Reconsideration of the proposed luteotropic and luteoprotective actions of ovine placental lactogen in sheep: *in vivo* and *in vitro* studies

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Abstract

Ovine placental lactogen (oPL) is produced by the conceptus trophoderm and is secreted into both the maternal and fetal circulations. The present study was designed to examine *in vivo* the luteotropic effect of recombinant oPL (roPL), as determined by monitoring progesterone concentration and cycle length (experiment 1), and the antioxidative and antiapoptotic effects of roPL, as determined respectively by monitoring antioxidant enzymatic activity and apoptosis in the corpus luteum (CL) of cyclic ewes (experiment 2). We also studied whether roPL is capable of stimulating progesterone secretion *in vitro* by cultured luteal tissue of functionally active CL obtained from day-10 cyclic ewes (experiment 3) and day-60 pregnant ewes (experiment 4). Circulating concentrations of progesterone and cycle length were not affected by treatment of ewes with 80 µg/kg body weight per day of roPL ($n=4$ ewes) for 10 days beginning on day 11 post-estrus, as compared with saline-treated ewes ($n=4$ ewes). Luteolysis occurred between days 15 and 16 post-estrus in the four saline-treated ewes and in 3/4 roPL-treated ewes. The activities of the key antioxidant enzymes

copper-zinc superoxide dismutase (Cu,Zn-SOD), manganese SOD (Mn-SOD), glutathione peroxidase (GPX), glutathione reductase (GSR) and glutathione S-transferase (GST) were unaffected by treatment of ewes with 80 µg/kg per day of roPL ($n=4$ ewes) for 3 days, between days 11 and 14 post-estrus, as compared with saline-treated ewes ($n=4$ ewes). *In situ* TUNEL method revealed that the number of apoptotic cells was not different between the two groups of ewes. There was no significant change in progesterone secretion by explants from day-10 estrous cycle ($n=3$ ewes) or day-60 pregnancy ($n=3$ ewes) CL cultured with different concentrations (10, 100 and 1000 ng/ml) of roPL, whereas treatment with oLH at the concentration of 100 or 1000 ng/ml caused a significant increase in progesterone secretion by explants from day-10 estrous cycle CL ($P<0.05$) and by explants from day-60 pregnancy CL ($P<0.01$). In conclusion, our results demonstrate that oPL has no luteotropic and/or luteoprotective actions in sheep, either *in vivo* or *in vitro*.

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Introduction

Despite recent advances in reproductive technology, farm producers are still faced with the persistent problem of low fertility. Inadequate corpus luteum (CL) production of progesterone is likely to be a presumptive cause of early embryonic loss and pregnancy failure in sheep and cattle (Ayalon 1978, Ashworth *et al.* 1989, Shelton *et al.* 1990, Thatcher *et al.* 1994). Thus, researchers have specifically proposed progesterone supplementation after breeding to compensate for poor luteal steroidogenic activity in beef cattle (Garrett *et al.* 1988), dairy cows (Robinson *et al.* 1989), ewe lambs (Davies & Beck 1992) and mature ewes

(Kleemann *et al.* 1994, Nephew *et al.* 1994). An alternative strategy to increase progesterone concentrations is the use of luteotropic hormones. Pituitary luteinizing hormone (LH) is well known as the main luteotropin in domestic ruminants (Denamur 1968, Kaltenbach *et al.* 1968, Denamur *et al.* 1973, Niswender 2002). Human chorionic gonadotropin (hCG), the main luteotropic signal produced by the primate trophoblast, has activity similar to that of LH. Thus, researchers have used hCG in their attempts to increase reproductive performance in ruminants. Treatment of cattle (Rajamahendran & Sianangama 1992, Schmitt *et al.* 1996, Santos *et al.* 2001) and sheep (Beck *et al.* 1999, Khan *et al.* 2003,

Akif Cam & Kuran 2004) with hCG induces formation of accessory CL, enhances plasma progesterone concentration and improves reproductive performance. In spite of the encouraging results obtained, the use of hCG in ruminants may create problems in animal fertility. For example, repeated use of equine (e)CG has been shown to decrease fertility in goats (Roy *et al.* 1999a) and ewes (Roy *et al.* 1999b). The production of antibodies seems to explain the adverse effect of eCG on fertility in domestic ruminants (Chimineau *et al.* 1999, Roy *et al.* 1999a,b). Therefore, to minimize unwanted immunologic responses, alternative strategies using luteotropins to improve pregnancy rates require investigations to be conducted in homologous species.

