PN-ABH-945.
15536
CS-101

Final Report for AID Grant No. DPE-5544-G-SS-6046-00

Title of Proposal:
Isolation of a luteotropic substance from the bovine placenta as a basis for a new pregnancy test in cattle.

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Rec'd In SCI AUG 13 1990

Final Report

AID Grant No. DPE-5544-G-SS-6046-00

June 30, 1990

Title: Isolation of a luteotropic substance produced by the blastocyst and early pregnancy diagnosis in cattle.

Objectives:

1. Determination of the FSH-like activity of the bovine chorionic gonadotropin (bCG).
2. Determination of LH-like activity of the bCG.
3. Define the source of bCG.
4. Purification of the bCG by use of granules preparations and tissue culture media of early trophoblasts.
5. Purification of the bCG by use of HPLC.
6. Purification of the protein by isoelectric focusing.
7. Preparation of antibody to bCG.
8. Testing of the bCG in vivo on its effect on prolonging the lifespan of the corpus luteum.

1. Determination of the FSH-like activity of the bCG.

The technique of culturing trophoblast cells for several weeks proved to be a reliable method for detection of small amounts of bCG in the culture medium. To define the endocrine activity of the bovine trophoblast with respect to bCG gestational age (30-36 embryos) were cultured in Medium 199 supplemented with 2.5% rabbit serum. Cultured media were changed every 4 days, and biological potency of FSH-like substance in the 3M ammonium sulfate precipate was determine by radio-receptor assay (RRA; fetal calf testicular membranes, iodionated hFSH) and also by an in vitro asay in which androgens agument FSH-induced progesterone secretion by cultured rat granulosa (Armstrong and Dorrington, Endocrinology 99:1411,1976; Nimrod and Lindner, 1976, Mol. Cell. Endocrinol. 5:315,1976), using immature, hypophysectomized, diethylstilbestrol-treated rats.

As measured by RRA, secretion of FSH-like substance increased gradually from N.D. at 4 days to 60±12 ng/mg protein at 8 days and to 460±220 ng/mg at 12 days of culture, reaching a peak of 1180±410 ng/mg protein at 16 days of culture (Fig. 1). Furthermore, when 40% ammonium sulfate precipitated tissue culture medium from trophoblast 18-35 days of gestation were extracted on Sephacryl 300 columns and activity measured by radioreceptor assay (RRA), two main peaks, one of 70 KD and one of 30-35KD, which inhibited binding of labelled hCG to its receptor were noted (Data not shown). Male rat testicular membranes were used as a source of receptors (Ailenberg and Shemesh, Biol. Reprod. 28:517, 1983). It was also found that primary cultures of trophoblasts could serve as a source of bCG for at least six weeks.

To determine the biological activity of the FSH-like substance harvested from the tissue culture medium, we studied the synergistic effect of testosterone on the stimulation of progesterone secretion by FSH and bCG in cultured rat granulosa cells. Androgenic steroids have marked synergic effect on the stimulation of progesterone production by FSH in rat granulosa cells. Thus the cultured rat granulosa cell can be used as model system for the study of the FSH action of trophoblast extract.

As shown in Fig. 2, rat granulosa cells were cultured for 72h in the presence of testosterone, trophoblast secretory extract or combination of testosterone and extract. Both testosterone and the extract increased progesterone formation significantly ($P<0.01$; $n=4$). However, in the presence of testosterone (200 ng/ml), a significant stimulatory effect of the extract ($P<0.01$) was apparent. In contrast,

extracts from 246-260 day (N=3) placentae, alone or with testosterone, failed to stimulate progesterone formation. However, both trophoblast secretory extract and placental extract significantly stimulated the conversion of testosterone to estradiol by the cultured immature hypophysectomized rat granulosa cell.

2. Determination of the LH-like activity of the bCG.

We next examined the tropic effect of trophoblast secretory extract on progesterone secretion by the bovine corpora lutea cells to determine if bCG has LH activity (Fig. 3). Dispersed luteal cells (day 12 of the cycle) were incubated with 1 mg of the trophoblast secretory extract alone, or with the phosphodiesterase inhibitor, methyl-isobutyl-xanthine (MIX; 0.5mM). Both 8Br-cAMP and the trophoblastic extract increased progesterone production significantly ($P<0.01$). Furthermore the combination of MIX with trophoblastic extract increased progesterone production over that seen with MIX or extract alone ($P<0.01$).

