Evidence against a role for blastocyst-secreted oxytocin in early pregnancy maintenance in sheep


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ABSTRACT

Analysis of ovine conceptus RNA by slot blotting, Northern analysis and nested polymerase chain reaction failed to detect oxytocin–neurophysin prohormone mRNA. Probes used hybridized with both the 3′ end of the prohormone mRNA and the oxytocin-coding sequence. Northern analysis of bovine and porcine conceptus RNA was also negative, and polymerase chain reaction demonstrated oxytocin–neurophysin mRNA in ovine corpus luteum, but not in human corpus luteum or decidua, or in ovine endometrium. Infusion of oxytocin into the uterine lumen in cyclic ewes between days 9 and 19 or 20 after oestrus failed to prolong the luteal phase of the cycle and had no effect on endometrial oxytocin receptor concentrations or uterine prostaglandin F secretion. Oxytocin administered systemically prevented luteolysis and reduced uterine prostaglandin F secretion. Taken together, these data suggest that blastocyst-derived oxytocin is unlikely to contribute to corpus luteum maintenance in early pregnancy. They are inconsistent with a previous report that the ovine blastocyst synthesizes and secretes oxytocin.

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INTRODUCTION

In domestic ruminants, the maintenance of corpus luteum function in early pregnancy involves secretion of a type I interferon (IFN) of the (α11 or IFN-α) family by the trophoblast of the developing conceptus (Imakawa, Anthony, Kazemi et al. 1987; Stewart, McCann, Barker et al. 1987; see Flint, Lamming & Stewart, 1988 and references therein). Trophoblast IFNs interact with receptors on endometrial cell membranes to reduce prostaglandin production, thereby preventing the secretion of prostaglandin F2α, the uterine luteolysin (Godkin, Bazer & Roberts, 1984; Stewart et al. 1987; Thatcher, Hansen, Gross et al. 1989). The effects of endogenous trophoblast IFNs are mimicked by intrauterine infusion of purified IFNs, which reduce uterine prostaglandin secretion and delay luteal regression in non-pregnant sheep and cattle (Plante, Hansen & Thatcher, 1988; Stewart, Flint, Lamming et al. 1989a; Martal, Degryse, Charpigny et al. 1990). Infusion of conceptus secretory proteins elicits identical effects, and removal of trophoblast IFNs from conceptus secretory proteins by immunoaffinity chromatography renders them inactive (Vallet, Bazer, Fliss & Thatcher, 1988).

However, while administered IFNs delay luteal regression, they frequently block this process for less than the 50 days during which luteal function is required for pregnancy maintenance in the sheep (Casida & Warwick, 1945; Plante et al. 1988; Stewart
et al. 1989a). Furthermore only large doses of purified IFNs elicit a delay in luteolysis (see references cited above). Therefore it has been suggested that a second, as yet unidentified, trophoblast product also contributes to luteal maintenance at this time.

Oxytocin potentially represents one such compound. Continuous infusion of oxytocin into the systemic circulation in sheep between days 13 and 21 after oestrus delays luteal regression for up to 43 days, presumably by preventing the formation of the uterine oxytocin receptor and inhibiting endometrial prostaglandin secretion (Flint & Sheldrick, 1985; Sheldrick & Flint, 1990). A similar phenomenon occurs in cattle (Gilbert, Lamming, Parkinson et al. 1989). Furthermore, it has recently been suggested, based on radioimmunoassay and chromatography, that the sheep blastocyst synthesizes and secretes oxytocin between days 14 and 21 after oestrus (Lacroix, Charpigny & Reinaud, 1988).

The experiments described here were designed to test the hypothesis that oxytocin secreted by the blastocyst contributes to corpus luteum maintenance in early gestation. We have used slot blotting, Northern analysis and the polymerase chain reaction to determine whether the oxytocin–neurophysin gene is expressed in the ovine blastocyst, and have determined the effect of intrauterine infusion of oxytocin in non-pregnant sheep. Reproductive tissues from women, cattle, sheep and pigs were also investigated as positive and negative controls.