The placenta of ruminant species produces regulatory factors, including placental lactogens (PLs), also known as chorionic somatomammotropin (oCS). In sheep, the presumed luteotropic action of ovine PL (oPL) was first suggested by Kelly *et al.* (1974), who showed that a rise in oPL during late pregnancy paralleled the increase in progesterone secretion observed at that time. Shortly after the discovery of ruminant PL, it was purified from term placentae of ewes (Martal & Djiane 1975) and cows (Bolander & Fellows 1976). oPL is synthesized by the trophoblast as early as day 16 of pregnancy (Carnegie *et al.* 1977, Martal & Djiane 1977) and is found to be present in the peripheral circulation in increasing amounts as pregnancy progresses (Chan *et al.* 1978a). Specific binding sites for oPL have been detected in various tissues, including the CL, obtained from nonpregnant and pregnant ewes (Chan *et al.* 1978b). Evidence indicates that the ovine prolactin (oPRL) receptor transduces signals by oPL, because this hormone homodimerizes the oPRL receptor and activates signaling pathways (Herman *et al.* 2000). In addition, one property of ruminant PLs is their ability to bind both PRL and growth hormone (GH) receptors (for review, see Gertler & Djiane 2002). In spite of these investigations, little attention has been given so far to studying the luteotropic role of oPL in sheep. The lack of definitive evidence of this role has been due to the difficulty in obtaining sufficient quantities of the native hormone required for homologous *in vivo* experiments. The oPL has been cloned, and the recombinant form of this hormone has been produced (Sakal *et al.* 1997). It is expected that this will greatly facilitate investigations on the function of PL in sheep. For example, delineating the luteotropic action of oPL may provide new opportunities to minimize the incidence of early embryonic mortality in current production systems.

The present study was designed to examine *in vivo*:

1. the luteotropic effect of recombinant oPL (roPL), as determined by monitoring progesterone secretion from the CL of cyclic ewes treated with roPL (experiment 1).
2. the antiapoptotic and antioxidative effects of roPL, as determined respectively by monitoring apoptosis and antioxidant enzymatic activity in the CL of cyclic ewes treated with roPL (experiment 2).

We also studied whether roPL can stimulate progesterone secretion *in vitro* by cultured luteal tissue of functionally active CL obtained from day-10 cyclic ewes (experiment 3) and day-60 pregnant ewes (experiment 4).

Materials and Methods

Animals

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to French regulations for animal experimentation (authorization no. 78–34). The study involved cyclic and pregnant ewes of the Préailles-du-Sud breed. The ewes were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronize estrus, as previously described (Menegatos *et al.* 2003). After sponge removal, each ewe received an intramuscular injection of 400 IU eCG (Intervet). Ewes assigned to the pregnant group were mated twice at the time of the synchronized estrus with fertile rams, at an interval of 12 h. All ewes used were housed under conditions of natural day length and temperature.

Experiments 1 and 2: *in vivo* effect of roPL on CL functional life span and structure

Experiment 1 was designed to examine the luteotropic effect of roPL as determined by monitoring progesterone secretion from the CL of cyclic ewes. Experiment 2 was designed to examine the antiapoptotic and antioxidative effects of roPL as determined by monitoring apoptosis and antioxidant enzymatic activity in the CL of cyclic ewes respectively. Sixteen ewes were randomly assigned to one of two treatments: 80 µg roPL/kg live weight/day ($n=8$ ewes) or saline ($n=8$ ewes). In experiment 1, the ewes were treated with an intramuscular injection of 1 ml saline ($n=4$ ewes) or roPL ($n=4$ ewes) twice daily (at 0800 and 2000 h) for 10 days. In experiment 2, the ewes were treated with saline ($n=4$ ewes) or roPL ($n=4$ ewes) for 3 days. In both experiments, treatments were started on day 11 of the estrous cycle. The roPL used in the present study has mammogenic and lactogenic effects in ewes (Kann *et al.* 1999), and the dose used was selected because it had been shown to mimic the peripheral concentration of this hormone during midpregnancy (Kann *et al.* 1999). Blood samples (3 ml) were taken daily from the jugular vein into evacuated heparinized tubes, beginning on day 11 of the estrous cycle. After centrifugation (3000 g, 4 °C)

