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Plasma hormone concentrations associated with early embryo mortality in heifers

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Summary. Results of a detailed study involving 18 pregnant, 17 cyclic and 12 inseminated but non-pregnant Holstein heifers indicated the following. (1) Jugular plasma progesterone concentrations were higher ($P < 0{\cdot}05$) in pregnant than in cyclic or in inseminated–non-pregnant animals from Days 10 through 18. (2) Jugular plasma progesterone concentrations of inseminated–non-pregnant animals could be related to heifers in which (a) fertilization may not have occurred (N = 5); (b) embryonic death may have occurred between Days 6 and 9 (N = 5); and (c) embryo mortality may have occurred shortly before Day 18 (N = 2). (3) Jugular plasma oestradiol concentrations were higher ($P < 0{\cdot}05$) in pregnant than in cyclic animals between Days 10 and 18; the oestrogen values of the inseminated–non-pregnant animals resembled those of the pregnant animals. (4) The steroid-synthesizing capabilities of incubated luteal tissues from inseminated–non-pregnant heifers more closely resembled those of tissues from pregnant animals than those of cyclic animals. (5) At Day 18 PGF concentrations in endometrial and luteal tissues and ovarian arterial plasma did not differ among the 3 groups. (6) PGF and progesterone concentrations of luteal tissues were negatively correlated ($r = -0{\cdot}77$, $P < 0{\cdot}05$) in cyclic animals and positively correlated ($r = 0{\cdot}73$, $P < 0{\cdot}05$) in pregnant animals, implying a luteotrophic effect of PGF in pregnant animals.

These results suggested a luteotrophic effect of the embryo, beginning as early as Day 10 of pregnancy, and the results of further experiments suggested that (i) homogenates and extracts of Day 18 bovine embryos contain one or more substances capable of stimulating progesterone synthesis in dispersed bovine luteal cell preparations; (ii) the luteotrophic activity was proportional to the protein contents of the embryo homogenates; and (iii) the luteotrophic activity was heat labile and was removed from homogenates by dialysis, indicating a structure of one or more relatively small molecules.

Introduction

Hawk (1979) estimates that, of each 100 first services in dairy cattle, fertilization failure occurs in 13 cases, embryo mortality in 15 cases and fetal death in 3 cases. Higher embryo mortality rates are associated with several previous infertile services and with fertilization of aged ova. It therefore appears that early embryo mortality is a most important cause of reproductive loss in dairy cattle, accounting for about 18% of pregnancy failures.

The bovine blastocyst appears to signal its presence to maintain the corpus luteum by Day 17 or 18 of pregnancy, but little is known about the mechanism(s). Although the patterns of hormone concentrations during the oestrous cycle have been reported extensively, less

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information is available for early pregnancy. Practically no data are available for animals that are inseminated and then experience early embryonic loss. Several observations suggest that embryos can be detrimentally affected before the corpora lutea begin to regress. Degenerative changes have been seen in embryos at 7 days after insemination and so-called 'repeat breeder' cows are reported to have smaller than normal embryos at 16 days.

Lukaszewska & Hansel (1980) have conducted a detailed study of the plasma hormone concentrations of Holstein heifers and of the steroidogenic capabilities of their corpora lutea during the first 18 days of pregnancy. They found evidence that the embryo may produce one or more luteotrophic substances that stimulate progesterone secretion by the corpus luteum. The purpose of the present study was (1) to examine the plasma hormone concentrations and the steroidogenic capabilities of the corpora lutea of animals that were inseminated but were not pregnant 18 days later, (2) to compare these values with those obtained for pregnant and non-pregnant control animals, and (3) to consider the nature of the proposed luteotrophic substance(s) of embryonic origin.

Materials and Methods

In initial experiments there were 3 groups of Holstein heifers: Group 1 contained 17 untreated cyclic controls; Group 2 contained 18 inseminated heifers which remained pregnant; and Group 3 contained 12 animals inseminated but not pregnant on Day 17 or 18. The day of insemination was Day 0.