MATERIALS AND METHODS

Animals

Oestrus was synchronized in 25 Dorset Horn ewes using a flugosterone-impregnated intravaginal sponge (Chronogest; Intervet U.K. Ltd, Cambridge, Cambs, U.K.) for 13 days, followed by i.m. injection of 320 IU pregnant mare serum gonadotrophin (PMSG; Intervet U.K. Ltd) and 250 µg cloprostenol (Estrumate; Coopers Animal Health Ltd, Crewe, Cheshire, U.K.) on the day of sponge withdrawal. Oestrus, which was detected using a raddled vasectomized ram introduced 24 h after sponge withdrawal, occurred between 12 and 48 h after introduction of the ram; day of oestrus = day 0. Jugular venous blood samples (5 ml) were collected daily from the day after oestrus, for progesterone determination. Cattle and sows were killed after mating at spontaneous oestrus.

Messenger RNA hybridization

Blastocysts flushed from the uteri of sheep (days 13, 15, 16, 17, 19 and 23), cattle (days 16, 22 and 30) and sows (days 14 and 18) with sterile 0-9% (w/v) NaCl were extracted in buffered guanidine isothiocyanate, and total RNA was prepared by centrifugation through CsCl (Glisin, Crkenjakov & Byus, 1974; Chirgwin, Przybyla, MacDonald & Rutter, 1979). All animals were slaughtered at a commercial abattoir and reproductive tracts flushed within 20 min of death. RNA concentrations were determined spectrophotometrically. For Northern analysis, 20 µg total RNA/track was subjected to electrophoresis on 1% agarose gels containing 0.02 mol 3-(N-morpholino)-propanesulfonic acid/l, 0.0005 mol sodium acetate/l, 0.001 mol sodium EDTA/l, pH 7.4 (1 × MOPS) and 6% (w/v) formaldehyde, and blotted on to Hybond N nylon membranes (Amersham International plc, Amersham, Bucks, U.K.) using 20 × SSC (1 × SSC = 0.15 mol NaCl/l, 0.015 mol sodium citrate/l). For application to gels, RNA was dissolved in 41.5 µl containing 21 µl deionized formamide, 4 µl 10 × MOPS and 6.5 µl formaldehyde and denatured at 65 °C for 10 min. Hybridization was performed at 65 °C in 50 mmol Tris–HCl/l (pH 7.6) containing 1 mol NaCl/l, 6% (w/v) polyethylene glycol, 10 × Denhardt’s solution (100 × Denhardt’s = 2% (w/v) bovine serum albumin, 2% (w/v) Ficoll 400 and 2% (w/v) polyvinylpyrrolidone), 0.1% (w/v) sodium pyrophosphate and 100 µg denatured salmon sperm DNA/ml. Washing was at 65 °C in 6 × SSC containing 0.1% (w/v) sodium dodecyl sulphate (SDS).

Slot blots were prepared using nitrocellulose membranes equilibrated in 10 × SSC; 40 µg total RNA was denatured as for Northern analysis and diluted with an equal volume of 20 × SSC before application. Membranes were washed with 20 × SSC, dried in air, baked under vacuum at 80 °C for 2 h and probed as described above. After hybridization, washing was in 6 × SSC containing 0.1% SDS, followed by 2 × SSC containing 0.1% SDS.

Three DNA probes were used. One was a 172 bp cDNA derived from the 3’ end (positions 260–431) of the oxytocin–neurophysin prohormone mRNA, hybridizing to part of the neurophysin mRNA sequence (kindly supplied by Professor G. Schütz, Insitut für Zell- und Tumoriobiologie, Deutsches Krebsforschungszentrum, Heidelberg, Germany; Jones & Flint, 1988). This probe and an actin cDNA (Stewart, McCann, Northrop et al. 1989b) were labelled using the multiprime method (Amersham International plc). The third probe was a 27-mer oligonucleotide corresponding to the bovine cDNA sequence of the oxytocin moiety of the prohormone as described by Ivell & Richter (1984). This sequence, which was synthesized using a Biosearch 8750 four-channel DNA synthesizer (Biosearch, San Raphael, CA, U.S.A.), was end-labelled using T4 polynucleotide kinase. All probes were labelled with 32P.
Control mRNA extracted from mid-cycle ovine corpora lutea was quantified in terms of oxytocin prohormone mRNA as described by Jones & Flint (1988).

