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Mouse Leydig cell bioassay shows lack of LH in the bovine preimplantation conceptus*

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Summary. A mouse Leydig cell bioassay was established and validated to determine whether the bovine conceptus (age: 16–20 days after oestrus) synthesized and secreted a substance having luteinizing hormone (LH) activity. The response measured was testosterone production after incubating Leydig cells with various doses of LH. The assay was specific for LH as compared to other protein hormones, precise (an interassay CV of 21%) and sensitive (minimal detectable level 15·5 pg NIH-LH-B4/ml). The application of this assay to bovine conceptus homogenates or media in which similar conceptuses had been cultured for 0·5 to 20 h resulted in no LH activity being detected. Furthermore, concentrating the conceptus homogenate or culture media by freeze–drying and reconstitution in a minimum amount of buffer resulted in a similar finding. Because this bioassay is more specific and sensitive to the presence of LH-like activity than are other assays, and yet no activity was detected when conceptuses were manipulated in several ways, it is concluded that the bovine conceptus probably does not produce an LH-like substance at this stage of development.

Introduction

Regression of the corpus luteum (CL) and oestrus (Day 0) occur in the cow between Days 17 and 22 of the cycle unless a conceptus is present. Henricks, Lamond, Hill & Dickey (1971) reported that mean progesterone concentrations were significantly greater at 9, 12 and 15 days after mating in animals later verified to be pregnant than in mated animals that did not conceive. Lukaszewska & Hansel (1980) reported similar findings. Northey & French (1980) reported that uterine infusions of 17- and 18-day-old, but not 15-day-old, bovine conceptus homogenates lengthened the interval between oestrous periods and delayed CL regression.

The sheep has been used to study pregnancy maintenance. Moor & Rowson (1966) demonstrated that a conceptus must be present in the uterine lumen on Day 13 after oestrus to prevent the luteolysis that normally occurs by Day 16. Ellinwood, Nett & Niswender (1979) infused into the uterus saline or conceptus homogenates collected 14–15 days *post coitum* and significantly extended CL lifespan. Additionally they reported that this effect on the CL was not exerted via a luteinizing hormone (LH) or prolactin-like molecule in the conceptus homogenate. In a related study, Godkin, Bazer, Moffatt, Sessions & Roberts (1982) reported that cultured sheep blastocysts released a protein in significant quantities (50–100 μg/24 h) between Days 13 and 21 which had a molecular weight of about 21 000 (gel filtration). Martal, Lacroix, Loudes, Saunier & Wintenberger-Torrès (1979) reported the presence of a proteinaceous material (named trophoblastin) in Day 14–16 sheep conceptuses that was absent by Day 21 and antiluteolytic in cyclic sheep. Few such studies have been reported for cows. In 3–5-month-old fetuses an LH-like

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substance was detected using the rat ventral prostate weight assay. The activity was present in an ammonium sulphate extract of fetal cotyledons, but disappeared after heat treatment (Lunnen & Foote, 1967). Beal, Lukaszewska & Hansel (1981) reported that Day-18 conceptus homogenates and aqueous supernatants increased progesterone production of dispersed luteal cells: this activity was destroyed by heating, but since the stimulating activity of the embryo was lost when dialysed with a membrane having a 120 000 molecular weight cut-off, Beal *et al.* (1981) suggested that the luteotrophic activity was not LH.

Our present understanding is that bovine conceptuses produce a substance having luteotrophic activity, but the evidence as to its chemical nature is conflicting. This experiment was conducted to determine whether the luteotrophic activity in the bovine conceptus is LH-like by submitting 16- to 20-day-old conceptuses to a specific and sensitive mouse Leydig cell assay for detecting LH-like molecules.

Materials and Methods

After checking a group of cows of mixed breeding for oestrus with the aid of K-Mar (K-Mar, Inc., Steamboat Springs, CO) heat patches and a bull with a surgically deviated penis, 22 of the cows were injected with PMSG (Organon, Inc., West Orange, NJ) subcutaneously on Day 16 of the oestrous cycle (Day 0 = oestrus). The dose of PMSG (1600 i.u.) was just sufficient to give several ovulations (Henricks & Hill, 1978). At oestrus, the cows were mated to a fertile bull; 13 cows became pregnant. On Days 16 to 20 after mating, the cows were slaughtered and the conceptuses to be tested for possession of an LH substance were aseptically flushed from the uterus with Medium M-199 with Hanks' salts (Gibco, Grand Island, NY) and deep frozen in a minimum volume of the medium in liquid nitrogen or in a Revco Ultra Low Freezer (−70°C) until assayed.

