitary, several reports show that these peptides also have direct antigonadal effects both *in vitro* and *in vivo*.[7,9,15,21]

DIRECT INHIBITION OF TESTICULAR STEROIDOGENESIS AND LH RECEPTOR LEVELS

Rippel and Johnson were the first to demonstrate that a LHRH agonist has direct inhibitory effects on the ovary without requiring the pituitary.[21] Similar actions of these peptides have been demonstrated on the testes; Figure 24-4 illustrates a representative experiment. hCG caused a significant decrease in the testicular LH receptor concentration and a significant increase in serum T levels in hypophysectomized rats. D-His also produced a significant decrease in testicular LH receptor levels, but had no effect on the serum T concentration which was already low owing to hypophysectomy. When D-His was administered with hCG, the steroidogenic action of hCG was inhibited and testicular LH receptor concentration was lower than when each hormone was administered alone. Similar inhibitory effects of LHRH and its agonists directly on testicular LH receptors and steroidogenesis in hypophysectomized rats have been reported by others.[1,9]

To further understand the effect of D-His on the testes, we studied the time course of its extrapituitary actions. Hypophysectomized rats were given 50 μg oFSH daily for 5 days. Some of the rats also received 10 μg D-His daily for 1, 2, 3, or 5 days. On the following day, the rats were given hCG (5 IU, IP); 2 hours later their serum testosterone levels were measured. As shown in Fig. 24-5, hCG produced more than a tenfold increase in serum testosterone levels. Concomitant administration of 10 μg D-His (0 days) did not alter

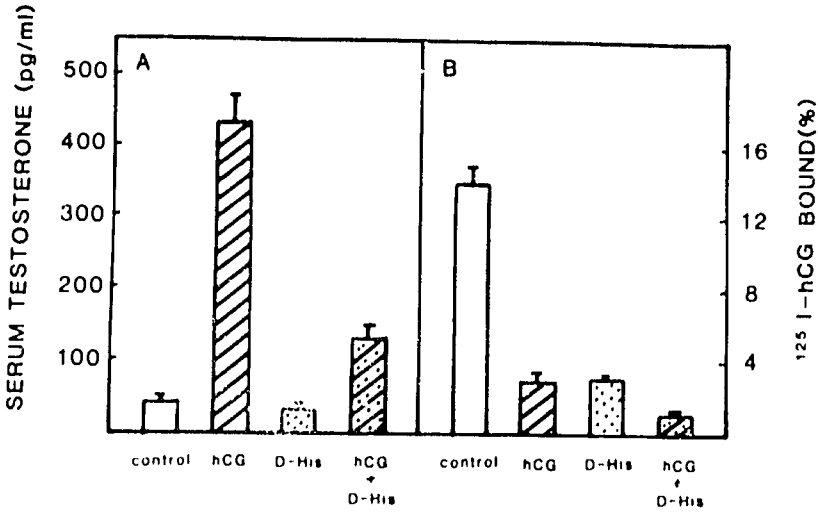

FIG. 24-4. Inhibitory effect of [(im-Bzl)-D-His[6],des-Gly[10]]-LHRH-EA(D-His) on testicular LH receptor levels and steroidogenesis. Immature hypophysectomized rats (25–26 days old) were treated with 5 IU hCG with or without 10 μg of the agonist daily for 4 days and killed on day 5. Each bar represents mean ± S.E. of six to eight animals.

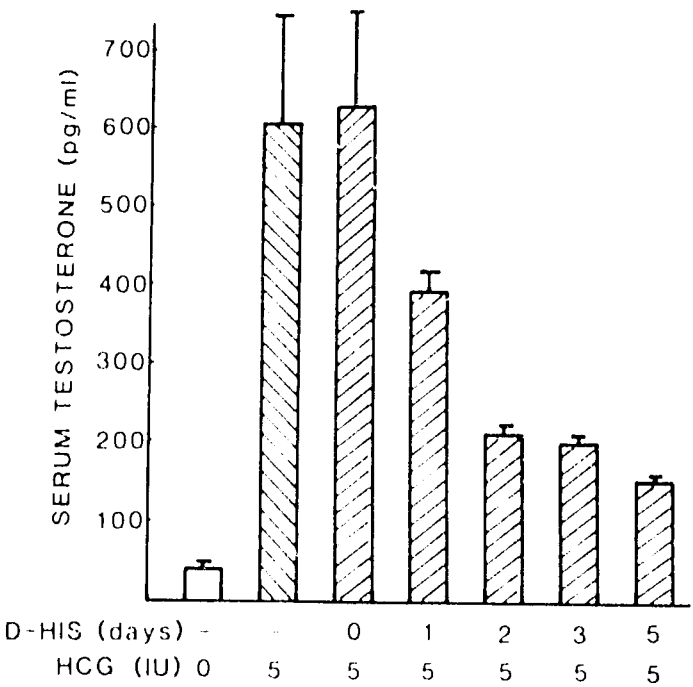

FIG. 24-5. Time course of action of [(im-Bzl)-D-His[6],des-Gly[10]]-LHRH-EA(D-His) on testicular steroidogenesis in immature hypophysectomized rats. Rats were given 50 μg oFSH for 5 days. Some rats were also given D-His (10 μg daily) for 0, 1, 2, 3, or 5 days. On the following day, hCG was administered, and 2 hours later, serum testosterone levels measured. Each bar represents mean ± S.E. of five to eight animals.

the response to hCG. However, when D-His was administered 1 day earlier, response to hCG was diminished. When the duration of treatment with D-His was extended, its inhibitory action on testicular steroidogenesis was progressively increased up to 5 days. These observations suggest that LHRH analogs require at least 24 hours to exhibit a direct effect on the testis and may explain the inability to demonstrate an effect on Leydig cells during short-term incubation.[3] In order to determine whether the direct antitesticular effects of LHRH analogs are unique to rats, similar experiments were done in mice.

SPECIES DIFFERENCES IN SENSITIVITY TO DIRECT ANTITESTICULAR EFFECTS OF LHRH AGONISTS

Hypophysectomized rats and mice were compared in an experiment in which each animal received oFSH (50 μg) with or without D-His (10 μg) daily for 5 days. On day 6, both mice and rats were given hCG (5 IU, IP) and serum testosterone was measured 2 hours later. The results of this study are presented in Figure 24-6. As expected from previous experiments, hCG caused a tenfold increase in testosterone levels in FSH primed rats. In rats receiving D-His along with FSH, the steroidogenic response of the testes to hCG was dramatically reduced (Fig. 24-6, left panel). FSH-primed mice also showed a testicular steroidogenic response to hCG (Fig. 24-6, right panel). In contrast to the effect in rats, however, D-His did not affect the hCG-stimulated testosterone secretion in mice. This unexpected finding was confirmed using [D-Trp^{6},des-Gly^{10}]-LHRH-EA.

Almost all reports showing the direct antigonadal effect of LHRH agonists have utilized rats. These results, which demonstrate the differences between

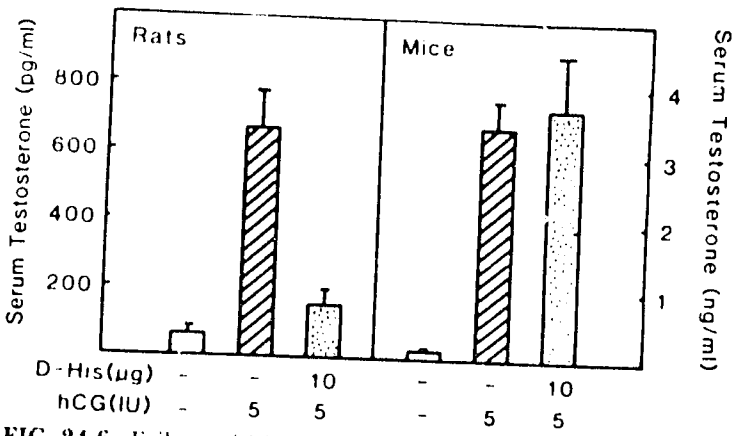

FIG. 24-6. Failure of [(im-Bzl)-D-His^{6},des-Gly^{10}]-LHRH ethylamide (D-His) to inhibit testicular steroidogenesis in hypophysectomized mice. Hypophysectomized rats and mice were given oFSH (50 μg) with or without D-His for 5 days. On day 6 they were given hCG (5 IU, IP), killed 2 hours later and serum testosterone levels measured. Each bar represents mean ± S.E. of six to ten animals.

rats and mice in the sensitivity to the direct-effects of LHRH analogs, suggest possible differences in other species. Such differences may play an important role in the mechanism of antifertility action of these peptides. For example, [D-Ser(TBU^{6}),des-Gly^{10}]-LHRH-EA was a much more effective antifertility agent in dogs than in rats.[23]

ANTISTEROIDAL ACTION OF D-HIS

Recent evidence suggests that apart from their effects on the pituitary and gonads, LHRH agonists may have antisteroidal activity in both male and female rats.[27] Administration of D-His to castrated rats along with testosterone propionate (TP) inhibited androgen-induced increase in ventral prostate and seminal vesicle weights (Fig. 24-7). That this is not a pituitary-mediated effect is illustrated by an experiment in which hypophysectomized-castrated rats were given TP with or without D-His. The results summarized in Figure 24-8 indicate that D-His has an antiandrogenic effect even in the absence of the pituitary. Analogs of LHRH have also been shown to inhibit the uterotropic action of estradiol in rats[20,27] and to inhibit the decidual response in pseudopregnant, ovariectomized rats receiving optimal doses of progesterone and estradiol.[19]

To assure that the antiandrogenic action of LHRH and its agonists were not unique to the male reproductive tract, we chose a different end point for further study. In this experiment, the androgen-induced increase in β-glucuronidase in mouse kidney was examined. One week after castration, male mice were given TP with or without D-His or LHRH for 7 days. Figure 24-9 shows that the androgen-induced increase in kidney glucuronidase concentration was inhibited by D-His as well as by LHRH.

Most steroidal and nonsteroidal compounds that have antiandrogenic activity are believed to exert their effects by competing with testosterone for binding sites or androgen receptors.[5,17] However, studies from our laboratory indicate that LHRH does not bind to androgen receptors in the ventral prostate of the rat or kidneys of the mouse. Whatever the mechanism of action, chronic treatment with potent LHRH agonists may exert significant antisteroidal effects to enhance or modify their antifertility effects.

ANTIFERTILITY EFFECTS

Chronic administration of [D-Ala^{6},des-Gly^{10}]-LHRH-EA to rats for 4 weeks has been reported to decrease testicular weight and disrupt spermatogenesis.[10,11] Administration of 5 μg [D-Trp^{6},des-Gly^{10}]-LHRH-EA to rats caused extensive disruption of spermatogenesis within 7 days.[22] However, [D-Ser(TBU^{6}),des-Gly^{10}]-LHRH-EA, another potent agonist, had minimal effect on spermatogenesis in rats but caused azoospermia in dogs administered for 4 weeks.[23,24]

We investigated the effect of chronic administration of D-His (20 μg/kg of body weight twice daily for 30 days) in rats. There was an 85% decrease in serum testosterone levels and a 50% decrease in ventral prostate weight. The

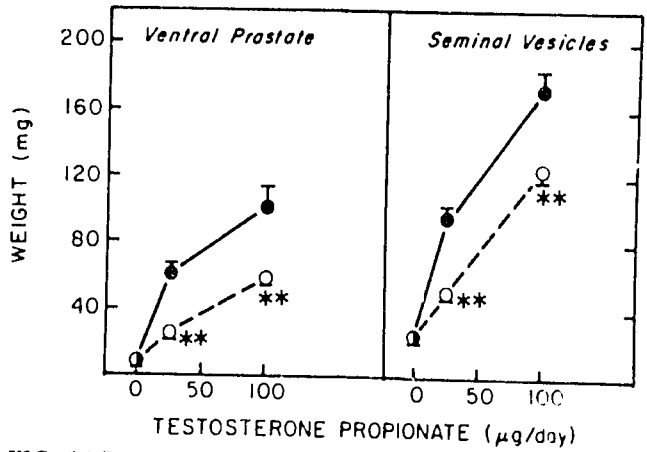

FIG. 24-7. Antiandrogenic action of [(im-Bzl)-D-His[6],des-Gly[10]]-LHRH-EA(D-His). One week after castration, rats were given 25 or 100 μg testosterone propionate (closed circles) with or without 10 μg of the agonist (open circles) for 5 days and killed on day 6. (** = $P < 0.01$. (Adapted and reprinted with permission from Sundaram K et al: Inhibition of the action of sex steroids by gonadotropin-releasing hormone (GnRH) agonists: A new biological effect. Life Sci 28: 83–88, 1981, Pergamon Press, Ltd)

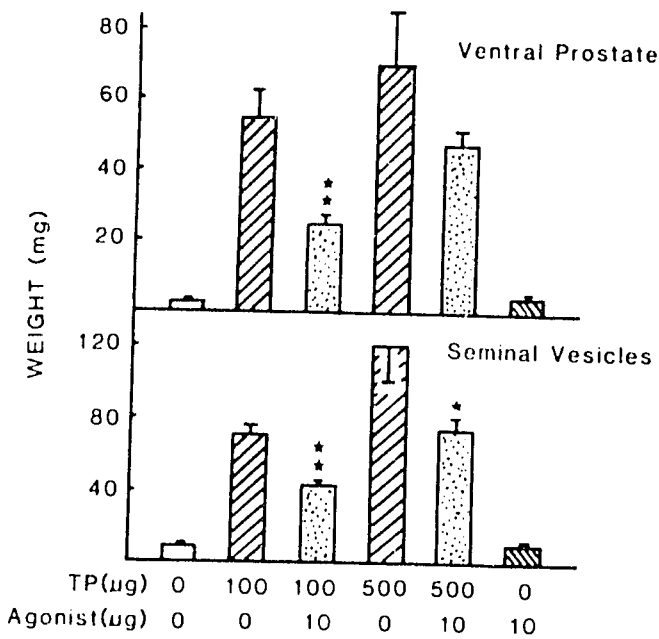

FIG. 24-8. Effect of [(im-Bzl)-D-His[6],des-Gly[10]]-LHRH ethylamide (D-His) on ventral prostate and seminal vesicle weights in hypophysectomized castrated rats treated with testosterone propionate (TP). Immature hypophysectomized rats were castrated and given TP with or without 10 μg of D-His for 5 days. (* = $P < 0.05$; ** = $P < 0.01$.) (Adapted and reprinted with permission from Sundaram K et al: Inhibition of the action of sex steroids by gonadotropin-releasing hormone (GnRH) agonists: A new biological effect. Life Sci 28: 83–88, 1981, Pergamon Press, Ltd)

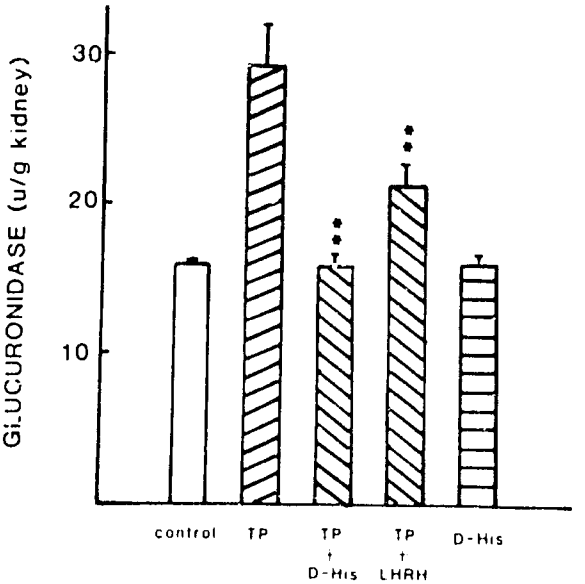

FIG. 24-9. Antiandrogenic effect of [(im-Bzl)-D-His[6], des-Gly[10]]-LHRH-EA(D-His) or LHRH on kidney β-glucuronidase in castrated male mice. Each animal received a daily injection of testosterone propionate (TP) and twice-daily injections of D-His or LHRH. (** = $P < 0.01$.)

testicular LH receptor concentration was reduced to 30% of control levels. However, the epididymides contained motile spermatozoa, and mating tests showed no impairment of fertility. Testicular weights were only slightly reduced (91% of control) and the histology of the seminiferous tubular epithelium appeared normal.

The results of this chronic study in rats and those of Sandow and colleagues[24] showing minimal effects of LHRH agonists on spermatogenesis are in contrast to other reports[4,11,22] showing extensive disruption of spermatogenesis. This may be due to differences in the sensitivity of the rats to the antifertility effects of various analogs. Alternatively, the differences may result from biological potency, dosage, and frequency of administration of the analogs. A systematic comparison of the different analogs will be necessary to understand the reasons for the diverse findings.

The antifertility effects of LHRH analogs have not been extensively tested in the male primate. Chronic administration of [D-Ser(TBU)[6],des-Gly[10]]-LHRH-EA (5 μg daily) to men caused a decrease in serum gonadotropin and testosterone levels, but spermatogenesis was unaffected.[6] It is possible that the lack of an effect was due to the low dose.

In our studies, we have also investigated the effects of chronic administration of large doses (500 μg) of [D-Trp[6]]-LHRH on pituitary and testicular function in male rhesus monkeys. Because the administration of LHRH agonists at 4-day intervals was reported to disrupt spermatogenesis in male rats,[18] a twice-weekly treatment regimen was first selected. Four animals were given 500 μg of [D-Trp[6]]-LHRH subcutaneously for a period of 12 weeks. The pituitary and testicular response to an injection of agonist was evaluated by measuring the serum LH and T levels at 0, 2, 4, 8 and 24 hours after the initial injection and at different times during treatment. Results from a representative animal are shown in Figure 24-10. The maximal serum LH levels

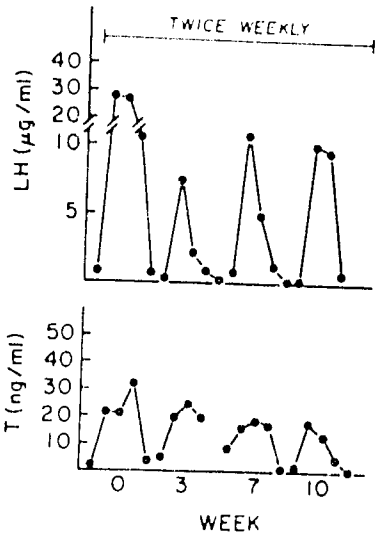

FIG. 24-10. Serum LH and testosterone (*T*) responses to 500 μg [D-Trp^6]-LHRH in monkey 125 at the beginning and after 3, 7 and 10 weeks of twice-weekly administration. The points on each curve represent values at 0, 2, 4, 8, and 24 hours after treatment.

obtained in weeks 3, 7, and 10 were only 25% of those obtained in week 1. In spite of the decrease in the LH response to the agonist, the serum T responses remained undiminished. Evaluation of electroejaculated semen showed no effects on sperm count or motility in any of the animals.

After it was demonstrated that biweekly treatment with [D-Trp^6]-LHRH did not inhibit testicular function, the same four animals were placed on a

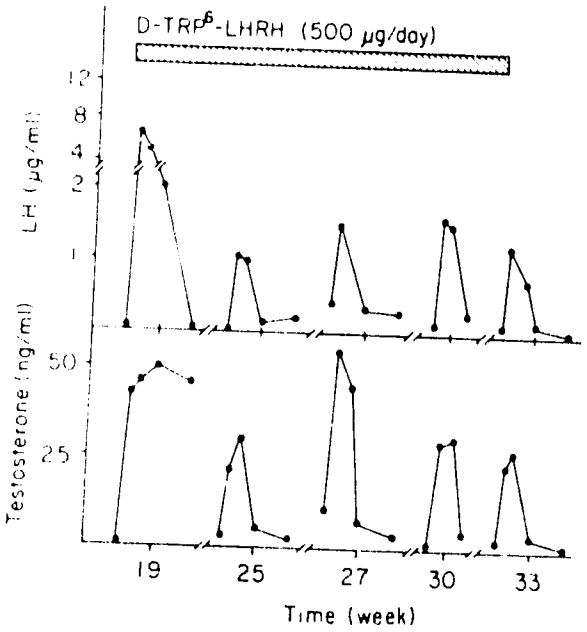

FIG. 24-11. Serum LH and testosterone responses to 500 μg [D-Trp^6]-LHRH at the beginning and at different times during daily treatment in monkey 312. The first week of daily treatment is week 19 after the beginning of twice-weekly treatment. The points represent values at 0, 2, 4, 8, and 24 hours after injection of analog.

daily regimen of [D-Trp^6]-LHRH (500 μg, subcutaneously) for a period of 3 months. Two types of response were obtained.

Two animals responded as shown in Figure 24-11. In these animals, the serum LH response to the first of the daily treatments (week 19 from the beginning of the study) was similar to that seen during the latter part of the biweekly treatment. Thereafter, LH response showed a further decrease during the daily treatment—the maximal increases being only 5% of the untreated control response. In spite of a 95% decrease in the LH response resulting from daily treatment with [D-Trp^6]-LHRH, there was only a slight diminution in T response

The other two animals responded as shown in Figure 24-12. In these monkeys, there were further decline of LH response and complete loss of T response. Thus, the small increases in serum LH produced by the agonist failed to increase serum T, which remained below 1.0 ng/ml.

Throughout the study, the animals were electroejaculated at 2-week intervals and the sperm count and motility determined. Treatment with [D-Trp^6]-LHRH twice weekly for 12 weeks had no effect on the sperm count or motility in any of the four animals studied. Daily treatment did not affect the sperm count or motility in the two animals that maintained an adequate testosterone response. In the other monkeys, there was a loss of ejaculatory response which coincided with a complete loss of serum T response to the analog treatment. In these latter animals, the first two ejaculates obtained 4 to 8 weeks after the cessation of treatment contained no spermatozoa. The next two ejaculates contained spermatozoa of decreased motility. Subsequent ejaculates contained normal numbers of sperm with good motility suggesting a reversibility of the antitesticular effects.

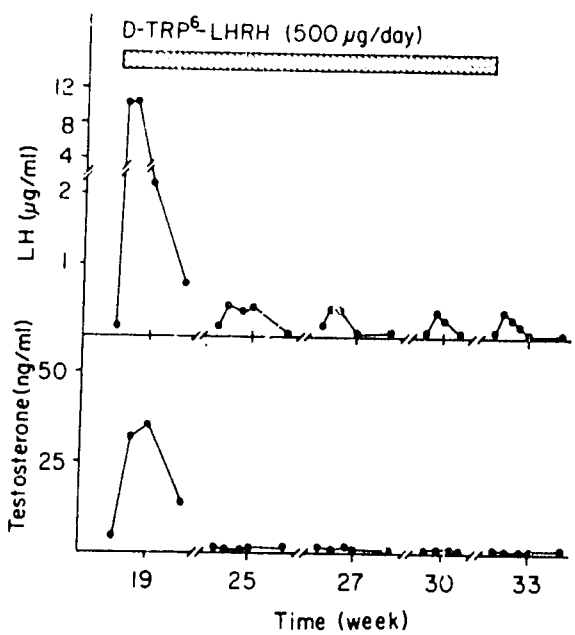

FIG. 24-12. Serum LH and testosterone responses in monkey 125. (See Fig. 24-11.)

The results show that twice weekly treatment of rhesus monkeys with [D-Trp^6]-LHRH led to desensitization of the pituitary but not of the testes. Daily treatment caused a drastic reduction in pituitary response in all animals. In two of the four animals, however, the testicular response to each administration of [D-Trp^6]-LHRH was adequate to provide serum T levels which were higher than levels seen in normal monkeys and, therefore, could be expected to maintain all androgen-dependent functions at normal levels.

Because these two animals continued to provide normal ejaculates, it must be assumed that the treatment had no antifertility effect even though it cannot be stated if there was an effect on libido or the fertilizing ability of the ejaculated spermatozoa. In contrast, the two other animals showed a complete loss of testosterone response associated with very low serum T levels. This decrease in serum T levels was followed by the failure of these two animals to respond to electroejaculation suggesting that libido was most probably lost. Whether spermatogenesis was affected in these animals is unclear. If it was, however, it might be a consequence of a severe androgen deprivation. Daily administration of 5 μg of [D-Ser(TBU)^6,des-Gly^{10}]-LHRH-EA (a relatively small dose) to men caused a decrease in basal T levels, but spermatogenesis and potency were unaffected.[6] Since, in these studies, the acute T responses after analog administration were not measured, it cannot be ascertained whether the lower basal T levels represent actual decrease in daily androgen supply.

Species variations in sensitivity to antifertility effects of LHRH analogs may be due to differences in the gonadal sensitivity to the gonadotropin-mediated effects on the testes or to differences in the sensitivity of the testes to the direct antigonadal effects of these peptides. The studies of rats and mice suggest that there may be differences among the species in their sensitivity to the direct antitesticular effects of LHRH agonists. The studies of rats suggest marked differences in the sensitivity of the same species to different analogs, and that a potency estimate based on LH-releasing activity may not reflect their relative antifertility activity.

SUMMARY

Superactive analogs of LHRH have antifertility effects in males. Though the mechanism of the antifertility action is not well understood, multiple sites of action are possible (Fig. 24-1). These include inhibitory effects on the pituitary, testes, and reproductive tract. Upon repeated administration, the analogs cause desensitization of the pituitary and a decrease in gonadotropin secretion. At the testis, the agonists produce a loss of gonadotropin receptors and an impairment of steroidogenic activity. These latter actions may in part be mediated via the pituitary and in part through a direct action of the agonist. LHRH and its agonists also possess antiandrogenic activities that do not appear to be mediated by the androgen receptors. The relative importance of each of these mechanisms of action remains to be elucidated.

In addition to the effects of LHRH analogs just summarized, the results presented in this chapter suggest that there are species differences in the

sensitivity to the direct antigonadal effects of LHRH agonists and that some of the potent LHRH agonists may be much more effective as antifertility agents than others.

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Physiology and Antifertility Effects of LHRH and Agonistic Analogs in Male Animals

BRIAN H. VICKERY

The history of extraction, identification, sequencing, and synthesis of LHRH has recently been described.[15,36] The physiologic function of LHRH was confirmed and extended following establishment of radioimmunoassays[1] that identified its presence in hypophysioportal blood.[7] The obligatory role of LHRH in gonadotropin release and synthesis and in the reproductive axis was amply demonstrated, during the generation of antibodies, by the associated testicular atrophy.[1] Claims have been made for detectable levels of LHRH in peripheral blood.[23]

Many groups have attempted synthesis of substituted analogs of LHRH in efforts to derive more potent agonists. Thus, substitution of the C-terminal glycinamide with an alkylamide, particularly ethylamide ([Pro-NHEt^9]-LHRH; LHRH ethylamide) leads to analogs with a three- to fivefold increase in agonist potency.[13,14] Independently, substitution of the sixth amino acid of LHRH (glycine) by D-amino acids also led to increased agonist potency.[22] Coupling of these two modifications may or may not result in multiplicative increases, depending upon the lipophilicity and/or bulk of the amino acid in position 6.[38,41] This is particularly noticeable for ([6-D-(2-naphthyl)-alanine])-LHRH, ([D-Nal(2)]^6-LHRH), which has *in vivo* approximately 200× the potency of LHRH.[24] The corresponding ethylamide is actually less potent.

Beginning in 1975, a series of studies began to develop data on actions of LHRH and its superactive analogs which appeared contrary to its physiologic function. Independently in males[25] and in females,[18,29] paradoxical antisteroidogenic and antireproductive effects were observed. Testicular atrophy was then claimed for male rats, golden hamsters, and dogs treated with LHRH analogs,[32,33] however, more probably delay of puberty rather than gonadal atrophy was the case, as originally shown by Oshima and co-workers.[25] More recently, studies in mature animals and man have shown some commonality but also some puzzling differences. This present chapter reviews these studies

I acknowledge the collaboration with Ms. Georgia McRae in these studies. Thanks are due to Patricia Kirchgatter, Christa Tsai, Anne Worden, Richard Seidenberg, Karen Bergstrom, and Susan Rinne for excellent technical assistance. I am grateful to Agnes Bajka and William Briones and to the laboratories of Drs. Swerdloff and Heber for radioimmunoassays. Some of these studies were supported in part by NIH contract (Nol-HD-0-2811) to Syntex Research.

in comparison with the observed and anticipated effects in men so that the relative merits of the different animal species as models for men may be evaluated.

CHOICE OF MODEL SPECIES

In the first report of paradoxical effects of LHRH in male animals, 0.5 μg per day administered to prepubertal male rats delayed the maturation of the accessory sexual organs and significantly increased testicular Δ^4-5α-hydrogenase activity, *ex vivo*.[25] Since that time, studies in mature male rats,[6,9,10,17,19,28,30,31] dogs,[42,43] baboons,[44] and men[8,20] have demonstrated reduction in circulating levels of testosterone. Attempts to reproduce this effect in mice,[43] rhesus,[43,47] and cynomolgus monkeys* have not been successful. Inhibition of spermatogenesis has been noted in rats[19,28,30] and dogs.[42,43] It appears not to have been investigated in baboons, and has not been achievable in rhesus[44] or cynomolgus monkeys.*

In men the results are equivocal. An early study using 5 μg per day of [D-Ser(TBU^t)^6]-LHRH ethylamide reportedly showed no effect on ejaculated sperm count. More recently, 50 μg per day of [D-Trp^6]-LHRH ethylamide induced oligospermia within 5 weeks of treatment.†

It therefore appears that the choice of laboratory models to demonstrate interference with male reproductive parameters lies between the rat and dog. A large body of literature on the effects of LHRH and its analogs has been developed with the rat as the subject, including the majority of studies into the mechanism of action in the male.[19,20] However, there is some question as to the applicability of this species. Of all species reported, the mature rat is unique in that a variable percentage of animals treated with even a single dose of LHRH agonists responds with an irreversible testicular infarct (Fig. 25-1). The lesion appears to be vascularly mediated but the incidence and extent of the fibrotic, calcified lesion does not vary over a wide range of doses. Spermatogenesis can be reversibly suppressed in rats, but the effect is always focal, with aspermatogenic seminiferous tubules existing side by side with euspermatogenic tubules. Inhibition of spermatogenesis in all tubules has not been reported.

Again, suppression of circulating levels of testosterone during chronic LHRH agonist treatment rarely exceeds 70% (Fig. 25-2). These results are thus consistent with the fact that even after 12 weeks of continuous daily treatment, some of the treated rats will still successfully impregnate female rats (Fig. 25-3). Although LHRH treatment of castrated rats routinely lowers circulating gonadotropin levels,[15] this does not seem to be the case in intact rats.[40] Chronic LHRH agonist treatment of normal or hypogonadotropic men routinely results in lowered circulating gonadotropin levels.[11,45] Thus the fact that testosterone replacement therapy does not reverse the antispermatogenic

*Vickery B, McRae G: Unpublished data.
†Rabin D: Personal communication, 1980.

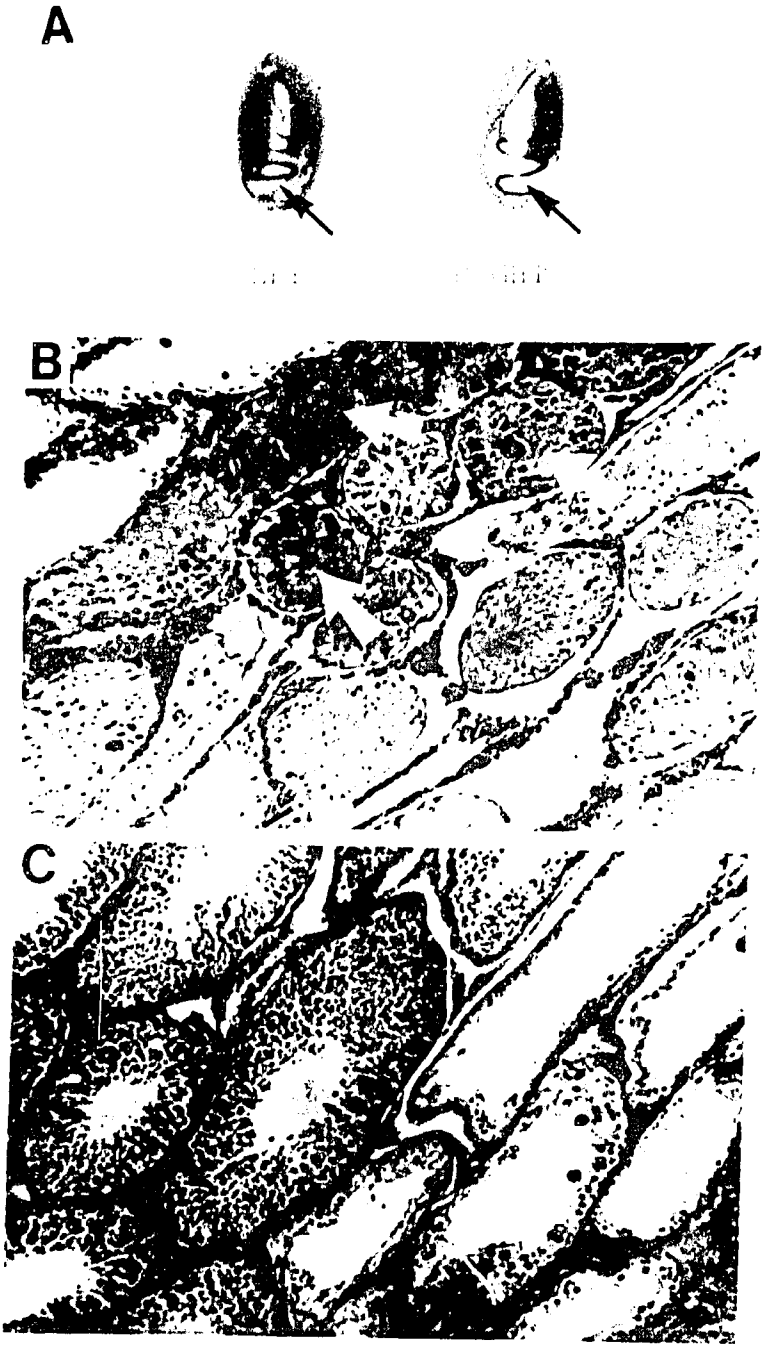


FIG. 25-1. Effect of 14 days' administration of [D-Nal(2)^6]-LHRH to mature male rats upon macroscopic and microscopic appearance of testis. (*A*) View of testes. Arrows indicate testicular infarct. (*B*) Testicular section in the plane of the infarct. Arrows indicate necrotic, calcified seminiferous tubules. (*C*) Testicular section showing coexistence of aspermatogenic and euspermatogenic tubules.