Polymerase chain reactions

Samples of RNA (extracted as described above for Northern analysis) from a number of ovine blastocysts and other control tissues (in which oxytocin–neurophysin mRNA was expected to be either present or absent) were reverse transcribed and subjected to nested polymerase chain reactions as follows. For reverse transcription 1 µl samples of RNA (containing 1–10 µg) were mixed with 2 µl oligo(dT) (100 µg/ml final concentration) and 5 µl H2O, before denaturing at 68 °C for 5 min and hybridization by chilling on ice.

To each sample were added 2 µl 10 × reverse transcriptase buffer (500 mmol Tris–HCl/l containing 400 mmol KCl/l, 60 mmol MgCl2/l and 0-4 mmol dithiothreitol/l (pH 8.3) at 42 °C), 2 µl 10 mmol deoxyribotrimonucleotides/l (dNTPs), 0-5 µl RNAsin (20 U, Promega, Southampton, Hants, U.K.) and 0-5 µl Super reverse transcriptase (10 U, Anglian Biotec, Colchester, Essex, U.K.). After incubation at 42 °C for 1 h, the reaction was stopped by heating to 80 °C for 10 min.

For the first round of polymerase chain reaction, to 1 µl of the cDNA solution obtained as above, were added 34 µl H2O, 5 µl 10 × polymerase chain reaction buffer (100 mmol Tris–HCl/l containing 500 mmol KCl/l, 15 mmol MgCl2/l and 0.1% (w/v) gelatin, pH 8.3, at 25 °C), 2.5 µl each of primers A and B (see below; 10 µmol/l initial concentrations in each case), 5 µl of 1 mmol dNTPs/l and 0.3 µl Tag polymerase (2.5 U, USB, Cleveland, OH, U.S.A.). This mixture was then cycled as follows: 95 °C, 90 s; 95 °C, 20 s; 55 °C, 30 s; 72 °C, 30 s) × 35; 72 °C, 3 min. For the second round of polymerase chain reaction, 1 µl of the first-round product was subjected to further cycling in a fresh reaction mixture (50 µl, as above) using primers C and D. Temperature cycles were as for the first round, but were repeated 20 times, not 35.

For analysis of the polymerase chain reaction product, 10 µl of the secondary product solution was subjected to 1.5% agarose gel electrophoresis; all samples were run concurrently in parallel tracks together with a 123 bp ladder.

Synthetic oligonucleotides used as primers were as follows:

A 5'-TC GTC GAC AGC CCA AGC GCG TCT GCA CCA T-3'

B 5'-TG GTC GAC GGG GAT GAT TAC AGA GGG AGT GAG GCC GGC-3'

C 5'-TGA CCT CCG CCT GCT ACA T-3'

D 5'-GGG GCC GCA GGG GAG ACA-3'

The predicted distance on the cDNA between primers C and D was 90 bases, and this was therefore the predicted size of the nested polymerase chain reaction product. These oligonucleotides were chosen to span intron 1 of the oxytocin–neurophysin gene, so that any contamination with genomic DNA would be detected in all samples. In order to confirm that negative control samples contained adequate cDNA following reverse transcription, all samples were subjected in parallel to polymerase chain reaction using universal positive control primers for His-tRNA synthetase, which resulted in production of the predicted band. This procedure showed that all samples contained good quality cDNA.

Effects of oxytocin infusion and radioimmunoassays

Ewes mated after induction of oestrus by administration of progestagen and PMSG as described above were distributed between four treatment groups. Group A (n = 5) received 150 mmol oxytocin/24 h (Hoechst U.K., Milton Keynes, Bucks, U.K.; purity confirmed by high-performance liquid chromatography) administered by osmotic minipump (model 2 ML 2; Alza Corp., Palo Alto, CA, U.S.A.) from day 9 until death on day 19 or 20. Pumps were preincubated at 37 °C for 12 h prior to s.c. insertion in the axilla. Samples of 0.9% (w/v) NaCl, in which the pumps were incubated for 1 h before insertion and after post-mortem removal from the ewes, were assayed for oxytocin content to ensure that the minipumps were active over the whole period of the experiment. Group B (n = 4) ewes received vehicle alone (0.9% NaCl plus 0.01% (v/v) acetic acid) via similarly placed osmotic minipumps over the same period as group A. Group C (n = 8) had a cannula placed in one uterine horn ipsilateral to a corpus luteum on day 7 following oestrus, as described by Stewart et al. (1989a) and, from day 9 until death, received 10 nmol oxytocin/24 h (in 7.5 ml 0.9% NaCl plus 0.01% acetic acid), constantly infused into the uterus. Group D (n = 8) had uterine cannulae similarly placed, but received vehicle alone.