Jugular blood samples taken at 08:00 h daily from Day 0 until Day 16 were analysed for luteinizing hormone (LH), progesterone and oestradiol-17β (Lukaszewska & Hansel, 1980). From Day 16 until Day 18, blood samples were collected at 6-h intervals. On Day 17 or 18 all animals were subjected to mid-ventral laparotomies and ovarian arterial, uterine venous and ovarian venous blood samples were collected. Corpora lutea were removed for subsequent incubation experiments, and the uterine horn ipsilateral to the corpus luteum in each animal was removed and flushed, to detect embryos. Plasma samples collected at laparotomy were analysed for progesterone, oestradiol, LH, arachidonic acid and prostaglandin (PG) F by previously described methods (Lukaszewska & Hansel, 1980). The corpora lutea were then sliced and incubated for 2 h in the presence or absence of LH (3 μg) or arachidonic acid (30 μg) (Hansel, 1971). After incubation, tissue and media were frozen and measurements of progesterone, PGF, testosterone and oestradiol were made at a later date.

Three additional experiments were conducted to determine whether Day-18 bovine blastocysts could stimulate synthesis of progesterone by dispersed bovine luteal cells *in vitro* (Beal, Lukaszewska & Hansel, 1981). In these experiments, embryos were collected from Holstein heifers bred artificially after a spontaneous oestrus or after induction of superovulation. Hysterectomies were performed 18 days later. Blastocysts were flushed from the excised uterine horns with cold 0·9% (w/v) NaCl, frozen on solid CO_2 and stored at −20°C. Later, the blastocysts were thawed and homogenized in 1 ml cold redistilled water and extracts and aliquots of the homogenates were tested for their ability to stimulate progesterone synthesis in 1×10^6 viable luteal cells dispersed and incubated as described by Hixon & Hansel (1979).

In the first of the three experiments, individual incubation tubes containing dispersed bovine luteal cells were treated with 100 μl Medium 199 (control); 1 ng luteinizing hormone (LH; NIH-LH-B9) in 100 μl Medium 199; 100 μl blastocyst homogenate; 100 μl aqueous extract of the blastocyst homogenate; or 100 μl heat-treated aqueous extract of the blastocyst homogenate. Each incubation treatment was replicated 4 or 6 times.

In the second experiment 18-day blastocysts were thawed and homogenized in 1·5 ml water and an aliquot of each homogenate was assayed for total protein (Miller, 1959). The following treatments were added to individual incubation tubes containing dispersed luteal cells: 100 μl

Medium 199; 1 ng LH in 100 μl Medium 199; 1 mg gelatin in 100 μl water and 100 μl bovine blastocyst homogenate. Each incubation treatment was replicated 5 times.

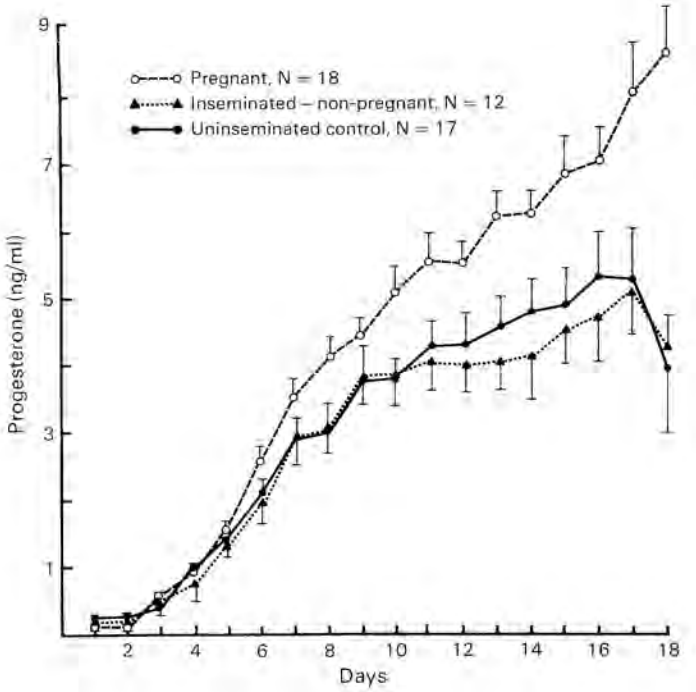
In the third experiment, blastocysts collected from heifers induced to superovulate were pooled and homogenized in 5 ml water as described above. Protein concentrations of the homogenate were determined and the homogenates were diluted so that each 100 μl contained 1 mg blastocyst protein. Half of the homogenate was placed in dialysis tubing (mean pore radii 0·24 nm) and dialysed against 18 litres distilled water for 24 h at 4°C. The remaining homogenate was stored at 4°C. Three incubations were carried out with corpora lutea collected from 3 different Holstein heifers. The following treatments were added to individual incubation tubes containing dispersed bovine luteal cells: 100 μl Medium 199; 1 ng LH in 100 μl Medium 199; 1 mg gelatin in 100 μl water; 100 μl dialysed blastocyst homogenate; or 100 μl non-dialysed blastocyst homogenate.