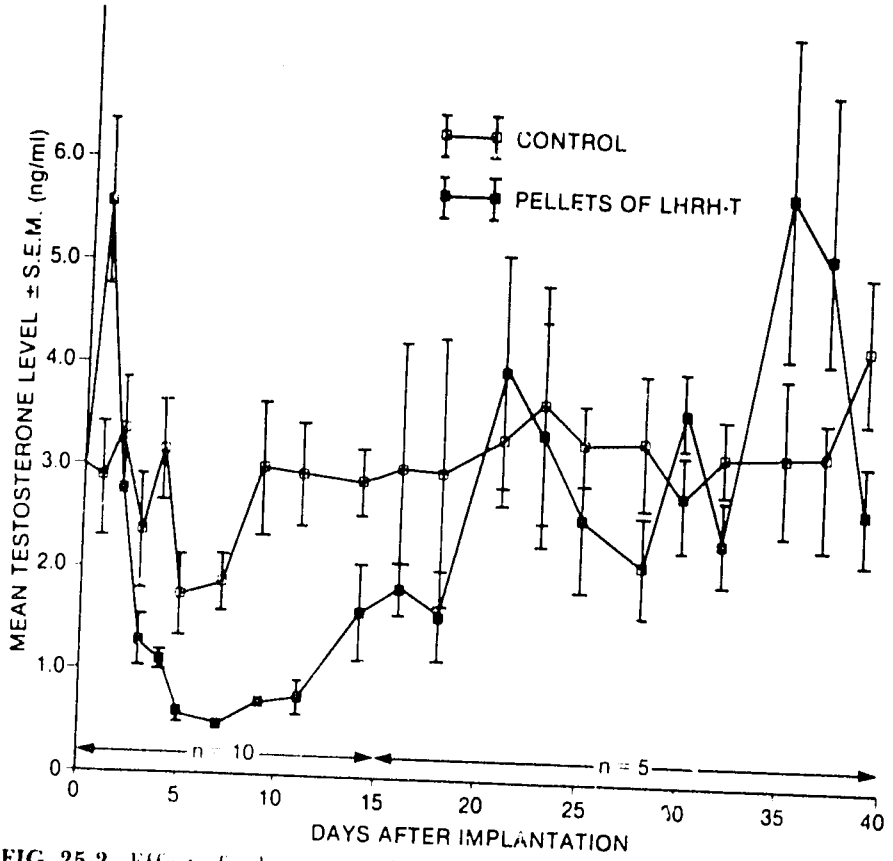


FIG. 25-2. Effect of subcutaneous implantation of pelleted [D-Nal(2)^6]-LHRH (LHRH-T) (125 μg/rat) on circulating plasma testosterone levels in mature male rats.

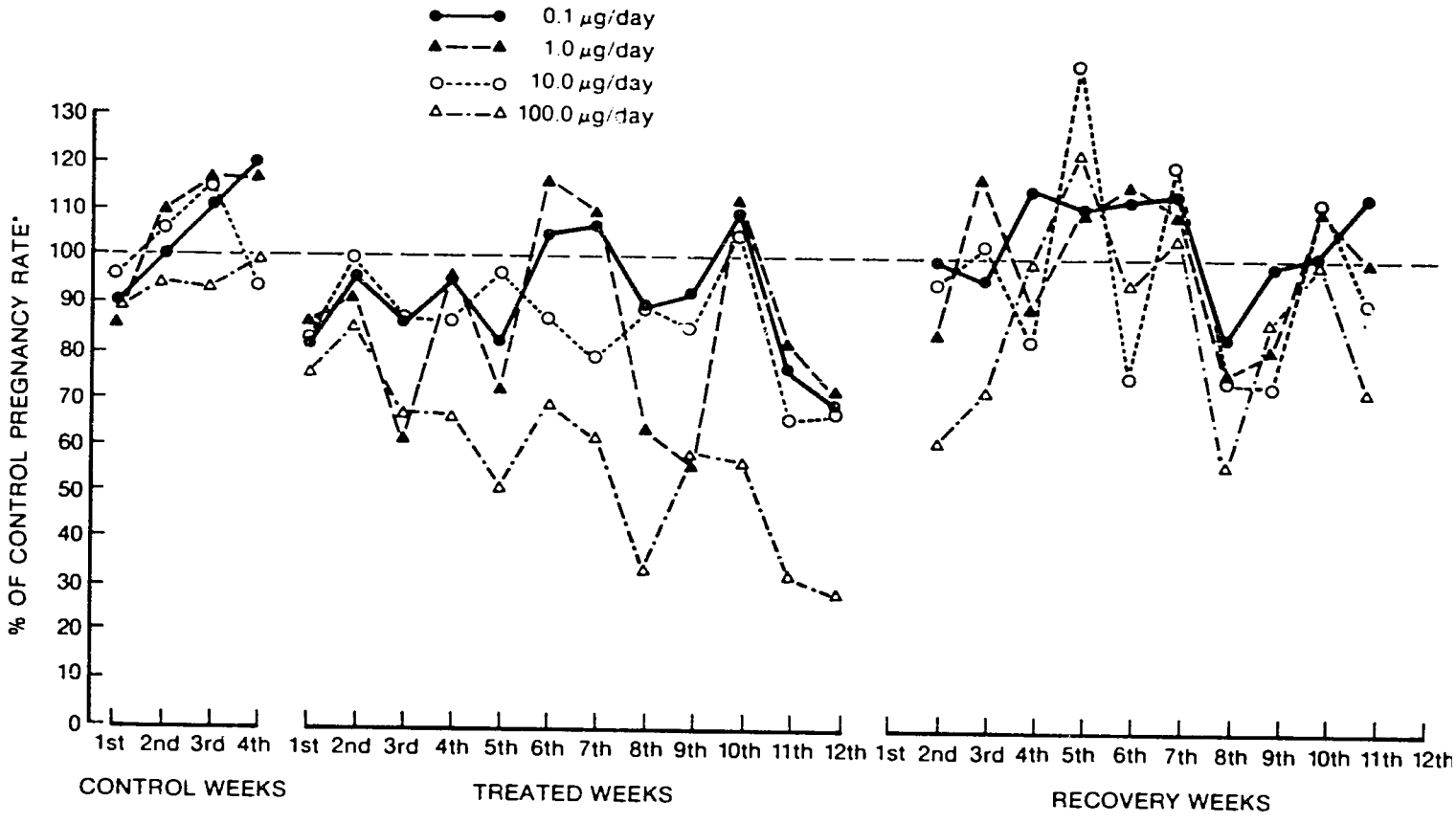
effects of these agents in rats,[30] and in fact may even be synergistic,* may not be relevant to humans.

Strong correlation of LHRH analog effects has been found for the limited parameters presently evaluated in both men and dogs. Because dogs do not possess seminal vesicles, effects on some semen constituents (such as fructose) cannot be evaluated; however, the ease of obtaining ejaculates has influenced our choice of the dog as a model species.

EFFECTS OF LHRH AGONISTS IN MALE DOGS

In the dog, chronic administration of LHRH agonists has rapid effects on circulating testosterone levels.[42,43] Subcutaneous implantation of pelleted agonist ([D-Trp^6]-LHRH ethylamide) elevates levels of testosterone for about a week, after which they fall to about 5% of normal (Fig. 25-4). Daily injection

*Heber D, Swerdloff RS: Personal communication, 1980.



$$* \frac{\text{\% Pregnancy of all females in treated group}}{\text{\% Pregnancy of all females in control group}} \times 100$$

FIG. 25-3. Effect of 12 weeks of continuous daily subcutaneous injection of [D-Nal(2)[6]]-LHRH on fertility of mature male rats, assessed by pregnancy rates in females housed with the males and changed weekly. (Four females were housed with each male and there were six males in each treatment group, making a total of 24 females in each group for each time period.)

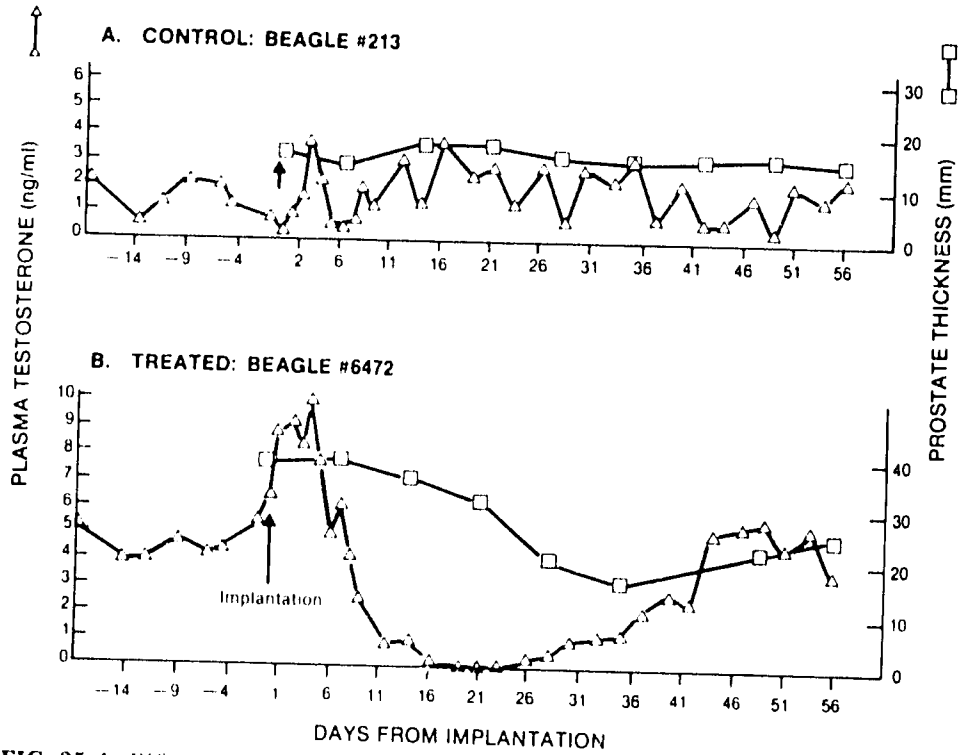


FIG. 25-4. Effect of subcutaneous implantation of 125 μg/kg [D-Trp^6]-LHRH ethylamide on circulating plasma levels of testosterone and on prostate size in geriatric male beagles. The prostate was visualized by pneumocystography. (Vickery B, McRae G, Bergstrom K et al: Suppression of sexual function in male dogs with a new agonistic analogue of LHRH: Potential for male contraception without need for androgen replacement? J Androl 2: 30, 1981

of 10 μg/kg of [D-Nal(2)^6]-LHRH, on the other hand, causes only transient elevation in testosterone levels, a response that declines day by day so that after 9 to 16 days no elevation is obtained (Fig. 25-5). As early as 24 hours after the first injection, basal, prechallenge, serum testosterone can be down to a fraction of normal levels (Fig. 25-6). This withdrawal of testosterone leads to decline in prostate volume and secretory function resulting in progressive diminution in ejaculate volume. Spermatozoa obtained at ejaculation after 2 weeks or more of treatment have poor to nonexistent motility, show visible cytoplasmic droplets on the tail or midpiece, and are accompanied by progressively increasing numbers of exfoliated germinal cells. Spermatozoa continue to be found through 4 weeks in the ejaculates of treated dogs, after which ejaculation is not achieved although erection is still observed.

Inhibition of spermatogenesis is rapid.[42] By 10 days of treatment, partial suppression of spermatogenesis is obvious (Fig. 25-7), as is some breakdown of the cytoarchitecture of the seminiferous tubules. The effect on the seminiferous tubules may be mediated in part through the Sertoli cells. This is reflected also by the presence and passage of early spermatids in the tubules

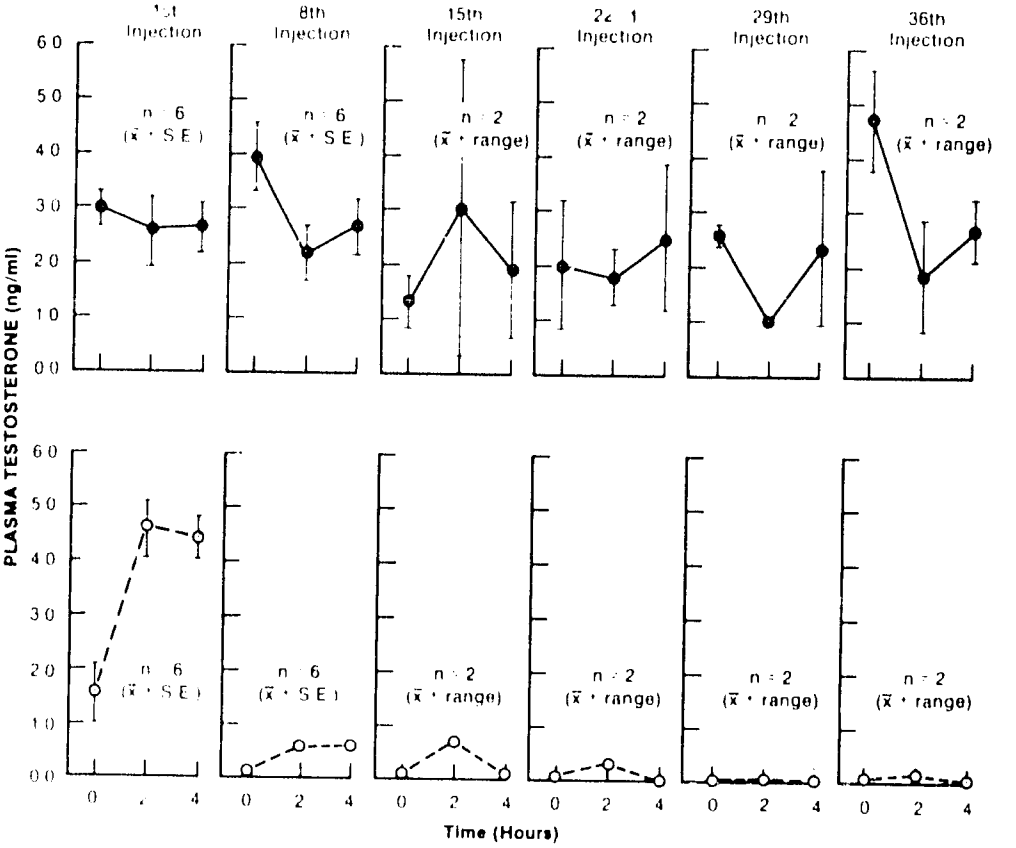


FIG. 25-5. Acute responses in plasma testosterone to daily subcutaneous injections of 10 μg/kg [D-Nal(2)^6]-LHRH in mature male beagles. (*Closed circles* = controls; *open circles* = treated animals.)

of the epididymis. (Exfoliation of germ cells appears to be a consequence of inhibition of spermatogenesis[21,26,27] rather than uniquely associated with these peptides.) By 38 days of treatment, the seminiferous tubules contain only Sertoli cells and a few germinal elements.

Early studies on reversibility investigated a 2-week course of treatment (Fig. 25-8). Basal testosterone levels had returned to normal by 4 days and ejaculate volume increased by 10 days after cessation of treatment. Sperm count continued to decline, reaching a nadir of 2% of pretreatment levels 24 days after last injection. Sperm count was the last parameter to return to pretreatment levels with a lag time of 45 days from last injection. The differences between the times of return to normal of plasma testosterone levels and ejaculate volume on the one hand, and of sperm count on the other, suggests that intermittent administration of LHRH agonists (perhaps one month out of three) might lead to male contraception without the need for androgen replacement.[42]

Recent studies in men suggest that unacceptable side-effects of testosterone withdrawal as a result of LHRH agonist treatment (*e.g.*, loss of libido and

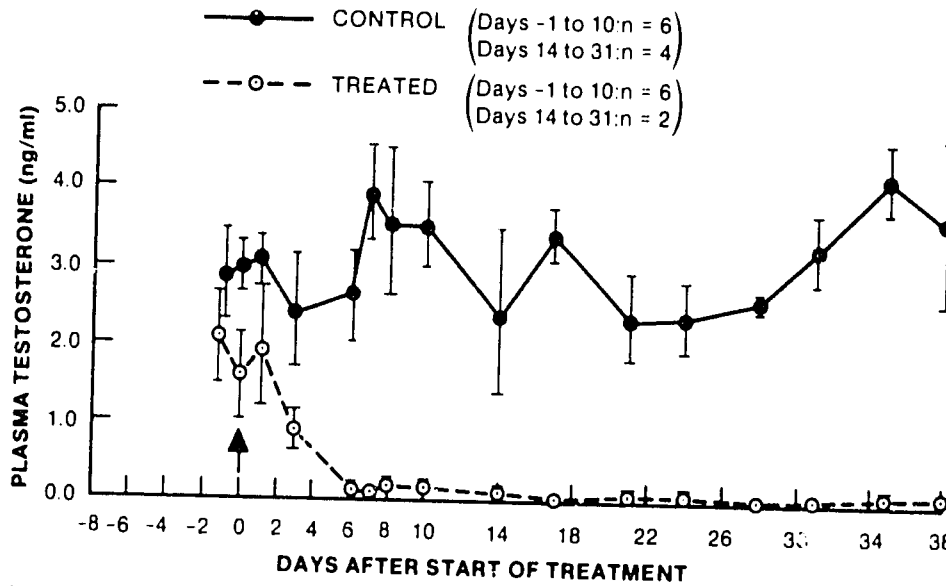


FIG. 25-6. Plasma testosterone in mature male beagles receiving daily subcutaneous injections of 10 μg/kg/day [D-Nal(2)^6]-LHRH. Blood samples were taken immediately prior to agonist injection.

potency, and appearance of hot flashes) are manifested very rapidly.* Accordingly, studies are presently under way to evaluate the effect of testosterone replacement upon the antispermatogenic effects of [D-Nal(2)^6]-LHRH.

EFFECTS OF TESTOSTERONE REPLACEMENT

In these studies, the testosterone was administered from subcutaneously implanted Silastic tubing, 20 cm in length, previously shown to maintain adequate levels of testosterone in the castrate beagle.† Concurrent treatment with 10 μg/kg/day of [D-Nal(2)^6]-LHRH and testosterone-filled Silastic tubes resulted in peripheral plasma testosterone levels of 1.25 to 1.5 ng/ml (Fig. 25-9). Those values are at the low end of the normal range but significantly above the levels in animals receiving [D-Nal(2)^6]-LHRH alone. Whereas values were higher each day before injection of agonist, the acute response measured at 2 and 4 hours after each injection suggests that the evolution of down-regulation is unchanged (Fig. 25-10). Analysis of the data produced at weekly semen collection from these dogs revealed that testosterone replacement restored potency (Fig. 25-11). Motility of sperm was maintained at a high level and no increased incidence of sperm with cytoplasmic droplets was detected, in marked contrast to the situation in the dogs treated with [D-

*Rabin D: Personal communication, 1980.
†Dunbar I: Personal communication, 1981.

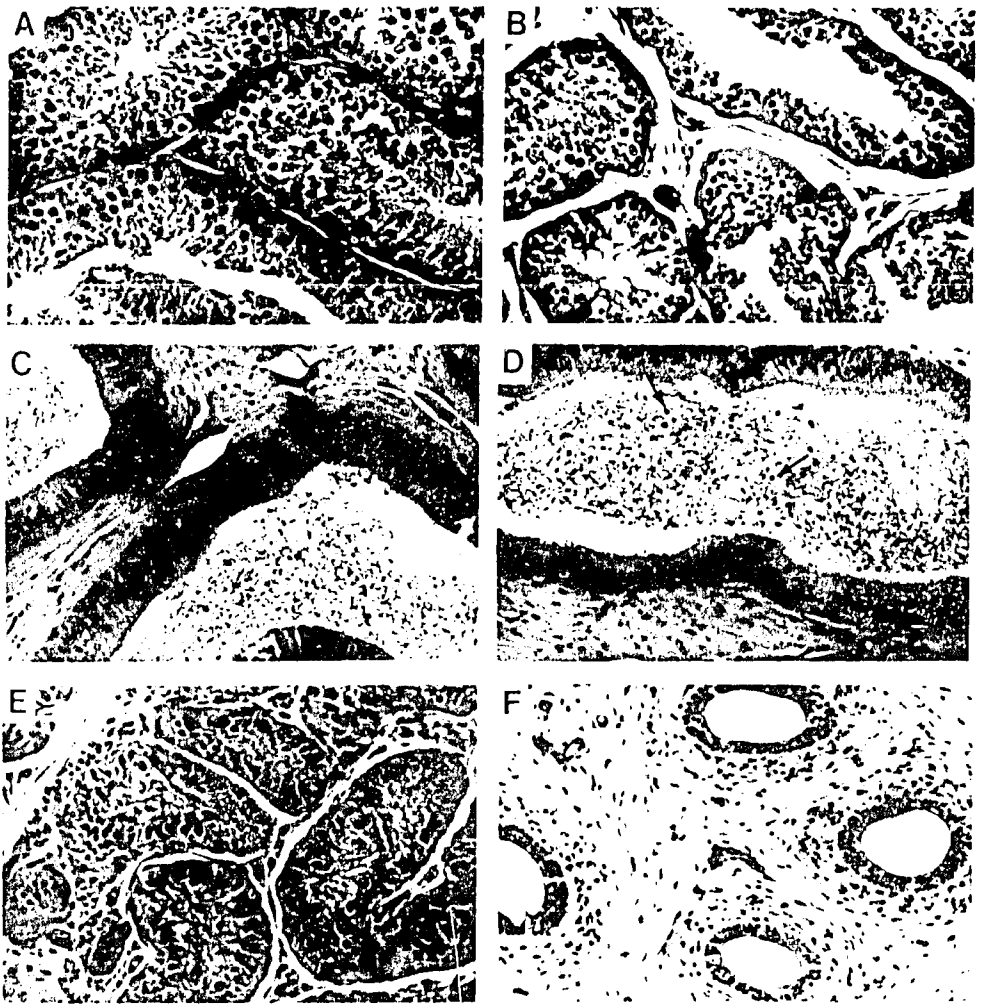


FIG. 25-7. Effect of daily subcutaneous injections of 10 μg/kg/day of [D-Nal(2')]-LHRH on spermatogenesis in mature male beagles. (*A*) Section of testis from control animal. (*B*) Section of testis from animal treated 10 days. (*C*) Section of epididymis from control animal. (*D*) Section of epididymis from animal treated 10 days. Arrows indicate spermatids. (*E*) Section of testis from animal treated 38 days. (*F*) Section of caput epididymis from animal treated 38 days. Note absence of sperm in epididymal lumen.

Nal(2')]-LHRH alone. Ejaculate volume in the dogs receiving combined treatment stayed in the normal range for the first 3 weeks of treatment but then fell to approximately 20% of normal. No ejaculate could be obtained from one of these animals in the sixth week of treatment. Sperm count declined, apparently at a somewhat slower rate than in ejaculates from animals receiving agonist alone. However, this could be an artifact resulting from the flushing effects of maintained ejaculate volume.

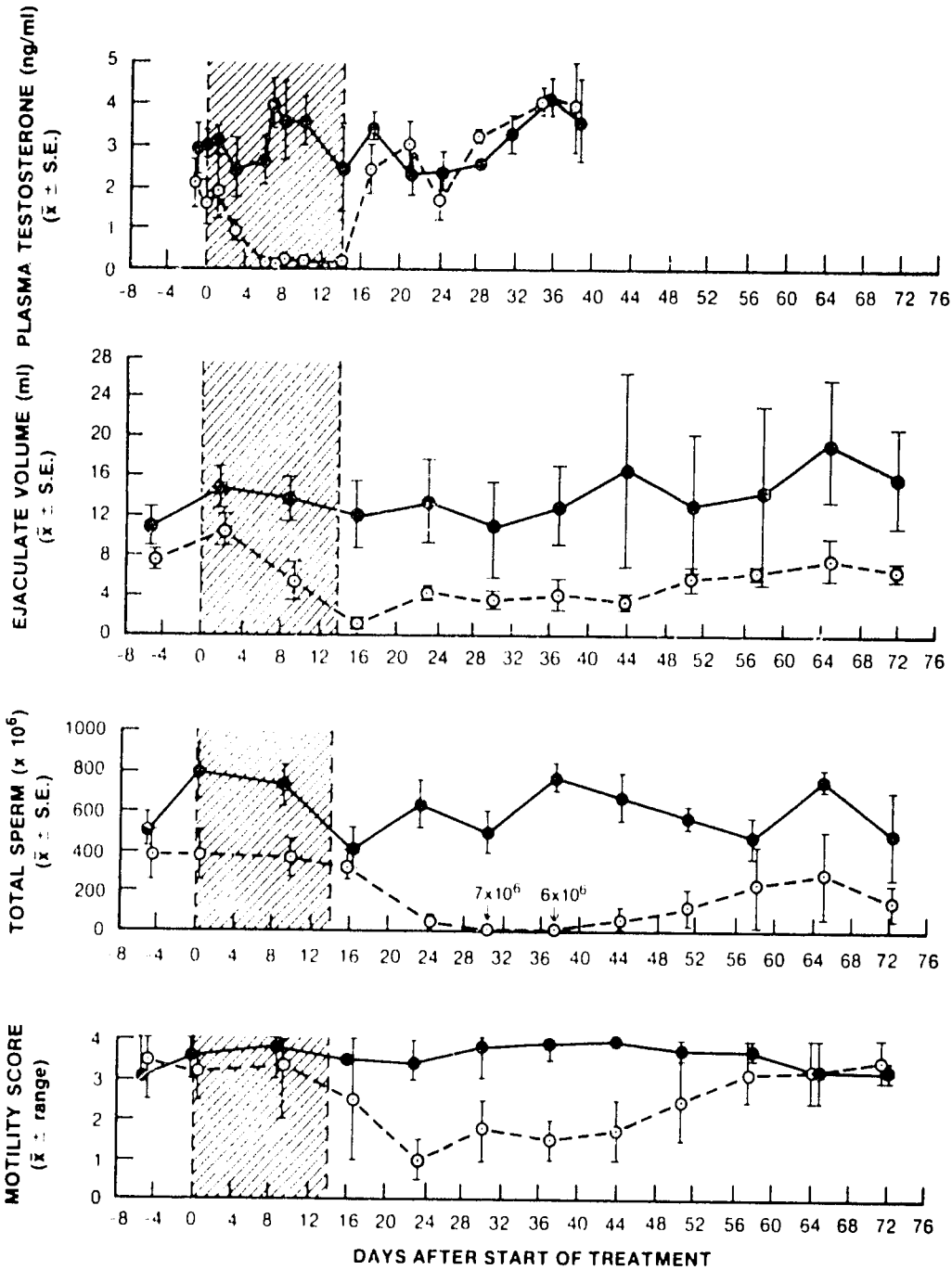


FIG. 25-8. Effect of 13 daily doses of 10 μg/kg [D-Nal(2)⁶]-LHRH on basal plasma testosterone, ejaculate volume, sperm count, and sperm motility in mature male beagle dogs. (*Closed circles* = controls; *open circles* = treated animals.)

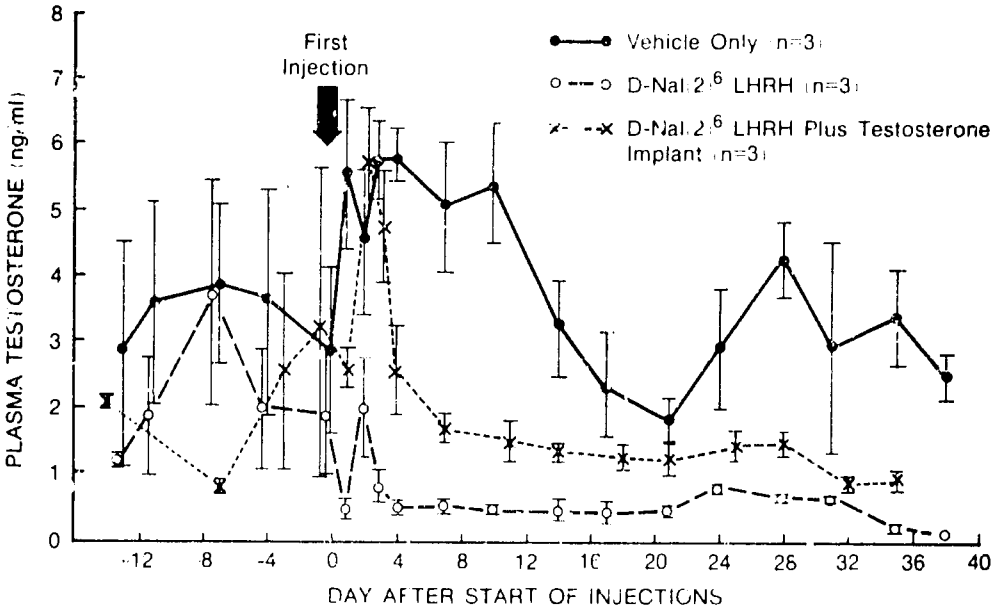


FIG. 25-9. Effect of subcutaneous implantation of testosterone-filled Silastic tubes on plasma testosterone in male beagles receiving daily subcutaneous injection of 10 μg/kg [D-Nal(2)^6]-LHRH.

The animals receiving combined treatment were sacrificed after 6 weeks of treatment. The weights of the reproductive organs demonstrated that testosterone replacement at this level did not counteract the effect of [D-Nal(2)^6]-LHRH on testicular or epididymal weight (Table 25-1). In addition, and in keeping with effects on ejaculate volume, prostate weight was not significantly increased. Pituitary weight was not changed from that in untreated dogs.

The discrepancy between the effects of replaced testosterone on sperm motility and maturity (which would indicate restoration of epididymal function and the limited or absent effect on prostatic size and secretory function) is puzzling. The discrepancy may reflect differing requirements of epididymis and prostate and may disappear with greater testosterone supplementation. Another possibility is that [D-Nal(2)^6]-LHRH exerts a direct effect at the level of the prostate, as has been suggested for other LHRH agonists in the rat by Bardin and co-workers.[37,38] Of course, direct effects of LHRH agonists have also been documented in rat testis, mediated through heterologous receptors;[2,3,16] an LHRH-like material has even been extracted from rat testis.[36]

As stated, the relevance of these rat studies to the dog is presently unknown. Preliminary data produced in the author's laboratory would indicate that the dog testis will still respond to exogenous gonadotropic challenge with steroid production while under the suppressive influence of LHRH agonists. However, in the absence of dose response titrations with gonadotropins, direct testicular effects cannot be ruled out.

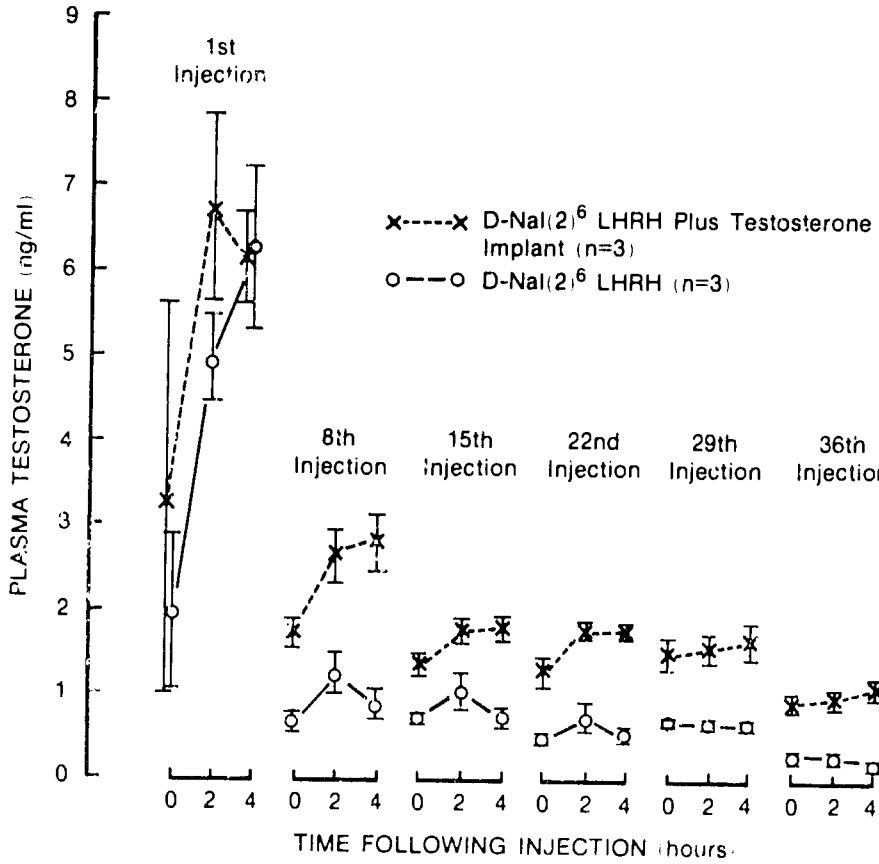


FIG. 25-10. Effect of subcutaneous implantation of testosterone-filled Silastic tubes on acute responses in plasma testosterone to daily injection of 10 μg/kg [D-Nal(2)^6]-LHRH in mature male beagles.

POTENTIAL USE OF LHRH AGONISTS AS MALE CONTRACEPTIVE AGENTS

In contrast to effects in females, there appear to be marked species differences in the response to LHRH agonists in males. One extreme is represented by macaques, which have traditionally been the accepted prognosticator of drug effects in humans, but which in this case are very resistant to the spermatogenesis-suppressive effects of LHRH agonists. Rats occupy a midground in that some interference with spermatogenesis is detectable at low doses for short periods of time. Even heroic dose levels for periods of 3 months, however, have not been able to abolish fertility. The dog responds with extreme oligospermia (1% of normal). Early reports of work in humans indicate that this is also the case, making the dog the experimental model of choice.

A number of questions remain before the contraceptive potential of LHRH agonists can be assessed properly. Some of these questions may be answered in studies using dogs, such as whether longer treatment periods will result in

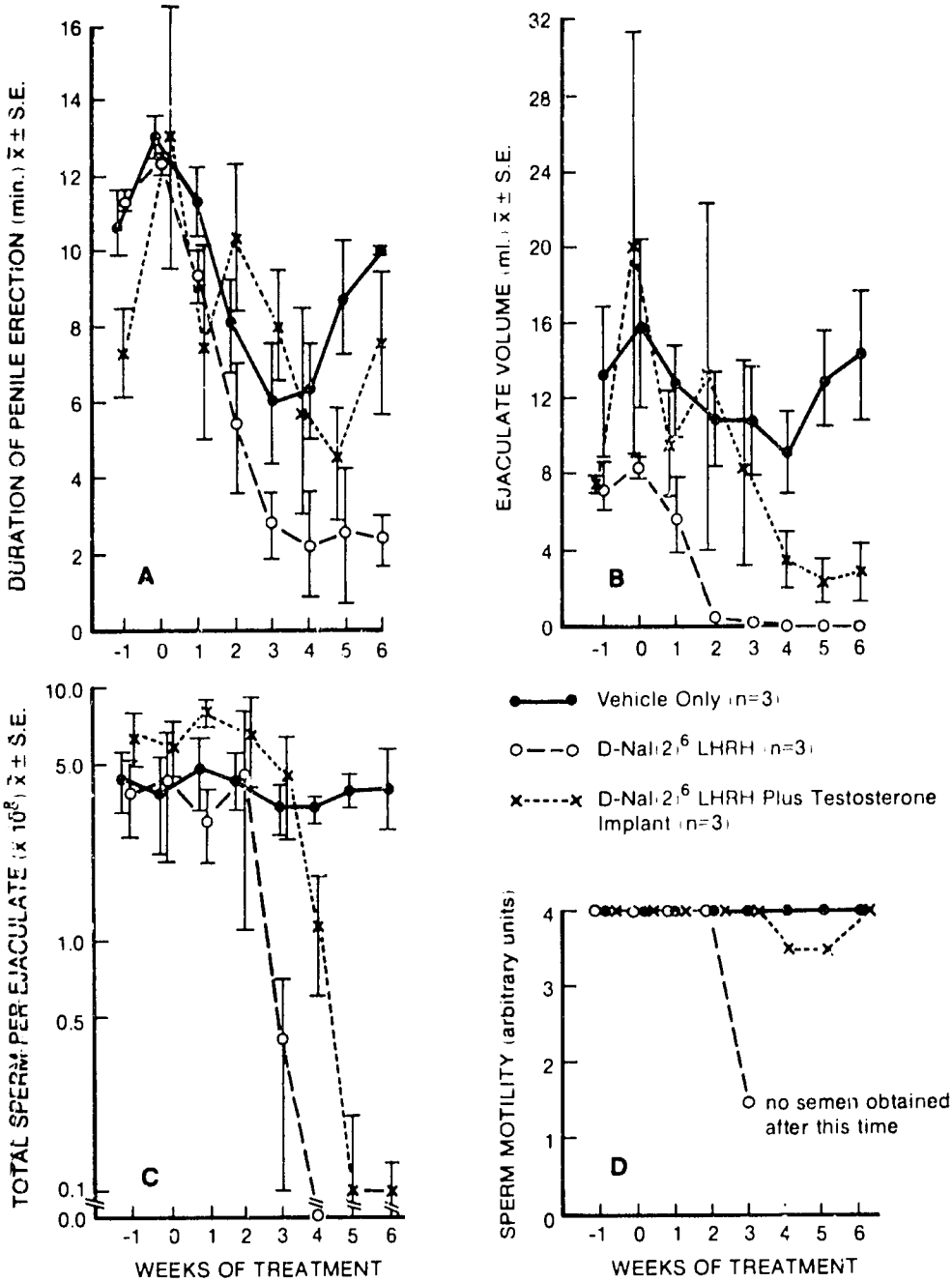


FIG. 25-11. Ejaculatory parameters at weekly intervals in male beagles with and without testosterone supplementation while under concurrent treatment with daily subcutaneous injection of 10 μg/kg [D-Nal(2)^6]-LHRH. (*A*) Duration of erection. (*B*) Total ejaculate volume. (*C*) Total sperm count. (*D*) Motility score for the sperm assessed 5 minutes after collection.