for 30 min, plasma was stored at -20°C until assayed. In experiment 2, the ewes were killed on day 14 post-estrus at a local abattoir in accordance with protocols approved by the local institutional animal use committee. After slaughter of ewes, the reproductive tracts were collected immediately and transported to the laboratory, and the CL were dissected from the surrounding ovarian tissue. The CL was snap-frozen in liquid nitrogen and then stored at -80°C until processed for the activities of copper-zinc superoxide dismutase (Cu,Zn-SOD), manganese SOD (Mn-SOD), glutathione peroxidase (GPX), glutathione reductase (GSR) and glutathione S-transferase (GST). For the determination of apoptosis, a piece of each CL was fixed overnight in freshly prepared 4% paraformaldehyde in PBS (pH 7.4), washed in PBS, dehydrated through a series of increasing concentrations of ethanol (70–100%), cleared in xylene and embedded in paraffin wax.

Experiments 3 and 4: in vitro effect of roPL on luteal progesterone secretion

These experiments were designed to examine the effect of roPL on progesterone secretion by cultured luteal tissue obtained from day-10 cyclic ewes ($n=3$ ewes, experiment 3) and day-60 pregnant ewes ($n=3$ ewes, experiment 4). Ovaries were collected from each ewe within 5 min after slaughter at a local abattoir and were transported immediately to the laboratory. From each ewe, one CL was dissected from the ovarian stroma, freed from its surrounding connective-tissue envelope, and cut into 1 mm^3 explants, each weighing 15–30 mg. They were then pooled and distributed randomly among treatments. Explants were placed (1 explant/ml per well in a 24-well culture plate) in DMEM:F12 serum-free medium (Sigma-Aldrich) containing 25 mM Hepes, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.1% BSA. In experiment 3, luteal tissue explants from each cyclic ewe were cultured alone (basal) and with different concentrations (10, 100 and 1000 ng/ml) of roPL or oLH. In experiment 4, luteal tissue explants from each pregnant ewe were cultured alone (basal) and with different concentrations (10, 100 and 1000 ng/ml) of roPL or oLH. In both experiments, incubations of luteal tissue explants from each ewe were performed in triplicate at 37°C for 24 h under a humidified atmosphere of 95% air and 5% CO_2 . At the end of the incubation period, the weight of each explant was recorded, and the media were stored at -20°C until assayed. Progesterone secretion over the 24-h incubation period by luteal explants was normalized with tissue weight, and secretion was expressed as ng/mg of tissue.

Progesterone assay

Plasma samples and incubation media were analyzed for progesterone concentration in duplicate by direct RIA, as previously described in Schanbacher (1979) with

some modifications. Charcoal-dextran solution was used instead of polyethylene glycol for the separation of bound and free radioactivity. Tritiated progesterone (1,2,6,7- ^3H -progesterone, sp act 88 Ci/mmol) was obtained from Amersham, and a specific antiprogesterone antibody was obtained from the Institut Pasteur (Paris, France). Hormone preparations, progesterone tracer and other reagents were diluted in 0.1 M PBS (pH 7.25). Approximately 3000 c.p.m. tritiated progesterone in 100 μl buffer, 100 μl progesterone antiserum (1/20 000 dilution), 20 μl progesterone standard, plasma samples or incubation media, and 80 μl buffer were dispensed into the assay tubes. After an initial 2-h incubation at laboratory temperature (21°C) followed by 24-h incubation at 4°C , 2.2 ml cold charcoal-dextran solution containing 0.1% gelatin was added, and the tubes were centrifuged at 3000 g for 45 min. The supernatant was decanted into vials, and 3 ml scintillation fluid (Scintillator Plus; PerkinElmer Life & Analytical Sciences, Boston, MA, USA) were added to each vial. The radioactivity was then counted in a Packard Tri-Carb Liquid Scintillation analyzer (model 2100 TR; Groningen, The Netherlands). To minimize assay variability, all plasma samples or incubation media were analyzed in a single RIA. The limit of assay sensitivity was 0.1 ng/ml, and the intra-assay coefficient of variation was less than 10%.