Testing of conceptus homogenates. Before bioassay, 17 bovine blastocysts were pooled according to 3 age groups: Day 16–17 (5 conceptuses), Day 18–19 (5 conceptuses) and Day 16–20 (7 conceptuses). Homogenates of the conceptuses were prepared in a glass homogenizer on ice in Medium M-199 with Hanks' salts, L-glutamine and 25 mM-Hepes buffer (Gibco). From each homogenate aliquant volumes ranging from 25 to 300 μl were used for the bioassay. Extracts of the homogenized conceptus were obtained by centrifugation for 15 min at 1900 ***g*** and were also assayed. In addition, the remainder of the Day 16–20 homogenate was concentrated by freeze-drying, resuspended in Medium M-199 with Hanks' salts and 25 mM-Hepes buffer (Gibco). For the bioassay, aliquant volumes of 25 to 100 μl of the samples were used. Each aliquant volume of the 3 homogenates of pooled conceptuses was tested in triplicate.

Testing of cultured conceptuses. Conceptuses used for culture were flushed from the uterus using Eagle's medium with Earle's salts (Gibco) and immediately incubated. Two Day-16 and 2 Day-17 conceptuses were incubated at 37°C in a Petri dish (8 mm) containing approximately 15 ml Eagles medium supplemented with glucose (5 mg/ml; Fisher Sci. Co.), bovine insulin (0·2 Units/ml; Eli Lilly), 1% (v/v) non-essential amino acids and 4% (v/v) penicillin (Gibco). The Petri dishes were placed on a rocking platform in an incubator under an atmosphere of 45% N_2, 5% CO_2 and 50% O_2. Samples of the medium were taken after incubation for 0·5, 4, and 20 h. In addition, 3 Day-18 conceptuses were recovered from cows not treated with PMSG and incubated at 37°C in Medium M-199 with Earle's salts under 5% CO_2 and 95% air for 12 h.

As a positive control the uptake of [^{3}H]leucine by the conceptus tissue was used to determine conceptus viability during the culture period. Intact tissue cut from the conceptus was cultured simultaneously beside the test culture. Depending upon the experiment, the intact tissue incorporated 5- to 7-fold more [^{3}H]leucine than did companion pieces that were first heat-treated; a significant ($P < 0·05$) difference.

Bioassay for LH. The presence of biologically active LH in aliquants of conceptus homogenates and medium from cultured conceptuses was tested with a dispersed mouse Leydig cell bioassay similar to that described by Ellinwood & Resko (1980). Testes were dissected from 6-week-old mice (Swiss ICR strain, Hilltop Labs, Scotsdale, PA), decapsulated on ice and mechanically disrupted (no collagenase) in ice-cold Medium M-199 with Hanks' salts, L-glutamine, 25 mM-Hepes buffer and 0·2% BSA. Leydig cells were separated from seminiferous tubules by filtration through 4 layers of nylon mesh and preincubated at 34°C for 60 min in a Dubnoff shaking waterbath. The cells were then centrifuged (130 ***g***) for 15 min and resuspended in ice-cold bioassay buffer with no BSA.

Viability was determined by the Trypan Blue method (Phillips, 1973) and exceeded 80% in all assays. Cell number, estimated by the Basic Blue method (Absher, 1973), was determined in 7 of 9 bioassays and averaged $56{\cdot}3 \pm 2{\cdot}7 \times 10^3$ (mean ± s.e.m.) cells per assay tube. Similarly, Murphy & Moger (1982) found a range of 43·0 to $62{\cdot}0 \times 10^3$ cells per 100 µl dilution medium by enzymic (collagenase) preparation of the cells. After resuspension in Medium M-199, 100 µl of the cells were added to 12 × 100 mm plastic incubation tubes (Falcon Plastics, Oxnard, CA) which contained either 100 µl LH standard preparation in bioassay buffer or an equivalent volume of sample plus bioassay buffer without LH standard. The cells were incubated for 2 h at 37°C in a shaking water bath. At the end of the incubation, 1·8 ml ice-cold phosphate-buffered saline (0·1 M, pH 7·0) with merthiolate was added to the tubes, and centrifuged (15 min, 1300 ***g***). The supernatant was decanted into RIA culture tubes (Fisher) and frozen at − 20°C or prepared for testosterone assay by heating at 70°C for 30 min.

