In-vitro synthesis of a low molecular weight lipid-soluble luteotrophic factor by conceptuses of cows at Day 13–18 of pregnancy

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Summary. Two culture systems for maintenance of Day 13–18 conceptus tissue were developed. Harvested culture media were assayed for luteotrophic activity by determining their ability to stimulate progesterone synthesis by dispersed bovine luteal cells. Significant luteotrophic activity was found in 80% of the 31 tissue culture media studied. A series of experiments carried out to determine the nature of the luteotrophic activity indicate that it is a small ($M_r < 10\,000$), heat-labile, lipid-soluble substance that is adsorbed by dextran-coated charcoal. The nature and activity of this factor, together with its synthesis by the early bovine conceptus, suggest that it may have a significant role in stimulating progesterone synthesis by the corpus luteum during early pregnancy.

Introduction

Day 16 is often cited as the day of maternal recognition of pregnancy in the cow, since the transfer of viable Day-16 bovine conceptuses into suitable recipients up to and including Day 16 after ovulation results in maintenance of the corpus luteum and a normal pregnancy (Betteridge *et al.*, 1980). The Day-16 conceptus is therefore capable of blocking the luteolytic mechanism(s) of the normal oestrous cycle after spending only a short period of time within the uterus. Henricks *et al.* (1971) and Lukaszewska & Hansel (1980), however, reported that plasma progesterone concentrations of pregnant cattle were significantly higher than those of non-pregnant cattle as early as Days 9 and 10 after oestrus. Bulman & Lamming (1978) reported a difference in milk progesterone by Day 13. These findings suggest that some form of maternal recognition of pregnancy occurs during the second week. Thatcher *et al.* (1985) were able to prolong the life span of the corpus luteum in cycling cattle by intrauterine infusion, on Days 15–18 of the cycle, of 'conceptus secretory proteins' harvested from cultures of Day 17–18 bovine conceptuses. However, the plasma progesterone profiles reported indicated a retarded luteal regression, or an antiluteolytic effect, rather than the increased steroidogenesis of the corpus luteum of early pregnancy described by Henricks *et al.* (1971) and Lukaszewska & Hansel (1980).

We have attempted, in this study, to identify luteotrophic factors produced by Day 13–18 bovine conceptuses in culture that could be responsible for the increased plasma progesterone concentrations found in early pregnancy.

Materials and Methods

Superovulation. Superovulation was initiated between Days 8 and 12 of the oestrous cycle by intramuscular administration of 5 mg of a follicle-stimulating hormone preparation (FSH: Burns Biotech Laboratories, Omaha, NE, U.S.A.) twice daily for 4 consecutive days. Prostaglandin (PGF-2 α) (Upjohn, Midland, MI, U.S.A.) was administered on the evening of the 3rd and morning of the 4th day of FSH treatment. The superovulated cattle were artificially

inseminated within 12 h of the onset of oestrus; usually they were inseminated again 12 h later and occasionally a third time after an additional 12 h. The inseminated animals were checked daily for signs of oestrus until the day of embryo collection.

Embryo collection and culture. At 13–18 days after insemination the uterus of each cow was flushed with Dulbecco's phosphate-buffered saline (D-PBS, GIBCO, Grand Island, NY, U.S.A.), containing 0.3% bovine serum albumin (BSA; Sigma Chemical Company, St Louis, MO, U.S.A.), 100 i.u. penicillin/ml and 100 µg streptomycin/ml.

The conceptuses were removed from the flushing medium and resuspended in fresh Dulbecco's PBS plus 0.3% BSA in sterile 90 × 50 mm Pyrex evaporation dishes (Corning Glass Works, Corning, NY, U.S.A.). Each conceptus was examined and damaged portions were excised and discarded. The remaining conceptus tissue was washed several times in BSA-free Dulbecco's PBS and resuspended in 5–10 ml Hams F-10 medium (GIBCO) plus penicillin (100 i.u./ml)/streptomycin (100 µg/ml) in Falcon 60 mm × 15 mm Petri dishes (Falcon, Oxnard, CA, U.S.A.), which were used for culture of the conceptus tissue. Fetal bovine serum (FBS, GIBCO), at a concentration of 2.5% was added to the medium used for the first day of culture only.