We conclude that the cultured trophoblast cell in tissue culture medium 199 produces bCG with both FSH and LH-like activities as determined by (1) RRA, (2) the synergistic effect of testosterone on the stimulation of progesterone secretion by bCG in cutured rat granulosa and (3) the increase in estradiol production over that produced in response to exogenous testosterone alone caused by the cultured granulosa cells. (4) the increase in progesterone production by the bovine luteal cells and rat granulosa cells.

3. Defining the sources of bCG.

It was found that trophoblastic cells present in the chorion, which produced bCG from 18-35 days of gestation, did not produce bCG after 45 days of gestation. However, the newly formed feral cotyledonary cells of 50 days and older do produce bCG as shown by direct extraction using 40% ammonium sulfate precipitation.

4. Develop a method of extracting bCG from granules.

We found that extraction of granules was a good source of bCG. Total granule extract was prepared as described by Izhar and Shemesh, 1989 (attached). Separation of the granule extract on Sephacryl S-300 colums yielded two major peaks, one of high mol wt of 70 KD and a second peak of low mol wt (30-35 KD). The two peaks were active in both RRA and bioassay (stimulation of progesterone production by luteal cells; Fig. 1 in text of Izhar and Shemesh, 1989).

5. Develop a method of purifying bCG on HPLC.

To further purify the bCG from the granule, it was found that acid treatment of the granule extract removed the high mol wt protein but enriched the 30-35 KD protein by three fold in the bioassay compared to total extract. Further purification on HPLC resulted in an 100 fold increase in activity (Fig. 2,3,4 in text of Izhar and Shemesh, 1989).

6. Develop a method of purifying bCG by electrophoresis.

Preparative isolectric focusing was carried out using a flat-bed LKB system (Multiphor). A circulation cooling bath maintained the cooling place at a temperature of 4°C. A pH gradient of pH 3-10 was prepared using ampholytes purchased from Pharmacia (Uppsala, Sweden), on a support of Ultradex (LKB). Samples to be subjected to preparative isoelectric focusing (300 mg protein were mixed with an ampholyte/ Ultradex slurry and added to the flat-bed plate. Isoelectric focusing was then carried out as described in Shemesh et al., Biol. Reprod. 29:856, 1983. After isoelectric focusing, a grid was applied to the bed, dividing it into 15 fractions. Each section was removed, mixed in a tube with 1 ml of deionized water and poured into a 5x100 mm column (Econo-column, Bio-Rad, Richmond, CA). The gel was then eluted with 5 ml of deionized water. The pH of each eluate was determined; the eluates were then dialyzed against deionized water to remove ampholytes and 0.1 ml aliquots of the fraction were examined for binding in the radioreceptor assay.

It was found that the 20% ammonium sulfate precipitate that was separated on the gel yielded two principal peaks; one at pH 4.5 and one at pH 9.5. Fig. 4 represents the isoelectricfocusing of bCG as determined by RRA in 9 separate experiments.

7. Preparation of antibody.

We have produced an antibody to the actives high mol. wt. and low mol. wt fractions. This antibody was found to cross-react in a dose-dependent mannor with oFSH and bLH but not with bovine prolactin using concentrations of 0.4 to 10 µg (Fig.5).

8. Biological activity in vivo

We investigated the effects of intrauterine infusion of cuture media from 18 day old embryos into cycling heifers. Donor heifers were superovulated, inseminated and hysterectomized on Days 17-19 post breeding to recover embryo. The embryos were then isolated and cultured in Medium 199, 5% rabbit serum and antibiotics. The media were changed every 4 days and the spent media were frozen. Six heifers with histories of normal cycles had the horn of the uterus ipsilateral to the CL cannulated on day 10 of the estrous cycle. The uteri were then infused with culture media, beginning on Day 12-13 and continuing for 9-14 days. Six control heifers were infused for 13 days with fresh media containing antibiotic. Jugular progesterone concentrations were deterimined in samples colllected daily from day 10 to 26 of the estrous cycle.