Data were analysed by analysis of variance and Student's *t* test.

Results and Discussion

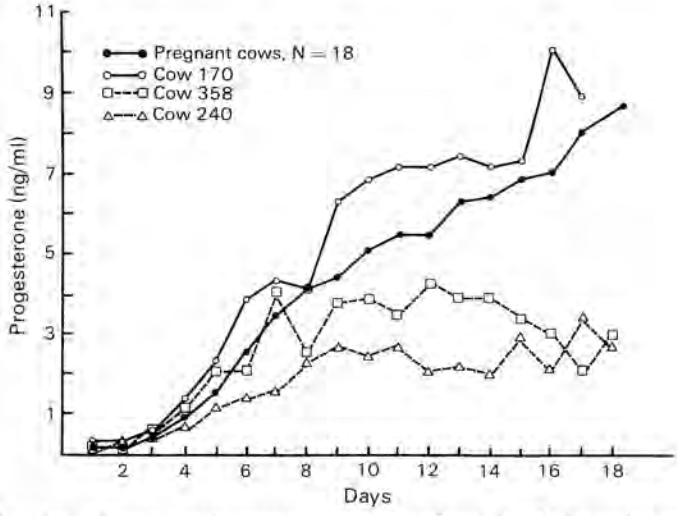
Plasma hormone concentrations

The concentrations of progesterone in jugular venous plasma were higher ($P < 0{\cdot}05$) in pregnant than in cyclic non-pregnant animals between Days 10 and 18 (Text-fig. 1). Mean daily progesterone concentrations during the 18-day period were similar in the cyclic control animals and in those that were inseminated but non-pregnant. These results indicate that the bovine blastocyst is able to stimulate progesterone synthesis by the 10th day of pregnancy and possibly earlier.



Text-fig. 1. Jugular plasma progesterone concentrations in pregnant, cyclic and inseminated–non-pregnant Holstein heifers during an 18-day period. Values are mean ± s.e.m.