Two methods of culture were attempted. In the first the conceptus was cut at right angles to its long axis into sections 1–4 mm in length. If the tissue was viable the open ends of the trophoblast sealed within 24 h in culture and trophoblast vesicles resulted (suspension culture). In the second method the conceptus tissue was supported on a stainless-steel grid platform and medium was added to the level of the lower surface of the grid (organ culture). Attachment of the conceptus tissue resulted within the next 24–48 h. In both systems the media were changed daily, the harvested media centrifuged at 1000 g, and the supernatant collected and stored at –20°C.

Dispersed luteal cell incubations. The luteotrophic effects of the harvested culture media were tested in the dispersed bovine luteal cell incubation as described by Hixon & Hansel (1979). Corpora lutea were enucleated from Holstein heifers under epidural anaesthesia between Days 8 and 12 of the oestrous cycle (oestrus = Day 0) through a small incision in the anterior vagina and immediately placed in chilled tissue-culture medium, Medium 199 (GIBCO). The luteal cells were dispersed in Medium 199 with collagenase. The dispersed cells were washed several times with fresh medium and filtered through artist's silk for removal of undissociated tissue and clumps of luteal cells. A representative sample of the cells was stained with 0.2% trypan blue in balanced salt solution for determination of viability based on dye exclusion (Tennant, 1974). The live cells were diluted to a final concentration of $0.25\text{--}1.0 \times 10^6$ cells/ml Medium 199, and 1.2 ml was pipetted into each incubation tube.

After a preincubation of 15 min in a 37°C water bath with gentle shaking in an atmosphere of 95% air and 5% CO₂, an aliquant of cell suspension (200 µl) was collected and centrifuged at 1000 g to concentrate the cells. The supernatant was then decanted and stored at –20°C until determination of initial progesterone concentration in the medium was carried out. This was taken as the progesterone concentration at time zero.

In each experiment there were two standard treatments consisting of addition of (a) 100 µl Medium 199 and (b) 5 ng LH in 100 µl Medium 199. The test substances were added in 100 µl volumes (8% of final volume). After the incubation period, the samples were centrifuged at 1000 g to concentrate the cells and the supernatants were decanted and frozen at –20°C.

The differences between the concentrations of progesterone in samples collected at time zero and the concentrations in samples collected after 2 h of incubation were considered to represent secretion of progesterone by luteal cells during the incubation.

Radioimmunoassay for progesterone. The method described by Milvae (1978) was used for progesterone radioimmunoassay in unextracted incubation media. The lower limit of sensitivity was 0.125 ng/ml, the interassay coefficient of variation was 14.2% at 120 pg/100 µl, and the intra-assay coefficient of variation was 3.9% over a concentration range of 12.5 to 1600 ng.

Progesterone concentration in the harvested culture media. To establish that the mean progesterone concentration measured in the luteal cell incubation samples was not due to progesterone already present in the conceptus tissue culture medium being tested, the harvested media were assayed: 23 samples of 100 µl each were tested in duplicate.

Luteotrophic activity of conceptus tissue culture media. Luteotrophic activity of each medium sample was measured in 3–5 replicates of 100 µl each added to a luteal cell incubation.

All characterization procedures outlined below were limited to culture media that showed significant luteotrophic activity ($P < 0.05$) when assayed in the dispersed luteal system and to which no FBS had been added.