TABLE 25-1. Effect of 38 Days' Treatment with Either 10 μg kg/day [D-Nal(2)^6]-LHRH Alone or Combined Treatment with Testosterone Administered from a Subcutaneously Implanted Silastic Tube

Group	No. of Animals	Testes (g)	Epididymides (g)	Prostate (g)	Pituitary (mg)
Untreated	3	14.1 ± 0.98	3.9 ± 0.25	10.6 ± 1.97	70.3 ± 4.0
Testosterone plus [D-Nal(2)^6]-LHRH	3	6.3 ± 0.85	2.5 ± 0.24	4.7 ± 1.07	67.0 ± 6.7
[D-Nal(2)^6]-LHRH alone	2	6.9 ± 0.22	2.4 ± 0.10	3.0 ± 0.61	

azoospermia; however, the infrequency of estrus in female dogs makes it unlikely that fertility per se will ever be addressed in the dog. If azoospermia is achieved, this may not be a problem; if oligospermia, no matter how severe, persists, then fertility assessment becomes pivotal. Of course, the oligospermia already noted in the early phases of treatment and, probably, in the recovery phases also requires assessment. Although oligospermia is often associated with infertility in men, there may be multiple factors involved. Recent studies following treatment of hypogonadotropic individuals suggest that sperm counts of $< 10 \times 10^6$ are compatible with fertility.[7]

The association of poor to nonexistent motility and immaturity of sperm with the oligospermia in dogs receiving daily [D-Nal(2)^6]-LHRH would make for a more favorable prognosis. However, it appears likely that the effects of testosterone withdrawal will be unacceptable and some testosterone supplementation will be required. Early data suggest that this supplementation will restore epididymal function resulting in mature, motile sperm even in severe oligospermia. Another question to be answered asks: what are the advantages of combination treatment with LHRH agonists plus testosterone over progestagen/testosterone, danazol/testosterone, or even testosterone treatment regimens?

The present experimental status with LHRH agonists is assessment of feasibility. Should LHRH prove to be an effective male contraceptive agent, major investigations will still remain. Mutagenicity, teratogenicity, and reversibility of long-term treatment will occupy the resources of investigators for some time to come.

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Discussion: Physiology and Antifertility Effects of LHRH and Analogs in Male Animals

D. J. PATANELLI, MODERATOR

LHRH and various analogs are being proposed as agents to regulate fertility, not only in females, but in males also. It is interesting that clinical studies with these agents have actually preceded animal studies. Yet the observed responses from animal studies have shown wide species variability and complexity. The mechanisms through which LHRH and agonists alter fertility are unclear. They appear to have numerous sites of action contributing to their antifertility effects, although the extent to which each site is involved in the overall effect remains to be determined.

Several mechanisms have been identified for which there is supporting evidence:

1. Loss of pituitary receptors (desensitization) leading to impaired gonadotropin release.
2. Loss of testicular gonadotropin receptors (down-regulation) leading to decreased steroidogenesis.
3. Inhibition of testicular steroidogenesis by direct effects on the gonads.
4. Inhibition of androgen action on the male reproductive tract.

Because of the great species variability, we are left in a quandary as to which animal to use in predicting or extrapolating effects in humans. Nominal to severe testicular atrophy has been reported in the rat, the animal model in which most of the studies have been undertaken. More consistent suppression of spermatogenesis appears to be achievable in the dog; however, this is a more difficult and expensive laboratory animal to work with. Rhesus and cynomolgus monkeys have demonstrated remarkable resistance to the effects of LHRH on spermatogenesis, although in rhesus monkeys, there is some evidence that this may be a reflection of dose levels; that is, doses higher than 500 μg may be required to exert an effect. Recent investigations in human males have revealed that short-term treatment will induce a significant lowering of sperm counts, thus indicating a greater sensitivity to these agents

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Panelists: Fernand Labrie, Kalyan S. Sundaram, Brian H. Vickery. *Discussants:* Thomas M. Badger, Theodor Braun, Alan Corbin, Elsimar Metzker Coutinho, William F. Crowley, Horacio B. Croxatto, Roberto Rivero Damm, Kenneth T. Kirtin, Kamran S. Moghissi, Catherine Rivier, Jurgen K. Sandow, Emil Steinberger, Gerald I. Zatuchni

than shown by other primates. It is interesting to note that histologic sections of the seminiferous epithelium of rat testes reveal tubules containing an essentially normal complement of germinal elements, alongside tubules containing Sertoli cells and spermatogonia only. At present, there appears to be no explanation for this observed focal effect. Whether or not spermatogenesis is similarly focally interrupted in human males has not been determined.

Of concern is the report of calcium deposition in seminiferous tubules of rat testes treated with LHRH and its analogs. The deposition may be of sufficient magnitude to break a standard microtome knife. These calcium crystals have been observed within a few days of a single administration of a few nanograms of compound. This phenomenon has not been reported in other species and may be a characteristic of the rat only, independent of the nature of the agonist. However, there appears to be some question of its reversibility. One investigator claimed complete disappearance of these concretions by 10 weeks following cessation of treatment. In contrast, a pathology report has stated that despite the animals' recovery to a fertile state, histologic sections of the testes still demonstrated tubules with *permanent* calcifications. Apparently, these crystals have been observed in testes of rats treated with hCG as well. Although this calcification is a more transient event with hCG, permanently calcified tubules in the testes have also been noted. In evaluations of children being treated with LHRH analog for precocious puberty, ultrasound studies of testes in the male and ovaries in the females have not detected any abnormal concretions. Nonetheless, it is a totally inexplicable phenomenon, and since the effects of long-term treatment in the human are still unknown, its occurrence must be carefully watched for.

Diverse findings have also been reported on testosterone levels in animals treated with LHRH and its analogs. Reduction of circulating levels of testosterone in the rat is slow and rarely exceeds 70%, even after many weeks of treatment. Similarly, only slight diminutions of serum testosterone levels have been reported in rhesus and cynomologus monkeys and in mice. Conversely, very rapid effects are observed in the dog, baboon, and human male. Surprisingly, even with considerable testosterone withdrawal, erections have been achieved in dogs, as have matings in rats. The latter may be explained by the fact that in the rat, with the decrease in testicular androstenedione and testosterone, there is a corresponding increase in the production of DHT, thus providing sufficient androgenic stimuli to allow mating to continue. In the human male, however, libido and potentia are severely affected, indicating that androgen replacement therapy will be required if LHRH agonists are to prove feasible as contraceptive agents.

Although gonadotropin levels are lowered in the castrate male rat with LHRH administration, levels in the intact male rat are either normal or only slightly diminished. This may indicate that in the rat, the major mechanism is a direct effect at the testicular level. Gonadotropins are decreased in treated dogs, as well as in mice, but the mechanism of action appears to be a downregulation at the level of the pituitary. This fact is supported by the observation that decreased gonadal steroidogenesis under LHRH treatment can be reversed or prevented with an exogenous gonadotropic challenge. It has been reported that in normal men, as well as in hypogonadal men, serum LH and FSH levels are routinely lowered to about 50% of basal mean levels.

There is some evidence to suggest that pituitary desensitization may be operating here also.

The antisteroidal activity of LHRH and its analogs has been noted in both male and female rats. Evidence for a direct extrapituitary effect of LHRH agonist on the prostate in rats seems clear. In the dog, on the other hand, such activity is not clearly defined. Nonetheless, dogs with enlarged prostates responded dramatically to treatment with LHRH agonists. Prostate size had regressed to an immature state and could no longer be visualized by pneumocystography. Whether the observed effect is due to competition with androgen receptors in the prostate or to impaired steroidogenesis as a result of down-regulation of the pituitary—or both—is still open to question at this time.

Although there appears to be a commonality of effects in the various animals, the total picture is complicated by the fact that the degree and extent of observed effects of LHRH, as well as the return to viable reproductive status, appear to be a function of the dose, duration of treatment, administration modes, and the particular agonist. Recent data on humans suggest that the dog and perhaps the baboon may be more pertinent models. It may well be that no one animal can be used as a prototype for the human male, and that the observed responses in a number of species may be necessary to provide an understanding of the effects in the human. Clearly, more animal studies will be required before extensive clinical trials can be performed. Thus, it is useful to evaluate studies that have already been performed in animals, look at what still needs to be examined, and consider how these studies correlate with known responses in the human.

STUDIES OF LHRH ANALOGS IN THE HUMAN MALE

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Experience with a Potent Gonadotropin Releasing Hormone Agonist in Normal Men: An Approach to the Development of a Male Contraceptive

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Development of a reliable form of reversible male contraception that does not interfere with sexual function has been less than successful. A variety of research efforts have concentrated on the development of agents that either inhibit spermatogenesis or interfere with the normal pituitary–gonadal axis. Of considerable interest has been the Chinese report of the use of gossypol, an extract of cottonseed, as a reversible means of promoting male infertility.[18] Although toxic effects have been observed, the compound appears to warrant further study.

The isolation of LHRH by Guillemin[4] and Schally[22] was followed by the development of a variety of LHRH analogs that displayed powerful agonistic effects on pituitary release of LH when tested *in vitro*. By contrast, these same agents exhibited apparently paradoxical antifertility effects in a variety of experimental animals.[14,19,21] These effects have been used in studies of the potential application of this compound as a contraceptive agent in female subjects. Bergquist and colleagues,[3] administering [D-Ser(TBU)^6-EA^{10}]-LHRH, 400 to 600 µg intranasally once daily, to 27 regularly menstruating women, noted inhibition of ovulation in all but 2 of 89 treatment months.

Casper and Yen[7] gave [D-Trp^6-Pro^9-N-ethylamide]-LHRH (also known as LRF$_X$), to normal females on day 21 and 22 of the cycle. A dose of 50 µg

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subcutaneously for these 2 days of the cycle was associated with pronounced luteolysis, as evidenced by a shortening of the menstrual cycle and by a more rapid decline in serum progesterone levels than in a normal cycle. The results suggested a potential use as a contraceptive agent. However, in subsequent studies,[9] the same group reported that when pregnancy occurs in a cycle in which the LRF_V is used in the same manner, the corpus luteum is rescued by hCG produced by the placenta. Furthermore, exogenous hCG can similarly restore normal luteal function even in the presence of very high doses of exogenous LRF_V.[10] Our efforts have been directed toward establishing the biologic effects of LRF_V in a group of normal male subjects, with particular reference to the pituitary–gonadal axis and semen analysis. The present chapter covers our initial studies in two groups of subjects, one of which received LRF_V on a daily basis, the other on an every-fourth-day regimen.

METHODS

Healthy male volunteers who desired vasectomy and who showed normal sperm analysis were asked to defer vasectomy for approximately 30 weeks. All subjects came to the research clinic every other week, at which time blood was drawn for plasma levels of testosterone, progesterone, and 17-hydroxyprogesterone, and for serum levels of LH, FSH, and 17β-estradiol.

Following sampling of the blood, each man was asked to masturbate, saving the entire contents of a semen sample in a sterile plastic cup which was promptly transported to the laboratory. The men were requested to abstain from ejaculation for 48 hours prior to a clinic visit, with the understanding that recent ejaculation might cause lowering of the sperm count. The subjects became familiar with each investigator at the clinic and kept their appointments reliably. At each visit, they returned a calendar showing the frequency of ejaculation during the previous two-week period.

The two studies each consisted of three 10-week periods. During the first 10 weeks, six basal samples of semen and blood were collected. Physical examinations were performed, and a battery of toxicologic tests, including urinalysis, complete blood count, and automated blood chemical panel, were checked initially, as well as in each of the subsequent two periods of the study. During the fifth basal visit, the men were instructed individually on the technique of self-injection of LRF_V subcutaneously in the abdomen in areas which circumscribe the umbilicus. At the sixth visit, in the presence of one of the investigators, each man self-injected the first dose of a solution whose contents were known to be either LRF_V or saline.

Subsequently, the men were divided into three groups. One group received LRF_V subcutaneously, 50μg, each morning. The second group received 50 μg of LRF_V every fourth day, and the third group received saline, either daily or every fourth day. Each man was contacted at home periodically to check on compliance during the second, or treatment, period of the three 10-week intervals. This period lasted a maximum of 10 weeks. However, the studies were terminated if potentia was not maintained, if sperm density fell below 5×10^6/ml, or if toxic effects were noted on periodic checks of the

toxicologic battery. No toxicologic effects were observed; however, five of the eight men who received LRF_A daily had their treatment period terminated earlier than 10 weeks because of loss of libido or potentia.

In the recovery period, men were again observed at 2-week intervals for a minimum of 10 weeks, or until the sperm count had returned to pretreatment levels. All hormone determinations were performed in our laboratory by standard radioimmunoassays which had previously been in operation for at least 2 years.[17] Semen analysis was performed by a trained laboratory technologist who received the specimens on the same day of each week. The study was approved by the Vanderbilt University Committee for the Protection of Human Subjects, and all subjects gave written informed consent. The study was carried out with a personal IND awarded to Dr. David Rabin by the Food and Drug Administration, Rockville, Maryland.

RESULTS

EFFECTS OF DAILY ADMINISTRATION OF LRF_A, 50 μg SUBCUTANEOUSLY, IN NORMAL MEN

Figure 27-1 displays gonadotropin and testosterone levels in the eight men who received daily LRF_A. The figure shows the mean of all basal determinations performed during the initial 10-week study; the mean of the nadir of LH, FSH, and testosterone observed during the treatment period; and the peak levels of LH, FSH and testosterone observed during the recovery period. LH levels dropped to a nadir that was 50% that of the basal mean during treatment with LRF_A. Immediately following discontinuation of therapy, there was a rebound to levels exceeding normal basal concentrations. Serum FSH levels fell to 60% that of the basal mean by midtreatment period, and returned to normal promptly after discontinuation of LRF_A.

The response of plasma testosterone was particularly striking. All eight men experienced a rapid decline in plasma testosterone levels, with a mean nadir of 0.6 ng/ml by the fourth treatment week. The fall in testosterone occurred within 2 weeks of treatment in five men, and fell to 1.3 ng/ml or less by the fourth week in all eight subjects. Following cessation of therapy, testosterone levels tended to rebound above basal levels and returned to a mean level of 4.2 ng/ml by the fourth week after discontinuation of the analog.

Figure 27-2 displays the serum or plasma concentrations of selected steroids during the basal period, the nadir achieved during therapy, and the peak values observed during the recovery phase. For reference, serum testosterone levels shown in Figure 27-1 are again displayed in Figure 27-2. Circulating levels of progesterone, 17α-hydroxyprogesterone and estradiol all fell in a manner concordant with that observed for serum testosterone. Each of these steroids returned to or rose above basal values during the recovery period. Levels of progesterone, although falling significantly, dropped less than those of the other steroids. At no time during the 10-week treatment period did we observe a rise in 17α-hydroxyprogesterone, suggesting that the fall in testosterone accompanied by the fall of the other

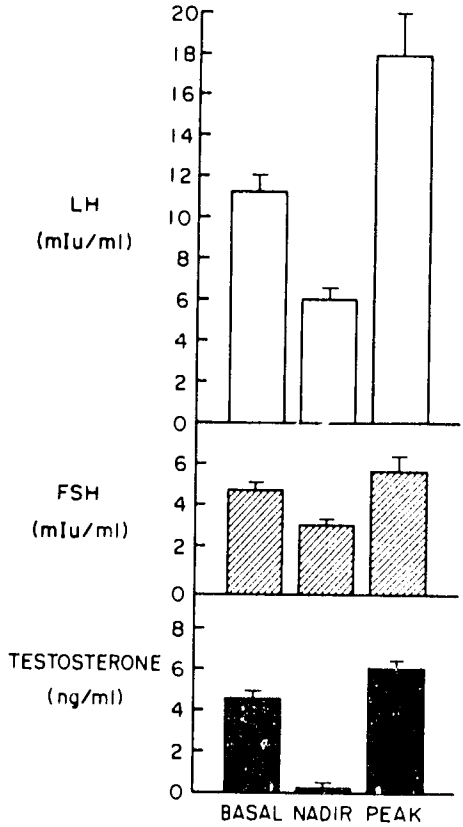

FIG. 27-1. Mean basal LH, FSH, and testosterone concentrations; mean nadir levels achieved during treatment; and mean peak recovery concentrations in subjects receiving LRF_A, 50 μg subcutaneously daily.

steroids shown in Figure 27-2 was the consequence of a blockade in an early step in steroidogenesis. Treatment with LRF_A was discontinued in five men at week 6 or 7 because of impotence. Each man regained normal libido and potentia within 2 weeks after stopping the injections.

Figure 27-3 displays the mean sperm density (number of sperm per milliliter of ejaculate) during the basal period, the nadir following treatment with LRF_A, and the peak value observed during the recovery period. Sperm density varied widely between subjects and in individual subjects during the basal period. For this reason, we made six estimations of basal sperm density in each subject. Sperm density fell to a mean nadir of 12×10^6/ml: six of the subjects experienced a drop in sperm to less than 6×10^6/ml, and the other two subjects displayed a fall of 70 and 86% in sperm density values. The three men who completed the entire 10 weeks of LRF_A therapy demonstrated a fall in sperm density to 4.6, 2.7×10^6/ml, and 0. Each of the eight men displayed recovery of sperm density to within the range of values observed during the basal period. Complete recovery was occasionally delayed, although it generally occurred within 10 weeks of cessation of therapy. Only one man showed a return 14 weeks after cessation of therapy. This subject had become azoospermic during the study, and was one of the three subjects who completed the entire 10 weeks of therapy.

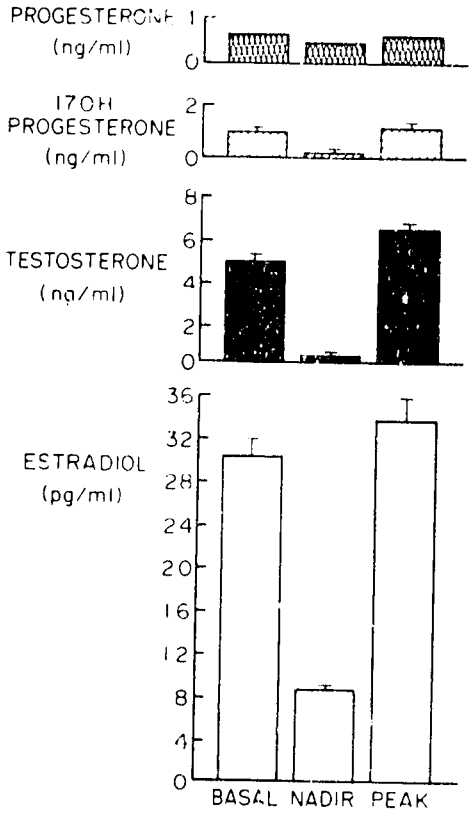

FIG. 27-2. Mean basal concentrations of progesterone, 17-hydroxyprogesterone, testosterone, and estradiol; mean nadir levels achieved during treatment; and mean peak recovery concentrations in subjects receiving LRF_A, 50 μg subcutaneously daily.

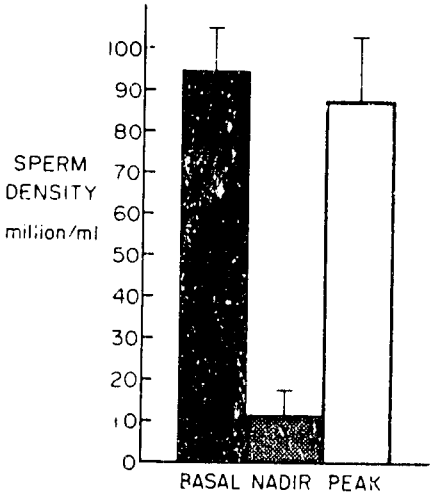

FIG. 27-3. Sperm density for subjects receiving LRF_A, 50 μg subcutaneously daily, showing mean basal value, mean nadir, and mean peak recovery level.

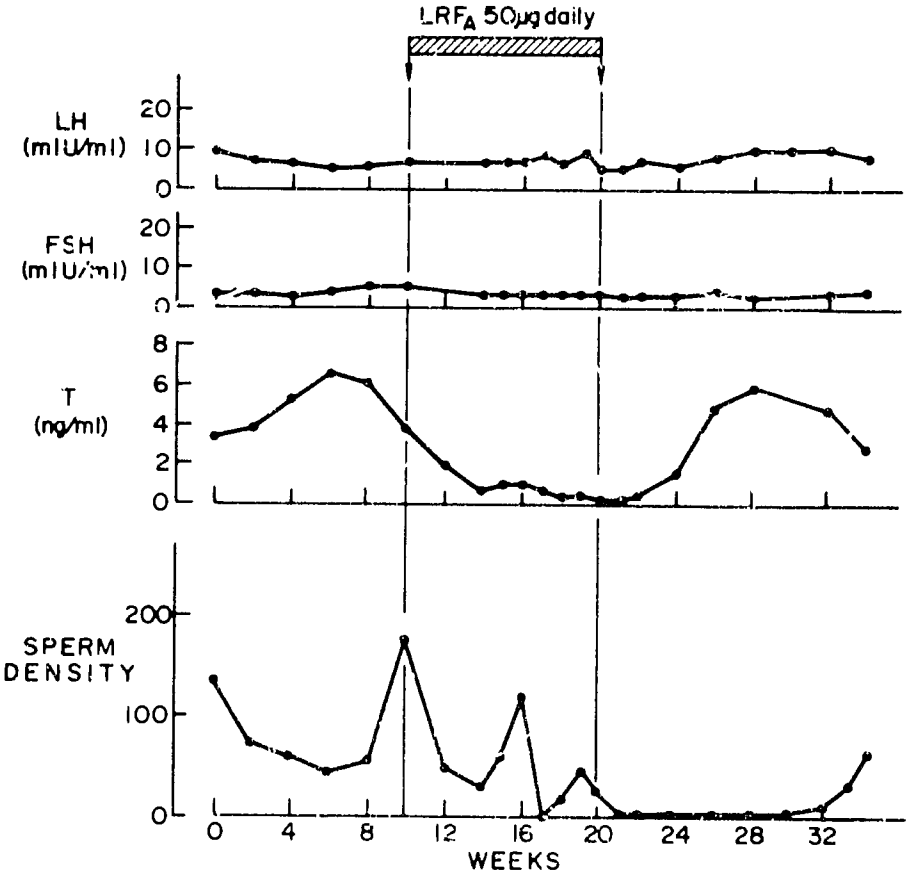

FIG. 27-4. Time course of LH, FSH, testosterone, and sperm density during basal, treatment, and recovery periods for a single subject receiving LRF$_A$, 50 μg subcutaneously daily.

An interesting observation made during the treatment period was the report of hot flashes in four men; this symptom was observed following decline in plasma testosterone and persisted until about 3 weeks after discontinuation of LRF$_A$. The time course of plasma testosterone, serum LH and FSH, and sperm density for one of the subjects is shown in Figure 27-4. It is of interest that the fall in plasma testosterone occurred much earlier than the fall in sperm density, and this feature is representative for all the subjects in the study.

TREATMENT WITH THERAPY EVERY FOURTH DAY

The effects on pituitary-gonadal function of LRF$_A$, 50 μg subcutaneously every fourth day for up to 10 weeks, were evaluated in seven normal men (Fig. 27-5). A modest rise in mean serum LH levels was noted during the treatment period, while mean serum FSH levels were unchanged. Mean

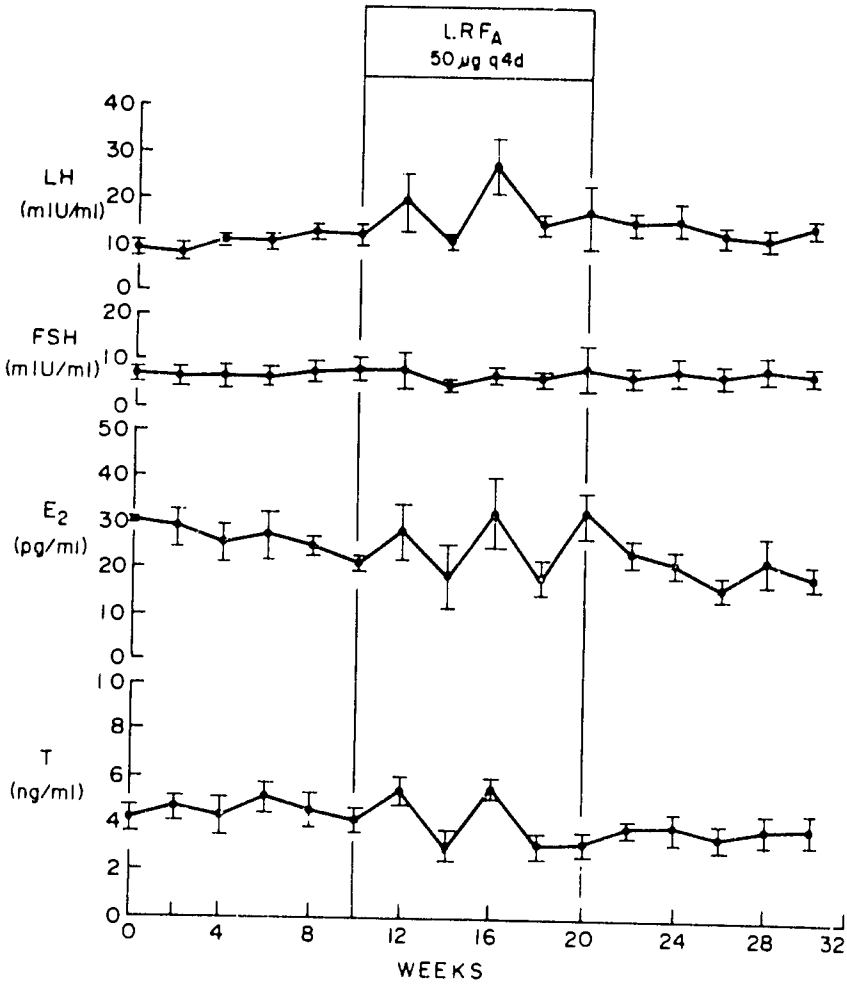

FIG. 27-5. Mean basal, treatment, and recovery period concentrations of LH, FSH, testosterone, and estradiol for seven men treated with 50 µg subcutaneously every fourth day for 10 weeks.

plasma testosterone concentration remained at 3.1 ng/ml or above. Sperm density during the control period varied widely within and between subjects, with a mean range of 69 to 137 × 10^6/ml. The mean sperm density fell to a nadir of 40 × 10^6/ml during treatment, but no consistent pattern was observed for each subject. Values varied between 4 and 368 × 10^6/ml. Elevated LH and testosterone values were observed on eight and seven occasions, respectively, in five subjects, and corresponded to blood drawn 24 and 48 hours after the last LRF_A injection. Unequivocally depressed testosterone values were observed on ten occasions in six patients, and in all but one corresponded to blood drawn 72 and 96 hours after the last injection. One subject had daily blood drawn at the start of, and 4 weeks after initiating

therapy. An agonistic effect on LH, FSH, testosterone, and estradiol was observed both times, although the effect was blunted on the second occasion.

DISCUSSION

Crowley and co-workers[8] reported a linear dose–response relationship between a single injection of LRF_A and a rise in serum FSH and LH over the dosage range of 10 to 100μg. Casper and others demonstrated a similar potency of LRF_A to discharge FSH and LH acutely and sustain their release in normal women throughout the menstrual cycle.[6] On the other hand, several studies have indicated that the prolonged administration of LRF_A may be associated with paradoxical effects on steroid levels in man, as previously reported in experimental animals. Thus, Smith and associates noted that the administration of [D-Ser(TBU)^6-EA^{10}]-LHRH, 5 μg subcutaneously daily for 1 week, produced a fall in plasma testosterone levels in normal men.[23]

Bélanger and others followed plasma testosterone and 17-hydroxyprogesterone concentrations after the single intranasal administration of 500 μg of the same analog.[1] They reported a lowering of these steroid levels to 40 to 50% of basal by the third day after medication, with a return to normal thereafter.

Bergquist and colleagues, in what appears to be the most comprehensive study of effects of the LHRH analog in normal men, administered 5 μg of [D-Ser (TBU)^6-EA^{10}]-LHRH by subcutaneous injection to healthy men for 17 weeks.[2] Serum FSH, LH, and plasma testosterone levels all fell modestly during the treatment period; the acute effects of the analog on gonadotropin release were decreased but not obliterated. Gonadotropin and testosterone levels returned to pretreatment concentrations within a week after discontinuation of therapy. The authors reported no significant changes in the composition of seminal fluid during the study period, although they did not provide individual data in their paper.

Our results varied significantly from those of the Bergquist study. Testosterone levels fell to considerably lower values, with a mean nadir in our subjects of 0.2 ng/ml, and there was a comparable decline in 17-hydroxyprogesterone and 17β-estradiol levels. Furthermore, loss of potentia was observed in five subjects. LH and FSH levels fell to approximately 50 and 60% of basal during the treatment period. This change is particularly noteworthy, since in the light of the very low circulating steroid levels a profound rise in circulating gonadotropin levels would have been expected. The fact that serum gonadotropin levels remained at or below basal values strongly suggests that the administration of the analog in our subjects was associated with desensitization at the pituitary level; the analog in some way seemed to prevent the rebound rise in the level of secretion of LH and FSH normally anticipated when steroid levels are acutely lowered.

Sperm density fell by 70% or greater in each subject, including the five subjects in whom therapy was discontinued after only 6 to 7 weeks. Because three subjects were unable to provide samples on one or more visits owing to impotence, the values given are upper estimates. The decline in sperm density was accompanied by a significant fall in sperm motility. The patho-

genesis of the fall in sperm motility is unclear to us, although it may be related to the profound inhibition of testosterone biosynthesis. Of great importance was the time course in the fall of plasma testosterone and in sperm density. The former occurred within 2 weeks of starting therapy; the latter, generally after 6 to 10 weeks. The recovery of plasma testosterone and the return of sperm density to normal showed a similar dissociation, with steroid levels returning to normal promptly and sperm density remaining depressed for up to 14 weeks in one subject. These results may reflect the very long interval required for spermatogenesis relative to that for steroidogenesis. MacLeod and co-workers had observed that healthy young men exposed to high temperature and humidity for several hours experienced a fall in sperm density,[16] although the latter was delayed for a time period quite similar to that observed in our experiments. These comparable temporal relationships in two very disparate experimental situations may reflect storage of spermatozoa in the epididymis.

Our results do not provide definitive answers as to the mode of action of LRF_V. However, the subnormal LH and FSH levels associated with extremely low ambient steroid levels strongly support LRF_V-initiated pituitary desensitization. Clearly, under normal circumstances, the fall in circulating steroid levels would have initiated a profound rise in both gonadotropins, and by contrast, we observed a modest decline in ambient levels of both LH and FSH. Hsueh and Erickson have reported a direct effect of LHRH analogs on the gonad, specifically, inhibitory effects on steroidogenesis.[11,12] Our data do not preclude a direct effect of LRF_V on the gonad in addition to what we believe to be convincing evidence for pituitary desensitization produced by the analog.

Side-effects were confined to those that might be anticipated from a rapid decline in plasma testosterone levels; that is, loss of libido and potentia were almost ubiquitous. A second interesting side-effect was noted in four of the men, namely the appearance of hot flashes. These occurred frequently each day during the treatment period and resolved within 3 weeks of discontinuation of therapy. The flashes were clinically similar to those experienced by postmenopausal women and were not associated with other symptoms. Of interest is the report by Huggins and others, who noted hot flashes among some subjects with cancer of the prostate who were surgically castrated.[1] The pathogenesis of the hot flash remains to be established in more detail.

We studied the effect of LRF_V administration on an every-fourth-day schedule in the light of data obtained in the male rat by Rivier and Vale.[20] Findings from their study suggested greater antigonadal activity of similarly administered analog. When LRF_V was administered to male rats every fourth day for 13 to 16 days, more pronounced elevations in plasma progesterone were observed 2 hours after an LRF_V injection. The authors suggested that the increased levels of progesterone, whether cause or effect, might represent greater inhibition of gonadal function.

In the seven men we examined, no consistent suppression of the pituitary–gonadal axis was achieved, and agonist effects of LRF_V were retained. This was seen clearly in a single subject from whom daily blood samples were obtained on days 1 through 4 and 29 through 32 of the 10-week treatment period. Levels of LH, FSH, testosterone, and estradiol all increased at 24

hours following LRF_A injection, variably returning to basal over the subsequent 3 days. Analysis of blood samples obtained at 2-week intervals from the remaining subjects verified this phenomenon. Thus, during every fourth day of LRF_A therapy, wide fluctuations in gonadotropin and steroid levels contrasted with the consistently suppressed values resulting from daily administration of the agonist. These results confirm those of Crowley and associates, who demonstrated LH responsiveness and increased testicular size during alternate day LRF_A therapy in men with hypogonadotropic hypogonadism.[9] These effects were not observed with daily administration of the analog.

A modest decline in sperm density, as assessed by the lowest value to which each man dropped during the treatment or posttreatment period, was noted in all seven men during the study. The decrease, however, was inconsistent and ill-sustained, contrasting with the consistent and sustained fall in sperm density observed in the daily treatment group.

In summary, our results clearly support the biologic effects of an LHRH analog in producing reversible inhibition of testicular steroidogenesis and spermatogenesis in normal men. Per se, this therapeutic mode does not provide a satisfactory male contraceptive because of the attendant loss of libido and potentia. It is possible that the addition of testosterone enanthate therapy may preclude the latter and provide the framework for a practical male contraceptive. Studies pursuing this combined avenue are in progress at the present time.

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28

Inhibition of Androgen Biosynthesis in the Human Male by Chronic Administration of [D-Ser(TBU)^6-des-Gly-NH_2 ^{10}]-LHRH Ethylamide (Buserelin)

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Isolation and synthesis of LHRH has greatly facilitated our understanding of the control of LH and FSH secretion.[11,24] Moreover, the availability of this neurohormone has raised new hope for the treatment of male and female infertility.[25,45] At the same time, new approaches for contraception are able to be considered.