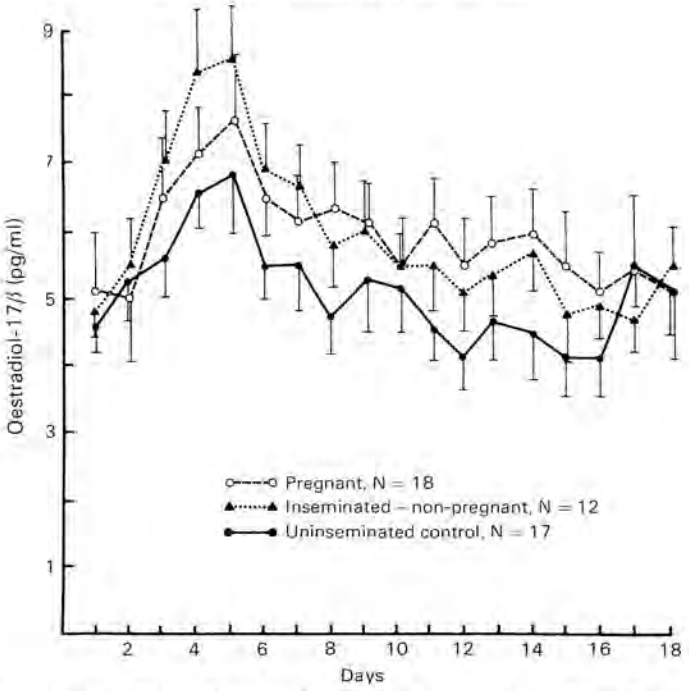
Examination of the plasma hormone profiles for the 12 inseminated–non-pregnant animals revealed that they fell into three classes. Five of the 12, exemplified by Heifer 240 (Text-fig. 2), had plasma progesterone concentrations similar to those found in the non-inseminated control animals in that the values were consistently below those found in pregnant animals during the entire 18 days, suggesting that fertilization did not occur. A second group of 5 animals (see Heifer 358, Text-fig. 2) exhibited plasma progesterone profiles that were identical to those of pregnant animals until Days 7–10; thereafter, the plasma progesterone concentrations closely resembled those seen in the cyclic control group. These may represent animals in which early embryo mortality occurred. The remaining 2 animals exhibited progesterone profiles that were indistinguishable from those seen in the pregnant animals (see Heifer 170, Text-fig. 2). We suspect that the embryos in these 2 animals died within 1–2 days of the time that the uteri were flushed; the flushings of one of the heifers did contain tissue fragments.



Text-fig. 2. Jugular plasma progesterone concentrations in 3 inseminated–non-pregnant Holstein heifers compared with mean values for the 18 pregnant heifers shown in Text-fig. 1. Heifer 240 represents an animal in which fertilization may not have occurred. Heifer 358 is representative of those in which early embryonic mortality may have occurred. Heifer 170 represents an animal in which the embryo may have survived until near the 18th day.

These data suggest that the concept of the bovine corpus luteum of the cycle being 'rescued' by the presence of an embryo at Day 16–18 needs to be revised. Furthermore, the data suggest that the embryo produces a luteotrophin(s) that begins to act on the corpus luteum as early as 8–10 days after fertilization.

Concentrations of plasma oestrogen were significantly higher ($P < 0{\cdot}05$) in pregnant than in cyclic control animals during Days 6 through 16 (Text-fig. 3). The plasma oestrogen profiles of the inseminated–non-pregnant animals resembled those of the pregnant animals more closely than those of the cyclic animals. Although plasma oestradiol concentrations appeared higher in the inseminated–non-pregnant animals than in the other two groups at Days 2–6, these differences were not significant ($P > 0{\cdot}05$). Concentrations of jugular venous plasma LH were not significantly different among the three groups at any time during the 18-day period. However, two of the inseminated but non-pregnant animals exhibited remarkably high plasma LH concentrations (10 and 15 ng/ml) on Days 3 and 4. Both of these animals were in the group that had plasma progesterone profiles suggesting that embryonic development may have been terminated between the 7th and 10th days.



Text-fig. 3. Plasma oestradiol-17β concentrations in pregnant, cyclic and inseminated–non-pregnant Holstein heifers during an 18-day period. Values are mean ± s.e.m.