On days 14 and 15, all ewes had 10 ml jugular venous blood samples collected hourly for 10 h for determination of plasma concentrations of 13,14-dihydro-15-keto prostaglandin F2α (PGFM). Samples were also collected on days 9 (before commencement of treatments), 10, 12, 15 and the day of death for plasma oxytocin determination. Ewes were killed on either day 19 or 20, at which time the positions of any corpora lutea present on the ovaries were noted, the uterine cannulae tested for patency and the osmotic minipumps recovered. The uteri were removed, placed on ice and, within 10 min, endometrial tissue was excised for preparation of subcellular fractions.
for determination of oxytocin binding (Sheldrick & Flint, 1985).

Plasma progesterone concentrations were determined by radioimmunoassay using the method of Hunter, Southee, McLeod & Hareshign (1986). Intra- and interassay coefficients of variation were <10% and <15% respectively. Mean recovery of [3H]progesterone after extraction was 79% and the limit of sensitivity, using a 250 µl sample, was 0-29 pmol/ml. Plasma PGFM concentrations were measured using the method of Kaker, Murray & Dobson (1984). Intra- and interassay coefficients of variation were <10% and <20% respectively. Mean recovery of [3H]PGFM after extraction was 77% and the limit of sensitivity, using a 500 µl sample, was 126 fmol/ml.

Oxytocin concentrations were measured after extracting 4 ml plasma or media, using octadecylsilicate cartridges (Jones Chromatography Ltd, Hengoed, Mid Glamorgan, U.K.), as described by Wathes, Guldenaar, Swann et al. (1986). A single assay was used; the limit of sensitivity was 0-5 fmol/tube, and the intra-assay coefficient of variation was 8-5%. Samples of saline in which pumps had been incubated were assayed without prior extraction, but following appropriate dilution in assay buffer.

The length of the luteal phase was calculated as the interval from the day of oestrus until the day on which plasma progesterone concentrations declined to below 0-95 pmol/ml for the first time. For ewes whose luteal function was maintained at the time of death, the following day was taken for statistical purposes. Mean luteal phase lengths were subjected to one-way analysis of variance, using treatment group as the factor. The frequency, amplitude and area under the curve of PGFM episodes were calculated, defining an episode as any increase in concentration of at least 279 fmol/ml above the preceding nadir. The total episodic secretion was also calculated as the sum of the areas under the curves of all PGFM episodes occurring during both sampling periods. These data were log-transformed to normalize the errors and subjected to analysis of variance with respect to treatment and day.

RESULTS

Northern analysis of blastocyst RNA

Results of hybridization analyses of ovine blastocyst RNA using the 3'-neurophysin probe are shown in Pl. 1. No oxytocin prohormone mRNA was detectable on slot blots or Northern analysis. On slot blots, 40 µg total blastocyst RNA gave an undetectable signal, whereas 27 pg oxytocin mRNA extracted from ovine corpus luteum was detectable; therefore the blastocyst RNA samples contained 0-00007% oxytocin RNA. On Northern analysis, luteal oxytocin mRNA migrated as expected on the basis of previous results (608 bases in length; Jones & Flint, 1988). Even when the film was overexposed oxytocin mRNA was not detectable in days 13–23 ovine, days 16–30 bovine or days 14 and 18 porcine blastocysts. Probing the blots for actin showed that this mRNA was present in all blastocyst extracts.

Identical results (data not shown) were obtained when Northern analysis was performed using an oligonucleotide corresponding to the oxytocin-coding sequence.

Polymerase chain reaction

Results of nested polymerase chain reactions carried out with RNA from ovine blastocysts on days 15, 16 and 19, and from other tissues, are shown in Pl. 2. The only tissue extract shown to produce the expected 90 bp product was that from the ovine corpus luteum: all other samples, including ovine blastocysts and endometrium and human corpus luteum, were negative.