These potential clinical applications of LHRH have become even more promising with the availability of potent LHRH analogs.[14,41] The increased potency, as well as the longer duration of action of these agonists, has stimulated numerous studies in men and women in order to improve reproductive function; however, the results obtained have been somewhat disappointing.[20,32]

LHRH AGONIST STUDIES IN EXPERIMENTAL ANIMALS

Detailed studies of the effects of treatment with LHRH agonists in male rats showed, unexpectedly, decreased weights of the testes, ventral prostate, and seminal vesicles; marked loss of testicular LH receptors; and lowered plasma testosterone concentration.[2-4,13,21,28-30,39,40] These first studies offered an explanation for the disappointing results of treatment with LHRH and its agonists in oligospermic men.[32] Moreover, the loss of testicular LH and

These studies were supported in part by grants from the National Cancer Institute of Canada. Buserelin was generously supplied by the Hoechst Pharmaceutical Company.

prolactin receptors provided the first possible mechanism for the inhibitory effects observed.

Detailed time-course studies showed that the changes in testicular LH and prolactin receptors could be correlated with inhibition of steroidogenesis. Enzymatic blockages in the testicular steroidogenic pathway at the level of 17-hydroxylase and 17-20 desmolase activities were demonstrated.[6,7] The following sequence of events was then suggested: LHRH agonist-induced endogenous LH release caused a secondary loss of gonadotropin receptors and a blockage of steroid biosynthesis.[2-4,21-30]

However, the observations of an inhibitory effect of the agonists in hypophysectomized animals,[1,19-22,33] as well as in the presence of excess anti-LH serum,[31] indicate that LHRH and its agonists can also have direct effects on the testis. This was further supported by the demonstration of LHRH receptors in testicular homogenate and Leydig cells.[10,23,36] These testicular receptors have properties similar to those found in the pituitary. Moreover, recent reports describe "LHRH-like" activity in the testis as well as in the ovary.*[37] In addition to its inhibitory effect on steroidogenesis, long-term treatment of the male rat can inhibit spermatogenesis. Marked degenerative changes can be seen in the histology of the seminiferous tubules after 2 to 8 weeks of LHRH agonist administration. These changes can be partially reversed within 1 or 2 months after cessation of treatment.[27]

Although the detailed mechanisms of the effect of LHRH agonists on spermatogenesis are still unknown, there is, nevertheless, hope that such an action of LHRH agonists could eventually be used in man as a reversible approach for inhibition of spermatogenesis.

DOSE–RESPONSE TO INTRANASAL BUSERELIN ADMINISTRATION IN THE HUMAN MALE

These interesting findings of LHRH agonist-induced enzymatic blockage in the testicular steroidogenic pathway prompted us to study the possibility of a similar inhibition in man. Previous studies with LHRH and its agonists by other groups were aimed at finding a compound more potent and with a longer duration of action in order to improve its stimulatory action on gonadotrophs.[18,42,43] These dose–response studies were thus performed over limited time periods.

During our first study, our aim was to examine a possible inhibitory effect on testicular steroid biosynthesis after single administration of an LHRH agonist.[8] The maximal inhibitory effect on LH receptors in the rat testis being at 2 to 3 days, our studies in the human were extended over a period of 6 days. The intranasal route of administration of the potent LHRH agonist [D-Ser(TBU)^6-des-Gly-NH$_2$^{10}]-LHRH-EA (HOE 766, or Buserelin)[30] was chosen because of its ease of application and in view of its potential widespread utilization.[44] In order to obtain information on the optimal effective dose as

*Godbout M, Labrie F: Unpublished data.

inhibitor of testicular steroidogenesis, we studied the responses of serum LH and steroids to increasing doses of Buserelin.

To take into account the diurnal cyclicity of serum steroids, it was important to measure their blood levels at different time intervals during the day in order to assess fully the effect of the gonadotropin-releasing peptide.[5,15] Normal young men, ages 20 to 40, were studied over a 9-day period with blood samplings at 8 a.m., 4 p.m., and 10 p.m. On the third day (day 0), they received 50, 100, 200, 500, 1250, or 2500 μg Buserelin by nasal spray (six subjects per dose-group). Serum levels of LH and various steroids (pregnenolone, 17-OH progesterone, testosterone, DHT, and 17β-estradiol) were measured at various time intervals over the following 24-hour period (minus 30 minutes, minus 15 minutes, 0, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 11 hours, 14 hours, and 24 hours relative to the time of administration).

The serum LH response is minimal and of short duration at the two lowest doses used. A near maximal effect is already obtained at the 200-μg dose, usually two hours after its administration (Fig. 28-1). On the other hand, a longer duration of action of the peptide is seen with increasing doses, the elevation from baseline levels being still significant at 8 hours and 14 hours for the 1250- and 2500-μg doses, respectively.

Diurnal cyclicity, with a morning peak and an evening nadir, can be seen for all the steroids measured. At all the doses studied, there is some response with a loss of cyclicity but it is only at the 500-μg dose that the elevation of testosterone and 17-OH progesterone is significant. (Testosterone levels are shown in Figure 28-2; 17-OH progesterone data not shown.) Inhalation of 500 μg or more of Buserelin was followed, after an initial rise in testosterone and 17-OH progesterone levels, by a decrease of the level of these two steroids during the first to the third posttreatment days. Thereafter, there is a return to normal cyclicity.

The time course of the effect of Buserelin raises interesting questions concerning the mechanism of such an inhibition of androgen levels. Studies in adult male rats have shown a maximal loss of testicular LH receptors 2 to 3 days after single administration of an LHRH agonist with a progressive return to normal at 6 to 8 days.[3,4,21] This loss of LH receptors was accompanied by an enzymatic blockage at the level of 17-hydroxylase and 17-20 desmolase activities.[6,7,12] The finding of a return to normal testosterone and 17-OH progesterone levels after 4 to 5 days in men treated with the highest doses of Buserelin suggests that the time course of the inhibitory effects of LHRH agonists are closely similar to the kinetics described in much more detail in the rat.[3,4,21] Our data also show unchanged levels of pregnenolone, thus suggesting that a blockage of 17-hydroxylase activity can explain, at least partially, the inhibitory effect of Buserelin treatment on steroidogenesis.

CHRONIC BUSERELIN ADMINISTRATION IN MEN

Our findings of an inhibition of testicular androgen biosynthesis following single administration of Buserelin are in agreement with recent data showing that chronic administration of various LHRH agonists can induce inhibition

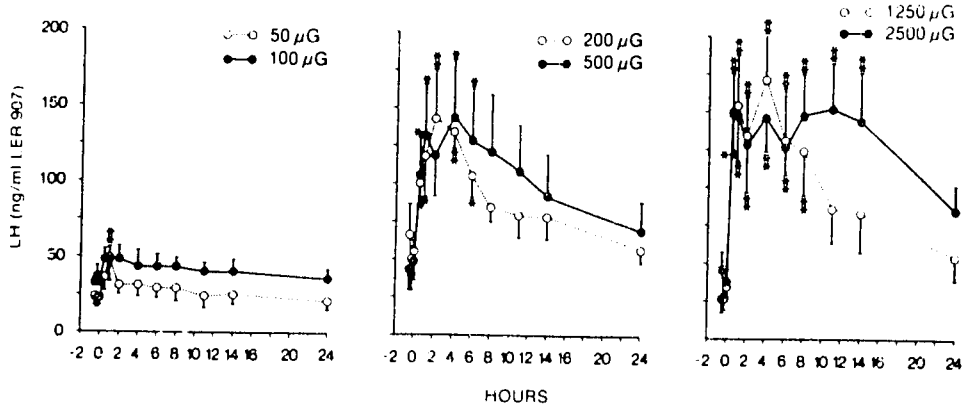

FIG. 28-1. Effect of single intranasal administration of increasing doses of Buserelin in young adult men on serum LH levels. Data are expressed as means ±S.E.M. of values obtained from six subjects per group. (* = $P < 0.05$; ** = $P < 0.01$.)

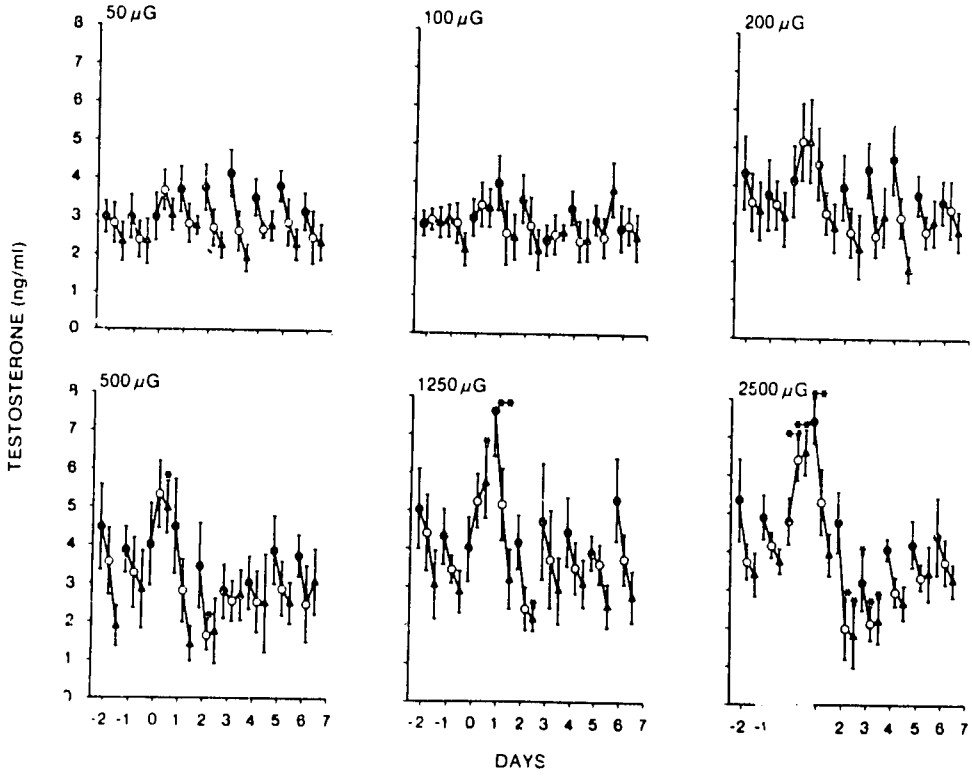

FIG. 28-2. Effect of single intranasal administration of increasing d[illegible] Buserelin in young adult men on serum testosterone levels measured [illegible] (●——●), 1500 (○——○) and 2200 (▲——▲) hours. Serum testosterone [illegible]vels were measured on two pretreatment, one treatment, and six posttreatment days. (* = $P < 0.05$; ** = $P < 0.01$.)

of serum testosterone levels in normal men.[9,17,38] Except for two subjects treated intranasally,[17] the volunteers received the LHRH agonists by subcutaneous injections (5 μg daily) over periods ranging from 1 to 17 weeks. Basal LH levels showed little or no change, but LH response to the peptide was partially inhibited after a period of 1 to 2 weeks of treatment. With the dose of the LHRH agonist used, plasma testosterone levels showed a decrease of 25 to 40%, except in Smith's study in which an inhibition of about 50 to 70% resulted after only 1 week of Buserelin administration in five subjects.[38]

Our next studies were aimed at studying the effect of long-term treatment with the LHRH agonist Buserelin, in patients with cancer of the prostate. We then wanted to know the dose and schedule of administration of Buserelin that could obtain an almost complete inhibition of testicular androgen biosynthesis. Such treatment could eventually replace surgical castration and the administration of high doses of estrogens, both treatments being associated with side-effects, mostly psychological and cardiovascular, respectively.[5]

Nineteen patients with a histologic diagnosis of prostatic carcinoma were treated for periods ranging from 1 to 12 months. After three control days during which blood samplings were performed at 8 a.m., 3 p.m., and 9 p.m., the patients received either 200 or 500 μg of Buserelin twice a day by nasal spray or one daily subcutaneous injection of 50 μg of the peptide. At the time of the first administration, as well as at regular intervals (usually every 2 months), a 24-hour response curve to the peptide was performed for measurement of LH and various steroids (pregnenolone, 17-OH progesterone, testosterone, DHT, and 17β estradiol). Blood was also drawn weekly, three times over a single day, to measure the levels of these same hormones.

Figure 28-3 illustrates the serum testosterone and LH levels measured three times a day before and during intranasal treatment with 200 μg of Buserelin twice daily. After 2 weeks, serum testosterone is already at 40% of control, then it decreases further over the next few weeks to reach 5 to 15% of control. LH levels, on the other hand, slightly higher than baseline for the first 2 weeks, are not different from the control levels and show a tendency to decrease over the last 4 weeks of Buserelin administration. However, at the twice-daily 200 μg dose, a somewhat variable response was observed.

Another case is presented in Figure 28-4. In this patient, serum testosterone levels show no significant change for the first 3 weeks before a clear decrease is seen for a similar time period. This is followed at 8 weeks by a return of serum testosterone to values not different from control levels. These fluctuations in serum testosterone levels appear to be independent of serum LH. Baseline LH levels show a tendency to a progressive decrease up to 8 weeks, although LH response is completely blunted as measured 7 and 13 hours after administration of the peptide.

A similar pattern of testosterone response was seen in another patient treated with 500 μg of Buserelin twice a day (Fig. 28-5); in this case, it is after 11 weeks of treatment and after a marked inhibition of testosterone levels that an increase of serum androgen levels is seen with a second almost complete inhibition at 18 weeks. However, LH response in this patient was very different from the previous one; a fairly close parallelism is seen between the LH and testosterone levels, both hormones showing the same fluctuations during the course of treatment.

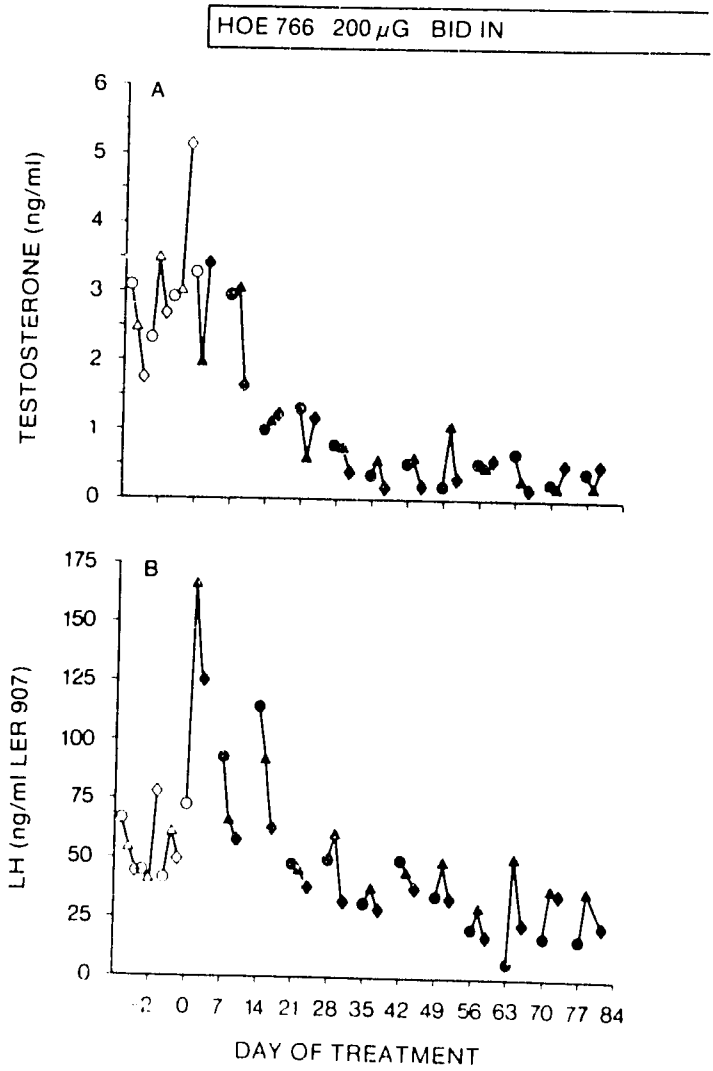

FIG. 28-3. Effect of twice-daily intranasal administration of 200 μg Buserelin in a patient with cancer of the prostate on (*A*) testosterone and (*B*) LH levels measured at 8 a.m. (○——○), 3 p.m. (△——△) and 10 p.m. (◇——◇). (Open characters represent values obtained before Buserelin treatment and closed characters, values obtained during the 11-week treatment period.)

Figures 28-6 and 28-7 illustrate the responses of two other patients treated with one daily subcutaneous injection of 50 μg of Buserelin. While in the first case (Fig. 28-6), testosterone levels were 60 to 75% inhibited after only 1 week of treatment, the second patient (Fig. 28-7) shows a clear decrease of plasma androgen levels—although most of the injections of the agonist elicit a small response. In this patient, even if the LH response is decreased compared with the first treatment, there is still some degree of response even

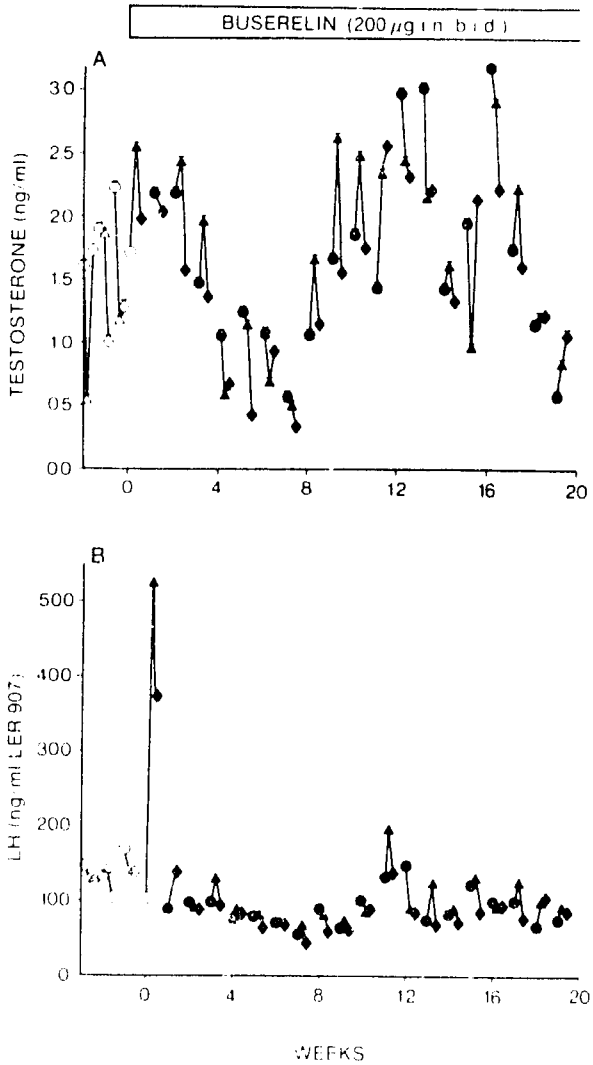

FIG. 28-4. Effect of twice-daily intranasal administration of 200 μg Buserelin in a patient with cancer of the prostate on (*A*) testosterone and (*B*) LH levels measured at 8 a.m. (○——○), 3 p.m. (△——△) and 10 p.m. (◇——◇). (Open characters represent values obtained before and 1 month after Buserelin treatment; closed characters, values obtained during 19-week treatment period.)

after more than 4 months of daily administration of Buserelin. The daily small response in testosterone levels may well be secondary to these low LH surges.

We also studied changes of the LH responsiveness to the peptide at various time intervals during treatment with Buserelin. Figure 28-8 clearly shows that chronic intranasal administration of Buserelin nearly completely abolishes the LH response to the peptide. That this pituitary desensitization is revers-

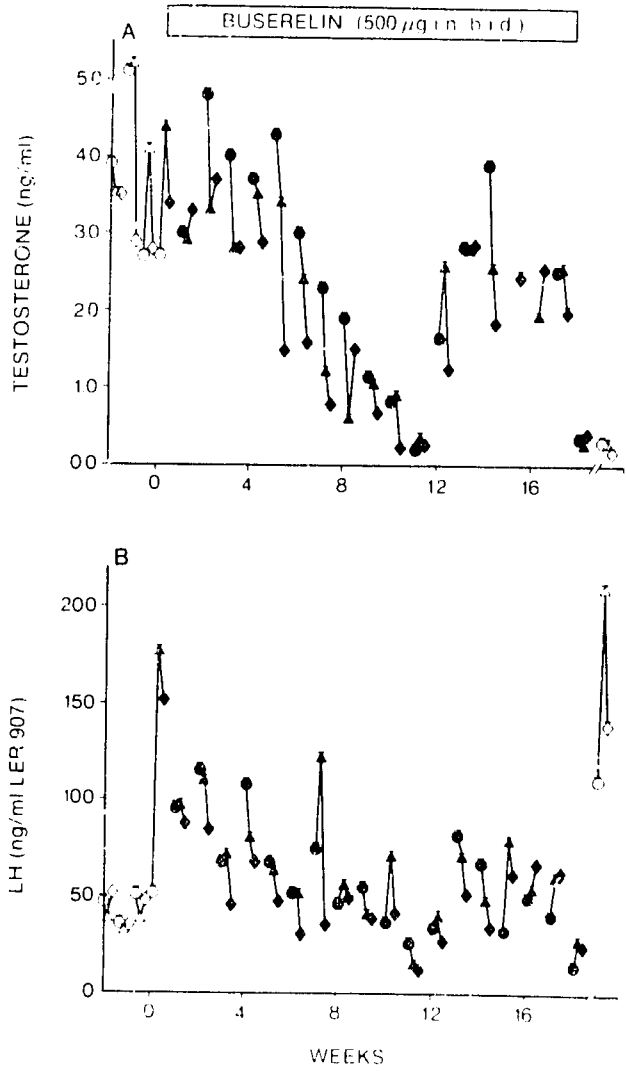

FIG. 28-5. Effect of twice-daily intranasal administration of 500 μg Buserelin in a patient with cancer of the prostate on (*A*) testosterone and (*B*) LH levels measured at 8 a.m. (○——○), 3 p.m. (△——△) and 16 p.m. (◇——◇). (Open characters represent values obtained before Buserelin treatment; closed characters, values obtained during 18-week treatment period.)

ible is shown by the return of the serum LH levels to normal 1 month after cessation of treatment. This type of response is found in the great majority of patients, with slight differences in the magnitude of the LH inhibition.

In all patients, the serum levels of the precursor pregnenolone showed no change, but levels of 17-OH progesterone responded to the agonist with a pattern similar to that of testosterone, thus suggesting that the blockage described at the level of 17-hydroxylase in rats also exists in men. On the other

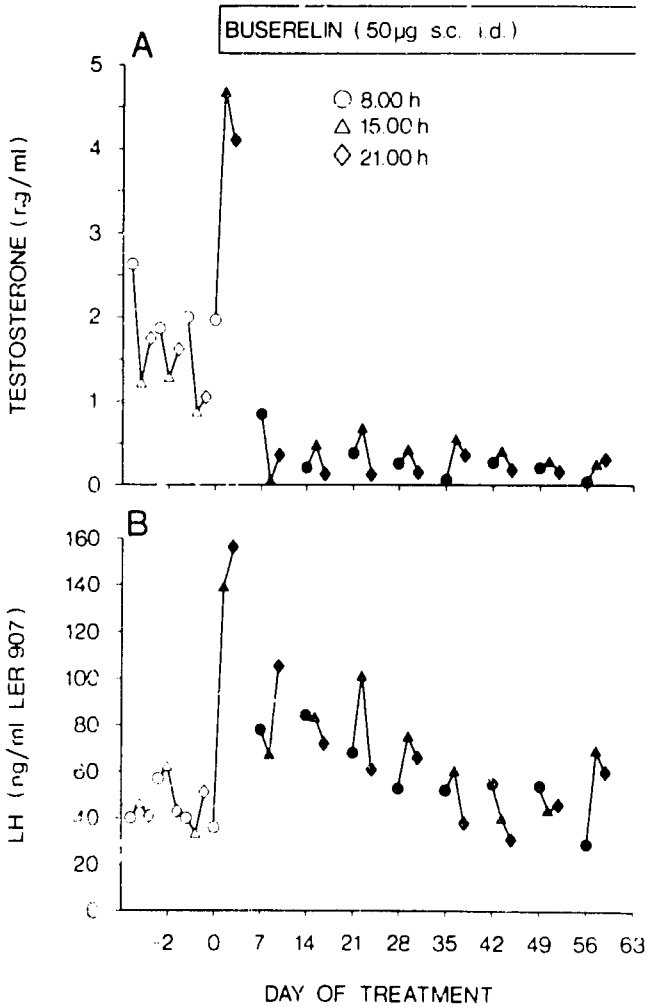

FIG. 28-6. Effect of daily subcutaneous administration of 50 µg Buserelin in a patient with cancer of the prostate on (*A*) testosterone and (*B*) LH levels measured at 8 a.m. (○——○), 3 p.m. (△——△) and 10 p.m. (◇——◇). (Open characters represent values obtained before Buserelin treatment; closed characters, values obtained during 8-week treatment period.)

hand, the situation in man differs from what is found in the rat. In this experimental animal, an increase in 5α-reductase activity (reflected by an increase in androstane-3α, 17β-diol) follows treatment with LHRH agonists,[12] but no such increase is found in men.

The apparent variability in individual responses can be attributed to various factors. There are clear differences among individuals in the period of time necessary to induce desensitization of the pituitary–gonadal axis to repetitive administration of LHRH agonists. As seen in the preceding figures, some

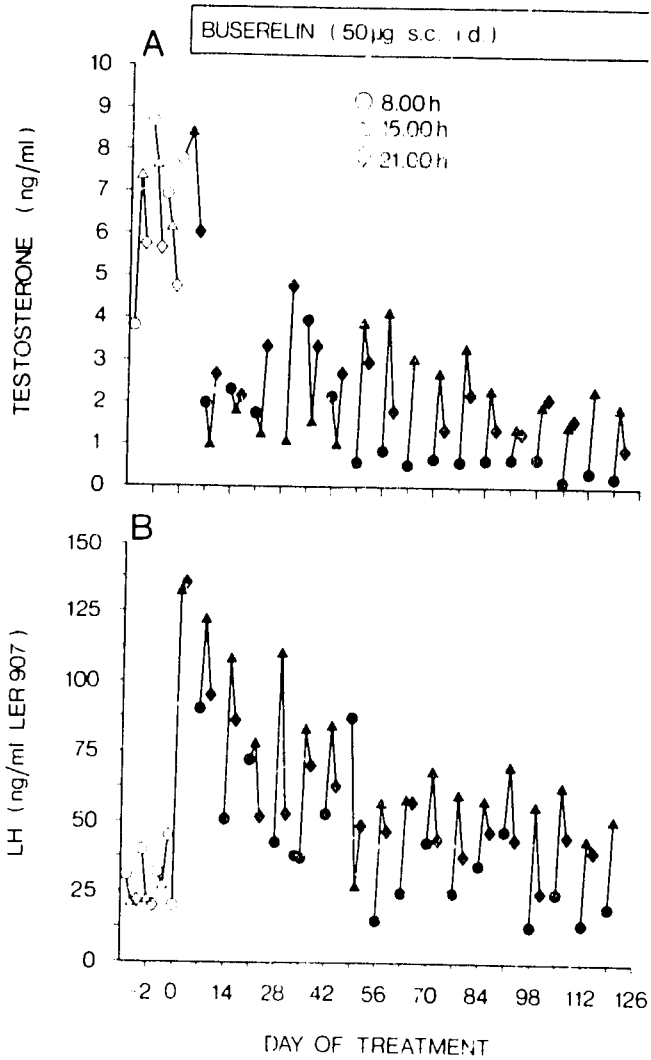

FIG. 28-7. Effect of daily subcutaneous administration of 50 µg Buserelin in a patient with cancer of the prostate on (*A*) testosterone and (*B*) LH levels measured at 8 a.m. (○——○), 3 p.m. (△——△) and 10 p.m. (◇——◇). (Open characters represent values obtained before Buserelin treatment; closed characters, values obtained during 17-week treatment period.)

patients show a nearly complete absence of the LH response to Buserelin after only seven administrations (Fig. 28-3), and others show some response even after months of treatment. The same is true for the decreased serum androgen levels. There are also some differences between the degree of inhibition at the pituitary and testicular levels as evaluated during the 24-hour response studies. Variation of the degree of desensitization at the pituitary level may well be reflected on the degree of blockage of the steroidogenic pathway.

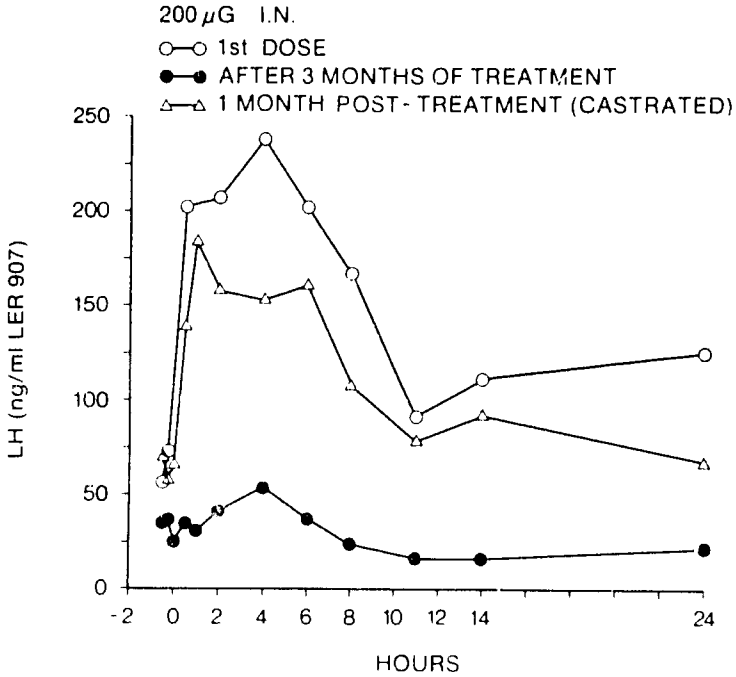

FIG. 28-8. Effect of chronic (3 months) twice-daily intranasal administration of 200 μg Buserelin on plasma LH response to LH-releasing peptide in a patient with cancer of the prostate. LH levels following stimulation at the first administration (○——○), after 3 months of treatment (●——●), and 1 month post-treatment (△——△) are shown.

It is significant that the same individual can show occasional fluctuations in response to the peptide during the course of treatment. This is well illustrated in Figures 28-4 and 28-5. After inhibition for some weeks, LH as well as testosterone levels rise, thus suggesting that the pituitary and/or the testis can escape from the inhibitory effect of the LHRH agonist. That escape seems to be temporary in some cases, with a second lowering of serum hormone levels.

Close examination of the data published by Bergquist[9] shows the same phenomenon after daily subcutaneous injections of 5 μg of the peptide. After an initial inhibition of testosterone levels which reach minimal values at 4 weeks, there was a progressive increase of the plasma levels with a plateau at values midway between baseline and nadir. In that study, in spite of the continuing subcutaneous injection of 5 μg of D-Ser[6], there was a second increase of serum testosterone for the last 3 weeks. In addition, the fluctuations of testosterone levels were parallel to those of serum LH levels.

After surgical castration, circulating levels of androgens originating from the adrenals and from peripheral conversion are still measurable.[31] Those androgens have a potentially adverse effect on the growth of prostatic carcinoma. The use of antiandrogens to neutralize these residual androgens acting at the cell receptor level would then be an interesting approach. Furthermore, the combined treatment with LHRH agonists and antiandrogens

would have the advantage of bypassing the limitations of antiandrogen treatment alone. Since high doses of antiandrogens have some hepatic toxicity, it should be possible to use low doses of these drugs to neutralize the remaining low levels of androgens during LHRH agonist treatment. Second, the long-term use of antiandrogens is associated with an escape phenomenon owing to the removal of androgen-negative feedback at the pituitary level.[26] With the pituitary being desensitized by the LHRH agonist, the antiandrogens should prevent the increase in LH secretion usually observed when antiandrogens are administered alone.

Such an association between an LHRH analog and an antiandrogen appears to be more effective than either treatment alone in diminishing prostate or seminal vesicle weights in the rat. The LHRH agonist at high doses or the antiandrogen RU 23908 alone decreases prostate and seminal vesicle weights by 30 and 65% respectively. However, after combination of both treatments, the secondary sex organ weights are decreased to 10 and 30% of control.[55]

In summary, administration of a potent LHRH agonist to men leads to inhibition of testicular steroidogenesis. These findings and the observation of a complete inhibition of spermatogenesis after chronic treatment with a similar peptide in the male rat[27] clearly suggest that chronic administration of LHRH agonists in men could be used to lower circulating androgen levels and offer the possibility of a new contraceptive approach. Such an approach should be useful in the treatment of androgen-dependent pathologies, such as cancer of the prostate and prostatic hyperplasia. Though subcutaneous administration of Buserelin leads to a marked and constant inhibition of serum androgen levels and could thus be useful alone in the treatment of cancer of the prostate, it appears that the incomplete inhibition of serum androgen levels after intranasal administration of the peptide (at the 200- and 500-μg twice-daily dose) will require combined treatment with an antiandrogen for acceptable efficiency.

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29

LHRH in Hypogonadotropic Hypogonadism

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Since the discovery of the gonadotropin-releasing factor, LHRH, considerable information has been acquired about its chemistry, metabolism, and diagnostic utility.[14,44,50] Considerably less is known about its physiology in humans. Current understanding of its role in pituitary control in primates is derived primarily from tracking LH pulsations in puberty and adulthood,[10,43] direct measurement of hypophysial–portal levels in man[2] and animals,[12] and gonadotropin responses attending its diagnostic use.[14] More recently, attention has turned to observation of patterns of gonadotropin release during prolonged administration of LHRH and/or its analogs in circumstances of presumed LHRH deficiency. Thus seasonal breeding animals,[35] arcuate nucleus-lesioned and prepubertal monkeys,[7,33,52] and patients with anorexia nervosa[36] have served as useful models for such studies.

To gain further insight into the nature of hypothalamic–pituitary interactions in humans, we employed another group of patients presumably deficient in endogenous LHRH, that is, men with idiopathic hypogonadotropic hypogonadism. By contrasting the responses of their pituitary gonadotrophs to repeated administration of a long-acting analog of LHRH with those encountered during pulsatile delivery of the shorter-acting natural sequence decapeptide, we have been able to delineate several important differences.

MATERIALS AND METHODS

Patient Population

Seven men with idiopathic hypogonadotropic hypogonadism participated in the protocol. Historically each demonstrated failure to undergo puberty by

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age 18, incomplete virilization prior to androgen therapy, and no systemic illness or psychologic reason to account for this delay. All had discontinued androgen replacement for a minimum of three months prior to the study. Laboratory assessment at the time revealed these conditions:

1. A low serum LH and FSH level
2. Serum testosterone concentrations in the female range
3. An XY karyotype
4. Normal baseline and stimulated levels of TSH, prolactin, ACTH and growth hormone following insulin-induced hypoglycemia (0.15 μ/kg) and thyrotropin administration (200 μg)
5. Normal sella polytomography
6. Gonads with histologic capability of response by testicular biopsy.

Four hypogonadotropic men who met all of these criteria participated in phases I through III of the protocol; six (including three of the original four) were studied in phase IV.