Ovine placental lactogen assay

Plasma concentrations of roPL were measured in duplicate by a previously described double-antibody RIA (Chan *et al.* 1978a) with slight modifications. The roPL was radioiodinated by the chloramine T method (Greenwood *et al.* 1963) and then purified on disposable PD-10 columns packed with Sephadex G25. Hormone preparations, roPL tracer and other reagents were diluted in 0.1 M barbital sodium buffer (pH 8.6), containing 0.25% BSA and 0.004 M EDTA. Approximately 22 000 c.p.m. ^{125}I -roPL in 100 μl buffer, 100 μl oPL antiserum (1/7700 dilution), 20 μl roPL standard or plasma samples, and 330 μl buffer were dispensed into the assay tubes. After a 96-h incubation at 10°C , 100 μl sheep anti-rabbit γ -globulin serum (1/10 dilution) were added to each assay tube. After 10-min incubation at laboratory temperature (21°C), 2 ml polyethylene glycol 6000 (4.4%) were added. The resulting precipitates were centrifuged at 3000 g for 45 min, and the supernatant was discarded. The radioactivity in the precipitates was then counted in the LKB automatic γ -counter (model 1272 Clinigamma). The limit of assay sensitivity was 2.5 ng/ml, and the intra-assay coefficient of variation was less than 10%.

Antioxidant enzyme assays

Frozen, unthawed luteal tissues corresponding to each CL were homogenized separately in 50 mM phosphate buffer

(pH 7.4) before centrifugation at 15 000 *g* for 30 min at 4 °C. The resulting supernatant was used for determination of protein concentration and measurement of activities of Cu,Zn-SOD, Mn-SOD, GPX, GSR and GST. Protein concentrations were determined by Lowry's method (Lowry *et al.* 1951). Enzyme activities in the supernatant of each CL were determined in duplicate on a Cobas-Bio centrifugal analyzer (Hoffman-LaRoche, Basel, Switzerland), according to a modification of the method (Wheeler *et al.* 1990), as previously described and validated (Ceballos-Picot *et al.* 1992). Activity of each enzyme was determined as previously described in detail (Al-Gubory *et al.* 2005).

In situ detection of apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method

Luteal apoptotic cells were identified with the *In Situ* Cell Death Detection Fluorescein Kit (Roche). This assay detects nuclear DNA fragmentation in apoptotic cells by the TUNEL method according to Gavrieli *et al.* (1992). Briefly, tissue sections (6 µm thick) were prepared, mounted on silane-coated slides (3-amino-propyl-triethoxysilane-coated slides; Sigma-Aldrich) and rehydrated through a series of decreasing concentrations of ethanol (100–70%) to water. They were then treated with 100 µg/ml Proteinase K (Sigma-Aldrich) in 10 mM Tris (pH 8.0), for 30 min at 37 °C and washed with PBS (pH 7.4). The slides were submerged in 0.1 M sodium citrate for 30 min at 70 °C and then washed with PBS. Sections were incubated for 60 min at 37 °C with the TUNEL mixture, washed with PBS and counterstained with an aqueous solution of propidium iodide (DNA-PREP stain; Beckman Coulter, Miami, FL, USA) for 60 min at room temperature, to detect red-staining nonfragmented cell nuclei. Tissue sections were analyzed under a fluorescent microscope. Negative control sections were processed identically except that the labeling enzyme (terminal deoxynucleotidyl transferase) was omitted. Four different optical fields (magnification 250) were selected in a random manner for each CL tissue section and used to calculate the percentage of apoptotic nuclei.

Statistical analysis

In vitro experiments were repeated three times, using a different ewe for each experiment. Since concentrations of progesterone secretion over the 24-h incubation by luteal explants varied widely between ewes, progesterone values were expressed as a percentage of the basal secretion rate. The data are given as the mean $\pm$ S.E.M. of triplicate treatments for each of the three experiments. Differences in concentration of progesterone in culture media between the control and treated explants were assessed by one-way ANOVA followed by Dunnett's multiple-range

Figure 1 Mean ($\pm$ S.E.M.) recombinant oPL (roPL) concentrations in jugular blood samples collected daily from four roPL-treated ewes. Ewes were treated with roPL at a dose of 80 µg/kg live weight per day twice daily (at 0800 and 2000 h), starting on day 11 of the estrous cycle. Results are from blood samples taken at 0900 h.

test (GraphPad Prism, San Diego, CA, USA). A level of $P < 0.05$ was accepted as statistically significant.