Effects of oxytocin infused in vivo

Plasma oxytocin concentrations were significantly (P<0.001) higher in ewes receiving systemically administered oxytocin (group A) than in control animals, from the sample following the insertion of the osmotic minipumps until the day of slaughter. Peripheral plasma concentrations were not elevated in ewes which received intruterine oxytocin (group C; Text-fig. 1).

The mean length of the luteal phase of ewes in group A (20±0.55 days) was significantly (P<0.01) greater than that of ewes in groups B, C and D (170±0.41, 17.3±0.36 and 17±0±0.57 days respectively). Differences between groups B, C and D were not significant. There was no statistically significant difference between the amount of [3H]oxytocin bound by endometrial tissue of ewes in the different groups, although there was a trend towards reduced values in ewes in group A (108±6±40.8 fmol/mg protein) compared with those in groups B, C and D (306.5±110.8, 240.9±59.6 and 178±7±45.8 fmol/mg protein respectively). The amplitude of PGFM episodes was significantly (P<0.05) lower in ewes in group A than in other animals and, although the number of PGFM episodes was not significantly different between groups, ewes in group A tended (0.10>P>0.05) to have fewer than those in other groups. The total episodic secretion of PGFM over the 20 h of sampling on days 14 and 15 was significantly (P<0.05) lower in ewes in group A (mean ± s.e.m. = 0.99±0.99 log pg/ml per h, corresponding to an untransformed value of 2.69 pg/ml per h) than in groups B (log: 4.34±0.32;
untransformed, 76.7 pg/ml per h), C (log.; 4.12 ± 0.85; untransformed, 61.6 pg/ml per h) or D (log.; 3.76 ± 0.63; untransformed, 42.9 pg/ml per h). Differences between days were not significant.

**DISCUSSION**

When our initial Northern analysis experiments with blastocyst RNA and the 3'-probe proved negative, we considered that blastocyst oxytocin synthesis may result from expression of an as yet unidentified gene lacking a neurophysin sequence related to the probe. On the basis of experiments carried out with the 3'-probe used here, there is thought to be only one oxytocin gene in cattle (Ivell & Richter, 1984) but the number of genes in sheep has not been determined. For this reason the Northern blots were also probed with an oligonucleotide derived from the region of bovine oxytocin–neurophysin mRNA which codes for the oxytocin amino acid sequence. This oligonucleotide has been shown to hybridize to oxytocin–neurophysin mRNA extracted from sheep corpora lutea. However, although we have confirmed the presence of oxytocin–neurophysin mRNA in corpora lutea, no hybridization to blastocyst RNA was seen. On the basis of the quantities of luteal oxytocin–neurophysin mRNA detected by Northern analysis in these experiments, the concentration of oxytocin–neurophysin mRNA in ovine blastocyst would be below 200 pg per conceptus. It is unlikely that degradation of mRNA during extraction accounts for these negative data, as the same sheep blastocyst RNA preparations have been successfully probed for interferon mRNA (Stewart et al. 1989b), and the blots initially probed for oxytocin–neurophysin mRNA were subsequently washed and successfully re-probed for actin. Van Vliet, Walton, Wildeman et al. (1991) have also failed to detect oxytocin–neurophysin mRNA in day 18 bovine conceptuses by Northern analysis with the 3'-probe used here.

The nested polymerase chain reaction was used to analyse blastocyst RNA, as this procedure is extremely sensitive. The probes used for the first cycle were oligonucleotides based on the 5' and 3' ends of the bovine oxytocin–neurophysin prohormone sequence; for the second cycle, probes spanning intron 1 were used, which were expected to yield a product of 90 bp. These primers generated a product of the predicted size in ovine luteal extracts, but not in blastocyst RNA preparations. There was no detectable contamination with genomic DNA. The absence of a positive signal in human decidua, or in ovine endometrium, is consistent with present understanding of the sources of extrahypothalamic oxytocin among reproductive tissues. Whether the human corpus luteum synthesizes oxytocin remains uncertain, although others have detected oxytocin–neurophysin mRNA in this tissue by polymerase chain reaction (see Auletta, Jones & Flint, 1988; Ivell, Furuya, Brackmann et al. 1990).