Protocol

Baseline Status. The patients were admitted to the Metabolic Research Center and informed consent was obtained. Blood was then drawn at 20-minute intervals for a period of 12 hours for subsequent determination of FSH and LH levels. Estradiol and testosterone values were determined every 8 hours for 24 hours.

Initial Responsiveness to [D-Trp^6-Pro^9-NEt]-LHRH. Following this baseline assessment, the patients were administered a dose of the $LHRH_A$ subcutaneously at time 0. Serum for LH and FSH determinations was obtained at 15-minute intervals for the first hour, 30-minute intervals for the next 2 hours, hourly for the next hour, and then 2-hour intervals for the remainder of the 24-hour period. Serum testosterone was measured every 2 hours for the first 12 hours and at 18 and 24 hours. All studies were initiated between 8 and 10 a.m.

Previous studies have documented that the initial pituitary responsiveness of hypogonadotropic subjects to the $LHRH_A$ is a function of prior exposure to the $LHRH_A$ rather than its actual dosage.[15] Consequently, the initial responsiveness to the analog was defined as that observed following the first exposure of each subject to [D-Trp^6-Pro^9-NEt]-LHRH. Two men received an initial dose of 10 μg and two others received 100 μg as the first dose. Since there was not a significant difference between these two sets of responses, their patterns were pooled as the initial responses to [D-Trp^6-Pro^9-NEt]-LHRH. Subsequently, all testing during chronic administration of the LHRH analog was performed with a standard 10-μg bolus based upon the previously observed ability of this dose to elicit a biphasic pattern of LH release in normal men and to facilitate comparisons between the various regimens of $LHRH_A$ administration.[15]

Treatment Schedule. Each subject was treated with [D-Trp^6-Pro^9-NEt]-LHRH ($LHRH_A$) and/or the native decapeptide according to the following four-phase protocol:

Phase I	$LHRH_A$—50 μg every 48 hours for 6 to 8 weeks
Phase II	$LHRH_A$—50 μg every 24 hours for 4 to 6 weeks
Phase III	$LHRH_A$—10 μg every 24 hours for 2 to 4 weeks
Phase IV	LHRH (natural sequence)—25 ng/kg every 2 hours for 7 days

The physiologic actions of the $LHRH_A$ chosen have been shown previously to differ from those of LHRH itself only in potency and duration.[35] The analog was self-administered subcutaneously with insulin needles and syringes. The natural sequence LHRH was administered subcutaneously or intravenously. Previous studies have shown these routes of administration to yield equivalent gonadotropin responses.[3,37] Subcutaneous administration of LHRH was accomplished by a portable, preprogrammed infusion pump (Autosyringe). Preliminary assessment of this device by radioisotopic dilution studies revealed capability of delivering the correct dosage with greater than 2% accuracy. The $LHRH_A$ was dissolved in normal saline/10% mannitol, and LHRH was administered in normal saline alone. Both were stored at −20°C until use and each batch was bioassayed prior to its administration to patients. No deterioration in $LHRH_A$ potency was detected after 1 year of storage under these conditions. Though LHRH appears to be equally stable under these circumstances,[12] it was never stored in solution for more than 3 months. The selection of the 50-μg dosage of the $LHRH_A$ for initial studies was based upon the previous finding that this dose caused a persistent elevation of the serum gonadotropins of normal subjects for 48 hours.[15]

Reevaluation. During the first three phases of the protocol, the patients returned to the Clinical Research Center for reevaluation every 14 days. The standardized 10-μg dose of the LHRH analog was administered subcutaneously. Serum for FSH, LH, testosterone, and estradiol levels was obtained according to schedule during the baseline assessment. An interval history was taken and testicular size was reevaluated. During the administration of pulsatile LHRH, the patients remained in the Clinical Research Center and blood was obtained for gonadotropin, testosterone, and estradiol determinations prior to the LHRH bolus at 8 a.m. A daily 2-hour LHRH pulse was monitored with blood determinations for LH and FSH at 0, 15, 30, 60, 90, and 120 minutes following the 10 a.m. or noon bolus.

Methods. Serum levels of LH, FSH, estradiol, and testosterone were measured by previously described radioimmunoassay techniques.[15] Testicular size was measured by the Prader orchidometer. Bioactivity of the serum LH levels was determined in the dispersed Leydig cell assay.[21] Testicular responsiveness to hCG was tested by determining serum testosterone levels on days 0, 3, and 5 following 5,000 IU of hCG administered intramuscularly on days 1, 2, and 3. LH pulses were determined according to previously established criteria.[13] All results are expressed as the mean ± S.E.M. Comparison of responses during phases I through III of the study were made to the initial responses

to the $LHRH_A$. Responses during phase IV were compared with the results of the baseline status as determined by LH and FSH levels every 20 minutes for 12 hours. Two-tailed *t* testing was employed to determine statistical significance.

RESULTS

Baseline Status and Initial $LHRH_A$ Response

During the 12-hour baseline monitoring prior to $LHRH_A$, mean LH level was 0.5 ± 0.02 mIU/ml; FSH, 1.3 ± 0.05 mIU/ml; testosterone, 55 ± 19 ng/dl; and estradiol, < 20 pg/ml. No discrete LH pulses were observed. Testicular size was 4.5 ± 0.8 ml. Testes biopsies revealed varying degrees of immaturity of seminiferous tubular development and Leydig cell morphology. In one subject, there was no distinct tubular formation but the specimen was quite small.

The initial response to $LHRH_A$ resulted in an LH rise to 1.1 ± 0.4 mIU/ml ($P < 0.01$ vs. baseline), which persisted for 18 hours (Fig. 29-1, Panel A). The FSH maximum of 2.8 ± 1.8 mIU/ml ($P < 0.01$ vs. baseline) occurred 30 minutes later and also persisted for the duration of the sampling period. Gonadal steroids did not change throughout the initial $LHRH_A$ exposure.

Chronic $LHRH_A$ Administration

Phase I: 50 μg $LHRH_A$ Every 48 Hours. Within 2 weeks of administration of 50 μg $LHRH_A$ every other day, several changes from the pretreatment status were evident. All subjects volunteered that testicular discomfort had occurred and was most evident on the same day as the injection. One subject noted descent of an abdominal testis into the scrotum by 14 days of therapy. Testicular size had begun to increase by 2 weeks and achieved a new plateau (6.5 ± 0.8 ml, $P < 0.05$ vs. baseline) by 6 to 8 weeks of treatment (Fig. 29-2). The only subject failing to experience a change in testicular size lacked distinct seminiferous tubular structures in his testicular biopsy.

The gonadotropin responses to the standardized 10-μg $LHRH_A$ test had similarly changed (Fig. 29-1, Panel B). The peak LH level achieved was now 5.0 ± 0.5 mIU/ml at 105 minutes ($P < 0.001$ vs. initial response peak) with an FSH peak of 2.0 ± 0.3 mIU/ml occurring 45 minutes later (NS vs. initial response peak, $P < 0.05$ vs. baseline). Serum testosterone and estradiol levels did not change throughout the 24-hour period. This pattern of pituitary–gonadal responsiveness was established after 14 days of $LHRH_A$ therapy and did not significantly vary from 2 to 8 weeks of treatment in any of the patients.

Phase II: 50 μg $LHRH_A$ Daily. Increasing the frequency of $LHRH_A$ to daily administration caused all signs of a response to disappear. All symptoms subsided and mean testicular volume returned to 5.2 ± 0.4 ml which was indistinguishable from baseline (Fig. 29-2). Gonadotropin responses were

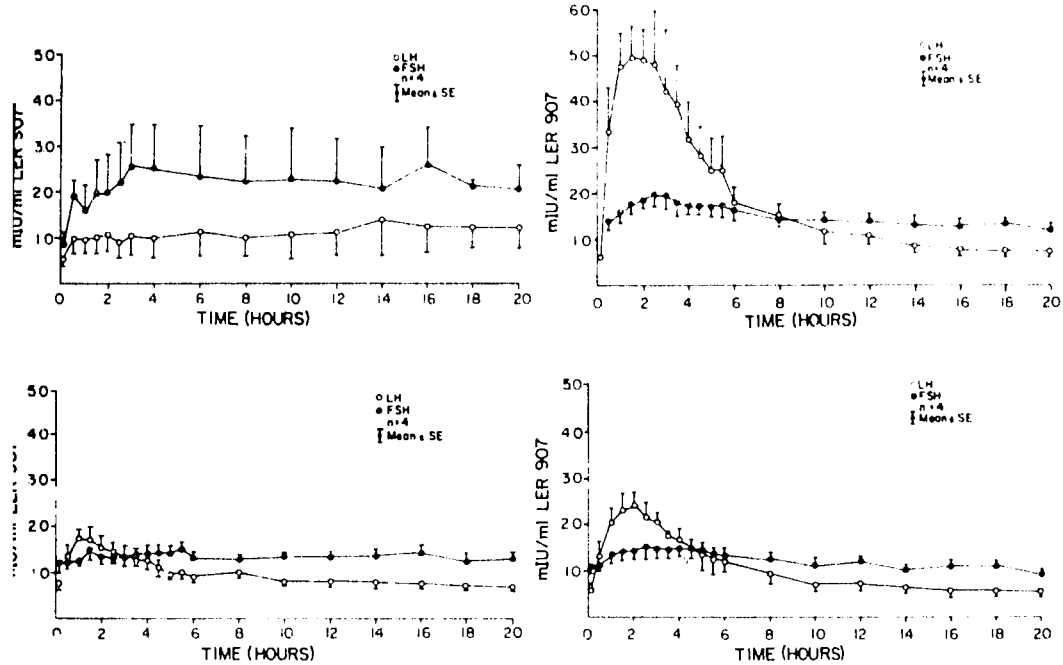

FIG. 29-1. LH (○) and FSH (●) responses of four men with hypogonadotropic hypogonadism to 10 μg [D-Trp⁶-Pro⁹-NEt]-LHRH ($LHRH_A$) following various treatment schedules. (*A, Upper left panel*) Initial responsiveness to $LHRH_A$ prior to treatment. (*B, Upper right panel*) Responsiveness following administration of 50 μg $LHRH_A$ on alternate days for 6 to 8 weeks. (*C, Lower left panel*) Responsiveness following administration of 50 μg $LHRH_A$ daily for 2 to 4 weeks. (*D, Lower right panel*) Responsiveness following administration of 10 μg of $LHRH_A$ daily for 2 to 4 weeks. All results are expressed as mean ± S.E.M. (See text for details.)

similarly depressed. An LH peak of 1.7 ± 0.3 and an FSH peak of 1.5 ± 0.2 mIU/ml occurred at 90 minutes. Neither of these differed significantly from the initial $LHRH_A$ response (Fig. 29-1, Panel A vs. C). Gonadal steroid levels also remained unchanged.

While $LHRH_A$ administration was continued at 50 μg daily, Leydig cell responsiveness to hCG was reassessed and compared with that obtained pretherapy in three of the four patients. Figure 29-3 compares the pretreatment hCG response with that observed during daily administration of 50 μg $LHRH_A$. The peak testosterone on day 5 of 230 ±80 ng/dl did not differ significantly from that obtained prior to $LHRH_A$ administration.

Phase III: 10 μg $LHRH_A$ Daily. When the dosage of $LHRH_A$ was reduced to 10 μg daily for 2 to 4 weeks, no further clinical changes were evident. Testis volume did not vary significantly (5.0 ± 0.4 ml) from pretreatment size. Response to the 10 μg $LHRH_A$ bolus, however, was significantly different. LH responsiveness was partially restored with a peak LH level of 2.4 ± 0.3 mIU/ml at 2 hours and a peak FSH response of 1.5 ± 0.3 mIU/ml (Fig.

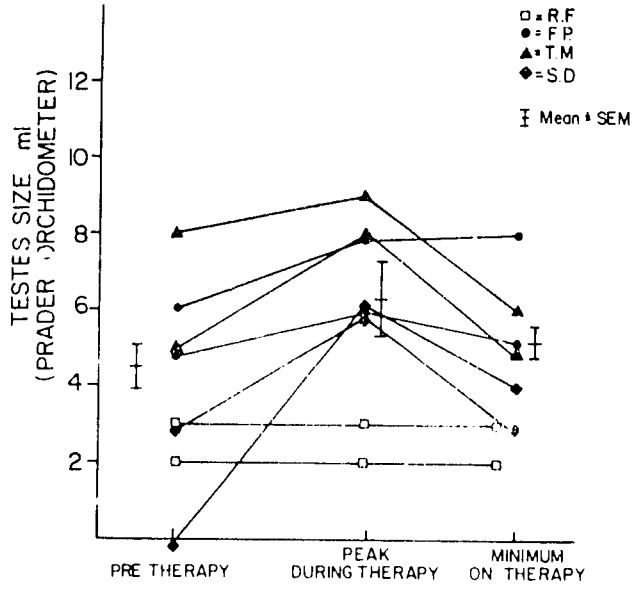

FIG. 29-2. Testicular size in four hypogonadotropic males before treatment (pretherapy), with 50 μg $LHRH_A$ on alternate days for 6 to 8 weeks (peak during therapy), and with 50 μg $LHRH_A$ daily (minimum on therapy). All results are mean ± S.E.M. (See text for details.)

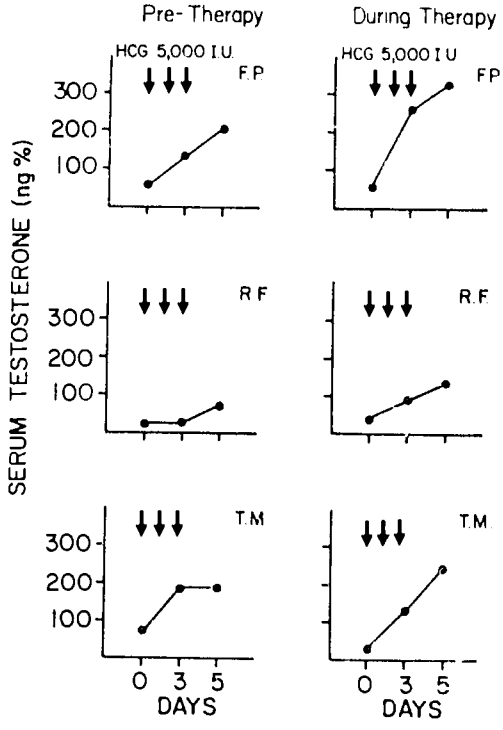

FIG. 29-3. Leydig cell responsiveness to hCG (5,000 IU × 3 days) in three hypogonadotropic men before (pretherapy) and during daily administration of 50 μg $LHRH_A$. All results are expressed as mean ± S.E.M. (See text for details.)

29-1, Panel D) occurring 60 minutes later. This represents a significant return of LH responsiveness ($P < 0.05$ vs. initial peak) while FSH response remained unchanged. No change in serum testosterone or estradiol levels occurred.

Phase IV: Pulsatile Native LHRH, 25 ng/kg Every 2 Hours. Following cessation of $LHRH_A$ therapy for a minimum of 3 months, the patients received LHRH in a pulsatile fashion at a dose of 25 ng/kg every 2 hours for 7 days. The pituitary response to this mode of administration of the natural sequence decapeptide was markedly different from that attending $LHRH_A$ administration.

Figure 29-4 depicts the 8 a.m. levels of gonadotropins obtained immediately prior to a pulse of LHRH. The baseline LH of 0.6 ± 0.1 rose to 1.0 ± 0.2 mIU/ml, the increase becoming significant ($P < 0.05$) only on the seventh day of therapy. In contrast, the basal FSH levels of 1.1 ± 0.1 mIU/ml rose significantly to 2.4 ± 0.3 mIU/ml ($P < 0.01$) following 2 days of treatment and to a final level of 6.4 ± 1.5 mIU/ml by day 7 ($P < 0.01$). Figure 29-5 contrasts the gonadotropin responses to the first bolus of 25 ng/kg of LHRH on day 1 (NS vs. baseline) with those to the last bolus on day 7 ($P < 0.001$ for both LH and FSH vs. baseline). In five of the six men, the first significant pulse of FSH following an LHRH bolus was evident within 48 hours of therapy. The establishment of significant LH pulsations in response to LHRH, however, required 4 days of administration in five of the six men. The pretreatment testosterone levels of 48 ± 11 ng/dl rose linearly to a maximum of 296 ± 114 ng/dl ($P < 0.05$) on day 7.

DISCUSSION

Previous evidence indicating a hypothalamic defect in hypogonadotropic hypogonadism includes anatomic changes in the hypothalamus of some postmortem specimens,[20] clomiphene responsiveness of a subset of hyposmic

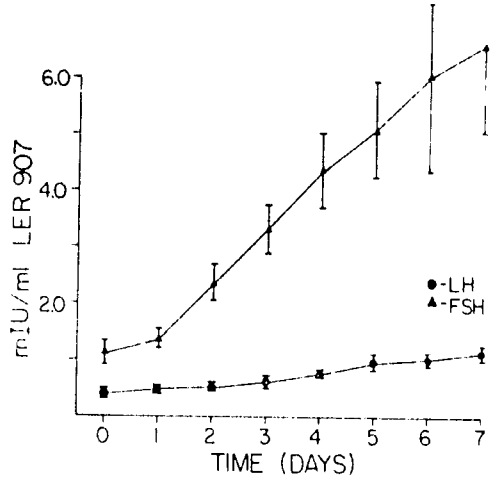

FIG. 29-4. LH (●) and FSH (▲) levels at 8 a.m. for six hypogonadotropic men, obtained immediately prior to a pulse of 25 ng/kg LHRH during 7 days of 2-hour administrations. Results are expressed as mean ± S.E.M. (See text for details.)

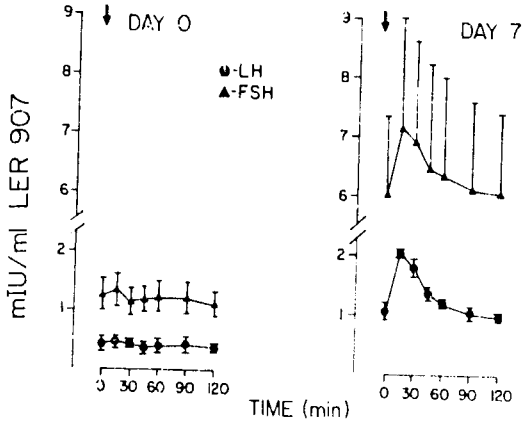

FIG. 29-5. LH (●) and FSH (▲) responses to a bolus of 25 ng/kg LHRH at the beginning (day 0) and end (day 7) of 1 week of 2-hour administrations of LHRH in six hypogonadotropic men. All results are expressed as mean ± S.E.M. (See text for details.)

men,[22] and increases in LH and FSH following administration of LHRH and/or its analogs.[11,21,26,27,31,32,34,38,40,46,48,49,53] To these observations, we added the absence of endogenous LH pulsations in each of our subjects. Accordingly, these patients appear to have a selective defect in the physiology of their endogenous LHRH. As such, they offer a unique circumstance in which to define the physiology of LHRH in man, since interpretation of their gonadotropin responses following administration of gonadotropin releasing factor or its analogs is not obscured by consideration of the role of their endogenous LHRH.

The protocol employed was designed to explore systematically the nature of dosage, frequency of administration, and duration of LHRH effect in determining pituitary and gonadal responses. Initially a dose of 50 μg of the long-acting agonist, [D-Trp^6-Pro^9-NEt]-LHRH, was administered at 48-hour intervals. Definite clinical and biochemical changes ensued but were transient in nature (16 hours or less). Within 14 days, modest increases in testicular size and changes in pituitary responses were apparent. The initially low LH/FSH ratio of gonadotropin secretion, characteristic of the "prepubertal" state, was converted to the predominant LH secretion more typical of the adult male pattern. Moreover, this secretory response of the gonadotrophs appeared stable over 2 to 8 weeks of therapy and did not exhibit the declining responsiveness described by others during chronic LHRH$_A$ or high-dose LHRH administration.[8,11,19,21,25,34,46,49,51] Once a plateau had been achieved, the frequency of LHRH analog administration was increased to a daily regimen while the dosage was held constant. All symptoms promptly abated, testicular size returned to baseline, and gonadotropin responsiveness was nearly extinguished. Finally, the frequency of analog administration was held constant while the dosage was decreased to 10 μg daily. Although this regimen proved insufficient to cause clinical changes, it did permit a clear-cut restoration of LH responsiveness.

The final phase of the protocol then employed pulsatile delivery of the natural sequence LHRH. The schedule of administration every 2 hours was designed to simulate the frequency of endogenous LH pulses observed in normal men.[43] The dosage employed had been demonstrated to achieve levels

of LH peaks in the peripheral circulation similar to those observed in adult men.[30] With this regimen, a strikingly different pattern of gonadotropin release was observed. Initially, there was no response to the first bolus of LHRH. Thereafter, a predominantly FSH response was observed during the first 7 days. FSH pulsations became evident on the second day of treatment, whereas significant LH pulses did not appear until treatment had been continued for 4 days. Further evidence accumulated during longer periods of administration of 2-hour pulsatile LHRH confirms that declining pituitary responsiveness is not observed with this regimen.[10] It thus appears that pituitary responsiveness to the gonadotropin releasing factor is critically dependent upon dosage, frequency of its administration, and duration of action of the LHRH species utilized.

Comparison of these results with previously published experience with higher doses of LHRH and or its agonistic analogs is complicated by differences in protocol design and compounds used. Additionally, none of the prior studies has attempted to decrease the dose of the analog or LHRH used; nor has the effect of pulsatile delivery of the natural sequence peptide been contrasted in the same patients. Nonetheless, previous observations appear consistent with these conclusions. Following a transient period of improved gonadotropin secretion, decreasing responsiveness of first FSH and then LH is usually observed in both normal and hypogonadotropic subjects.[5,11,19,21,23,31,39,46,49,51] With most investigators employing an interval of 24 hours or less between doses, they encountered the same pituitary refractoriness that we observed with daily administration of 50 μg of [D-Trp[6]-Pro[9]-NEt]-LHRH. Significantly, the only study resulting in no decrease in gonadotropin secretion during LHRH analog administration employed a 48-hour dose interval.[41] This observation is consistent with the responses observed during phase I of our protocol.

Our exception to the universal failure of large doses of LHRH and its analogs to restore pituitary and gonadal secretions is the initial report of effective treatment of hypogonadotropic men with thrice-daily administration of 500 μg LHRH.[48] However, subsequent workers have found such successes to be the exception rather than the rule.[11,31,49] It may be, as suggested by Smals and co-workers,[47] that in some hypogonadotropic subjects the predominant deficiency is one of LH. In such individuals, the requirement for FSH secretion may be modest and mere restoration of LH secretion may suffice to permit return of normal gonadal function. Total normalization of gonadal function with hCG alone has occasionally been reported in hypogonadotropic patients, suggesting that such may be the case.[43]

The cause of the pituitary refractoriness induced by large doses of LHRH or its analogs is unclear. Several hypotheses have been suggested. Antibody formation[48] or altered metabolism of the compounds[19] are precluded by one demonstration of return of partial pituitary responsiveness with lowering of the $LHRH_A$ dose. Blunting of response due to steroidal feedback appears unlikely, since this phenomenon occurred despite castrate levels of gonadal steroids. Depletion of pituitary gonadotropin pools,[19] observed during chronic LHRH administration in some species,[4] has not been observed in others.[9,41] Moreover, even in species in which exhaustion of gonadotropin stores has been documented, the onset of pituitary refractoriness to further

LHRH stimulation occurs prior to depletion of the gonadotrophs.[41] Evidence for down-regulation of pituitary LHRH receptors has similarly been lacking. When LHRH receptor kinetics have been quantitated during treatment with pharmacologic doses of LHRH or its analogs, receptor affinity appears unaltered while their numbers have remained unchanged or have increased slightly.[28] Thus, it appears that a postreceptor defect in gonadotropin synthesis or release, or perhaps an imbalance between these two processes, may account for this phenomenon. Such a differential effect of a releasing factor upon synthesis and release of prolactin has been demonstrated during TRH administration to cloned pituitary cells,[18] suggesting these two processes may be differently controlled despite their direction by a single trophic hormone.

Preliminary experience with pulsatile LHRH delivery is more limited. Initial reports indicated that 50 μg LHRH administered at 4-hour intervals to hypogonadotropic men resulted in modest improvements of gonadotropin secretion but absent gonadal responses.[30] Hourly administration of 1 μg/kg of LHRH to castrate monkeys stereotactically rendered LHRH deficient successfully reestablished their hypergonadotropic state.[7] This same regimen has also established normal menstrual cycles in similarly lesioned monkeys with intact ovaries and in prepubertal rhesus monkeys.[31,32] The pituitary–gonadal quiescence of seasonal breeding rams has similarly been overcome by pulsatile delivery of 100 to 500 ng of LHRH at 2-hour intervals.[33] A 5-day course of pulsatile LHRH in doses and frequency similar to that employed in phase IV of the present protocol restored gonadotropin and partial gonadal secretion in three patients with anorexia nervosa.[36] An FSH-predominant pattern of gonadotropin secretion analogous to that observed during phase IV of the present study was observed by these workers. Nocturnal delivery of pulsatile LHRH at a dosage of 40 μg/hour for 10 days in two men with Kallman's syndrome was successful in establishing nighttime elevations of gonadotropins in both and testosterone in one.[34]

It thus appears that the gonadotroph can respond in at least two ways to stimulation with LHRH or its analogs, depending upon its dosage and frequency of administration. Low-dose pulsatile administration of the shorter-acting LHRH elicits the more physiologic secretory pattern, and high doses of LHRH or longer-acting analogs extinguish gonadotropin secretion.

Given the two distinctly different responses of the pituitary–gonadal axis to LHRH stimulation, it may be possible to exploit both therapeutically. When the more physiologic program is desired, as in induction of ovulation or initiation of puberty, the low-dose pulsatile mode of LHRH administration is required.[17] Failure of previous efforts to succeed in these circumstances[23] may well be attributed to the relatively unphysiologic regimen of LHRH administration. Conversely, when pituitary suppression is desired, as in treatment of precocious puberty, contraception, or hormonal withdrawal therapy of malignancies, higher doses of LHRH or its long-acting agonist are indicated. Investigation into both of these aspects of the gonadotroph response is presently under way in this laboratory.

The lack of gonadal steroid responses to $LHRH_A$ administration is remarkable. While the absence of testosterone (and estradiol) responses permitted a unique opportunity to study gonadotropin responsiveness to the $LHRH_A$ without concern for the role of changing steroid concentrations, this

phenomenon occurred in the face of high levels of endogenous LH. Several possible explanations were considered. That the LH bioactivity was diminished was excluded by the finding that biologic and immunologic activity were proportional as examined in the dispersed Leydig cell assay over a wide range of serum levels.[21] Previous experience has also demonstrated that the LH secreted following $LHRH_A$ stimulation is capable of elevating endogenous gonadal steroids in normal men.[15] Finally, the gonadotropins secreted in response to pulsatile LHRH stimulation proved capable of elevating testosterone levels in the same subjects. All of these observations indicate that the gonadotropins secreted under the influence of the $LHRH_A$ are biologically active.

Since previous studies have documented a direct gonadal effect of LHRH and its analogs,[4,9,13,29] a repeat hCG stimulation test was performed during daily administration of 50 μg of the $LHRH_A$ and compared to the pretreatment test. Since the two tests revealed similar responses, no direct gonadal effect was apparent employing these criteria. The possibility remains, however, that any direct gonadal effect could have been overridden by the large doses of hCG. A similar ability of hCG to overcome the direct inhibitory effect of LHRH on steroidogenesis in dispersed luteal cells has been observed.[13] Alternatively, the direct gonadal effects of these analogs may not be mediated primarily by Leydig cells.[5]

An attractive explanation for the failure of gonadal responses during $LHRH_A$ therapy is attributable to the relatively unphysiologic mode of LH delivery to the gonad. Several authors have noted a desensitization of testicular steroidogenesis during hCG administration or the prolonged endogenous LH stimulation following $LHRH_A$ use.[4] Certainly, the testosterone rises following pulsatile LHRH administration were quite impressive, suggesting that the testis, like the pituitary, might prefer episodic to continuous trophic stimulation. It appears more data will be required to clarify this issue.

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Discussion: Studies of LHRH Analogs in the Human Male

ELSIMAR METZKER COUTINHO, MODERATOR

Clinical trials with LHRH analogs have confirmed the expected effects on the secretion of pituitary gonadotropins and secondary effects of gonadal steroidogenesis. The discussion dealt mainly with the significance of the endocrine changes resulting from down-regulation of the pituitary and alterations in gonadotropins and testosterone secretions. One investigator indicated that LHRH treatment results in variable decline in basal gonadotropin levels, while testosterone is markedly depressed throughout treatment. He pointed out that in his patients, gonadotropins remain within the basal level and would be considered normal except that they are inappropriately low for ambient steroid levels. On the question of dosage, however, LHRH can be administered in a pulsatile form, thus eliminating some of the down-regulating effects. Leydig cells seem to respond to pulsatile LH.

How does hCG work? There is some evidence of desensitization of acute testicular response to hCG in terms of testosterone production. If hCG levels remain elevated, serum testosterone levels will eventually decline to prepubertal levels. In speculating on species differences in the various responses of male animals to GnRH agonists, one investigator reported that the administration of GnRH agonists in rats was accompanied by lowered prolactin secretion and elevated progesterone levels; those changes might mediate some of the antagonistic effect of the agonist. In a study in humans, on the other hand, no elevation of progesterone was seen during the 30-week treatment period. However, if more blood samples had been taken, some transient elevations in progesterone might have been detected.

An investigator in Sweden found that LH responses to GnRH tests increased following the discontinuation of treatment with Buserelin. Therefore, GnRH agonists may induce the down-regulation of receptor levels in the target organ, and in this aspect they differ from steroids. Prolonged therapy of GnRH agonists results in the deficiency in gonadotropin utilization. In the Swedish study, the fall in testosterone levels was moderate, and a complete suppression of LH responses to the agonists was never observed. Some of the differences between that work and American studies could be due to the dose and time of administration. One participant was intrigued by a report that

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Panelists: William F. Crowley, Jr., Nacia Faure, David Rabin. *Discussants:* Joseph W. Goldzieher, Fernand Labrie, Kamran S. Moghissi, Catherine Rivier, Jurgen K. Sandow

there were reproducible increases in estradiol in the presence of low testosterone levels; results may vary according to how often blood is drawn, however. In long-term treatment with the analogs, no elevation in estradiol is observed. In fact, a fall in estradiol levels has been found. Some of these effects may be due to inhibition of 17α-hydroxylase activity. Regarding timing and dosage, estrogens have a profound inhibitory effect of 1α-hydroxylase and 7,20-desmolase so that the link between the two steroidogenic blocks may be due to the fact that the aromatase is continually stimulated by LH and desensitized at a slower rate.

Major conclusions of the discussion were that investigators with considerable experience in clinical trials involving men have learned that the first and most pressing question to be raised even before starting the treatment is whether, and in what manner, the treatment affects sexual behavior. The problem of loss of libido and potency is difficult to solve in most treatments and it stands alone as the main block to development of an acceptable male contraceptive. Certainly, drugs that inhibit spermatogenesis are available. But, with the exception of gossypol, most of them inhibit gonadotropin secretion, reduce testosterone levels, and cause loss of libido and potency.

From the presentations considered in this section, it seems that LHRH analogs have the same potential and unfortunately the same undesirable side-effects displayed by previous candidates for a male contraceptive. Unquestionably, some LHRH analogs may effectively suppress spermatogenesis and render a man infertile for as long as the treatment lasts. However, loss of libido will be common, because of the inhibition of gonadotropin secretion and lowered testosterone levels. Therefore, unless we decide to turn men into seasonal breeders, these analogs cannot be recommended as male contraceptives. As mores and morals undergo rapid changes, however, this possibility may be explored by the leaders of our "brave new world." Seasonal breeding is widespread in animal species and offers advantages to numerous animals whose survival depends on this special reproductive strategy. Men treated with an LHRH analog seem to enter a rest phase that is as sexless as that of a seasonal breeder during the winter. The buck, during this phase of low sexual activity, will abandon the female, shed its antlers, and develop testicular atrophy. He fails to recognize a female as such and refuses to fight another male. He concentrates his activity on eating and resting. Between two mating seasons, he accumulates energy. At the end of the rest phase, his testicles are reactivated, atrophy subsides, testosterone-dependent behavior is reestablished, the animal searches for females, marks territories, fights other males for the possession of the females, and copulates frequently. The rutting season of many hoofed mammals is a time of intense and continuous sexual activity. As soon as the breeding season is over, no more mating occurs. Would men and women accept having a season for sex? It seems that depending on the circumstances, seasonal sex may become acceptable and even desirable. For the time being, however, this will probably be a difficult idea to sell. In order to make the present-day LHRH analogs acceptable, we must continue the treatment with androgen replacement.

A final comment regards the mode of administration of LHRH analogs as contraceptives. Various reports indicate the need for daily administration. If

we are trying to develop a substitute for the presently available contraceptives, however, we must aim at alternatives that offer substantial advantages over the pill. One such advantage would be long-lasting action, which would obviate the need for daily administration. None of the analogs discussed here, which are the first compounds to reach the clinical trial stage, presents long-acting possibilities. It is likely that some of those in earlier stages of development will offer this advantage.

SAFETY AND SECONDARY PHARMACOLOGIC STUDIES OF LHRH ANALOGS

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Metabolic and Dispositional Studies with LHRH Analogs

JURGEN K. SANDOW

GERTRAUD JERABEK–SANDOW

BIRGIT KRAUSS

WILFRIED STOLL

The control of fertility by new methods requires characterization of the therapeutic agents with regard to the mechanisms of drug distribution and drug inactivation in the body. The highly active LHRH nonapeptide agonists studied today in the development of a peptide contraceptive were derived from the LHRH decapeptide by chemical substitution.[23] The result has been a striking increase in biologic activity, so that doses of 1 to 2 μg subcutaneously or 100 to 200 μg intranasally are given as therapeutically effective doses in the human. In experimental animals, the biologically active doses are minute, and in the rat, potent LHRH nonapeptide-ethylamide agonists elicit LH-release at doses of 1 to 5 ng subcutaneously.[27]

We have studied the pharmacokinetics, organ distribution, and metabolic inactivation of LHRH (Fig. 31-1) in comparison with the highly active agonist, [D-Ser(TBU)^6]-LHRH (1-9) nonapeptide-ethylamide (Buserelin, Hoe 766). This agonist will be discussed as an example for a group of similar highly active peptides substituted in position 6 (D-Ala, D-Leu, D-Trp) and position 10 (Pro-ethylamide), or containing additional substitutions in position 7 (N-Me-Leu) or amino acids with protected side-chains like Ser (TBU) or Lys (BOC). Similarly, to the strategy used in our laboratory for the biologic evaluation of the contraceptive effects, we have systematically compared the distribution and inactivation of the LHRH decapeptide with several agonists. It was found that the increased enzyme resistance of agonists facilitated studies on enzymes degrading the LHRH decapeptide.[7,10,11,28] Thus, the agonists serve as model peptides for a better understanding of LHRH inactivation (because some of the enzyme cleavage sites in the LHRH molecule are blocked in agonists), for the regulation of hormone action at the receptor site,[6] and also provide important information for the contraceptive application of the agonists.