Luteal tissue responses

Corpora lutea of pregnant animals contained a higher initial concentration of progesterone and synthesized more progesterone when incubated alone, with LH or with arachidonic acid than did corpora of cyclic control animals. Corpora lutea of the inseminated–non-pregnant animals (Group 3) responded more like those of pregnant (Group 2) than cyclic (Group 1) animals (Table 1). Luteal tissues of Group 2 and Group 3 animals synthesized progesterone in the absence of added LH (17·6 and 13·4 μg/g), but the tissue from Group 1 animals had a very limited ability to synthesize progesterone (5·0 μg/g) in the absence of added LH ($P < 0{\cdot}05$). Therefore, the presence of an embryo, even for a relatively short period of time, 'programmes' the luteal tissue to synthesize progesterone at an increased level. The LH-stimulated progesterone synthesis (value for incubated with LH minus incubated alone) was about equal for luteal tissues from each of the three groups (14·6 ± 3·2, 17·5 ± 4·5 and 13·0 ± 4·2 μg/g). Loss of response to LH cannot therefore be the first event in luteolysis. Indeed, in many instances, LH caused increased progesterone synthesis in luteal tissue from animals in which plasma progesterone levels had already declined.

Prostaglandin F concentrations were nearly identical in both the luteal and endometrial tissues of the three groups of animals (Table 2). As had been previously shown (Shemesh & Hansel, 1975), addition of free arachidonic acid caused large increases in PGF concentrations in tissues from all three groups, but there were no differences ($P > 0{\cdot}05$) among the groups in their abilities to convert arachidonic acid to PGF. A particularly interesting feature of these data was the negative correlation found between PGF concentration and net progesterone synthesis ($r = -0{\cdot}77$, $P < 0{\cdot}05$) for cyclic animals and the positive correlation ($r = 0{\cdot}73$, $P < 0{\cdot}05$) for pregnant animals. The same relationships held after addition of arachidonic acid to the tissues. These results imply a luteotrophic effect of PGF in the pregnant animal (Lukaszewska & Hansel, 1979). There were no differences ($P > 0{\cdot}05$) in concentrations of PGF in endometrial tissues of the three groups.

Table 1. Corpus luteum weights and progesterone synthesis by luteal tissue removed from pregnant and non-pregnant Holstein heifers at Day 18

	Group 1 (cyclic)	Group 2 (pregnant)	Group 3 (inseminated–non-pregnant)
No. of animals	10	10	8
Corpus luteum weight (g)	4·1 ± 0·4	5·1 ± 0·3†	3·9 ± 0·3
Progesterone synthesis (μg/g)			
Unincubated	27·2 ± 5·8	48·9 ± 8·6*	38·1 ± 5·8
Incubated	32·2 ± 5·9	66·5 ± 9·3*	51·5 ± 6·5‡
Incubated with LH (3 μg)	45·2 ± 7·1	81·1 ± 8·4*	66·4 ± 8·7
Incubated with arachidonic acid (30 μg)	33·9 ± 5·7	66·3 ± 9·4*	52·0 ± 5·2‡

Values are mean ± s.e.m.
* Values differ from those for Group 1, $P < 0·05$.
† Values differ from those in Groups 1 and 3 combined, $P < 0·05$.
‡ Values differ from those in Group 1, $P < 0·05$.

Table 2. Prostaglandin F concentrations (ng/g) in the corpus luteum and endometrium of Day-18 pregnant and non-pregnant Holstein heifers

	Group 1 (cyclic)	Group 2 (pregnant)	Group 3 (inseminated–non-pregnant)
No. of animals	10	10	8
Endometrium			
Unincubated	197·5 ± 28·4	257·9 ± 34·4	181·3 ± 28·1
Corpus luteum			
Unincubated	27·7 ± 5·5	22·1 ± 4·3	23·4 ± 2·6
Incubated	27·5 ± 5·5	22·5 ± 4·5	29·6 ± 2·9
Incubated with LH (3 μg)	27·2 ± 6·7	22·1 ± 4·7	27·9 ± 2·8
Incubated with arachidonic acid (30 μg)	73·4 ± 6·5*	82·4 ± 13·1*	83·6 ± 12·2*

Values are mean ± s.e.m.
* Values different from those for tissues incubated alone and incubated with LH, $P < 0·01$.

Oestradiol-17β concentrations were very low (0·32–0·59 ng/g) in luteal tissues from all three groups; there was no evidence of synthesis of oestradiol *in vitro*, or of stimulation of oestradiol synthesis by LH.