Ammonium sulphate precipitation of conceptus tissue culture medium. Precipitations with 20% and 60% ammonium sulphate ((NH₄)₂SO₄) were carried out on 4 different culture media at 4°C. These media had been harvested on Day 2 (2), Day 4 and Day 10 of culture. The resulting fractions (20%, 60% and residual volume) were dialysed against distilled water for 24 h at 4°C and further dialysed against 0.9% NaCl using dialysis tubing with a molecular weight cut-off of 14 000 (Spectrapore 2, 12 000–14 000 M_r cutoff; Spectrum Medical Industries, Los Angeles, CA, U.S.A.). Each of the precipitates was resuspended in 0.9% NaCl to the original medium volume and, with the residual fraction, was assayed for luteotrophic activity in 4 replicates of 100 µl each when added to a dispersed luteal cell incubation as described above.

Heat treatment. Medium harvested on Day 3 of culture was maintained at 60°C for 30 min in a water bath and tested for luteotrophic activity.

Further characterization studies on conceptus culture media. Conceptus culture media harvested on Day 4 and Day 7, which had significant luteotrophic activity, were pooled and separated into fractions of $M_r < 10\,000$ and $> 10\,000$ using an Amicon C-10 Centrificon filter (Amicon Corp., Danvers, MA, U.S.A.). The $M_r > 10\,000$ fraction was concentrated to 500 μl in 0.9% (w/v) PBS, resulting in a 4-fold increase in concentration and assayed for luteotrophic activity. Half of the $M_r < 10\,000$ fraction (which remained at its original concentration) was assayed for luteotrophic activity. Both fractions ($M_r < 10\,000$ and $M_r > 10\,000$) were tested in 4 replicates of 100 μl each.

The remainder of the $M_r < 10\,000$ fraction was subjected to the Folch procedure for lipid extraction (Folch *et al.*, 1951, 1957). Lipid extraction was also carried out on Hams F-10 medium and Hams F-10 medium plus 2.5% fetal bovine serum. Each lipid was resuspended in Medium 199 by sonication and tested for luteotrophic activity in 5 replicates of 100 μl each.

Media harvested on Days 4 and 7 of culture were divided into fractions of $M_r < 10\,000$ and $> 10\,000$. Dextran-coated charcoal was added to an aliquant of the $M_r < 10\,000$ fraction and left for 15 min at 4°C. The suspension was centrifuged at 2000 g for 15 min and the supernatant was collected and tested for luteotrophic activity, along with the untreated $M_r < 10\,000$ and $M_r > 10\,000$ fractions in 4 replicates of 100 μl each.

Enzymic digestion was carried out on the remainder of the $M_r < 10\,000$ fraction of culture media. Lyophilized trypsin (Worthington Biochemical Corporation, Freehold, NJ, U.S.A.) and chymotrypsin (Sigma) were dissolved in D-PBS in equal amounts and added in a 10:1 (w/w) protein:enzyme ratio to culture media and incubated for 3 h at 37°C. Protein in the culture media was determined from spectrophotometric absorbances at 260 nm and 280 nm. Lyophilized trypsin inhibitor (Millipore Corporation, Freehold, NJ, U.S.A.) dissolved in D-PBS was added to the incubation mixture and allowed to stand for 30 min at room temperature before assaying for luteotrophic activity in 4 replicates of 100 μl each.

Statistics. One- and two-way analyses of variance were used to assess the data. Whenever possible, data for the same treatments carried out in different experiments were combined to increase the number of replicates per treatment. To combine data in this manner, the results of the treatments for the 2 or more experiments were compared using two-way analysis of variance. The data were converted to units of 'stimulation index' (progesterone treated/progesterone control) to reduce the variation in response between batches of corpus luteum cells. If there was no statistically significant difference present in the response for treatments across experiments, the data were combined. A one-way analysis of variance was then carried out on the combined data sets. If the differences between treatments were significant, the individual means were compared by Duncan's multiple range test. The results are given as mean and s.e. and the significance of response of each treatment over the control response is given at the $P < 0.05$ or $P < 0.01$ levels. The number of different conceptus tissue culture media is indicated by n .