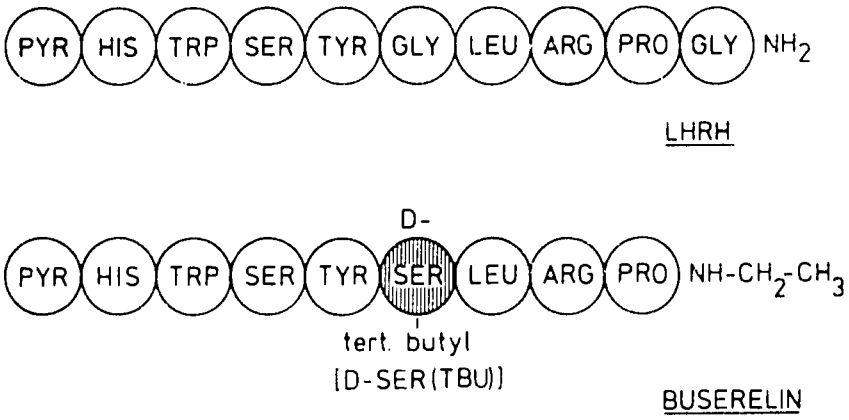

FIG. 31-1. Structure of the LHRH decapeptide and of the LHRH nonapeptide agonist, [D-Ser(TBU)^6]-LHRH (1-9) nonapeptide-ethylamide (Buserelin, Hoe 766).

PHARMACOKINETICS OF LHRH AGONISTS

After intravenous injection, LHRH is rapidly cleared from plasma. The plasma concentrations reached after high doses of LHRH are sufficient for bioassay. Serum samples of animals injected with LHRH are tested for the LH-release induced in ovariectomized, estrogen-progesterone treated rats.[8,16,20] The LH-releasing activity in serum decreases rapidly, with a half-life of 4 to 5 minutes. This bioassay procedure is unequivocal, because all LHRH fragments have very low biologic activity. Similar studies with agonists have confirmed that the biologic activity in serum decreases with the same half-life as LHRH.[8,28] Bioassay determination requires doses of 100 μg LHRH IV in rats, to reach serum levels detectable in bioassay, or doses of 2 to 5 μg of nonapeptide agonists. In contrast to IV injection, serum elimination is considerably prolonged when agonists are infused. After termination of an infusion of [D-Trp^6]-LHRH, the serum half-life was 17.3 minutes.[2] However, in sheep, the activity of infused LHRH or [D-Leu^6]-LHRH (1-9) nonapeptide-ethylamide decreased with identical half-life.[8]

Agonists labeled with ^{125}I are suitable tracer preparations for pharmacokinetic studies, because the [mono ^{125}I-Tyr^5] peptides retain their biologic activity.[17,26,28] After IV injection of 1 μCi (5 ng peptide) of ^{125}I Buserelin, plasma clearance is rapid during the first phase of elimination, followed by a second slower phase. This type of elimination is identical with the biphasic pattern observed after ^{125}I LHRH.[27,29] The physical nature of the labeled material circulating in serum can be assessed by thin-layer chromatography of serum extracts (Fig. 31-2). During the first phase of elimination, intact ^{125}I Buserelin is identified in serum extracts, whereas during the second phase no peptide material can be detected. This phase is characterized by high uptake of ^{125}I into the thyroid, and the radiolabel in serum corresponds to circulating ^{125}I and ^{125}I tyrosine resulting from metabolic inactivation.[22,29]

When serum concentrations after injection of unlabeled Buserelin are measured by radioimmunoassay, multiphasic elimination is also noted. Dur-

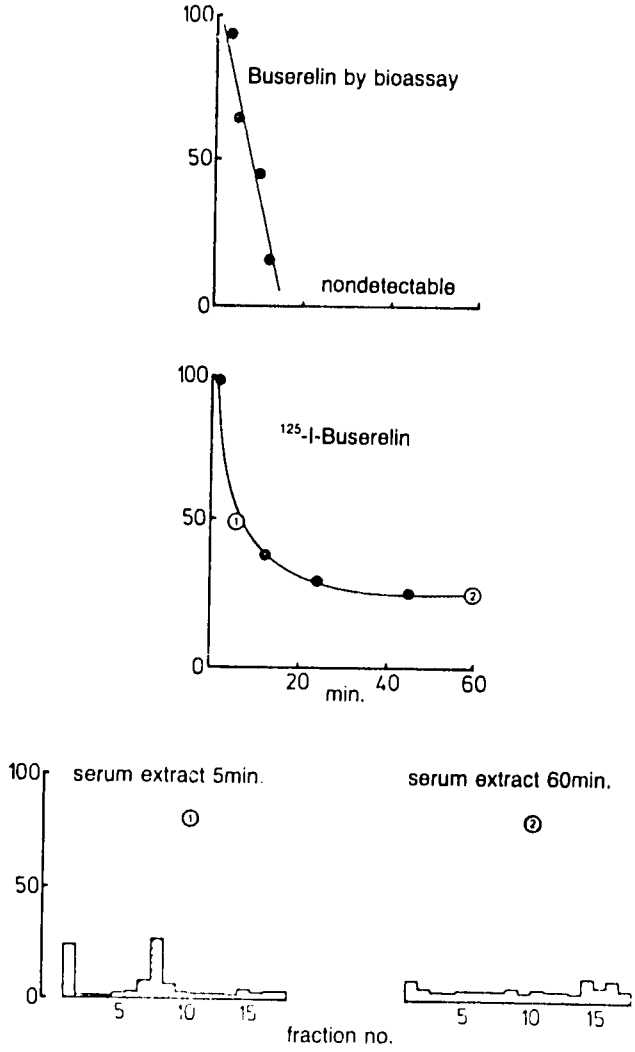

FIG. 31-2. Plasma elimination of Buserelin after intravenous injection: Unlabeled Buserelin measured by bioassay (percent initial activity, LH-release induced in rats). Radiolabel resulting from ^{125}I Buserelin injection measured by gamma counting (percent initial radioactivity). Serum extracts analyzed by thin-layer chromatography (percent radiolabel per fraction). Serum extract 5 minutes, contains intact Buserelin (shaded area), serum extract 60 minutes, does not contain labeled peptide material. Circulating radiolabel is ^{125}I-Na resulting from metabol inactivation.

ing the initial phase, immunoreactive Buserelin decreases with a half-life of 3 to 6 minutes, followed by a second slow component between 18 and 30 minutes in sheep.[27,28] The slow phase of elimination is due to the presence of newly formed Buserelin metabolites, which are measured together with intact Buserelin by certain nonapeptide antisera. When antisera with prefer-

ential affinity for Buserelin metabolites are used,[21] elimination is considerably prolonged. During infusion experiments in rats with 2.5 to 25 μg Buserelin per hour, a gradually increasing level of immunoreactive material is found. This material consists of C-terminal Buserelin metabolites with greatly reduced biologic activity. After the infusion, the concentration of Buserelin metabolites decreases with a half-life of 60 to 90 minutes. When Buserelin was infused from osmotic minipumps at concentrations of 5 to 5,000 ng per day, we were unable to find a detectable level of immunoreactive Buserelin in serum, indicating that at biologically effective concentrations, elimination of fragments proceeds rapidly. Greatly increased rates of infusion (60 μg/day) are required for measurable accumulation of Buserelin fragments in serum. After nasal administration of 4 mg Buserelin in rabbits, prolonged elevation of Buserelin serum levels was noted with a half-life of 30 to 60 minutes.

The physical half-life of intact Buserelin in serum is clearly different from the duration of induced gonadotropin release. After IV or subcutaneous injection, Buserelin in serum decreases to nondetectable levels, even though LH and FSH release is maintained for 6 to 8 hours. The prolonged biologic action is maintained by binding of a small fraction of peptide to anterior pituitary receptors,[5,6,13] independently of detectable quantities of Buserelin in serum. This dissociation of drug level and biologic effect is also found with other oligopeptide hormones (*e.g.*, vasopressin and oxytocin).

ORGAN DISTRIBUTION STUDIES

In studies with ^{125}I LHRH and agonists (Fig. 31-3), labeled material accumulates in four organs of rats: anterior pituitary (specific receptors, biologic target organ); liver and kidneys (main inactivating organs); and thyroid gland (from uptake of ^{125}I resulting from metabolic breakdown). Uptake into ovaries of rats has been reported in the context of a direct ovarian site of action of agonists.[15] In our own studies, we have not found uptake into the testes of mature male rats. Organ distribution and metabolic inactivation were studied in male rats injected with 10 μCi (50 ng peptide) of ^{125}I Buserelin or ^{125}I LHRH. At autopsy, 60 minutes after IV injection of ^{125}I LHRH, the liver contained 1.61% of the dose injected, the kidneys contained 1.33%, and pituitary accumulation was not detectable. The thyroid contained 0.27% of the dose. In contrast, after ^{125}I Buserelin, significantly higher fractions of label accumulated in liver (21%), kidneys (5.3%), and anterior pituitary (0.025%). In the thyroid gland, 0.24% of the dose was recovered. The finding of increased pituitary accumulation of agonists[15] is in agreement with our own studies on the correlation of organ accumulation and biologic activity of nonapeptide analogs of increased biologic activity.[26] In contrast, LHRH is only transiently accumulated in the pituitary.[19,27]

The physical nature of the material accumulated in the different organ tissues was assessed in organ extracts. The metabolites were extracted from boiled tissues by a stepwise solvent extraction procedure, using 1-N acetic acid, ethanol, acetone, triton-X 100, and 1-N NaOH, and identified by thin-layer chromatography, with ^{125}I labeled peptide fragments as reference com-

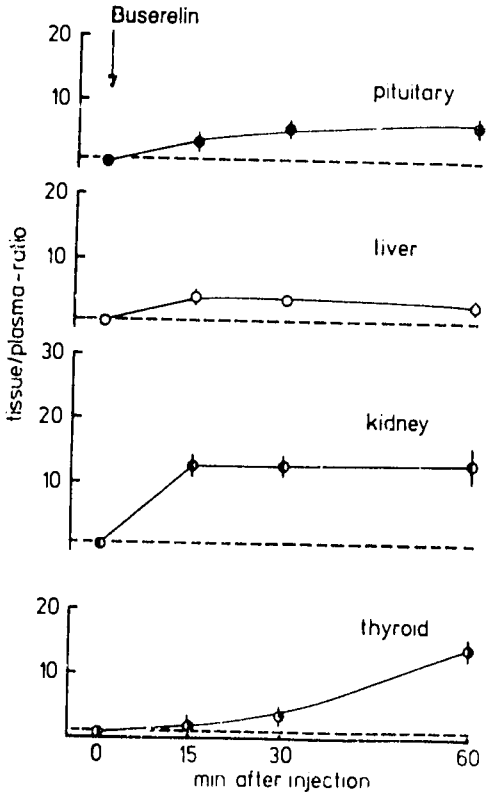

FIG. 31-3. Organ distribution of ^{125}I Buserelin in rats: After IV injection of 1 μCi (5 ng peptide), radiolabel is accumulated in anterior pituitary, liver, kidney, and thyroid. Radiolabel in pituitary, liver, and kidney was identified as peptide material. (Means and S.E. of four rats per group.)

pounds. For further analysis, metabolites were extracted from rat tissue in sufficient quantity for enzyme degradation studies, and additionally characterized by binding to specific nonapeptide antisera (Fig. 31-4).

IDENTIFICATION OF METABOLITES *IN VIVO*

At near-physiologic concentrations of LHRH agonists, the doses injected are minute and identification of metabolites is only possible by tracer techniques. In our own method, identification of metabolites was achieved using a combination of different techniques, namely stepwise solvent extraction, thin-layer chromatography, binding to peptide antisera, and enzyme degradation of the labeled material extracted from different tissues. After ^{125}I LHRH, there was no intact peptide material in either liver or kidney, at autopsy 60 minutes after injection. In contrast, 60 minutes after ^{125}I Buserelin injection, several metabolites were identified in extracts of anterior pituitary, liver, kidney and serum. In the anterior pituitary, a metabolite M I was found,[5] which was not present in either liver, kidney or thyroid gland[1] (Fig. 31-5). This metabolite was also obtained *in vitro* after incubation with a pituitary arylamidase preparation. In liver and kidney, extraction with 1-N acetic acid followed by ethanol provided a material which did not contain metabolite M I

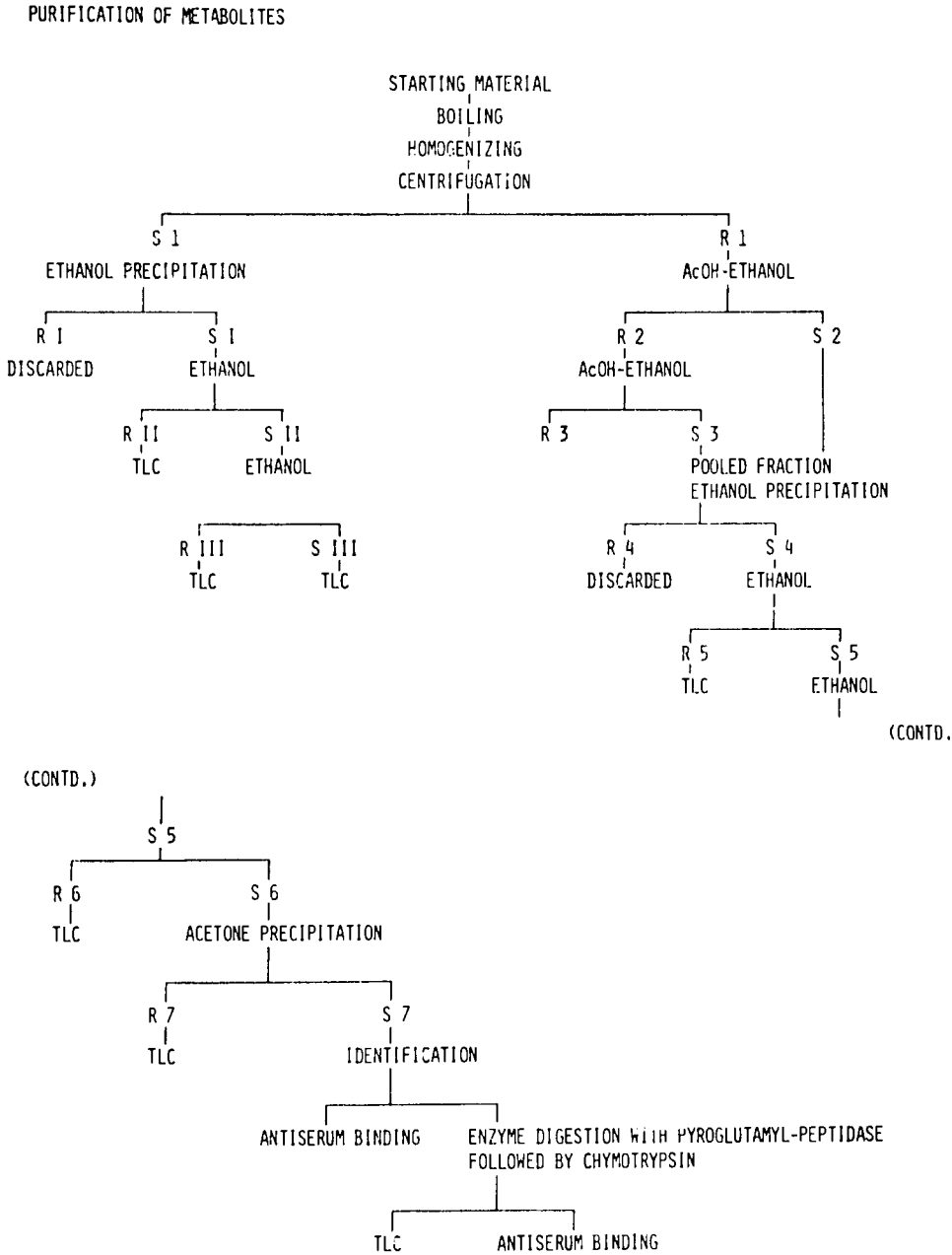

FIG. 31-4. Extraction of peptide metabolites from liver and kidney of male rats after treatment with [illegible] μCi (50 ng peptide) ^{125}I Buserelin. Additional identification at final step by enzyme digestion and binding to specific Buserelin antisera before and after enzyme digestion. (S = supernatant fraction, R = residue. TLC = identification by thin-layer chromatography on silica plates.)

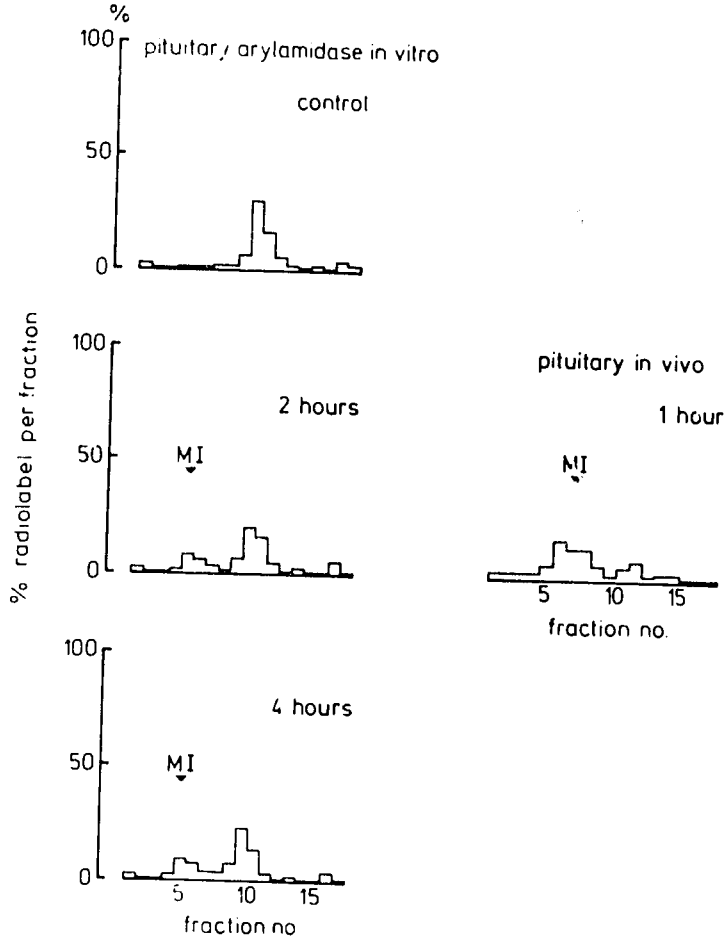

FIG. 31-5. Identification of a pituitary metabolite of Buserelin in rats *in vivo*. Degradation pattern on TLC plates is identical with degradation pattern after incubation with pituitary arylamidase *in vitro* (10,000 × g supernatant fraction of pituitary homogenate).

and had Rf values compatible with either unchanged Buserelin or the Buserelin (4-9) hexapeptide. Subsequent identification by enzyme degradation with pyroglutamyl-aminopeptidase (PGP)[1] confirmed the absence of intact Buserelin, and identified this material as the (4-9) hexapeptide. In the thyroid gland, labeled material was extracted by 1-N NaOH. This material was identified as ^{125}I.[25] No labeled peptides were found in the serum 60 minutes after injection of ^{125}I Buserelin. However, when degradation was competitively inhibited by pretreatment with 5 μg of unlabeled [D-Phe^2,Phe^3,D-Phe^6]-LHRH, a competitive LHRH antagonist, a metabolite similar to M I previously identified in the anterior pituitary was also found in serum (Fig. 31-6).

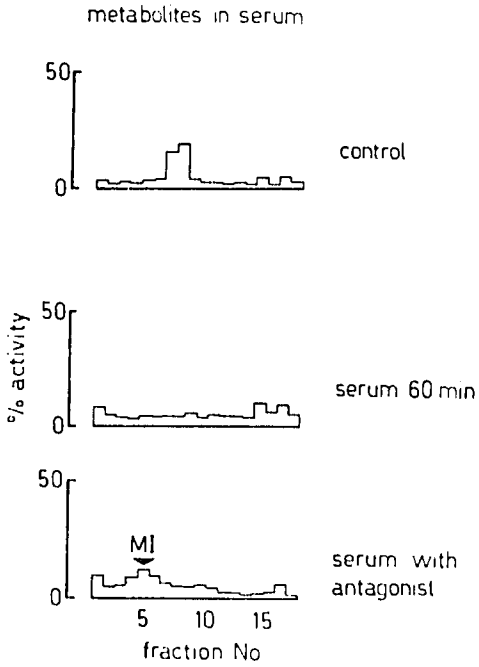

FIG. 31-6. Buserelin metabolites in serum. Serum extract 60 minutes after IV injection of ^{125}I Buserelin (percent radiolabel per fraction). No peptide material is recovered 60 minutes after IV injection. After pretreatment of rats with the LHRH antagonist [D-Phe^2,Phe^3,D-Phe^6]-LHRH, degradation of ^{125}I Buserelin is competitively inhibited and a metabolite M I is found in serum, corresponding to the pituitary metabolite M I.

METABOLISM OF LHRH AGONISTS *IN VITRO*

As a preparatory step for the identification of metabolites of LHRH agonists and the enzymes involved in degradation, *in vitro* studies were performed with homogenates of various organ tissues. It is well established that peptidases present in hypothalamus, pituitary, and other tissues can rapidly degrade LHRH.[9-14] The residual LHRH concentrations in incubation media can be measured by bioassay in ovariectomized, estrogen-progesterone treated rats.[18,20] With this method, we found that LHRH is rapidly inactivated by crude enzyme preparations (10,000 × g supernatant fraction) of liver, kidney, and neural tissues (anterior pituitary, posterior pituitary, hypothalamus, brain tissue), as well as ovary, testes and uterus.[18] The rapid decrease in biologic activity is due to the chemical specificity of the LHRH molecule. Both C-terminal and N-terminal LHRH fragments are devoid of significant biologic activity.[23] This is also true for LHRH nonapeptide agonists. In a study on synthetic C-terminal Buserelin fragments,[22] we found that removal of the N-terminal PyroGlu greatly reduces biologic activity, although at high concentrations, C-terminal agonist fragments retain some ovulatory activity, in contrast to similar LHRH fragments. The metabolism of Buserelin was studied by inactivation *in vitro* with enzyme preparations of various organ tissues, and by degradation of ^{125}I Buserelin with identification of the metabolites on TLC. Buserelin was rapidly inactivated by enzymes in homogenates of liver, kidney, and salivary gland. Inactivation by enzymes contained in hypothalamus, anterior pituitary, cerebral cortex, spleen, and muscle was less

rapid. This is in agreement with reports on reduced rates of degradation by hypothalamic and anterior pituitary homogenates.[10,28]

During inactivation by homogenates of rat liver, kidney, testis and ovary, there was a concentration-dependent and time-dependent loss of immunoreactivity, confirming the presence of potent Buserelin-degrading enzymes. However, the quantitative contribution of these inactivating tissues *in vivo* differs greatly, and final proof by identification of metabolites in rats was only obtained for liver, kidney, and anterior pituitary.

Degradation by purified enzyme preparations provided a more specific answer for the nature of the Buserelin-degrading enzymes. PGP (EC 3.4.11.8), chymotrypsin-A4 (EC 3.4.21.1), aminopeptidase-M (EC 3.4.11.2) and arginine-specific protease from mouse submaxillary gland (not yet classified) were obtained from Boehringer Mannheim, Germany F.R. After incubation for 30 to 120 minutes at +37°C, enzyme activity was destroyed by boiling, and the recovery of Buserelin and Buserelin fragments was determined by RIA. PGP was found to inactivate intact Buserelin, but not its C-terminal fragments. In contrast, chymotrypsin also inactivated C-terminal Buserelin fragments, and converted M I formed by PGP degradation to M II (Fig. 31-7). Aminopeptidase-M and arginine-protease were ineffective in studies with analytical TLC.

The degradation products of ^{125}I Buserelin in incubation media or tissue extracts were analyzed by thin-layer chromatography on silica plates in butanone:pyridine:water:acetic acid (70:15:15:2 by volume) as reported previously[19,21,24] and compared with ^{125}I labeled Buserelin fragments (Fig. 31-8). Incubation with PGP or pituitary arylamidase resulted in formation of the Buserelin (2-9) octapeptide, previously identified *in vivo* in pituitaries of rats (Rf 0.33). Sequential inactivation by PGP followed by chymotrypsin resulted in formation of Buserelin metabolite M I, identified as the (2-9) octapeptide. Subsequent chymotrypsin treatment abolished this peak and resulted in a new product different from Buserelin, but with an Rf similar to the intact peptide. The characteristic susceptibility of intact Buserelin to PGP (resulting in formation of M I as the intermediate product), followed by chymotrypsin incubation (removing this metabolite M I and converting it to M II), formed the basis for subsequent identification of the Buserelin fragments found in liver and kidney in rats *in vivo* (Fig. 31-9). None of the smaller C-terminal fragments is degraded by PGP (Fig. 31-10), and antiserum binding of liver and kidney extracts does not change after PGP incubation, indicating the absence of intact peptide.

MEASUREMENT OF LHRH AGONISTS BY RADIOIMMUNOASSAY

A nonapeptide RIA was developed for the measurement of Buserelin. Peptide antisera were raised in rabbits by intradermal immunization.[19,27] The antigenic determinant of the antisera was evaluated by displacement curves of fragments of Buserelin and of structurally related nonapeptide analogs[27] substituted with different amino acids in position 6 instead of D-Ser(TBU)^6. Buserelin was iodinated by the chloramine-T method and purified on sephadex LH 20.[22] The specificity of the antisera was sufficiently different to

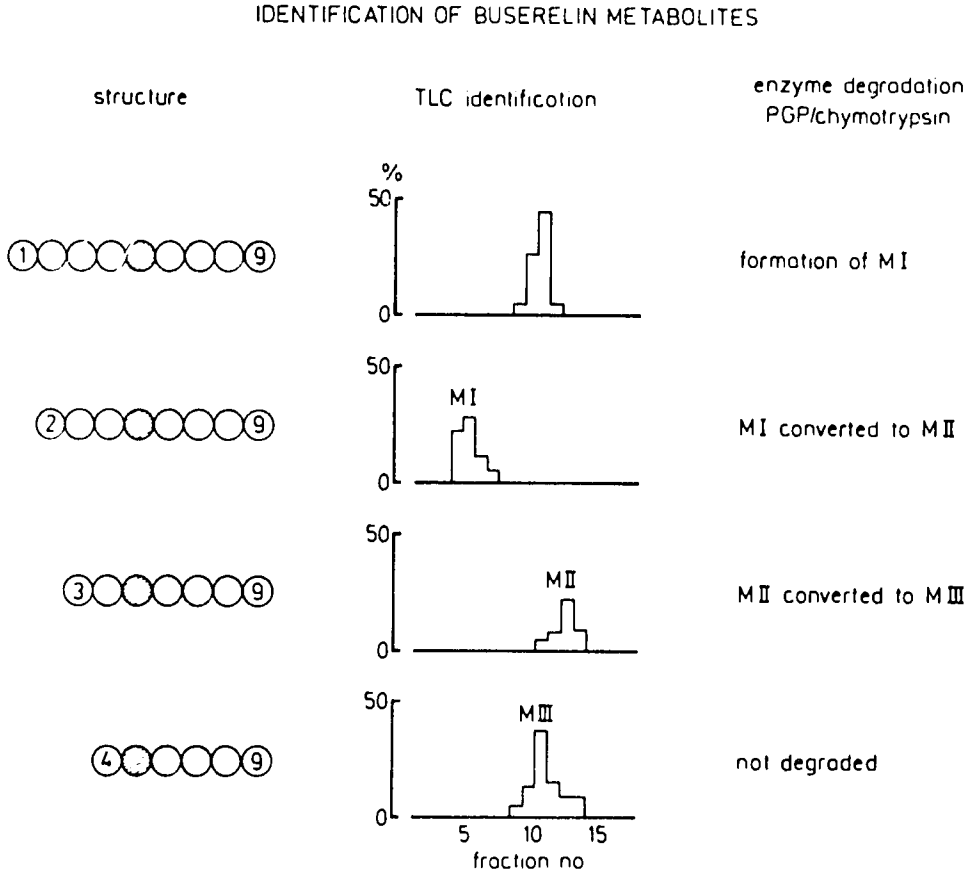

FIG. 31-7. Identification of Buserelin metabolites in tissue extracts. (*Left Panel*) C-terminal Buserelin fragments of decreasing chain length, labeled in Tyr^5 with ^{125}I. (*Middle panel*) Chromatographic mobility on TLC plates (percent radiolabel per fraction). (*Right panel*) Products formed after sequential degradation with pyroglutamyl-aminopeptidase, followed by chymotrypsin.

permit preferential measurement of intact Buserelin (sequence specific antiserum AS-14) or of C-terminal Buserelin fragments (fragment-specific antiserum AS-9). All antisera were specific for the C-terminal Pro-ethylamide moiety, and did not cross-react with LHRH decapeptide. Considerable chemical exchange of D-amino acids in position 6 did not reduce affinity, but substitutions in position 7 were critical.

This RIA was suitable for the measurement of Buserelin in buffer solutions. However, in serum there was a significant inhibitory effect of higher protein concentrations on tracer binding. Samples of 1 to 10 μl serum were measured without extraction. At lower concentrations, Buserelin was extracted from serum or biologic fluids by adsorption to silicate (Florisil or silicic acid). This extraction step prevents degradation in serum, a potential source of error in RIA determination of LHRH and Buserelin.[4,16,20] To eliminate

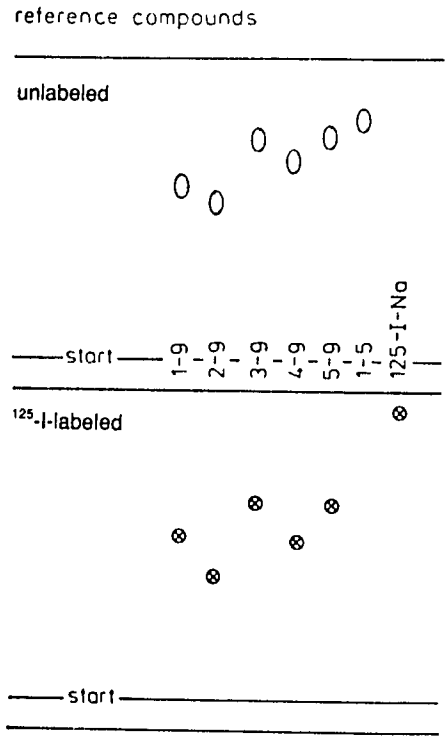

FIG. 31-8. Reference compounds used for identification of Buserelin metabolites by thin-layer chromatography on silica plates. Chromatographic mobility of unlabeled fragments (detected by spray reagents) and ^{125}I-labeled fragments (detected by gamma counting) is identical. (*1–9*) denotes intact Buserelin nonapeptide, (*1–5*) denotes the N-terminal LHRH (*1–5*) pentapeptide, identical in LHRH and Buserelin.

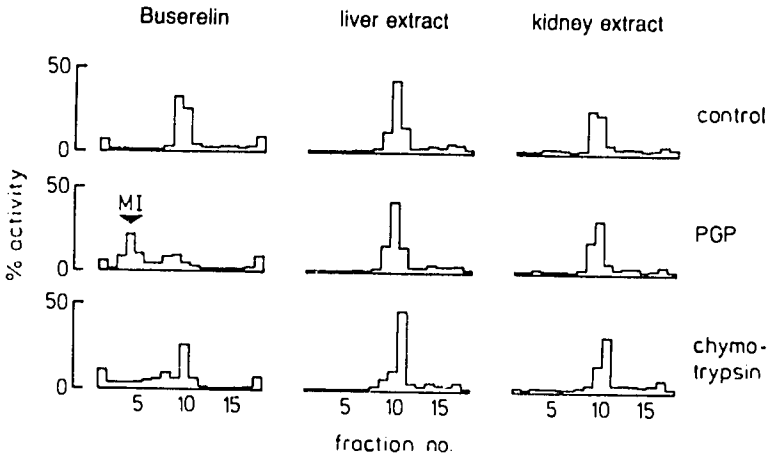

FIG. 31-9. Identification of Buserelin metabolites in liver and kidney of rats after treatment with ^{125}I Buserelin (extraction procedure indicated in Fig. 31-4). Intact Buserelin is degraded to metabolite M I by incubation with pyroglutamyl-aminopeptidase (PGP). M I disappears after subsequent chymotrypsin digestion. PGP treatment of liver and kidney extracts does not result in formation of M I, the Buserelin (2-9) octapeptide, indicating that these metabolites are C-terminal Buserelin fragments lacking pyroglutamic acid.

IDENTIFICATION OF BUSERELIN METABOLITES IN LIVER AND KIDNEY: ENZYME DEGRADATION BY PYROGLUTAMYLPEPTIDASE/ CHYMOTRYPSIN

TEST MATERIAL	CONTROL INCUBATION	INCUBATION WITH PGP/CHYMOTRYPSIN
BUSERELIN	40.1	1.0
(3-9)HEPTAPEPTIDE	36.6	0.4
LIVER EXTRACT	14.8	12.3
KIDNEY EXTRACT	11.3	7.1

% BINDING TO BUSERELIN ANTISERUM

FIG. 31-10. Identification of Buserelin metabolites in liver and kidney extracts. Antiserum binding after enzyme degradation of tissue extracts by pyroglutamyl-peptidase, followed by chymotrypsin. Binding affinity of Buserelin and the (3-9) heptapeptide is abolished by sequential incubation with PGP/chymotrypsin. In contrast, binding affinity in liver and kidney extract is not destroyed, indicating the presence of undegraded Buserelin (4-9) hexapeptide.

interference in the RIA by enzyme contamination, tissue extracts and incubation media were boiled for 10 minutes at +95°C before assay. For pharmacokinetic studies in rats and rabbits, sequential blood samples were drawn from indwelling catheters implanted into the jugular vein. Detection of immunoreactive Buserelin in serum by RIA requires administration of unphysiologically high quantities of peptide, much higher than biologically effective doses.

The material in serum consists of various peptide metabolites. Chromatographic separation of Buserelin metabolites prior to RIA was achieved by TLC on silica plates, measuring the Rf values of unlabeled Buserelin and its C-terminal fragments after elution of TLC segments. The (2-9) octapeptide and (3-9) heptapeptide were clearly differentiated from intact Buserelin by their different Rf values, whereas the (4-9) hexapeptide and (5-9) pentapeptide were indistinguishable by TLC. However, these fragments can be identified by their resistance to pyroglutamyl-peptidase degradation.

DEGRADATION OF LHRH AGONISTS BY ANTERIOR PITUITARY PLASMA MEMBRANES

The inactivation pattern of ^{125}I Buserelin was compared with the pattern of ^{125}I LHRH using pituitary membranes isolated by ultracentrifugation in a sucrose gradient.[3,7,13] Metabolites of Buserelin and LHRH were analyzed by thin-layer chromatography on silica plates. Degradation was found to be time- and temperature-dependent. The products formed from LHRH or Buserelin were different. Degradation of Buserelin resulted initially in formation of a

metabolite, M I, similar to the metabolite found previously in the pituitaries of rats treated with ^{125}I Buserelin (Fig. 31-11). This metabolite was subsequently converted to a further product, M II. Addition of 330,000 × excess of either unlabeled LHRH or Buserelin retarded membrane inactivation, and reduced conversion of M I to M II.