The mean (±s.e) time at which jugular progesterone concentrations fell below 1.0 ng/ml in the control heifers was 19.66±0.88 days. In 4 of the 6 treated heifers progesterone did not fall below 1.0ng/ml during the infusion period but did so in the first sample take after the infusion ended (Fig. 6,7).

In another series of experiments, the low mol. wt. active fraction of bCG obtained from fetal cotyledons of 50-150 days was infused under a similar protocol. It was found that the bCG prolonged the lifespan of the corpus luteum and delayed the return to estrus (Fig. 8). These in vivo experiments were done in cooperation with Prof. William Hansel of Cornell Univ. Preliminary evidence indicates similar effects with the high mol. wt. fractions but these experiments are not yet completed.

Summary

The pesent work demonstrate that bCG is present in the early trophoblast at 16 to 35 days of gestation and thereafter it is produced by the fetal cotyledon from 50 to at least 150 days of gestation (It is apparently not produced by fetal cotyledons near term.) The material has FSH and LH activity as demonstrated in radio-receptor assay, bioassay and antibody cross-reaction. The material was demonstrated to be active in vivo in at least that it will prolonged the life of corpus luteum.

Significance

We have confirmed our initial observations that a chorionic gonadotropin (bCG) is produced by the bovine placenta. Since we have also produced an antibody to this substance, it should now be possible to develop a pregnancy test and allow the study of the effects of purified bCG on fertility.

Cooperation

Dr. Jorge Camacho, the Costa Rican partner, came to Israel. During this time he learned radioimmunoassay for determination of hormones in bovine sera and other technique use in diagnosis of infertility in cattle which will be necessary to evaluate the use of bCG in cattle in Costa Rica. On his return to Costa Rica, he began to establish a diagnostic service for pregnancy and infertility in cattle which was not previously availble in Costa Rica. Dr. Shemesh travelled to Costa Rica to help him establish the laboratory. This work was also done in cooperation with Prof. William Hansel, Dept. of Physiology, Cornell University, with whom the in vivo experiments were done.

Appendix

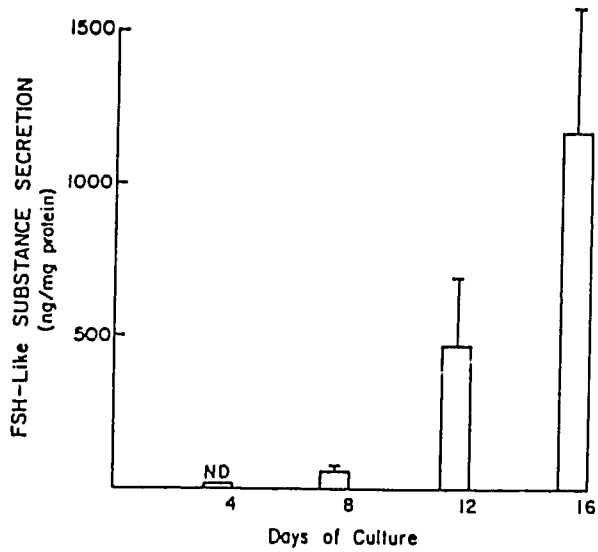

Figure 1. FSH-like substance secretion by cultured dispersed trophoblast cells from 18 days of gestation. Medium was changed every 4 days of culture and FSH-like substance secretion during the subsequent 4 days was determined by a specific RRA, using calf testes as a source of receptor. Values presented are means of ± S. E. M. of three different batches of each 10-12 embryos each.