Results

Altogether, 72 embryos ranging in age from 13 to 18 days were flushed non-surgically from donor animals and 31 cultures were initiated; 18 were organ cultures and 13 suspension cultures. All cultures remained viable, based on gross appearance, from a minimum of 2 to a maximum of 54

Fig. 1. Trophoblast vesicles from a Day 18 bovine conceptus after culture for 7 days: (a) single vesicle, (b) fusion of several vesicles resulting in a single multilobed vesicle. $\times 22$.

Fig. 2. Net progesterone synthesis (mean $\pm$ s.e., $n = 2$) by bovine luteal cells (ng/10⁶ cells/2 h) in response to Medium 199 (C), 5 ng LH (LH) or media collected during sequential days of tissue culture. Data are expressed in units of stimulation index (progesterone treated/progesterone control).

days with a mean of 12 days. Trophoblast vesicles developed in both systems; in the suspension culture they were freely floating in medium, with 2–3 vesicles often adherent (Fig. 1). In the organ culture, pedunculated trophoblast vesicles formed beneath the stainless-steel grid; these would occasionally bud off into the medium.

Progesterone concentration in the harvested culture medium

Progesterone was assayed in duplicate 100 μ l aliquants of 23 different samples. The concentrations ranged from non-detectable ($n = 8$) to 50.9 ng/ml with a mean of 12.8 ± 3.2 ng/ml. Since 100 μ l culture medium were added to all of the luteal cell incubations, these results indicate that a maximum of 5.1 ng progesterone would have been added to the culture medium. Contributions of this magnitude could not therefore explain the relatively large increases in progesterone synthesis (90 ± 18 ng/ml) that occurred during the 2-h incubation periods after addition of the embryo culture media.

Luteotrophic activity of conceptus tissue culture media

Significant luteotrophic activity ($P < 0.05$) was present, at some period, in the media of 80% of the 31 conceptus tissue cultures studied. Luteotrophic activity was most consistently found in media harvested during the first 5 days of culture. The culture media of both the organ culture system and the suspension culture system contained luteotrophic activity. The combined data for luteotrophic activity of harvested media from two different conceptus tissue cultures are shown in Fig. 2. Although Day 1 media (FBS present) contained the highest level of luteotrophic activity ($P < 0.01$), significant activity persisted during the first 5 days of culture in the absence of FBS ($P < 0.05$ for Days 3 and 5; $P < 0.01$ for Days 2 and 4). The presence of 2.5% FBS in the culture media had no effect on progesterone secretion over the control media level alone.

Ammonium sulphate precipitation of conceptus tissue culture media

The results obtained when the 20% and 60% (NH₄)₂SO₄ precipitates or the residual fractions of 4 culture media were added to incubations of bovine luteal cells are shown in Fig. 3. No significant stimulation of progesterone synthesis resulted, indicating that the active substance(s) is not a protein.

Effect of heat treatment on the luteotrophic activity of culture media

As shown in Table 1, maintaining a culture media at 60°C for 30 min resulted in a significant loss of the stimulatory activity present in the original sample (progesterone ng/10⁶ cells/2 h: control, 86 ± 1.7; culture medium, 131 ± 5.5; culture medium after heat treatment, 77 ± 4.5; $P < 0.05$).

Further characterization studies on conceptus culture media

Fractionation of the culture media containing luteotrophic activity revealed that the luteotrophic activity was present only in the $M_r < 10\,000$ fraction. Addition of the entire spent culture medium, or the $M_r < 10\,000$ fraction resulted in significantly increased progesterone synthesis over the control tissue ($P < 0.01$, Fig. 4). Figure 4 also shows that a lipid extract of the $M_r < 10\,000$ fraction contained all of the luteotrophic activity ($P < 0.01$). In a separate experiment, lipid extracts of Hams F-10 medium, with or without 2.5% FBS, failed to stimulate progesterone production above the control level (data not shown, $P > 0.05$).