The primary Buserelin metabolite M I was identified as the (2-9) octapeptide. M I has greatly reduced ovulatory activity in rats (Fig. 31-12), and does not affect the sensitivity of the pituitary to LHRH or Buserelin stimulation. The secondary Buserelin metabolite M II was similar to the metabolite found

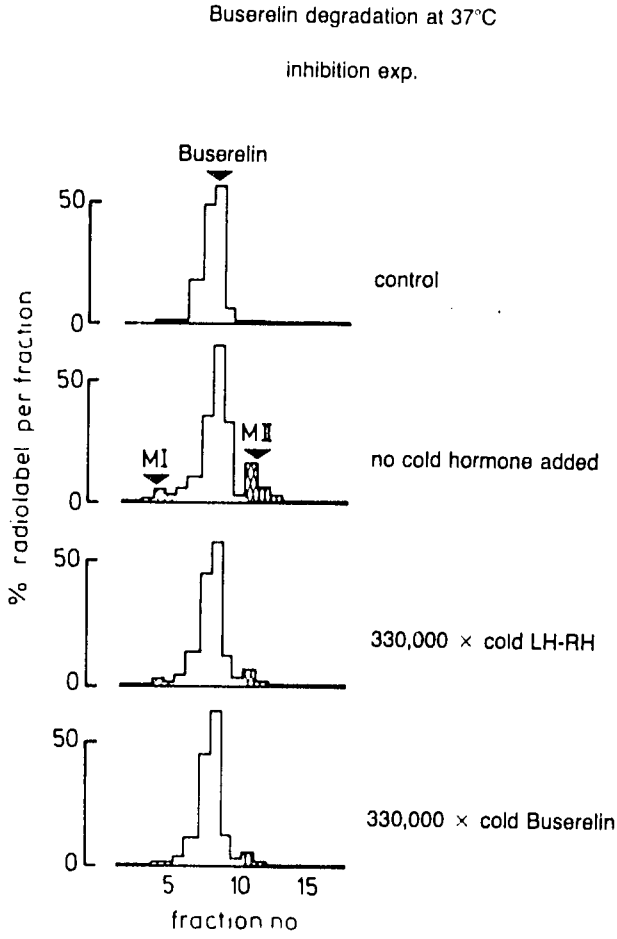

FIG. 31-11. Degradation of ^{125}I Buserelin by pituitary plasma membranes *in vitro:* 0.8 μCi (4 ng peptide) of ^{125}I Buserelin incubated with the 1.18/1.20 sucrose gradient fraction containing pituitary membranes. Pattern of degradation analyzed on silica plates (percent radiolabel per fraction). After 30 minutes' incubation, two metabolites, M I and M II, are formed (see Fig. 31-7), corresponding to the (2-9) octapeptide and (3-9) heptapeptide. A 330,000 × excess of unlabeled LHRH or Buserelin significantly inhibits degradation.

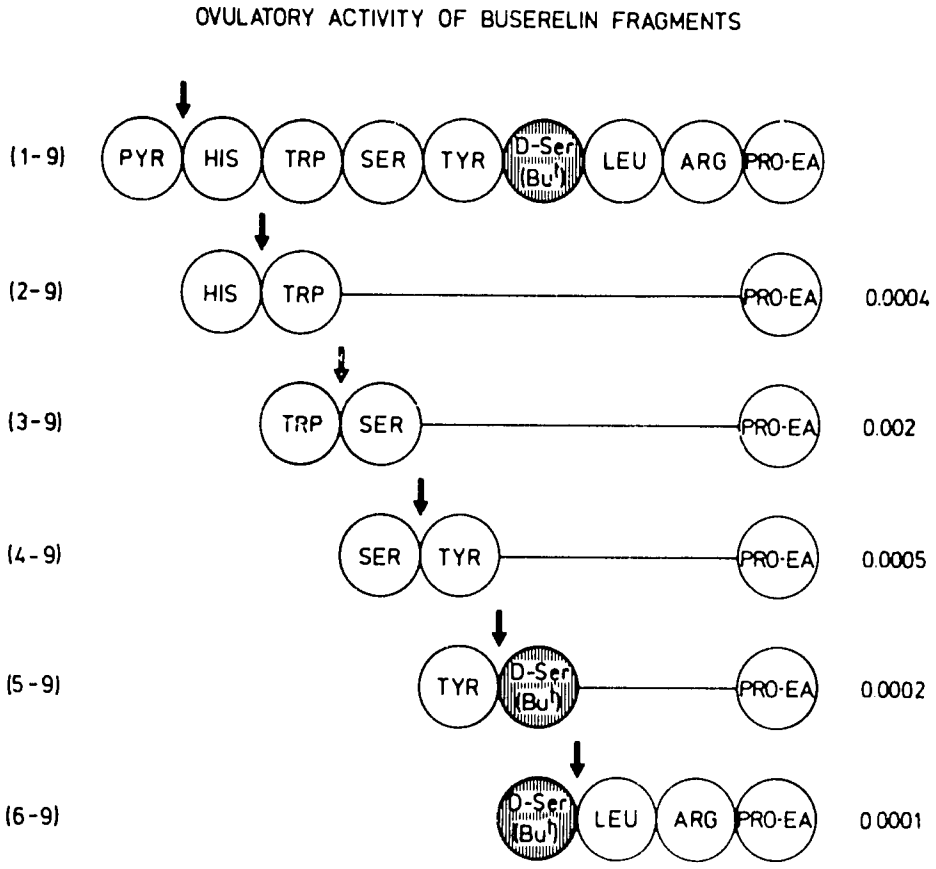

FIG. 31-12. Biologic activity of C-terminal Buserelin fragments: Relative potency to induce ovulation in phenobarbital-blocked rats. Cleavage of the N-terminal Pyr-His bond results in a dramatic decrease of ovulatory activity. At pharmacologic doses, these fragments retain the ability to release LH. (Sandow J, König W: Studies with fragments of a highly active analogue of luteinizing hormone releasing hormone. J Endocrinol 81:175–182, 1979)

in liver and kidney of rats *in vivo*, 60 minutes after treatment with ^{125}I Buserelin, or after chymotrypsin-treatment of the (2-9) octapeptide. Degradation of LHRH by pituitary plasma membranes was significantly more rapid than degradation of Buserelin. The reduced rate of degradation is due to protection of the Tyr^5-Gly^6 bond of LHRH by the [D-Ser(TBU)^6] substitution in Buserelin.

SUMMARY

Studies on the pharmacokinetics, organ distribution, and metabolic inactivation of LHRH agonists are of considerable importance for the assessment of drug safety. The studies reported here demonstrate that, similar to LHRH,

a highly active agonist, [D-Ser(TBU)^6]-LHRH (1-9) nonapeptide ethylamide (Buserelin), is degraded by enzymes present in liver, kidney, and anterior pituitary of rats. Metabolic inactivation is rapid; 60 minutes after IV injection of a near-physiologic dose of ^{125}I-labeled peptide (50 ng) in rats, extraction of the labeled fragments from liver and kidney, and subsequent identification by thin-layer chromatography and enzyme digestion/antiserum binding confirm that no intact Buserelin is present at this point in time. The metabolite M II in liver and kidney is not degraded by PGP, and exhibits reduced binding to a Buserelin antiserum. By chromatographic identification, M II corresponds to the Buserelin (4-9) hexapeptide, whereas antiserum binding excludes any fragment smaller than the (6-9) tetrapeptide.

In the anterior pituitary of rats, a specific metabolite, M I, is found. This metabolite is identical with the incubation product of Buserelin *in vitro* with pyroglutamyl-aminopeptidase, and M I is also identified after incubation of ^{125}I Buserelin with pituitary arylamidase, a crude enzyme preparation from rat anterior pituitaries. When the specific metabolism in the biological target organ is analyzed in more detail, using purified anterior pituitary plasma membranes, the inactivation pattern of ^{125}I Buserelin is similar to the metabolites found in liver and kidney. The primary product, M I, has been identified as the Buserelin (2-9) octapeptide, by comparison with labeled and unlabeled peptide fragments. Further degradation by chymotrypsin results in formation of M II, identical with the material found in liver and kidney of rats, 60 minutes after treatment.

Studies on metabolism of peptide are complicated by the minute doses given for therapeutic purposes. Classical pharmacokinetics using radioimmunoassay are not applicable, because the increasing concentration of Buserelin at the anterior pituitary receptor site is not reflected in a sustained serum level of peptide. After initial receptor binding, serum concentrations decline to nondetectable levels despite ongoing gonadotropin release. A more appropriate parameter of biologic action is the progressive accumulation in the anterior pituitary *in vivo*, which can be measured with ^{125}I labeled agonists. Pituitary uptake is in good correlation with the biologic potency, both with regard to duration of accumulation and quantity of peptide accumulated.[17,26,28]

The practical implications for a peptide contraceptive resulting from this study are related to the drug safety and to the reversibility of pituitary suppression by agonists. Rapid inactivation in liver and other tissues indicates that LHRH agonists are effectively handled by the same enzyme systems operative in degradation of endogenous LHRH. At near-physiologic concentrations *in vivo*, these enzymes are capable of splitting agonists into peptide fragments of greatly reduced biologic activity within 60 minutes. The primary cleavage site is the unprotected Pyr-His bond accessible to PGP, an enzyme present in mammalian liver in considerable quantity. This finding may in part explain the excellent reversibility of pituitary suppression by agonists, even after long periods of treatment.[29] In contrast to steroid contraceptives, a peptide contraceptive is inactivated much more efficiently and rapidly by endogenous peptidases and would thus carry a reduced risk of metabolic consequences.

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32

Secondary Pharmacologic Studies with LHRH and Its Analogs

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Evaluations of the possible nonreproductive actions of LHRH peptides offer some unique challenges to the pharmacologist. An important aspect of our approach is the appreciation of the rapidly expanding body of knowledge on the extrapituitary role of LHRH on reproductive organs.[6] One might even speculate that LHRH is a member of the "putative" peptidergic nervous system, and thus plays a physiologic role modulating neuronal function.[1] In fact, LHRH has been identified by immunochemical means in other tissues such as the adrenal gland[2] and placenta.[3]

The studies described in this chapter concern only the nonreproductive, secondary pharmacologic effects of a specific LHRH analog. [D-N-al(2)^6]-LHRH is a synthetic LHRH agonist possessing antifertility effects both in male and female laboratory animals.

It was decided that, because of the possible effects of [D-N-al(2)^6]-LHRH on the central nervous system (CNS), extensive neuropharmacologic and gross behavioral evaluation would be conducted. In addition, cardiovascular, autonomic, and pulmonary studies were undertaken in several laboratory animal species with this synthetic peptide.

BEHAVORIAL PROFILE IN THE MOUSE, RAT, AND DOG

Mice were given the peptide acutely at IP doses of 10 to 3,000 μg/kg and rats were given 1 to 100 μg/kg IP. A subacute study in rats consisting of daily IP injections of 1 and 10 μg/kg for 5 days was also carried out. Dogs were administered [D-N-al(2)^6]-LHRH at doses of 1 to 100 μg/kg IM. Observation of behavior lasted from several hours to several days (see Table 32-1). A comprehensive behavioral evaluation[7] included assessment of the animal's awareness, motor activity, and neurologic and autonomic profile after [D-N-al(2)^6]-LHRH administration. Altered awareness such as alertness, stupor, and passivity could be indicative of CNS stimulation, depression, or tran-

TABLE 32-1. Effect* of [D-N-al(2)[6]]-LHRH on Mice, Rats, and Dogs

ANIMAL	TYPE OF STUDY	ROUTE AND DOSE (μg/kg)	N PER DOSE	OBSERVATION PERIOD	RESULTS
Mouse (M)	Acute	10–3,000 IP	3	7 hrs	No change
Rat (M)	Acute	1–100 IP	3	7 hrs	No change
Rat (M)	Subacute (daily for 5 days)	1 and 10 IP	10	7 hrs	No change
Dog (F)	Acute	1–100 IM	1–3	3 days	No change

*Effect on gross behavior, activity, various reflex responses, coordination, posture, pupil size, heart and respiration rates, body temperature

quilization. Changes in behavior typified by increased grooming, restlessness, and irritability might point to central, sympathetic, or noxious stimulation.

Observations of spontaneous activity and reactivity give clues to the presence or absence of CNS stimulation. A decreased response to elicited pain could signify analgesia or sedation, whereas altered touch responses might indicate anesthetic activity. Motor incoordination such as deviation from normal body posture or muscle tone, or abnormal gait might point to central, myorelaxant, or neuromuscular activity. The elicitation of reflex responses is important in establishing effects on sensory nerves, spinal synapses, and efferent pathways.

Observing peptide effects on optical signs such as pupillary size or exophthalmos could indicate parasympatholytic or sympathomimetic activities. Secretory signs such as urination or salivation would suggest muscarinic or irritant effects. Other useful autonomic signs are piloerection, body temperature, skin color, and heart and respiration rates.

[D-N-al(2)[6]]-LHRH given acutely to mice, rats, and dogs or when administered subacutely to rats did not alter any of the behavioral signs or responses.

NEUROPHARMACOLOGIC TESTS IN THE MOUSE, RAT, AND CATS

Some tests were more specific (see Table 32-2). Given acutely at 1 to 100 μg/kg IP the peptide did not induce a neurologic deficiency such as loss of motor coordination or strength in mice during a traction test.

Seizures were elicited experimentally in mice either electrically by transcorneal electroshock or chemically with pentylenetetrazole. The synthetic peptide displayed no anticonvulsant activity in these models.

Barbiturates such as hexobarbital produce sleep in mice. Prolongation of this sleep might indicate potentiation of the central effects and/or interference with barbiturate metabolism. [D-N-al(2)[6]]-LHRH did not alter hexobarbital sleeping time.

TABLE 32-2. Acute Neuropharmacologic Tests with [D-N-al(2)[6]]-LHRH in Mice, Rats, and Cats

ANIMAL (SEX)	PURPOSE OF STUDY	ROUTE AND DOSE (μg/kg)	N PER DOSE	RESULTS
Mouse (M)	Induction of neurologic deficiency	1–1,000 IP	10	No change
Mouse (M)	Effect on seizures induced by electroshock, PT*	1–1,000 IP	10	No anticonvulsant activity
Mouse (M)	Hexobarbital sleep	10 IP	10	No effect
Rat (M)	Hyperphagia induced by stress	1–1,000 IP	8	Slight reduction of food consumed at 1 but not at 10–1,000 μg/kg
Cat (F)	Gross behavior, EEG†; EMG‡; RFMUA§ sleep	30 and 100 IM	1–3	No change

*Pentylenetetrazole—chemically induced convulsions.

†Cats with chronically implanted EEG electrodes in various parts of the brain.

‡Electromyogram.

§Reticular formation multiple unit activity.

Another central effect we studied was regulation of food intake elicited by stress.[8] The experimental animals (rats) consumed excessive amounts of milk while their tails were chronically pinched. The synthetic peptide at IP in doses of 1 to 1000 μg/kg reduced milk consumption by these rats at 1 μg/kg, but not at higher doses of 10 to 1000 μg/kg.

In a group of cats, electrodes were implanted chronically at various loci in the brain for recording of the electroencephalogram (EEG). Neck muscle tone (EMG) and reticular formation multiple unit activity (RFMUA) were also recorded. Effects on gross behavior and sleep patterns were noted as well. Doses of 30 or 100 μg/kg IM of the peptide did not alter any of the recorded parameters in the cat.

CARDIOVASCULAR, PULMONARY, AND AUTONOMIC EFFECTS

[D-N-al(2)[6]]-LHRH was given acutely by the IV route to anesthetized dogs, cats, and guinea pigs (see Table 32-3). The animals were instrumented for the measurement of blood pressure, ventricular contractile force, pulmonary airway pressure, heart rate, and, in the case of the cat, nictitating membrane contractility. The synthetic peptide was administered in IV bolus doses to guinea pigs at 1 to 100 μg/kg, to dogs at 10 to 100 μg/kg and cats at 3 to 100 μg/kg. [D-N-al(2)[6]]-LHRH did not alter blood pressure, heart rate, or ventricular muscle contractility. Since it failed to block challenges to exogeneous acetylcholine or catecholamines, it possesses no anticholinergic or adrenoreceptor blocking activity. Its lack of effect on the heart, peripheral vasculature, and cat nictitating membrane indicates an absence of sympathomimetic activity. [D-N-al(2)[6]]-LHRH did not possess ganglionic blocking activity, nor did

TABLE 32-3. Acute Cardiovascular, Pulmonary, and Autonomic Effects of [D-N-al(2)[6]]-LHRH in Male and Female Guinea Pigs, Cats, and Dogs

ANIMAL	PURPOSE OF STUDY	ROUTE AND DOSE (μg/kg)	N PER DOSE	RESULTS
Dog	Effect on heart rate, ventricular contractility, blood pressure, and responses to α-, β-adrenergic, cholinergic, and ganglionic stimulation and responses to other autacoids	10–100 IV	2	No alterations of cardiovascular parameters; no effect on α- or β-receptor stimulants or autacoids; no ganglionic blockade
Cat		3–100 IV	2	
Guinea pig	Effect on airways, heart rate, blood pressure	1–100 IV	4	No effect on histamine and methacholine responses

it modify cardiovascular reflex responses (carotid artery occlusion). It had no effect on the cardiovascular responses to autacoids such as histamine, bradykinin, and angiotensin. In the guinea pig, it did not alter the reduction of pulmonary airway tone owing to either histamine or methacholine challenges.

In conclusion, [D-N-al(2)[6]]-LHRH was remarkably free of nonreproductive acute or subacute pharmacologic effects in laboratory animals.

EXPANSION OF PHARMACOLOGIC KNOWLEDGE ON LHRH AND ANALOGS

The nature and scope of such work would be greatly affected by the rapidly expanding understanding of the role of peptides as neurotransmitters, and also by a more profound appreciation of the intimate interaction of neuroendocrine humoral factors with the CNS. It is of great interest that in some nerve terminals "putative" peptide neurotransmitters and biogenic amines coexist.[3] Whether a peptide such as LHRH could produce a sustained effect on neuronal excitability and on non-neuronal receptor function as already postulated for other peptides[2] can only be speculated. An LHRH analog administered chronically, for example, might be evaluated in pharmacologic tests for possible modification of central activity as well as peripheral, nonreproductive target organ functions (cardiovascular, nonreproductive endocrines). Possible interactions between LHRH analogs following their chronic administration and CNS drugs[4] such as neuroleptics, anxiolytics, antidepressants, anticonvulsants, opiates, and drugs of abuse such as alcohol and amphetamines need to be considered.

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33

Preclinical Toxicology Program for LHRH Analogs

LAKSHMIPATY PENUMARTHY

ROBERT HILL

LHRH and several other synthetic analogs of LHRH are currently being investigated as potential male and female contraceptives. When administered in supraphysiologic doses or for a prolonged period of time, these agents have been shown to interrupt the reproductive processes in various laboratory animals including nonhuman primates.[6,10,11,13,14] Limited clinical trials in women have also shown ovulation inhibitory properties of LHRH and its analogs.[2,8] The contraceptive effects in men were inconclusive; though marginal reduction was noted in gonadotropin and testosterone secretion, spermatogenesis was unaffected.[1] Though the LHRH agents appear very attractive as contraceptive agents, the initiation of clinical trials and the rapidity with which the safety and contraceptive efficacy of these agents can be established in human beings depend upon completion of certain toxicologic studies in animals. Identification of target organ toxicity and safety assessment are primary goals of these preclinical animal toxicity studies.

The objectives of preclinical animal toxicity studies for a new drug generally include the following:

1. Establishing the toxicity profile of the drug through evaluation of clinical condition, organ function, blood chemistry, hematologic parameters, urinary profiles, and gross and histopathologic organ changes.
2. Delineation of whether anatomic/functional changes, if induced, are reversible.
3. Determination of potential effects on fertility and reproduction, including teratogenicity and the postnatal development of the offspring.
4. Assessment of the carcinogenic/mutagenic potential.
5. Evaluation of the results in animals to assess the safety of administering the drug to humans.

PRECLINICAL ANIMAL TOXICITY STUDIES FOR LHRH ANALOGS

The amount of toxicologic testing and the types of toxicologic studies required for developing any new drug depend on factors such as its intended use, duration of treatment, and the characteristics of the human population that would be potential users of the drug. Chemical contraceptives, including

LHRH analogs, can be expected to be used by a significant proportion of the human population throughout their reproductive lives. Meaningful animal toxicity studies should therefore be performed to generate data showing adequate evidence of safety to the human user.

The studies should also meet the guidelines of regulatory agencies in individual countries where license for marketing of the product is desired. A comprehensive toxicologic program such as the one outlined below can be expected to be required for an LHRH analog for contraceptive use in males or females. This program is drawn from the general guidelines and requirements suggested by the U.S. Food and Drug Administration (FDA).[1]

Proper design of such studies cannot be overemphasized and no attempt is made in this chapter to outline in detail the various factors, such as the numbers of animals, purity of the chemical, formulation stability, and evidence of absorption, that need to be considered in each of the following studies. These studies are time consuming, complex, and expensive. Although the program to be presented is designed according to U.S. FDA guidelines, each country has its own guidelines and regulations. It is suggested that interested readers review the regulatory requirements of individual countries before initiating such studies.

Toxicity studies can be categorized into acute, subacute (subchronic), chronic, reproductive, and mutagenic/carcinogenic. These studies are conducted in a sequential fashion to allow progressive investigation of the drug in clinical trials. All of the toxicity studies, however, need to be completed prior to the approval for marketing of a new drug.

Acute Studies

These studies entail administration of a single high dose or a few high doses over a short interval to at least one rodent and one nonrodent species. In rodents, the toxicity is usually evaluated by at least two different routes of administration, one of which should be the intended clinical route. Data collection in conventional acute toxicity studies includes clinical observations, determination of the median lethal dose (LD_{50}) or the highest tolerated dose (LD_0) and an assessment of the probable cause of death. With compounds such as LHRH analogs that have minimal acute toxic potential, attempts to determine the LD_{50} would be fruitless; instead, efforts should be made to evaluate the effects on reproductive or endocrine organs.

Subacute (Subchronic) and Chronic Studies

These studies are designed to delineate the probable functional and/or anatomic changes that may result from repeated exposure to the drug. The test compound is administered at several dose levels to at least one rodent and one nonrodent species by the intended clinical route. Several studies are conducted in this category in which the duration of treatment ranges from 1 month up to 12 months. In long-term toxicity studies (such as 1 year), attempts are made to include the species most closely approximating human metabolism of or response to the drug. Routine data collection in these studies includes clinical condition, body weight, food intake, ophthalmology, blood

and urinary evaluation, and necropsy examination. Specific gonadal and gonadotropic hormones, plus other endocrine hormones, should be evaluated if considered appropriate. Histologic examination should include all organ systems including the reproductive and endocrine organs. Provision should also be made to determine the recovery potential of altered parameters.

Reproductive Studies

In the design of a toxicologic program for a contraceptive agent, considerations must be given to the following:

1. Is the induced sterility temporary and reversible?
2. Are there any alterations in secondary sexual characteristics including libido?
3. In the event of a possible contraceptive failure, does the agent cause teratologic or postnatal developmental defects in the resulting offspring?

Toxicologic studies aimed toward delineating the potential effects on the reproductive processes are not unique to contraceptive agents but in general are conducted for most new drugs. Reproductive studies are usually conducted in laboratory animals in three different segments as outlined below. For LHRH analogs, appropriate subcontraceptive dosages that would adequately maintain pregnancy are to be selected in conducting such studies.

Segment 1—Study of Fertility and Reproductive Performance: The rat is the animal most commonly used for evaluation of this segment. The general reproductive performance of both treated males and treated females is evaluated. Male rats are treated with the drug for at least 60 days and the females are treated for 14 days before initiation of mating. The treated animals are mated to untreated animals of opposite sex. Parameters usually evaluated in this segment include mating behavior, fertility, litter size, duration of gestation, nature of parturition, care of the offspring, growth, and postnatal development of the offspring. As part of the postnatal evaluation, various milestones of physical development and several behavioral tests are conducted on the offspring.[7,9] Generally, fertility of the offspring is evaluated at the attainment of sexual maturity. Male and female fertility studies can also be conducted as part of long-term toxicity studies.

Segment 2—Teratologic Evaluation: Mouse, rat and rabbit are the species commonly employed for these investigations. In such studies the animals are treated only during the period of organogenesis. Female mice and rats are treated from day 6 through day 15 of gestation; female rabbits are treated from day 6 through day 18 of gestation. The fetuses are harvested by cesarian section usually 1 or 2 days prior to the anticipated date of parturition and examined for skeletal and visceral anomalies.

Segment 3—Perinatal and Postnatal Study: The rat is commonly employed for this investigation. The pregnant dams are treated starting from the last trimester of pregnancy and continued until weaning of the pups. Parameters

evaluated in this segment include nature of parturition, effects on lactation, care of the offspring, growth and development of the offspring.

While reproductive segments 1 and 2 are appropriate for evaluation of LHRH analogs as female contraceptives, only segment 1 is applicable for male contraceptive evaluation purposes. During the course of treatment of male animals, additional studies may be designed to evaluate the possible male mediated teratogenic or postnatal development impairments in the offspring. Further, a modified version of these reproductive segments is appropriate to investigate the reversibility of reproductive processes following cessation of treatment. As an example, a scheme for reproductive evaluations in male rats treated with one of our LHRH analogs for 6 months is illustrated in Table 33-1.

Mutagenic/Carcinogenic Studies

Various short-term *in vitro* and *in vivo* tests are purported to predict the mutagenic/carcinogenic potential of chemicals.[3] Several test procedures appear promising for the evaluation of the mutagenic potential of a chemical.[12] However, controversy exists in regard to their reliability, repeatability, and relevance to the human population. Currently, most pharmaceutical industries employ these tests as a screening tool before embarking on extensive developmental work with a promising new drug candidate. The short-term tests that have been adequately validated for evaluating the carcinogenic potential are very few and regulatory agencies recommend and recognize them at present only as valuable adjuncts to long-term studies in animals.

The most commonly used animal species for the assessment of carcinogenic potential of a new drug are the rat and the mouse. These studies are conducted over the animal's average lifespan; for the rat, 24 months, and for the mouse, 18 months. The treatment is initiated in weanling animals, thus permitting a greater part of their life span for tumor development to coincide with exposure to the drug. Generally, the animals are given the drug by way of the intended clinical route. Selection of appropriate strain, familiarity with the spontaneous tumor incidence, and diet are some of the critical factors to be considered in the design and evaluation of such studies.

TABLE 33-1. Reproductive Evaluations in Male Rats Treated with LHRH Analog for 6 Months

	PROCESS FOR EVALUATION
Treatment month	
3	Mate to untreated females; sacrifice females on gestation day 13 and evaluate fertility
4–5	Mate to untreated females; sacrifice one-half of females on gestation day 20 and evaluate teratology; allow the other one-half of females to litter and observe postnatal development of the offspring
6	Mate to untreated females; repeat observations of treatment month 3
Posttreatment month	
1–2	Mate to untreated females; repeat teratology and postnatal observations as at treatment month 4–5
3	Sacrifice treated males; conduct blood chemistry, hematology, and necropsy

QUALITY ASSURANCE

Assurance of the quality and integrity of the data are necessary elements of any scientific experiment; however, they assume paramount significance in the conduct of all preclinical animal toxicity studies since such studies form an important component of safety assessment. All preclinical studies for new drugs intended for registration in the United States must comply with Good Laboratory Practice Regulations (GLPs) issued by the U.S. Food and Drug Administration.[7] Similar regulations are also being implemented by several other international regulatory agencies.

Some salient features of GLPs include the following:

1. Conduct of studies by responsible and qualified personnel
2. Proper animal care facilities
3. Adequate specimen and data storage and retrieval facilities
4. Proper maintenance of laboratory equipment
5. Satisfactory characterization of the purity of the test compound and stability of the test formulations
6. Availability of adequate standard operating procedures.

According to the GLP regulations, the FDA is authorized to inspect any testing facility (both within and outside the United States) where preclinical studies are conducted as part of safety assessment of a new drug intended for U.S. registration. The investigator should be thoroughly familiar with these regulations. Failure to comply with the GLP regulations, if determined to affect the validity of the study adversely, may lead to disqualification of the study; the investigator and the institution could also be disqualified from conducting such studies in the future. For a pharmaceutical firm, disqualification of a toxicity study could mean denial or delay in the approval of a new drug.

CONCLUSION

Identification of target organ toxicity and safety assessment are the primary objectives of the preclinical animal toxicity studies. A comprehensive toxicologic program is outlined for the development of LHRH analogs as contraceptive agents. In general, the preclinical animal toxicity studies routinely conducted for any new drug evaluate the reproductive/endocrinologic effects of LHRH analogs as well. Additional evaluation of gonadal and gonadotropic functions may be appropriate for the toxicologic evaluation of LHRH analogs. The studies should comply with regulatory requirements of individual countries. In addition, strict compliance with the GLP is necessary for approval of the drug in the United States.

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34

The Need for Controlling Variables Involved in Biologic Assays of LHRH

DO WON HAHN

AUDREY PHILLIPS–PROBST

JOHN L. McGUIRE

Following the structural elucidation of LHRH by the laboratories of Guillemin[1] and Schally[15] in 1971, LHRH and hundreds of analogs of the decapeptide have been synthesized and tested in biologic assays. It is known that LHRH stimulates secretion of pituitary gonadotropins both *in vitro* and *in vivo* and induces ovulation and changes in secondary sex organs. Both agonists and antagonists of LHRH have been identified, many having antifertility effects.[5,20] Data on these analogs have generally been reported in terms of potency and activity relative to LHRH.

It is well known that the biopharmaceutic properties of a compound (such as particle size, crystalline form, solubility characteristics, and partition coefficient), vehicle, and route of administration can influence the rate and extent of absorption of a compound and the resultant biologic response to that compound.[10] Reported studies with LHRH and its analogs have utilized different vehicles, including saline,[8] saline + 5% ethanol,[19] saline + 0.1% gelatin,[14] saline + propylene glycol,[9] corn oil,[3] and nonidentified oil.[6] Various studies have also utilized different routes of administration and have measured different biologic endpoints. These results are often compared in discussion by investigators with results obtained using other assay systems and different vehicles, even though consistency of results obtained using these different vehicles and assay systems has not been demonstrated.

As part of an ongoing program in our laboratories studying LHRH and its analogs, we initially conducted certain basic studies with LHRH in order to develop standard methodology for evaluating analogs. In this chapter, we present data clearly showing that the vehicle does influence the biologic response to LHRH, and different biologic assay systems utilizing different target organ responses can produce markedly different results with LHRH.

We thank Dr. Wylie W. Vale of the Salk Institute for Biological Studies and Drs. Marvin E. Rosenthale and R. M. Cohn of Ortho Pharmaceuticals for their helpful comments during the course of these experiments. We also thank M. T. Lai, L. Canton, and S. Klimek for their technical assistance.

EFFECTS OF VEHICLE

In addition to tissue culture assays, in our laboratories we have routinely utilized several *in vivo* biologic tests to describe the potency and endocrine activity of LHRH analogs. We report here three of these tests—effect on uterotropic activity in immature mice and the ability to induce ovulation in diestrous rats and in androgen-sterilized rats. Other laboratories have utilized these same animal models in studying LHRH.[4,17,22] Our initial efforts were directed at studying the effect of two different vehicles on the biologic response elicited by LHRH in these assay systems.

Induction of Ovulation in Androgen-Sterilized Rats

It is known that androgen treatment of newborn female rats produces a hypothalamic disorder resulting in persistent vaginal cornification, anovulation, and subsequent alteration of cyclic release of LH from the pituitary,[2,11,21] possibly as a result of altered hormone receptor populations.[16] LHRH will induce ovulation and estrous cycles in these animals,[13] and the androgen sterilized rat can thus be utilized as a test system for measuring the activity of LHRH analogs relative to LHRH. The procedure we utilized in these studies was the same procedure reported previously.[13] Androgen sterilization of Wistar strain rats was induced by subcutaneous injection of 100 μg testosterone propionate to female pups on the third day after birth. The animals were allowed to mature to a weight of 225 to 250 g (4–5 months of age) prior to use. To determine the ovulation inducing potencies of LHRH, sterilized rats showing persistent cornified vaginal cytology were treated with LHRH obtained from Chemalog (Plainfield, NJ) and dissolved in physiologic saline or sesame oil. Twenty-four hours after a single subcutaneous injection of LHRH at dose levels ranging from 0.31 to 10.0 μg/kg, animals were sacrificed and their oviducts were examined for the presence of ova under a light microscope.

Figure 34-1 demonstrates that different results can be obtained when the effect of LHRH administered in physiologic saline and in sesame oil is tested on ovulation in androgen-sterilized rats. The vehicles alone had no biologic effect, whereas LHRH administered in either vehicle induced ovulation. However, in various studies with LHRH administered in sesame oil, considerable intrastudy variability in results is routinely seen; less variability is seen with saline used as a vehicle. Frequently, this appears to lead to a difference in potency of the two LHRH preparations. For example, in the study shown in Figure 34-1, the material dissolved in physiologic saline appears more potent at lower doses than that dissolved in sesame oil. In this experiment, when dissolved in physiologic saline, LHRH began to induce ovulation in doses as low as 0.31 μg/kg; LHRH dissolved in sesame oil failed to induce this response at doses lower than 2.5 μg/kg. However, at higher doses, responses in the two vehicles were similar. In other studies not reported here, little difference was seen between the two preparations.

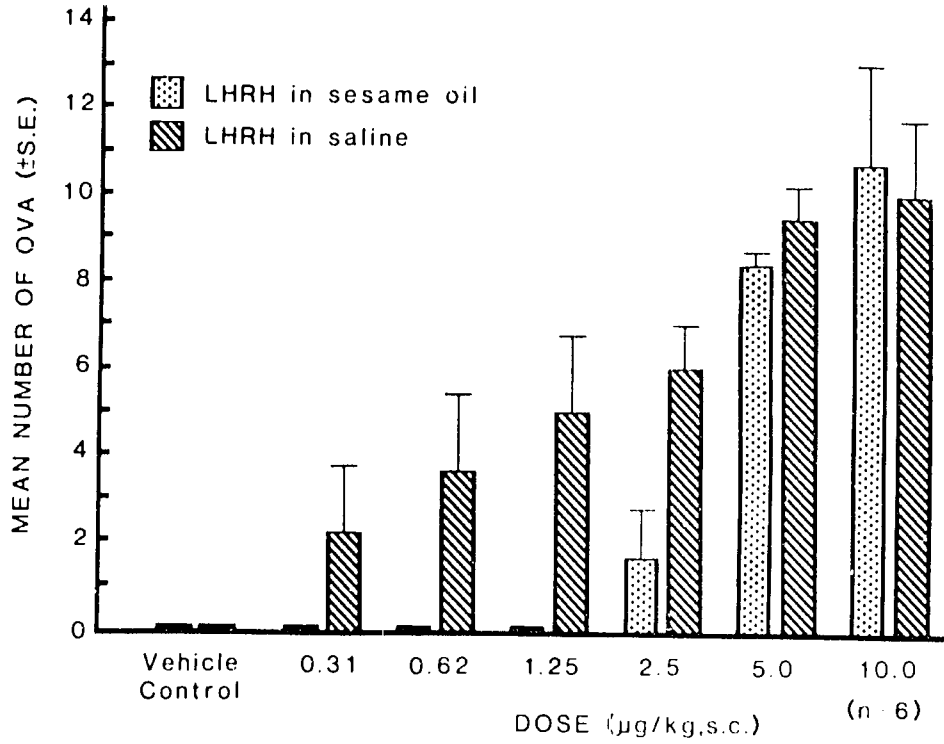

FIG. 34-1. Effect of vehicle on LHRH-induced ovulation in androgen sterilized rats.