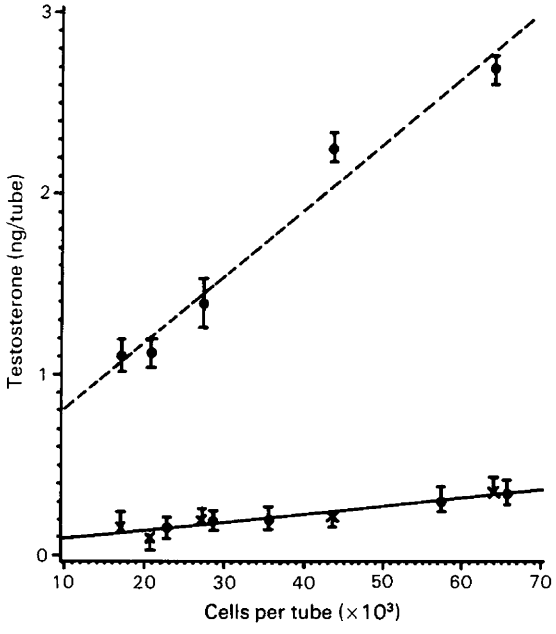
The standards of bovine LH (NIH-B-4, 2·4 NIH-S1 U/mg) which ranged from 7·8 to 250 pg gave a sigmoidal response curve ranging from 0·3 to 4·5 ng testosterone, respectively. The linear portion of the dose–response curve was consistent and ran from 15·6 to 125 pg LH; the sensitivity of the assay was therefore 15·6 pg. To check for non-specific stimulation of the Leydig cells by proteins capable of binding steroids, as shown by Ewing, Chubb & Robaire (1976) and Caffrey, Nett, Abel & Niswender (1979), tubes containing 100 µl 1·6% BSA (no LH standard added) in Medium M-199 were included in every assay. A plasma pool obtained from the jugular vein of cows treated with gonadotrophin-releasing hormone to induce a high concentration of LH was included in each assay. The interassay coefficient of variation for the pool based on testosterone produced per bioassay tube was $20{\cdot}8 \pm 0{\cdot}53\%$ ($n = 7$).

The testosterone RIA described by Karg *et al.* (1976) was employed using an antiserum (S-250) kindly provided by Dr G. D. Niswender (Colorado State University). The antiserum was produced by injecting rabbits with 4-androstene-11α,17β-diol-11-hemisuccinate–BSA. The antibody cross-reacted 5% with dihydrotestosterone, 4% with androstenedione, 0% with oestradiol-17β (1–10 ng) and 0% with progesterone (10 pg–10 ng). Interassay and intra-assay variation, measured by including a pool of plasma in the testosterone RIAs, were 16·3 and 11·6%, respectively.

To demonstrate that the bioassay was able to measure LH in a dose-related manner in tissue containing other trophic hormones, crude homogenates of anterior pituitaries of a cow and a steer were assayed. A pituitary extract was prepared by homogenizing the anterior pituitary with a Sorvall homogenizer in Medium M-199, Hanks' salts, L-glutamine and 25 mM-Hepes buffer. The homogenate was centrifuged (1900 ***g*** for 15 min), and the supernatant recovered and centrifuged (25 300 ***g***) for 30 min with a Beckman J-21B centrifuge. This precipitate was diluted $1{\cdot}5 \times 10^5$, $2{\cdot}25 \times 10^5$ and $3{\cdot}0 \times 10^5$ times. A sample (10 µl) of each of these dilutions was placed in the bioassay. The bioassay was also tested for specificity by incubating Leydig cells with purified preparations of other bovine pituitary hormones: FSH (K. W. Cheng, University of Manitoba), TSH and prolactin (L. E. Reichert, Albany Medical School) and GH (J. B. Mills, Emory University).

Preliminary experiments were conducted to ascertain the relationship between Leydig cell number and basal testosterone production in bioassay buffer only (no LH or BSA). Cell numbers in this assay ranged from 22·5 to $64{\cdot}5 \times 10^3$ per bioassay tube, which produced 0·15–0·39 ng testosterone per tube, respectively (see Text-fig. 1). Compared to this curve are testosterone production curves resulting from incubating an increasing number of cells in the presence of 1·6%

BSA or 125 pg bovine LH (NIH-B-4). Cell numbers ranged from 17·2 to 64·3 × 10^3 per bioassay tube. Over this range, BSA had no effect on testosterone production as seen by its coincidence with the basal curve, but at a constant LH dose testosterone (ng/tube) increased linearly with increasing number of cells. As cell number increased in the basal medium and medium containing BSA, production increased 0·05 ng per 10 000 cells, whereas bovine LH increased testosterone 0·37 ng per 10 000 cells ($P < 0·01$). Due to the effect of cell number on the assay, precise pipetting of the cells within an assay was done. Amongst all assays the number of cells ranged between 50·0 and 65·0 × 10^3 cells.