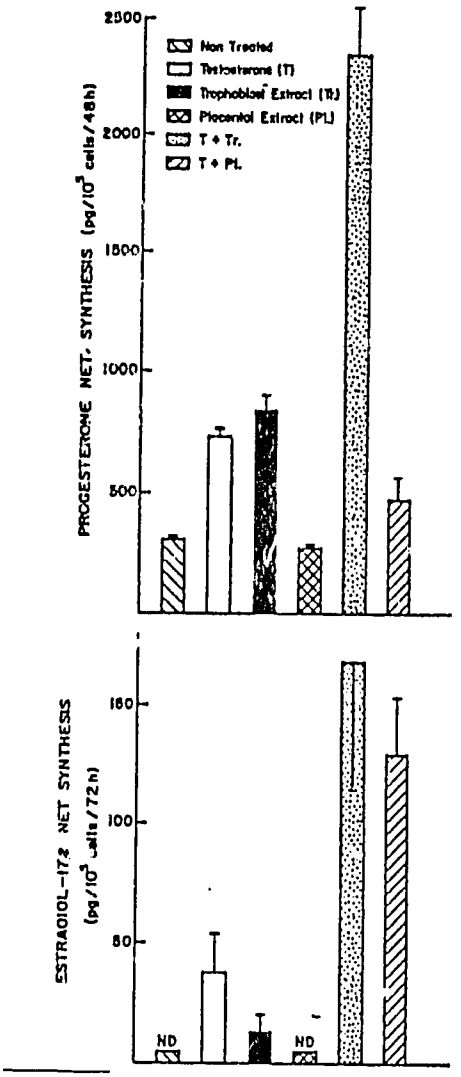

Figure 2. Stimulation of progesterone and estradiol-17 β accumulation by immature hypophysectomized DES treated rat granulosa cell cultures during 72 h of incubation by testosterone and an FSH-like substance in fetal trophoblast. The FSH-like substance was harvested from trophoblast collected at 12 and 16 days. Values presented are means $\pm$ S. E. M. of four different experiments.

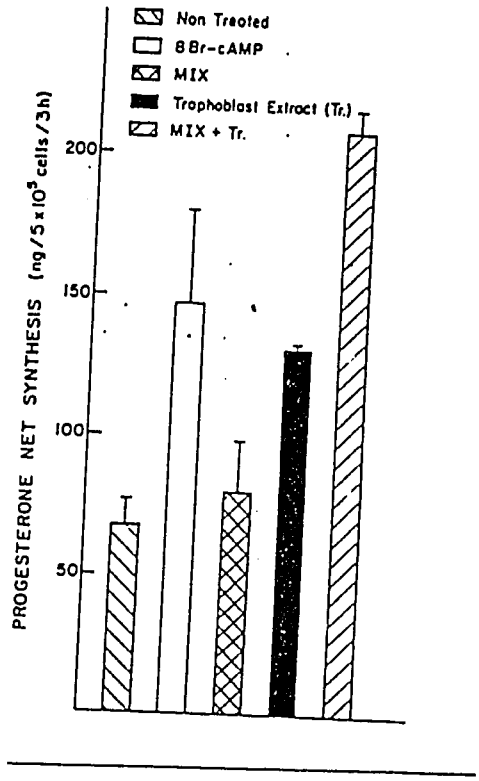

Figure 3. The effect of methylisobutyl xanthine (MIX) and trophoblast extract from 12 and 16 days of culture on progesterone biosynthesis by incubated dispersed corpus luteum (D12) cells during 3 h of incubation. Values presented are means ± S. E. M. for net synthesis by four corpora lutea.