Uterine vein and ovarian arterial PGF concentrations

Although uterine vein plasma PGF concentrations were slightly ($P < 0·05$) higher in the cyclic and inseminated–non-pregnant animals than in the pregnant animals, no differences were found in PGF concentrations in ovarian arterial, ovarian venous or jugular venous plasma among the three groups (Table 3). Thus, the data do not support the concept that the presence of an embryo causes luteal maintenance by depressing the concentration of PGF reaching the ipsilateral corpus luteum by way of the ovarian artery.

The failure to find differences between pregnant and non-pregnant animals in endometrial or ovarian arterial concentrations of PGF, and the positive, rather than negative, associations found between PGF and progesterone in luteal tissues of pregnant animals tend to reduce the possible importance of inhibition of a PGF-mediated luteolytic mechanism as a major factor in corpus luteum maintenance in early pregnancy. On the other hand, the increased progesterone concentrations found between Days 10 and 18 in jugular vein plasma of pregnant compared to cyclic animals and similarities in responses *in vitro* of luteal tissues of pregnant and inseminated–non-pregnant animals all suggest the possible involvement of a luteotrophic mechanism, triggered at a very early stage by the presence of the embryo.

Table 3. Prostaglandin F concentrations in plasma (ng/ml) of Day-18 pregnant and non-pregnant Holstein heifers

	Group 1 (cyclic)	Group 2 (pregnant)	Group 3 (inseminated–non-pregnant)
No. of animals	10	10	8
Jugular vein	0·10 ± 0·02	0·09 ± 0·01	0·10 ± 0·01
Ovarian vein	0·18 ± 0·05	0·13 ± 0·01	0·12 ± 0·01
Ovarian artery	0·15 ± 0·03	0·11 ± 0·01	0·15 ± 0·03
Uterine vein	0·65 ± 0·16*	0·31 ± 0·02*†	0·58 ± 0·20*

Values are mean ± s.e.m.
* Values differ from those in jugular vein, ovarian vein and ovarian artery ($P < 0·05$).
† Value differs from those in Groups 1 and 3 ($P < 0·05$).

The nature of the embryonic luteotrophic effects

Day-16 bovine embryos are known to synthesize testosterone, progesterone, PGE-2, PGF and, in some cases, oestradiol (Shemesh, Milaguir, Ayalon & Hansel, 1979). In view of these considerations, a number of experiments were carried out to test the abilities of Day-18 bovine embryos to stimulate progesterone synthesis *in vitro* by dispersed bovine luteal cells (Hixon & Hansel, 1979). This system is sensitive to added bovine LH (NIH-LH-B9) over the range of 0·1–10 ng/ml.

In the first experiment homogenates and extracts of whole Day-18 bovine embryos stimulated progesterone synthesis ($P < 0·01$) by the dispersed luteal cells (Table 4). Heat treatment (autoclaving at 120°C for 15 min) inactivated the luteotrophic principle(s) in the blastocyst extracts.

Table 4. Progesterone accumulation (ng/ml) in Medium 199 and dispersed luteal cells after addition of blastocyst homogenates and extracts (from Beal *et al.*, 1981)

Treatment	Progesterone concentration in medium	
	1 h	2 h
None	47·4 ± 19·6	158·4 ± 69·7
LH (1 ng)	171·3 ± 27·3*	330·8 ± 35·6*
Blastocyst homogenates	222·8 ± 31·1*	336·5 ± 48·4*
Blastocyst extracts	292·0 ± 30·5*	366·2 ± 18·2*
Heat-treated blastocyst extracts	110·9 ± 12·4	200·4 ± 24·8

Values are mean ± s.e.m.
* Different from values for no treatment and heat-treated blastocyst extracts ($P < 0·01$).