Results

Experiment 1

During roPL treatment (80 µg roPL/kg live weight per day), plasma concentrations of oPL were maintained between 200 and 400 ng/ml, and dropped to 42 ng/ml at 24 h after treatment was stopped (Fig. 1). Progesterone concentrations in jugular blood samples collected from the four ewes treated with roPL and the four saline-treated ewes are shown in Fig. 2. In all the saline-treated ewes and in 3/4 roPL-treated ewes, progesterone levels remained high (>2 ng/ml) until day 14 of the estrous cycle, when an abrupt decline occurred, dropping to low (<1 ng/ml) or undetectable levels on days 15–16. This indicates complete functional regression of the CL. Only one of the four roPL-treated ewes showed a prolonged cycle, with complete luteolysis occurring on day 18. The three other roPL-treated ewes showed normal cycles with a duration of 16 days. Spontaneous new ovulations occurred in all the ewes, except for one saline-treated ewe, as evidenced by the rise in progesterone concentrations (>0.5 ng/ml) 3–6 days after luteolysis. The mean duration ($\pm$ S.E.M.) from complete luteolysis to return of functional activity of the newly formed CL, as identified by the first elevation of plasma progesterone concentration (≥ 0.5 ng/ml), was shorter ($P < 0.05$, unpaired *t*-test) in roPL-treated ewes (3.8 ± 0.3 days) than in saline-treated ewes (5.8 ± 0.6 days).

Experiment 2

There were no significant differences between roPL-treated and saline-treated ewes in the mean ($\pm$ S.E.M.)

Figure 2 Progesterone concentrations in jugular blood samples collected daily from four ewes treated with recombinant oPL (roPL) and four saline-treated ewes. Ewes were treated with roPL or saline (NaCl) at a dose of 80 µg/kg live weight per day twice daily (at 0800 and 2000 h), starting on day 11 of the estrous cycle. Results are from blood samples taken at 0900 h.

activities (U/mg protein) of Cu,Zn-SOD (71.8 ± 12.0 vs 65.3 ± 12.9), Mn-SOD (93.0 ± 8.0 vs 99.5 ± 32.3), GPX (45.1 ± 6.1 vs 33.0 ± 2.9), GSR (25.4 ± 4.7

vs 24.5 ± 2.0) and GST (287.8 ± 19.7 vs 372.7 ± 81.6). *In situ* identification of DNA fragmentation in luteal cells of CL collected at day 14 of the estrous cycle from

Figure 3 *In situ* identification of apoptotic cells by fluorescence labeling of nicked DNA in CL on day 14 of the estrous cycle in a representative ewe treated with saline (NaCl; A) or recombinant oPL (roPL; B). Ewes were treated with saline or roPL at a dose of 80 µg/kg live weight per day twice daily (at 0800 and 2000 h), between days 11 and 14 post-estrus. Histologic sections (6 µm) were subjected to the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Apoptotic nuclei are indicated by arrowheads. Original magnification $\times 250$.

representative oPL-treated and saline-treated ewes is shown in Fig 3. The mean percentage ($\pm$ S.E.M.) of apoptotic nuclei of luteal cells in roPL-treated ewes (2.0 ± 0.9) was not different from that in saline-treated ewes (3.3 ± 2.9).

Experiment 3

Progesterone secretion by luteal explants obtained from CL of day 10 of the estrous cycle was not stimulated by

any dose of roPL added to the culture media (Fig. 4). Unlike roPL, addition of oLH to culture media caused a significant increase in progesterone secretion by luteal explants (Fig. 4). The mean progesterone concentration was significantly increased ($P < 0.05$) by the addition of 100 and 1000 ng oLH/ml as compared with controls.

Experiment 4

Progesterone secretion by luteal explants obtained from CL of day 60 of pregnancy was not stimulated by any dose

Figure 4 Progesterone secretion by explants of sheep CL from day 10 of the estrous cycle incubated for 24 h with different concentrations (10, 100 and 1000 ng/ml) of (a) recombinant oPL or (b) oLH. Values are mean $\pm$ S.E.M. of triplicate treatments for each of three separate experiments. * $P < 0.05$ versus controls.