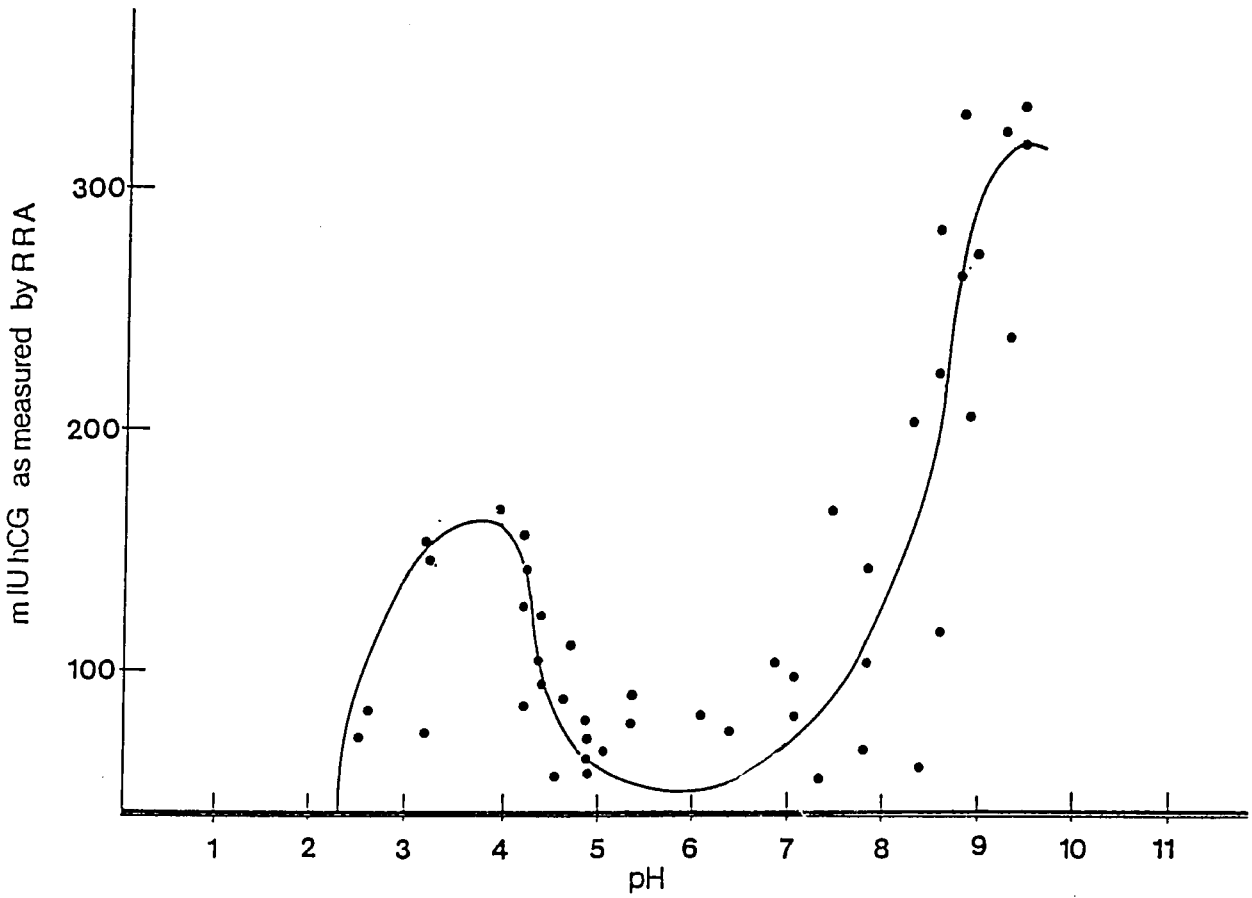

Fig. 4 hCG–like activity as measured in extracts of gel electofocused fractions of ammonium sulfate precipitate of fetal cotyledons from 50–150 days of gestation.

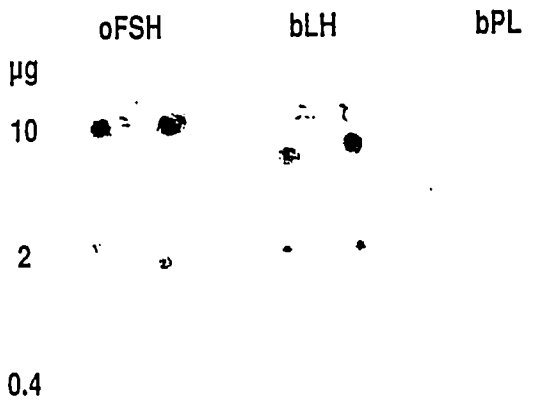

Fig. 5. Dot blot hybridization of polyclonal antibody against purified bCG with oFSH, bLH and bovine prolactin. The antibody cross-reacted in a dose-dependent manner with oFSH and bLH over the range of 0.4 to 10µg of protein. The antibody did not cross-react with bovine prolactin or hCG (not shown).