In the second experiment protein concentrations of blastocyst homogenates of 0·21, 0·26, 0·33, 0·43, 0·45, 0·57, 0·54 and 0·67 mg/ml were studied. In addition, control incubations (Medium 199), and incubations containing 1 mg gelatin and 1 ng LH were carried out. The homogenates stimulated progesterone synthesis ($P < 0·05$), as did added LH; addition of a non-specific protein (gelatin) had no effect. The effect of increasing concentrations of blastocyst protein (0–0·67 mg/ml) in the homogenates was linear (Table 5) and fitted the linear regression equation: $y = 129·4 + 305·0P + 104·0T$; where y is the predicted progesterone accumulation (ng/ml), P is the protein in the blastocyst homogenate (mg) and T is the sampling time in hours. These results established that the homogenates and extracts of Day-18 bovine embryos contain a luteotrophic principle(s) and that its concentration is proportional to the protein content of the homogenate.

Table 5. Multivariate analysis of variance and specific non-orthogonal comparisons for progesterone accumulation in medium and dispersed luteal cells treated with Day-18 embryo homogenates of varied protein concentrations (from Beal *et al.*, 1981)

Source	d.f.	MS	F-ratio
Protein concentration 0–0·67 mg/ml	8	$5{\cdot}66 \times 10^6$	
Linear	1	$2{\cdot}34 \times 10^7$	20·88***
Quadratic	1	$1{\cdot}16 \times 10^7$	1·04
Time	1	$1{\cdot}95 \times 10^7$	
Linear	1	$1{\cdot}95 \times 10^7$	17·12***
Treatment × time	8	$7{\cdot}32 \times 10^5$	0·67
Residual	54	$1{\cdot}10 \times 10^6$	

*** $P < 0{\cdot}001$.

The results of the third experiment clearly show that the dialysed blastocyst homogenate did not stimulate progesterone synthesis (Table 6), while the luteotrophic effect of the non-dialysed blastocyst homogenate was greater ($P < 0{\cdot}001$) than that of either of the control treatments or the dialysed homogenate. No differences were found between the effects of no treatment and gelatin. Under the conditions of this experiment, compounds having a molecular weight of <12 000 would be removed by dialysis.

Table 6. Progesterone accumulation (ng/ml) in Medium 199 and dispersed luteal cells after additions of LH and blastocyst homogenates before and after dialysis (from Beal *et al.*, 1981)

	Corpus luteum incubations					
	1		2		3	
Treatment	1 h	2 h	1 h	2 h	1 h	2 h
None	71·9	158·2	86·1	152·2	85·9	137·6
Gelatin (1 mg)	90·8	158·2	74·2	134·8	66·7	107·9
LH (1 ng)	189·8	307·5	143·8	200·0	115·3	234·2*
Blastocyst homogenate	192·1	208·6	215·8	233·8	102·9	142·0*
Dialysed blastocyst homogenate	88·1	133·5	91·4	113·6	75·7	121·4

* Value different from that for no treatment, gelatin or dialysed blastocyst homogenate ($P < 0{\cdot}001$).

Although the evidence suggests that the luteotrophic activity of the 18-day blastocysts is due to one or more small, heat-labile molecules, the possibility that the activity was due to a fragment of a larger protein molecule cannot be excluded. The linear increase in progesterone accumulation seen when luteal cells were incubated with increasing amounts of blastocyst protein suggests that the activity may reside in one or more small peptides. However, other small molecules (such as prostaglandins or steroids) could be involved. Oestradiol does not stimulate progesterone synthesis by dispersed bovine luteal cells, except in very high concentrations (Hixon & Hansel, 1979) and is luteolytic rather than luteotrophic when injected into cyclic cattle. PGF-2α is luteotrophic when added to the dispersed cell system (Hixon & Hansel, 1979), but the response is always much less than that seen after addition of embryo extracts or homogenates.

Prostacyclin (PGI-2), despite its short half-life, is luteotrophic when injected directly into the bovine corpus luteum. Injections of 1 mg into the corpus luteum maintained elevated jugular plasma progesterone concentrations for as long as 14 h (Milvae & Hansel, 1980).

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