Dextran-coated charcoal has the ability to bind steroids and other small molecules in solution. Since the luteotrophin appeared to be a lipid (i.e. not precipitated by ammonium sulphate and extractable by organic solvents), the effect of the addition of dextran-coated charcoal on the luteotrophic activity of the culture medium was studied. The combined mean progesterone concentrations for 2 separate experiments are shown in Fig. 5. The luteotrophic activity of the $M_r < 10\,000$ fraction of the culture medium was removed by the treatment ($P < 0.05$).

Fig. 3. Net progesterone synthesis (mean ± s.e., $n = 4$) by bovine luteal cells (ng/10⁶ cells/2 h) in response to Medium 199 (C), 5 ng LH (LH), ammonium sulphate precipitates (20%, 60%), or the residual fractions (R) of culture media. Data are expressed in units of stimulation index (progesterone treated/progesterone control).

Table 1. Net progesterone synthesis (ng/ml) by 10⁶ dispersed bovine luteal cells from 1 cow over 2 h when treated with Medium 199, LH, or conceptus tissue culture medium before or after heating to 60°C for 30 min

Treatment	Mean	s.e.
M 199	86	1.7
5 ng LH	**359	17
Culture medium	*131	5.5
Culture medium after heating at 60°C for 30 min	77	4.5

* $P < 0.05$; ** $P < 0.01$.

Fig. 4. Net progesterone synthesis (mean $\pm$ s.e., $n = 2$) by bovine luteal cells (ng/10⁶ cells/2 h) in response to Medium 199 (C), 5 ng LH (LH), culture media (C.M.), $M_r < 10\,000$ fractions of culture media (< 10), lipid extracts of the $M_r < 10\,000$ fractions (< 10 L.E.) or $M_r > 10\,000$ fractions of culture media (> 10). Data are expressed in units of stimulation index (progesterone treated/progesterone control).

Fig. 5. Net progesterone synthesis (mean $\pm$ s.e., $n = 2$) by bovine luteal cells (ng/10⁶ cells/2 h) in response to Medium 199 (C), 5 ng LH (LH), $M_r < 10\,000$ fractions of culture media (< 10), before or after addition of dextran-coated charcoal (< 10 DCC), or an $M_r > 10\,000$ fraction of culture media (> 10). Data are expressed in units of stimulation index (progesterone treated/progesterone control).

Enzymic digestion of the $M_r < 10\,000$ fraction was carried out to determine whether the activity may be due to a highly lipophilic peptide or small lipoprotein. The luteotrophic activity of the $M_r < 10\,000$ fraction of the culture medium was not significantly reduced by trypsin and chymotrypsin digestion (progesterone, ng/10⁶ cells/2 h: control, 92 ± 16 ; $M_r < 10\,000$, 192 ± 15 ; $M_r < 10\,000$ after digestion, 170 ± 14 ; $P > 0.05$ for $M_r < 10\,000$ vs $M_r < 10\,000$ after digestion).

Discussion

In the present study we report the successful culture of Day 13–18 bovine conceptus tissue for periods up to 54 days. These culture systems therefore permit examination of the potential role(s) of conceptus-derived factors in maintenance of the corpus luteum of early pregnancy.

Luteotrophic activity, as measured by ability to stimulate progesterone synthesis by luteal cells *in vitro*, was identified in the harvested media from the bovine conceptus tissue cultures. The

activity was found in the $M_r < 10\,000$ fraction, was not precipitated by $(\text{NH}_4)_2\text{SO}_4$ and was not destroyed by protease activity. The luteotrophin therefore does not appear to be proteinaceous in nature.

The activity was extracted by lipid solvents. Dextran-coated charcoal bound and removed the activity from the culture medium. These results suggest that the luteotrophin is lipid, in total or in part. Although heating the medium to 60°C for 30 min destroyed the activity, this is not an unexpected result for some lipids. The lipid class to which the luteotrophic substance belongs has not yet been identified. These results are in agreement with and extend the findings of Beal *et al.* (1981) who found a heat-labile dialysable luteotrophin(s) in homogenates of Day-18 bovine conceptuses.