Induction of Ovulation in Diestrous Rats

Induction of premature ovulation in adult rats is another test procedure used to evaluate the potency and activity of LHRH analogs. In these studies, female adult Wistar rats were kept in an environmentally controlled room maintained at 22 ± 1°C and a relative humidity of 50 ± 5%, with lights on from 6 a.m. to 6 p.m. Only those animals that demonstrated at least two consecutive 4-day estrous cycles were used. To determine the effect of vehicle on LHRH-induction of premature ovulation, selected rats in diestrous were treated with a single subcutaneous injection of LHRH dissolved in either one of the two vehicles. Twenty-four hours after treatment, animals were sacrificed and their oviducts were examined for presence of ova under a light microscope.

Figure 34-2 shows an experiment in which effects similar to those reported in Figure 34-1 are seen on LHRH-induced ovulation in diestrous rats. In our experience, higher doses of LHRH are generally needed to induce ovulation in diestrous rats than in androgen-sterilized rats. A second observation we made was, as described previously, variability in results when LHRH is suspended in sesame oil. In this experiment, both preparations of LHRH induced premature ovulation, but the potency of the two preparations appears different. LHRH dissolved in saline was more active at the lower doses but showed similar activity to LHRH dissolved in sesame oil at the higher doses.

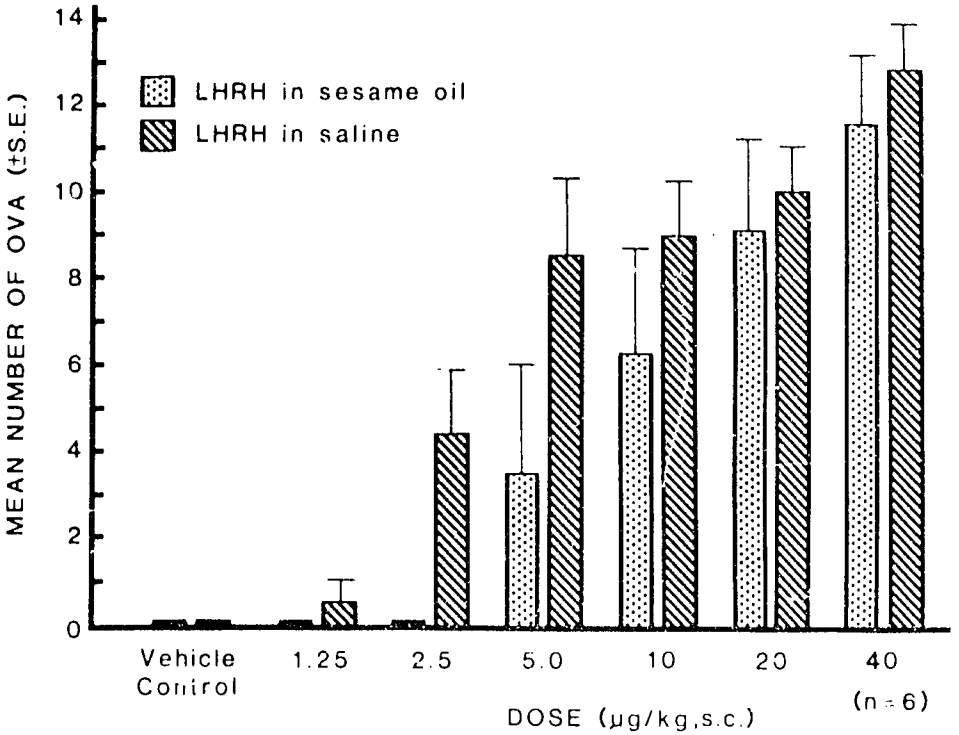

FIG. 34-2. Effect of vehicle on LHRH-induced ovulation in rats in diestrus.

Uterotropic Activity

The third bioassay system used in these studies was a measure of the uterotropic activity of LHRH. It has been reported that LHRH has both indirect and direct effects on the uterus.[4] In these studies, three groups of intact, immature female mice (21 days of age) were dosed subcutaneously once daily for three consecutive days, with 5, 50, or 500 μg/total dose of LHRH dissolved in either physiologic saline or sesame oil. Another three groups received estrone dissolved in sesame oil as a positive control at 0.03, 0.1, or 0.3 μg total dose, and a final group received vehicle alone. Mice were sacrificed approximately 24 hours after the last treatment, and uteri were excised, cleaned of surrounding tissue and weighed to the nearest 0.1 mg.

In our experience, data obtained with this model are generally consistent and reproducible. Figure 34-3 describes a typical experiment in which the effect of LHRH administered in the two different vehicles is measured. As can be seen from the positive control, estrone stimulated uterine growth in a dose-related manner. LHRH dissolved in sesame oil also produced a dose related uterine response. However, LHRH dissolved in physiologic saline had no effect on uterine growth at the doses tested. The explanation for the different responses in this animal model when LHRH is administered in the two vehicles is not clear. As discussed later in this chapter, however, we believe it may be related not only to differences in absorption rates of LHRH from

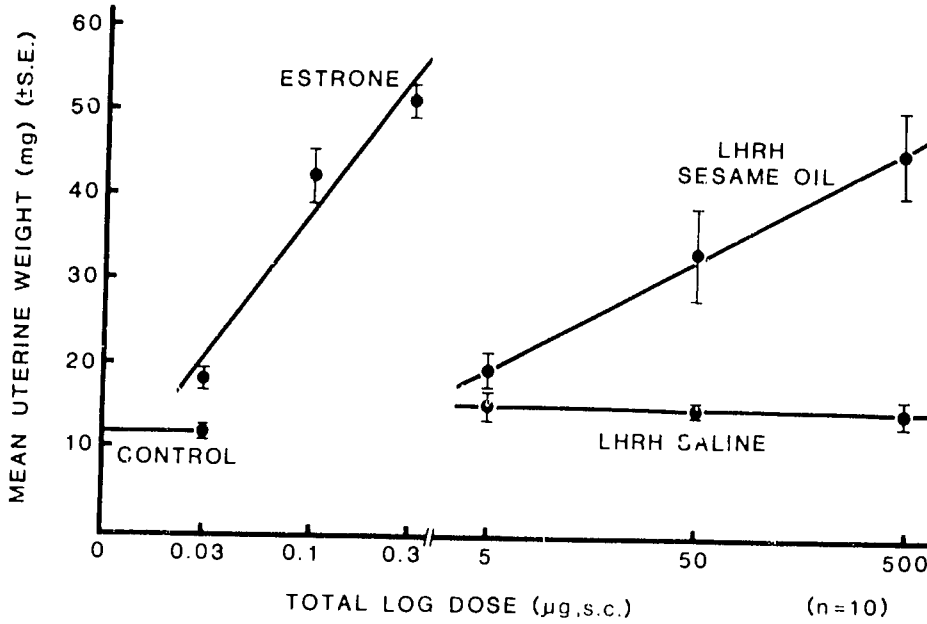

FIG. 34-3. Effect of vehicle on the uterotropic activity of LHRH in immature mice.

the two vehicles but also to the need for more prolonged serum levels of LHRH in order to stimulate uterine growth directly or indirectly. To test the latter hypothesis, we conducted an experiment in which we compared LHRH dissolved in saline administered to immature mice subcutaneously either once daily or five times daily at 500 μg total dose for 3 days. Vehicle control mean uterine weight was 9.0 ± 0.1 mg. The mean uterine weights of animals receiving LHRH once daily and five times daily were 14.5 ± 1.0 and 29.6 ± 2.4 mg, respectively. Though this is not proof of our hypothesis, it does support the concept.

One further point which we noticed, from a separate set of experiments which we conducted utilizing uterotropic growth as an endpoint, bears further discussion. Corbin and Beattie[4] have reported that LHRH in corn oil administered to either ovariectomized mice or hypophysectomized rats stimulates uterine growth. They suggested, based on these findings, that LHRH stimulated uterine growth directly. Utilizing ovariectomized immature female mice, we were unable to confirm that LHRH had this effect when dissolved either in physiologic saline or sesame oil. This difference may be due to variations between immature and mature animals, or in the composition of the vehicle or purity of the peptide. However, our data raise the question of whether LHRH does have a direct effect on the uterus.

DISCUSSION

Analysis of our data from the three experiments described suggests that vehicle was not the only variable affecting the biologic response to LHRH. If one compares the effect of LHRH in a common vehicle in the three bioassays

used, it is evident that different responses with respect to potency, activity, and slope are obtained from different assay systems. To illustrate this observation, we have replotted the data from the three studies described as percent maximum response. Although variations in responses to LHRH suspended in sesame oil are seen at lower doses, few differences are seen at higher doses. In Figure 34-4, androgen-sterilized rats were more sensitive than diestrous rats to LHRH in either vehicle. This difference was statistically significant ($P < 0.05$). In the uterotropic assay, LHRH was totally inactive in saline at the doses tested but showed a dose-related response when administered in sesame oil.

These data clearly demonstrate the need for caution in making comparative statements regarding potency and activity of LHRH, and presumably LHRH analogs, if the same assay or vehicle is not used to evaluate the compounds and if it is not certain that the relatively insoluble peptides are well distributed

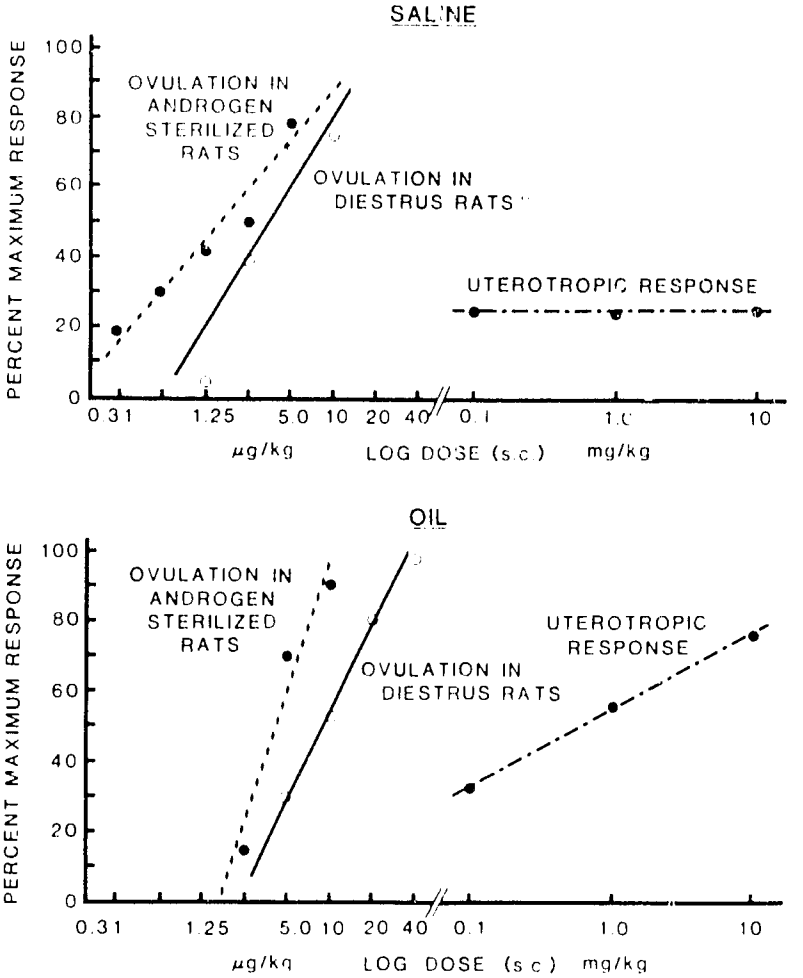

FIG. 34-4. Effect of vehicle and comparison of different bioassays of LHRH.

in vehicles such as sesame oil. The explanation for these differences in biologic response is not clear, but several possibilities exist.

First, the biopharmaceutic properties of peptides, such as solubility characteristics and partition coefficient, are such that LHRH would not be expected to be soluble in vehicles such as sesame oil. Thus, sesame oil becomes a suspending medium and one must assure uniformity of LHRH distribution prior to use. Second, LHRH may have several other biologic actions, such as a direct effect on other reproductive organs, in addition to stimulating secretion of pituitary gonadotropins.

Differences in the endocrine state may be important in the two animal models in which induction of ovulation is measured. One could speculate that because androgen-sterilized rats do not have a cyclic release of LHRH, these animals are more responsive to exogenous LHRH than are diestrous animals whose LHRH levels are cyclic and thus may be less sensitive to exogenous LHRH. It is believed that ovarian follicles in androgen-sterilized rats are mature[22] and primed to rupture upon stimulation by LH, whereas in diestrous animals, ovarian follicles are less mature, requiring time and perhaps more gonadotropin to rupture.

Finally, the different magnitude of response to LHRH in oil or saline in the uterotropic assay, as compared with the two test systems measuring induction of ovulation, may relate to a biologic need for a surge of LHRH and LH in order to induce ovulation, whereas the uterotropic response may require prolonged serum levels of LHRH. We suspect that the sesame oil is acting as a sustained-release form of LHRH and probably provides continual levels of LHRH to the circulation to impact either directly or indirectly on the uterus; whereas physiologic saline used as a vehicle provides a surge of LHRH to the circulation followed by a rapid drop in its plasma level owing to its short half-life.[18] It is apparent that the choice of vehicle utilized in the bioassay can either obtund the expression of LHRH action on the uterus or allow that activity to be expressed.

Vale and colleagues[20] have demonstrated that tissue culture assays and membrane binding assays of LHRH analogs do not always correlate, and the general relevance of these data to *in vivo* responses has yet to be proven.

We and others have shown that antifertility activity varies among species and may or may not correlate with LHRH agonistic or antagonistic activity *in vitro*.[7,12] The same is true for any anticipated therapeutic application for LHRH analogs. Our present data clearly demonstrate that variables inherent in bioassays used to study LHRH analogs, including vehicle, target tissue, or biologic endpoint being studied, must be controlled if valid conclusions are to be reached regarding the pharmacologic activity and potency of these peptides.

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Discussion: Safety and Secondary Pharmacologic Studies of LHRH Analogs

CARLOS A. SCHAFFENBURG, MODERATOR

Classical pharmacology in animals with LHRH or its analogs, to date, has revealed little in the way of activity of these peptides other than their effects on the endocrine organs. Direct cardiovascular, renal, hematopoietic, CNS, or other organ or system effects have not been demonstrated. Neither have these oligopeptide hormones shown acute or subacute toxicity in a variety of animal species even at high pharmacologic doses. This lack of organ or system toxicity in animals has been largely confirmed in clinical studies in men, women, and children, under a variety of physiologic conditions, albeit for relatively short periods of time.

In vivo and *in vitro* tissue distribution experiments in rats, using radiolabeled LHRH or its Buserelin analog, as well as pharmacokinetic studies, have demonstrated a quick peripheral enzymatic degradation of these substances into biologically inactive small peptides, thus largely allaying concerns about their persistent effects after withdrawal of administration. There is, unfortunately, a paucity of information concerning similar studies in other species, particularly in humans, but *in vitro* studies with human pituitary cells would seem to indicate that their response to LHRH and analogs is comparable to that obtained with rat tissues. Caution must be exerted, however, when interpreting data on *in vitro* binding to extrapituitary tissues, in view of the fact that *in vivo* studies with labeled material do not show either testicular or ovarian accumulation of the peptide. Down-regulation of the pituitary LHRH receptors seems difficult to demonstrate, even after administration of relatively large doses of the agonists, and such down-regulation seems to appear only when very high doses of the agonists are given by infusion over a period of several days.

As with all other hormones, the dose, the route, the manner and the duration of administration profoundly affect the response to LHRH and its analogs. The status of the subject at the moment of administration also affects the response, as amply demonstrated in the differences of response of women during the follicular as compared to the luteal phase, or whether the hormone is given to individuals lacking the endogenous hormone or showing evidence

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Panelists: William F. Crowley, Jr., Gabriel L. Garay, Do Won Hahn, Lakshmipaty Penumarthy, Jurgen K. Sandow. *Discussants:* Ricardo H. Asch, Kamran S. Moghissi, Carlos A. Schaffenburg

of dysfunctional endogenous secretion. Pulsatile administration of LHRH by a mechanically driven pump to individuals lacking the endogenous hormone, as is the case in Kallman's syndrome, has shown itself capable of inducing a hormonally nearly normal menstrual cycle, with normal ovulation and even pregnancy in some cases. We are, however, still largely ignorant of the effects of pulsatile LHRH administration to individuals possessing a degree of endogenous secretion of the hormone, such as in hypothalamic amenorrhea, where presumably an artificial rhythm aimed at mimicking the normal would be superimposed on an existing rhythm, albeit a pathologic one. The effect might be inhibitory rather than stimulatory, and the problem is compounded by our ignorance of the pulsing LH rhythm in normally menstruating women. A worthwhile endeavor is, therefore, for investigators to try to establish the pattern of gonadotropin pulses in normal women of various ages by a methodology comparable for the various institutions.

The experiments presented in this session point to the need for studying the pharmacokinetics of LHRH and analogs in men and women, with the ultimate aim of determining the most practical route of administration. While it is important to establish the response to these substances when given by methods mimicking as closely as possible what we deem to be the physiologic manner, such as the pulsing administration by pumps, such methods would be impractical for wide clinical application. It is important, from an academic and scientific point of view, to demonstrate the induction of ovulation and a normal hormonal cycle in women by methods such as the one mentioned. Present methods of administration of these substances, however, remain impractical when we contemplate their possible application for contraceptive purposes, particularly considering that more practical and fairly safe methods are presently available for this indication. It is, therefore, not entirely premature for investigators to undertake studies of practical, safe, and effective methods of drug delivery, if use of LHRH and analogs is to leave the realms of academic experimentation and enter the field of practical application. The safety of these substances, after long-range and wide application, remains a problem to be solved gradually and with caution.

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PANEL DISCUSSION 1: The Future of LHRH Analogs as Contraceptive Agents for Men

EMIL STEINBERGER, MODERATOR

The panel was charged with the discussion of several specific topics. Each topic is summarized and critically discussed by the moderator.

POTENTIAL OF LHRH AGONISTS OR ANTAGONISTS TO PROVIDE FULLY EFFECTIVE AND FULLY SAFE CONTRACEPTIVE AGENTS FOR MEN

The fundamental response to both the agonists and the antagonists in a number of species, with the possible exception of the monkey, is similar and akin to the responses observed in humans. Some investigators feel that, since certain differences in response to LHRH analogs have been noted among various species, these differences should be studied in greater detail. Furthermore, additional species should be investigated in order to delineate in a more precise fashion the physiologic and biochemical mechanisms associated with the action of the analogs. Some discussants supported the view that the rat serves as an adequate animal model for studies of LHRH analogs, pointing to the facts that most of the original investigations were conducted in the rat and a large body of information gained from those studies has been shown to apply to the human. Others cautioned that generalization based on results obtained in the rat, particularly in respect to toxicology, need careful evaluation in several species. The latter view has merit, but only when dissected into specific components. Undoubtedly, relative to studies of toxicology and pharmacology of any substance, no animal model will ever provide an answer to the question as to whether the substance will be fully effective and fully safe in the human. Since LHRH analogs have been administered to a relatively large number of human subjects over a fairly large dose range with no detectable detrimental effects, further studies should be focused on pharmacologic and toxicologic parameters in the human unless a specific contraindication can be demonstrated.

Panelists: Fernand Labrie, David Rabin, Catherine Rivier. *Discussants:* Elsimar Metzker Coutinho, William F. Crowley, Jr., Richard A. Edgren, Joseph W. Goldzieher, Kamran Moghissi, D. J. Patanelli, Roberto Rivera Damm, Jurgen K. Sandow, Carlos A. Schaffenburg, Brian H. Vickery

Although both the LHRH agonists and antagonists induce spermatogenic arrest, the damage to the seminiferous epithelium resulting from administration of agonists is characterized by patchy lesions in the seminiferous tubules; some tubules show complete disruption of the seminiferous epithelium with degeneration of most of the germ cells while others retain complete and orderly spermatogenic process. The testes of antagonist-treated animals show a more uniform picture of spermatogenic arrest involving all seminiferous tubules, very much akin to the picture of testes from hypophysectomized males. This uniformity of seminiferous tubule response is considered to be an important advantage of the antagonists over agonists as potential male contraceptives.

One area of investigation where the application of animal models is essential in the study of LHRH as a potential male contraceptive deals with the development of long-acting preparations. In a series of such studies, it has been shown that chronic administration of an agonist at a constant rate to the rat, utilizing a minipump, induces severe (90%) depletion of pituitary gonadotropins and an "inactive pituitary" state. This raises the question of the desirability of producing a chronic pituitary "involution" or "atrophy." Obviously this observation could not have been made in studies on human subjects; thus the details of this process—as well as its possible implication—will have to be further investigated in the lower species.

The original observation of antispermatogenic activity of LHRH agonists caught all the participants by surprise. However, the application of current knowledge of the molecular mechanism of hormone action in the testes provided a plausible explanation at least for some of the aspects of the antispermatogenic activity of the agonist. However, extrapolation of this knowledge to the human using the fundamental data on molecular mechanisms operating in the lower species is not warranted since the current understanding of the physiologic and biochemical events occurring in the human testes in response to hormones is at best conjectural and not supported by adequate direct data. Consequently, to elucidate the molecular mechanisms of the effects of LHRH analogs on the testes, further experimentation on lower species is essential; however, a concerted effort also needs to be exerted in the study of molecular mechanisms operative in normal human testes in preparation for the investigation of the effects of LHRH analogs in the human

SIDE-EFFECTS IN THE REPRODUCTIVE AND EXTRAREPRODUCTIVE TRACT WITH CHRONIC ADMINISTRATION OF LHRH ANALOGS

Discussion of this matter took an interesting turn. Apparently, no serious extragonadal side-effects of LHRH could be clearly demonstrated in any of the studies. This bears on the previous discussion concerning the advisability of expansion of these studies into the human on the basis of available toxicologic and physiologic data in the lower species.

The panel felt that the answer to this question should be affirmative. The primary concern in respect to side-effects of LHRH analogs in the human, is the suppression of androgen production with the attendant disturbance in

libido and potency. This leads to the obvious issue of substitution therapy with androgens and the questions of drug interaction. As one of the participants stated, as soon as a requirement is developed for administration of two drugs at the same time, the pharmacologic and toxicologic problems become astronomical, creating an entirely new series of problems. The question of androgen substitution therapy in the development of a male contraceptive has been discussed during the past several years in many publications dealing with the topic of male contraception.

Participants of this meeting also wondered about the following issues:

1. What will happen to Leydig cells after a period of chronic suppression?
2. Is chronic administration of testosterone over a long period of time advisable?
3. Will the fertility potential of men be affected by testosterone treatment?
4. Will there be any effect on the offspring conceived after discontinuation of testosterone therapy?
5. What form of androgens could and should be used?
6. Do we have appropriate androgen preparations for use as replacement therapy?

These questions should be, and frequently are, raised in respect to any pharmacologic agent, biologic substance or nutrient, exposure to any physical modality (*e.g.*, radiation, heat, altitude) or exposure to stress of any form. Actually, there is probably more clinical, pharmacologic, and toxicologic information in respect to the effect of long-term replacement therapy with testosterone than there is for most currently approved drugs used in a chronic manner in the human male. Physiologic aberrations and pathologic changes have been seen after chronic administration of androgens at doses creating marked elevation in its circulating levels, but most data on long-term administration of androgens at doses that result in relatively physiologic circulating levels have failed to uncover any serious side-effects resulting in a permanent health hazard. Similarly, no evidence has been uncovered to suggest permanent injury to Leydig cells or to the seminiferous epithelium resulting in either inadequate recovery of androgenic function of the testes or induction of permanent sterility. No increased incidence in congenital defects has been observed in offspring of men whose testes have been suppressed by or exposed to exogenously administered androgens.

Thus, although we must remain alert to such possibilities, at the same time we cannot be paralyzed by the thought that chronic administration of androgens in any amount over a long period of time may not be desirable. What we may wish to address, however, is the question of availability of appropriate long-acting preparations of androgens. It was forcefully pointed out by the panel participants that we ought to recognize that replacement therapy with testosterone has made no progress in the last 40 years. This probably is the most difficult and the most serious obstacle in the development of a "first-generation" systemic male contraceptive. It has been clearly demonstrated by a number of investigators for both the lower species and humans, that suppression of androgen production by endocrine means leads to reversible cessation of spermatogenesis, thus to an infertile state even when testosterone

is administered at doses which will result in normal circulating levels of androgens with the accompanying maintenance of normal libido and potency. The stumbling block in each of these approaches, whether with combinations of LHRH and analogs, progestins and androgens, or androgens alone, has been the need for unacceptably frequent parenteral administration of androgen preparations. Unquestionably, availability of appropriate parenteral androgen preparation that would maintain stable and precisely defined circulating androgen levels and could be administered at infrequent intervals would rapidly lead to the development of the first-generation male contraceptive. Development of an effective and safe oral preparation would probably be even more desirable. In other words, the issue at hand does not address a fundamental basic science question but deals with a purely pharmaceutical development issue. Neither the private sector nor the various agencies have placed a sufficiently enthusiastic endorsement behind the idea of such pharmaceutical development to expect positive results within the foreseeable future. Most likely, a breakthrough will result from the serendipity of some investigator who will "stumble" upon the answer to this question.

Another issue addressed by the panelists dealt with whether testosterone is an ideal androgen for replacement therapy. One question concerned the desirability of an aromatizable androgen for use in androgen replacement therapy. It has been suggested that investigators look at long-acting derivatives of nonaromatizable androgens such as dihydrotestosterone. This would be a very valid avenue for exploration, not only from the viewpoint of developing a male contraceptive, but also of developing more effective androgen replacement therapy for patients with hypoandrogenic syndromes.

MODE, SCHEDULE OF ADMINISTRATION, AND FORMS OF LHRH ANALOGS

Most studies conducted to date in the human male have been of short duration. This has precluded the development of "best" protocols for study of induction and maintenance of long-term suppression of sperm production. The development of impotence or loss of libido requires discontinuation of the study in agreement with FDA guidelines in respect to protocol. The protocol and experimental design restrictions mandated by regulatory agencies, while absolutely appropriate for this stage of human experimentation with LHRH, have restricted the investigators in their attempt to answer some of these mundane but absolutely essential questions dealing with long-term pharmacokinetics, biologic responses, and possible pathophysiologic sequelae. On the other hand, the question of LHRH preparations and route of administration could be approached in short-term studies as currently approved by the regulatory agencies.

Both the practical and the philosophical question of whether more potent analogs are needed evoked a lively discussion. One viewpoint could be summarized by the statement: A lot of the jugging with drugs is of very limited scientific and therapeutic importance. Although this is a valid practical and philosophical viewpoint, an opposite viewpoint has been voiced in respect to intranasal route of LHRH administration. Apparently, the amount of LHRH

required to obtain adequate biologic response by this route of administration is large. An analog with much greater potency (20- to 40-fold) would simplify this form of administration of LHRH.

Another issue in support for the need to develop more potent analogs is cost. Since the intranasal route requires inordinate amounts of drug, the cost of this therapy becomes prohibitive; synthesis of more potent analogs could bring the cost down into an economically more feasible range.

FUTURE STUDIES OF LHRH AS A CONTRACEPTIVE IN MEN

One of the major issues raised at all workshops concerned with the development of a male contraceptive is the question of acceptability. In respect to this issue, we are long on opinions and short on facts. One participant stated that investigators should not lock themselves into any preconceived ideas about what will be acceptable and what will not. He noted that in 1958 or 1959, he thought that giving women with limited education the option of taking a pill every day for 21 days would never succeed in a lower economic group, and he was proven wrong. Another participant pointed out that in talking about acceptability, they were talking in a vacuum, because acceptability in a clinic is one thing, demographic acceptability is another, cultural acceptability is another, and ethnic acceptability still another.

This viewpoint was eloquently illustrated by one of the participants from South America, who had considerable experience with administration of male contraceptives, after having treated more than 100 men. His experience with specific ethnic and socioeconomic groups led him to the conclusion that the men might take a contraceptive daily, twice daily, weekly, once a month, as an injectable or pill or other form if they are really motivated; if the man has 18 children but not enough food for them, he will accept any kind of contraceptive. He may even accept impotence. It appears that survey studies of acceptability of a male contraceptive may not be the first priority as far as the need for "future studies." Unquestionably, we must first develop an effective and safe contraceptive before we need to be seriously concerned about acceptability. It is likely that a man will accept a variety of forms and routes of administration of a contraceptive if he is appropriately motivated by his immediate personal needs and the environment.

If the investigators, the granting agencies, and the FDA accepted the idea that a segment of male population would be willing to live with a contraceptive to be administered by injection every 12 days or every 3 weeks, a number of avenues are open now; this includes the use of LHRH analogs. Since some FDA representatives indicated that the basic pharmacology and toxicology studies performed by now in lower species are impressive and that a number of analogs have had sufficient toxicology data to back up very considerable clinical studies, the obvious emphasis for future studies should center on investigations of the efficacy and safety of this contraceptive approach in the human male. These studies should lean heavily towards the investigation of fundamental pharmacology, physiology, and pathophysiology of LHRH in the human male. Wherever possible, then, clinical trials should not be constructed with the only purpose in mind to demonstrate efficacy and safety,

but should also be directed to answering questions dealing with molecular mechanisms of actions of LHRH in the human male.

The participants agreed that the investigation in the lower species should be supported vigorously with a focus on species' differences, particularly on the question of LHRH effects in the primates, on development of more powerful agonist, on long-acting agonist and on different forms and routes of drug delivery. The question of differences in the response of seminiferous epithelium to agonists vs. antagonists is of fundamental as well as clinical importance and needs to be approached via studies in lower species.

CONCLUSION

Optimism was evident among the participants in this workshop regarding the future of LHRH analogs as possible male contraceptives. A large body of data summarized at the workshop demonstrated that considerable insight has been gained in the past 3 years in respect to the paradoxical effects of LHRH agonists on testicular function. The relative freedom from serious toxic effects, as evidenced by results of extensive studies of lower species, as well as by limited studies in the human, is encouraging and should stimulate rapid expansion of the work to the human. Although it does not appear that currently available LHRH analogs, at least by themselves, will be effective and acceptable male contraceptives, formulation of an appropriate combination of LHRH with androgens or development of new and novel analogs appears to be a promising avenue of investigation which may lead to the development of a first-generation male contraceptive in the near future.

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PANEL DISCUSSION 2: Further Development of LHRH Analogs as Contraceptive Agents for Women

ALAN CORBIN, MODERATOR

It was only 10 years ago that Dr. Schally reported the identification and synthesis of LHRH, leading to the present generation of over 1000 analogs from numerous laboratories. We now have available many highly potent agonists that have been undergoing extensive pharmacologic, safety, and clinical evaluation as potential profertility and contraceptive agents. Only recently have the antagonists become available of an order of potency of the agonists that will now permit their detailed biologic study.

In terms of what are the needs to be fulfilled for the further development of LHRH analogs as female contraceptive agents, the following main points were discussed.

LABORATORY MODELS

There is no singular animal model that can fulfill the criteria of total reliability and predictability for contraceptive efficacy and safety in the human. The various animal species utilized (rodents, canines, subhuman primates) all have contributed (whether positively or negatively) to our understanding of the pharmacology of the analogs under investigation, as well as the reproductive physiology of the animal model when such compounds are used as pharmacologic tools. While costs, availability, ease of handling, reproductive cycle, responsiveness, and other factors are significant variables to be considered, it is important that as much information as possible be generated from a wide variety of species. Hopefully, this will provide a more coherent picture than the somewhat fragmented story presently available, and facilitate an educated guess, based on the total data, as to the potential clinical efficacy and safety of the analogs. Clearly, there is no substitute for actual clinical trials, and only after sufficient human data have become available will we be able to judge

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Panel: Ricardo Asch, Elsimar M. Coutinho, Manfred Schmidt-Gollwitzer. *Discussants:* Richard Edgren, Joseph W. Goldzieher, Fernand Labrie, Hugo Maia, Jr., Kamran S. Moghissi, Subir Roy, Jurgen K. Sandow, Carlos A. Schaffenburg, Brian H. Vickery

how appropriate the laboratory models actually were. This is a common problem that has always plagued pharmacologic research irrespective of the nature of the physiologic system or the therapy under design.

SAFETY AND UTILITY

To date, the toxicologic, pathologic and secondary pharmacologic studies clearly demonstrate the safety of many of the agonists, and especially those that are being evaluated in chronic human contraceptive studies. The animal studies, conducted by various routes of administration and regimens, at doses in considerable excess of anticipated human doses, support the wide safety margins already emerging. No untoward nonreproductive effects have been observed. However, human trials have not been extensive enough (in terms of numbers of subjects and years of continuous exposure) to rule out the potential for effects on metabolic and biochemical parameters. The safety data are indeed exceptionally encouraging; any unwanted effects have been restricted to the reproductive system and represent extensions and excessive manifestations of the basic reproductive properties of the agonists. Furthermore, the antireproductive effects of the agonists are reversible.

In terms of drug regulatory agencies, the safety studies thus far performed have been most acceptable in identifying risk liabilities, if indeed they exist. No hard-and-fast guidelines exist regarding the FDA, and safety studies generally will be carried out on a need basis to support, for the most part, the chronic (in excess of a year) studies in women that will be required to refine the clinical protocols.

In terms of utility, we have heard lively discussions on the ability of the agonists to inhibit ovulation or produce luteolysis, alter steroidogenesis, shorten the cycle, and induce premature menses, spotting, or amenorrhea. Concern has been expressed over possible resultant inappropriate endometrial function: atrophy due to impeding estrogen secretion; excessive proliferation due to luteolysis, and progesterone decline leading to a condition of unopposed estrogen.

This complex of reproductive effects, all of which represent a contraceptive event in one form or another, dictates that a careful analysis of the various strategies be made:

1. Do we administer the agonists chronically, only during the follicular phase, only during the luteal phase, or in combinations or variations thereof?
2. In spite of the inability of the agonists to terminate a 4- to 6-week pregnancy in the human (contrary to the effect in some animal models, and presumably because of the protective presence of hCG), would administration earlier in pregnancy (*i.e.*, pre- or peri-implantationally) be effective?
3. Would the potential for unopposed estrogen under certain dosing regimens provide risk for endometrial cancer?
4. Would the shortening of the estrogen phase create an osteoporotic liability?

5. Would poor cycle regulation (*i.e.*, spotting or chronic amenorrhea) be acceptable in spite of good efficacy?

Obviously, the questions that have arisen and that will continue to arise far exceed our ability to answer them at this time. We simply do not have enough clinical information to design a strategy that will provide us with a clear-cut contraceptive utility. We do know three things: the agonists can inhibit ovulation; they can induce luteolysis; and they are effective by nasal spray, an approach which has a high degree of patient compliance and which represents the most practical self-administered approach available. How to effectively use either of these two contraceptive methods in a predictable, acceptable, and cycle-regulating manner simply must await future clinical research on large female populations.

SYNTHESIS OF EVEN MORE POTENT AGONISTS AND ANTAGONISTS

Should this be encouraged? The consensus among the workshop participants was affirmative. Can more potent analogs be discovered? The feeling was that we should find out. Success has been achieved with the agonists to date, and the newly modified antagonists have spanned a potency increase of approximately 300 to 1000 since the advent of these traditional ovulatory inhibitors. Surely, any increase in potency presumes smaller utilizable doses, perhaps oral application and reduced cost of large-scale production.

In summary, we have learned a great deal in terms of animal models, efficacy, safety, mechanisms of action, chemistry, and clinical aspects. Most important, though, we have learned how complicated the LHRH contraceptive area really is, where our deficiencies lie in understanding and design, and how much more work is required to establish clear clinical guidelines and, hopefully, to bring this work to fruition.

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