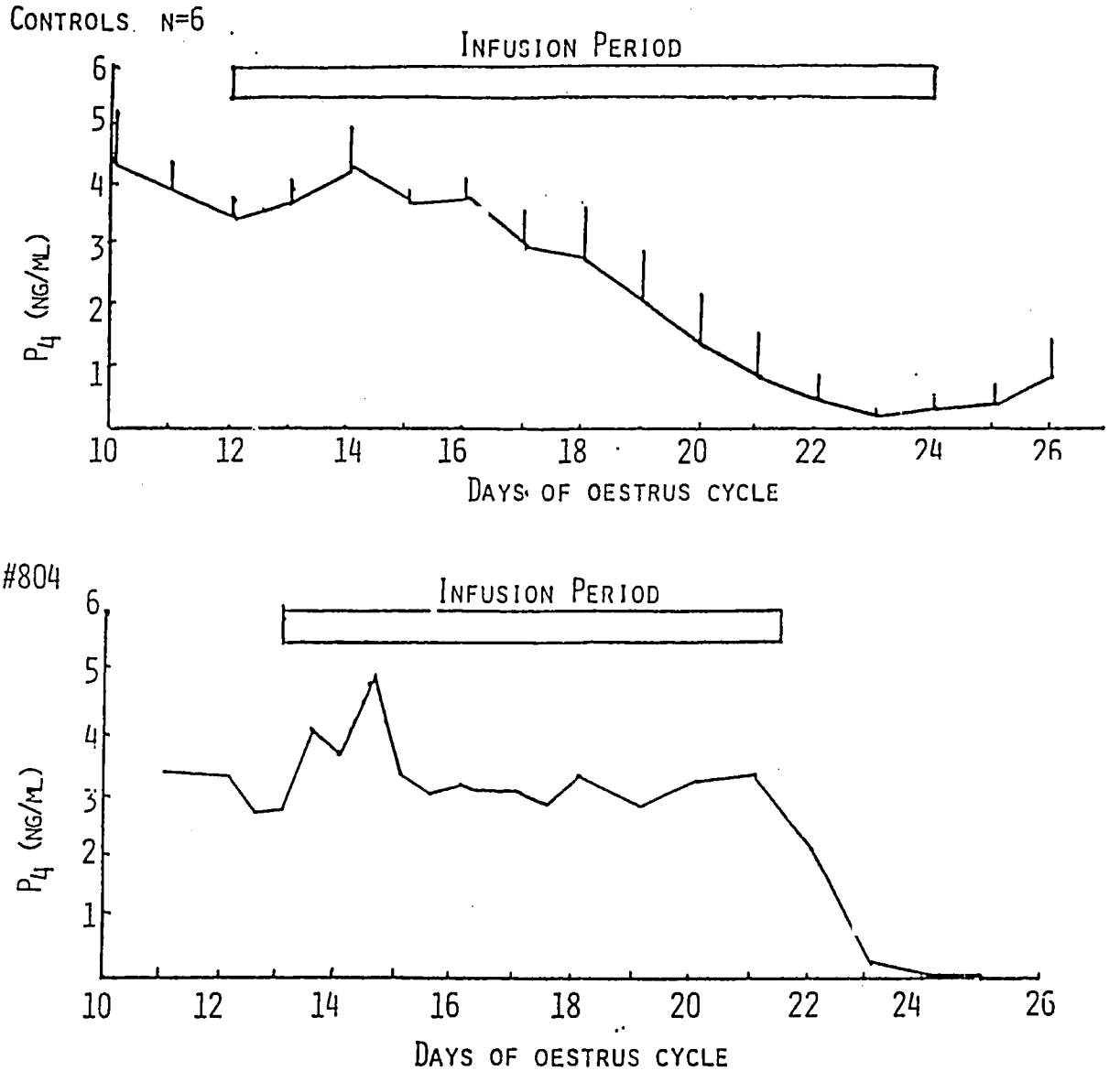

Figure 6 Upper panel. Mean (± S. E.) jugular progesterone (P_4) concentrations for 6 control heifers receiving intrauterine infusions of Medium 199 during Days 12-24 of the estrous cycle. Lower panel. Jugular plasma progesterone concentrations for a heifer (#804) infused during Days 13-21 of the estrous cycle with pooled media from cultures of 18 day trophoblast.