Text-fig. 1. Testosterone production as a function of various numbers of mouse Leydig cells per bioassay tube when stimulated by bovine LH standard, 125 pg per tube (--●--); 1·6% BSA, 125 pg per tube (—●—); or no protein added (—×—). Values are mean ± s.e.m.

Data analysis. All experimental data obtained from the assay are based on 3 replicates of each standard point and aliquant volumes of plasma, conceptus or pituitary homogenates. The linear portion of the dose–response curve was submitted to linear regression analysis using the SAS programme (Barr, Goodnight, Sall & Helwig, 1976) to obtain predicted LH values. To ascertain whether parallelism existed among the various preparations (standard, plasma, pituitary homogenates), the slopes of the standard and sample curves were calculated using linear regression, then tested for significant difference by *t* test (Steel & Torrie, 1960).

Maintenance of mouse colony. Mice were kept in plastic cages (34 × 20 × 12 cm) under the following conditions: light period from 06:00 to 18:00 h, 23°C, 50–60% relative humidity, and pelleted diet (5012 Rat Chow, Purina) and water *ad libitum.* Immature mice were weaned at 2·5 weeks of age and not more than 5 mice were maintained per cage.

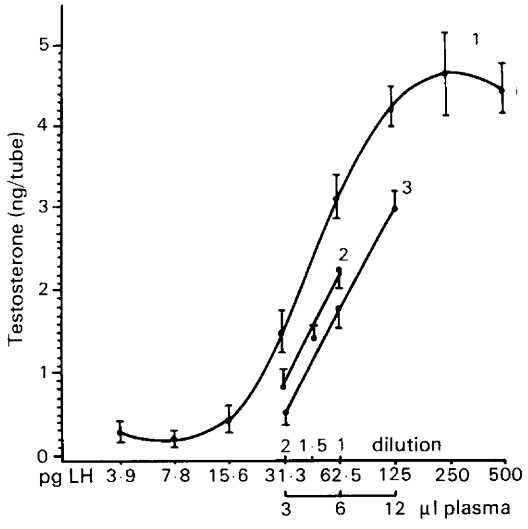
The basal level of testosterone production and the responsiveness of the Leydig cells to LH were altered by overcrowding and overhandling the mice, and the time of day at which the mice were killed for the bioassay. Gartner *et al.* (1980) reported that stress induced by moving cages, presence of an unfamiliar human or ether anaesthesia caused increased levels of gonadotrophins in rats.

The protocol for harvesting Leydig cells from mice was standardized as follows: on the day of bioassay a 5–6-week-old mouse was removed from its cage and the cervical vertebrae dislocated.

The body was taken immediately to the laboratory, and the testes removed quickly and prepared as described previously. Mice were always killed within 10 min of 09:00 h. Leydig cells from mice which were killed after 10:00 h exhibited higher basal levels of testosterone and reduced magnitude of response to LH.

Results

When tested in the bioassay, parallel response curves ($P > 0{\cdot}05$) were obtained between the LH standard preparation and dilutions of crude homogenates of steer and cow pituitary glands as shown in Text-fig. 2. There was also no significant difference ($P > 0{\cdot}05$) between the assay's response to increasing volumes of the plasma pool and the standard curve (Text-fig. 2). When highly purified preparations of bovine FSH, TSH, GH and prolactin were assayed at concentrations 10-fold greater than the highest concentration of the LH standard, no LH activity was detected.



Text-fig. 2. LH dose–testosterone response curves on a log scale for bovine LH standard (1), cow pituitary homogenate (2) and cow plasma (3). The standard was NIH-B-4 (3·9–500 pg). The pituitary preparation was tested at a dilution ratio of 1, 1·5 and 2. Three doses (3, 6 and 12 µl) of plasma were tested. Values are mean ± s.e.m.

The results of applying the bioassay to conceptus homogenates classified according to 3 age groups are shown in Table 1. None showed biological LH-like activity even though a wide range of volumes were assayed. Concentration of a pool of the Day 16–20 conceptuses by dialysis and freeze-drying also failed to elicit testosterone production by Leydig cells.

The results of applying the bioassay to media in which Day-16, -17 and -18 conceptuses had been cultured for 0·5, 4·0 and 12·0 h are shown in Table 2. No LH-like biological activity was detected, nor was it found in the viable conceptus at the end of 20 h culture.