The failure to elicit a prolongation of luteal function following uterine infusion of oxytocin indicates that even were the blastocyst to secrete oxytocin, this may not delay luteal regression. The blastocyst has not invaded the endometrium at the time at which the
antiluteolytic action of blastocyst oxytocin has been proposed (days 14–19; Lacroix et al. 1988) and therefore it is unlikely that the infusion failed as a result of the need for close trophectodermal-endometrial apposition for transfer of substances secreted by the blastocyst.

Oxytocin administered to the uterine lumen did not appear in the systemic circulation and failed to reduce oxytocin receptor concentrations in the endometrium. These findings are consistent since systemic administration does lead to receptor down-regulation (Flint & Sheldrick, 1985). Autoradiographic localization of the uterine oxytocin receptor in the ewe using an iodinated receptor antagonist has demonstrated that, at oestrus, binding sites are located on luminal and glandular epithelia and the caruncular stroma (Ayad, Guldenaar & Warthes, 1991). However, at the time of luteolysis, the majority of sites are on the luminal epithelium, and so should be accessible to intraluminal infusions. It is possible that proteolytic enzymes in the luminal fluid degrade the peptide.

It should be noted that uterine oxytocin receptor concentrations were relatively low in the control animals investigated in this study, as these animals had ovulated, resulting in receptor levels characteristic of the early luteal phase of the cycle (Sheldrick & Flint, 1985); for this reason the differences between treatment groups were less than had the control animals been killed at oestrus. Additional information supporting the lack of effect of intrauterine administration of oxytocin on receptor concentrations comes from measurements of circulating PGFM, the secretion of which was only affected by systemic administration of oxytocin.

Taken together these data do not support a role for blastocyst-secreted oxytocin in the regulation of uterine prostaglandin secretion in early pregnancy in the ewe. It is uncertain why we cannot confirm the conclusion of Lacroix et al. (1988); one possibility is that Lacroix et al. (1988) detected proteolytic degradation products of other proteins, which behave in a similar manner to oxytocin on high-performance liquid chromatography and cross-react in certain radioimmunoassays, as has been suggested may occur in some extracts of human corpora lutea (Flint, Auletta & Barker, 1988).

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REFERENCES


DESCRIPTION OF PLATES

Plate 1

FIGURE 1. Slot blot showing hybridization of the 3'-probe to various amounts of ovine luteal mRNA, and absence of hybridization to ovine blastocyst RNA on days 15, 16, 19 and 23 after oestrus and Escherichia coli ribosomal RNA (rRNA). Identical results were obtained using the oxytocin coding region probe (not shown). The slot blot has two rows of slots; those containing RNA are numbered as follows: 1, day 15 blastocyst RNA; 2, day 16 blastocyst RNA; 3, day 19 blastocyst RNA; 4, day 23 blastocyst RNA; 5, 50 µg rRNA; 6, 100 µg rRNA; 7, 2.7 pg luteal oxytocin mRNA; 8, 27 pg oxytocin mRNA; 9, 272 pg oxytocin mRNA.

FIGURE 2. Northern blot showing hybridization of the 3'-probe to ovine luteal mRNAs (amounts as for 7, 8 and 9 in Fig. 1) and lack of corresponding hybridization to 20 µg ovine (days 13, 16, 17 and 23), bovine (days 16, 22 and 30) and porcine (days 14 and 18) blastocyst RNA. 7S, 18S and 28S refer to positions of ribosomal RNA; OT indicates expected position of oxytocin–neurophysin mRNA. The lower panel shows the same blot overexposed in an unsuccessful attempt to detect minor bands. Similar results were obtained by probing the same blot (following removal of 3'-probe) with the oxytocin-coding region probe (data not shown).

FIGURE 3. Northern blot in Fig. 2 following hybridization with the actin probe.

Plate 2

Result of nested polymerase chain reaction. Lanes contained products obtained from: 1, ovine corpus luteum (day 5 after oestrus); 2–4, samples of human corpus luteum; 5, human decidua; 6–10, ovine blastocysts (days 15, 16, 19 and 19 after oestrus respectively); 11, ovine endometrium (during pregnancy); 12, ovine endometrium (from a non-pregnant animal); 13, blank sample without added mRNA; 14, 123 bp ladder (size markers); 15–18, lanes not loaded.