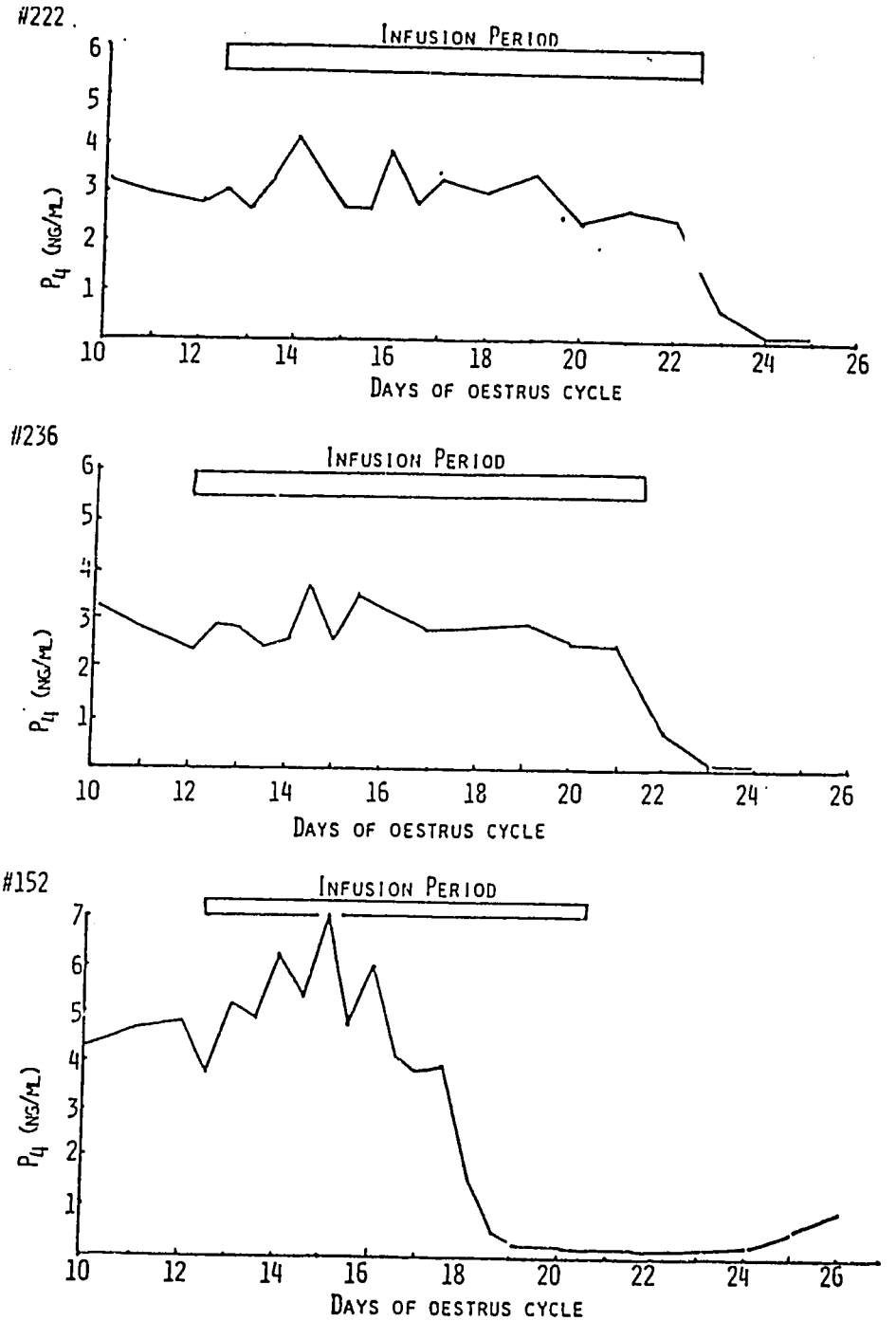

Figure 7 Plasma progesterone concentrations in heifers given intrauterine infusions of media harvested from the first four days of culture of Day 17-19 trophoblast.

Upper panel - undiluted cultured medium.