Discussion

The study of Dufau, Mendelson & Catt (1974) showing a dose–response curve between LH or hCG and testosterone produced by decapsulated rat testes cells set the stage for a sensitive, specific bioassay for LH in the presence of FSH and prolactin. A mouse Leydig cell bioassay was

Table 1. Assay for biologically active LH in bovine conceptuses

Age of conceptuses (days)	Conceptuses per homogenate*	Volume of homogenate (ml)	Aliquants of homogenate assayed (μl)	LH conc. (pg)
16–17	5	5·0	50, 300	ND†
18–19	5	4·5	50, 200	ND
16–20	6·5	3·0	25, 50, 100	ND
16–20‡	6·5	0·5	25, 50, 100	ND

* Conceptuses were glass homogenized in Medium M-199 with Hanks' Salts and 25 mM-Hepes buffer.

† Minimal amount of LH detectable was 15·6 pg per aliquant, thus for Day 16–17 conceptus using 300 μl of 1 conceptus per ml, more than 51·5 pg LH would be required per conceptus for detection. ND = non-detectable.

‡ The remainder (2·5 ml) of Day 16–20 homogenate was dialysed in M_r 1000 cut-off dialysis tubing against 0·14 M-ammonium acetate for 22 h, freeze-dried, resuspended in 0·5 ml buffer and assayed.

Table 2. Assay for biologically active LH in bovine culture medium and conceptus homogenates*

Conceptus age (days)	No. of conceptuses incubated	Culture medium volume (ml)	Conceptus homogenate volume (ml)	Length of incubation (h)	Final† volume for assay (ml)	LH conc. (pg)
16	2·0	15	—	0·5	0·5	ND‡
		15	—	4·0	0·5	ND
		—	15	20·0	0·5	ND
17	2·0	15	—	0·5	1·0	ND
		15	—	4·0	1·0	ND
		—	15	20·0	1·0	ND
18	1·0	4	—	12·0	0·5	ND
18	1·0	4	—	12·0	0·5	ND
18	1·0	4	—	12·0	0·5	ND

* Incubation medium was sampled after 0·5 and 4 h, freeze-dried, reconstituted and assayed. After 20 h, medium and conceptuses were homogenized, dialysed, freeze-dried and reconstituted for assay.

† Volume of buffer in which sample was resuspended after freeze-drying.

‡ ND = non-detectable in buffer (see second footnote in Table 1).

established which has the same advantages over a radioimmunoassay, but with a significant improvement in sensitivity over the rat bioassay (Van Damme, Robertson & Diczfalusy, 1974). Although specific for LH as compared to the activity of other protein hormones, the assay is responsive to a wide spectrum of LH-like molecules as shown by dose–response curves of LH standard preparations from pig, sheep and rabbit parallel to the bovine standard curve (unpublished data). For these reasons the mouse bioassay was established for the present study to determine whether the luteotrophic activity present in the preimplantation bovine conceptus is LH-like.

Since it has not been shown, the first task was to ascertain whether mouse Leydig cells responded to bovine LH in a dose–response manner. Valid mouse Leydig cell assays have been used to measure LH in fetal monkey plasma (Ellinwood & Resko, 1980) and rabbit plasma (Sundaram, Connell & Passantino, 1975). A consistent, sensitive dose–response curve was obtained for bovine LH after studying several variables such as Leydig cell number and then adhering to rigid conditions. The tests for specificity demonstrated that the bioassay could have detected the presence of LH-like activity in the conceptus if it were present. The similarity between the effects of the incubation medium without and with BSA ruled out any non-specific effect of large proteins capable of binding to steroids non-specifically.

In the present experiments LH was not detected in the preimplantation conceptus regardless of a number of manipulations of the conceptus tissue. Not only was it not detected in the tissue but the possibility that the LH activity was not stored, but secreted, was ruled out, although secreted LH activity may have been catabolized during culture. Discounting that remote possibility, the failure to detect activity in a bioassay tailored for LH suggests that the luteotrophin activity reported by others (Godkin, Cote & Duby, 1978; Beal *et al.*, 1981) is not LH. Evidence pointing to non-LH type luteotrophins can be cited. In sheep, PGE-2 has been tested and found to extend the life of CL in cyclic animals (Pratt, Butcher & Inskeep, 1977). In cattle, intrauterine infusion of PGE-2 into cows from Days 9 to 21 of the cycle resulted in continued secretion of progesterone until Day 21 and delay of oestrus (Gimenez & Henricks, 1983). There was no effect of PGE-2 on testosterone production in the mouse Leydig cell assay used (unpublished data).

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