Other evidence indicating the presence of a luteotrophin, attributable directly or indirectly to the conceptus as early as the 2nd week of pregnancy, includes the previously cited data of Henricks *et al.* (1971) and Lukaszewska & Hansel (1980), who found a significant difference in the plasma progesterone concentrations between pregnant and non-pregnant cattle by Day 10 after insemination. In sheep, there is indirect evidence for a conceptus-derived factor that increases the functional capacity of the corpus luteum in early pregnancy. Pratt *et al.* (1977) reported that exogenously administered PGF- 2α on Day 13 after oestrus was less effective as a luteolysin in pregnant than in non-pregnant ewes.

An early pregnancy luteotrophin may also exist in women, since Dlugi *et al.* (1984) reported finding significantly higher progesterone titres in pregnant than in non-pregnant women after in-vitro fertilization and embryo transfer. There is also increasing evidence for the existence of 'early pregnancy factors' in other species, including the observations of O'Neill (1985) and O'Neill *et al.* (1985) on embryo-derived platelet activating factors (PAF) in mice and women. Thrombocytopenia, a result of platelet activation, occurs during the first 7 days of pregnancy and is dependent upon the presence of fertilized ova and their production of PAF, PAF is a small molecule (1-0-alkyl-2-acetyl-Sn-glycerol 3 phosphocholine) extractable in chloroform; it is not known to possess luteotrophic properties.

The results of these experiments suggest that the active principle could be a steroid. Blastocysts of Day-16 pregnant cows produce progesterone, oestradiol- 17β and testosterone (Shemesh *et al.*, 1979). However, it has been shown in cattle that exogenous progesterone inhibits corpus luteum development, shortens the oestrous cycle and reduces CL weights if given early in the cycle before endogenous progesterone has begun to rise (Ray *et al.*, 1961; Woody *et al.*, 1967; Harms & Malven, 1969). Administration of oestrogen in the unconjugated (Greenstein *et al.*, 1958; Wiltbank *et al.*, 1961) or conjugated form (oestrone sulphate; Eley *et al.*, 1979a) is also luteolytic in the cow, an important point, since oestrone sulphate is the major form of oestrogen in the pregnant cow.

Androstenedione and testosterone have not been tested for luteotrophic activity *in vitro*. Immunization of ewes against androstenedione has been reported to result in increased early embryonic mortality (Boland *et al.*, 1986). Since there is extensive metabolism of androstenedione to 5β -reduced compounds by the bovine conceptus during early pregnancy (Eley *et al.*, 1979b) this group of compounds may warrant further study.

PGE-2 and PGI-2 have often been cited as possible luteotrophic signals from the conceptus to the dam to invoke maternal recognition of pregnancy in the cow and sheep (Pratt *et al.*, 1977; Silvia *et al.*, 1984). However, the luteotrophic factor described does not appear as labile as the prostanooids (half life of 15 sec *in vivo* for PGF- 2α and PGE-2; Green *et al.*, 1981; half life of 10 min at 37°C for PGI-2; Samuelsson, 1978). Harvested media maintained at room temperature for 1–2 h before addition to the in-vitro assay did not lose their activity.

It would appear that the early pregnancy conceptus-derived luteotrophin that we have identified in a conceptus tissue culture system is not essential for maintenance of the CL of pregnancy, since transfer of the Day-16 bovine conceptus to a cycling animal can result in maintenance of the CL of the recipient (Betteridge *et al.*, 1980). The low molecular weight and lipid-soluble properties of the luteotrophin, which would facilitate rapid absorption by the endometrium, and its produc-

tion during the accepted period of maternal recognition of pregnancy, suggest that it may be involved in increasing the functional capacity of the early CL and in embryo survival.

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