Middle panel - cultured medium diluted 1:1 with fresh medium

Lower panel - cultured medium diluted 2:1 with fresh medium

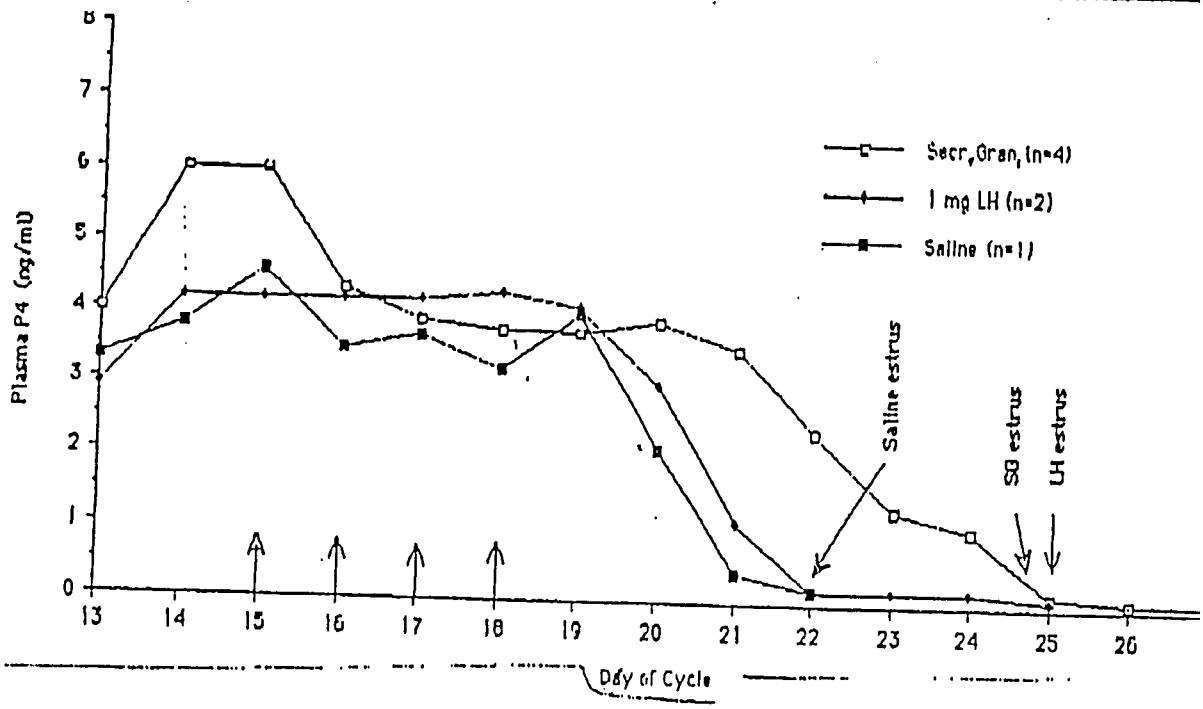

Fig. 8 Plasma progesterone concentrations and estrous cycle lengths in cattle receiving intraluteal infusion of a luteotropin isolated from placental secretory granule preparations, luteinizing hormone (LH) or saline.

Normal cycling heifers were used and the surgical cannulations were done on day 15 of the cycle. The treatments were given on Days 16, 17 and 18 of the estrous cycle (indicated by arrows). The treatments were administered via Vascular Access Ports, which are silicone and plastic "ports" fitted with long plastic tubing. The free end of the tubing is sutured into the CL while the "port" at the other end is placed under the skin over the back for ease of injection. After the treatment solution is injected, through the skin, into the "port", it is pushed into the CL with a calculated volume for heparinized saline. Figure 1 above shows the mean daily plasma progesterone values of the secretory granule (SG) treated and control cows in the CL infusions done to date. The progesterone levels for the SG treated animals was maintained longer than either the positive (LH) controls or the saline control. Two animals have been omitted from the data. One received the saline control treatment and had a long estrous cycle (28d) as well as progesterone levels above 1 ng/ml through day 24 of her cycle. Herd records indicate that this cow had a similar long cycle two months previously. Another cow given a very low dose of LH (100μg) had normal blood progesterone levels and a normal estrous cycle.

The mean estrous cycle lengths are also indicated by arrows. The 2 cows receiving LH and the 4 cows receiving the secretory granule preparation each had mean cycle lengths of 25 days. However, the LH treated animals had plasma progesterone levels similar to the saline treated control animals.