Figure 5 Progesterone secretion by explants of sheep CL from day 60 of pregnancy incubated for 24 h with different concentrations (10, 100 and 1000 ng/ml) of (a) recombinant oPL or (b) oLH. Values are mean $\pm$ S.E.M. of triplicate treatments for each of three separate experiments. * $P < 0.01$ versus controls.

of roPL added to the culture media (Fig. 5). Addition of oLH to culture media caused a significant increase in progesterone secretion by luteal explants (Fig. 5). The mean progesterone concentration was significantly increased ($P < 0.01$) by the addition of 100 and 1000 ng oLH/ml as compared with controls.

Discussion

In rodents, it is well established that luteotropic support of the CL during the second half of pregnancy depends on placental hormones. These hormones, particularly PLs in combination with estradiol, stimulate steroidogenesis by the rat CL (Glaser *et al.* 1984, Khan *et al.* 1987; for reviews, see Gibori *et al.* 1988, Gibori 1993). There is also evidence obtained by *in vivo* and *in vitro* studies that PLs play an important role in the regulation of CL function in the mouse (Galosy & Talamantes 1995). In sheep, the role of oPL in the regulation of CL function has been controversial until now. The presumed luteotropic action of oPL was first suggested by Kelly *et al.* (1974), who showed that a rise in oPL during late pregnancy in ewes paralleled the increase in progesterone secretion observed at that time. However, antibody neutralization of maternal oPL on day 131 of pregnancy in ewes did not affect plasma concentrations of progesterone (Waters *et al.* 1985).

Prostaglandin F- 2α (PGF $_{2\alpha}$) of uterine origin is known to be responsible for the periodic regression of the sheep CL (McCracken *et al.* 1972). At the time of initial functional luteolysis, the first significant rise in the secretion of PGF $_{2\alpha}$ occurred on days 12–13 of the sheep

estrous cycle (Baird *et al.* 1976). Administration of PGF $_{2\alpha}$ before the onset of luteolysis (on day 10 of the estrous cycle) has been shown to decrease progesterone secretion and induce luteal cell degeneration (Stacy *et al.* 1976). One of the proposed roles of oPL is its ability to protect the CL against the luteolytic action of PGF $_{2\alpha}$. However, constant infusion of purified oPL on day 12 of the estrous cycle directly into the arterial supply of the ovary for only 9 h at a rate of 0.6, 6 and 60 μ g/h or for 32 h at a rate of 30 μ g/h did not prevent a fall in the secretion rate of progesterone when a known luteolytic dose of PGF $_{2\alpha}$ was given as a continuous infusion in ewes (Schramm *et al.* 1984). Possibly the dose of oPL injected and/or the duration of infusion was not enough to counteract the luteolytic action of PGF $_{2\alpha}$. One can also speculate that after the initial rise in endogenous secretion of PGF $_{2\alpha}$ on day 12 of the estrous cycle (Baird *et al.* 1976), the CL can no longer be rescued from luteolysis even with oPL treatment. Therefore, in the present study (experiment 1), the luteotropic action of oPL was examined *in vivo* before the onset of luteolysis. We showed that treatment of ewes for 10 days with roPL at a dose of 80 μ g/kg live weight per day, which began before the initiation of luteolysis (day 11 of the estrous cycle), mimicked circulating concentrations of this hormone measured at days 90–100 of pregnancy (Chan *et al.* 1978a). However, this treatment did not alter luteal progesterone secretion and the functional life span of the CL. Of the four ewes treated with roPL, one ewe displayed an estrous cycle of 18 days in length. Since the duration of the estrous cycle in ewes varies from 16 to 18 days, it is unlikely that the delay in luteolysis in one roPL-treated ewe can be attributed to the

roPL treatment. After luteolysis, it is surprising to find that ovulation occurred earlier in roPL-treated ewes than in saline-treated ewes (present study). Although the significance of this finding is at present unknown, one might suggest that the short delay between luteolysis and subsequent ovulation in roPL-treated ewes may be related to developmental changes of follicles that ovulate after oPL treatment. Whether oPL has biologic action in the ovary is not yet clear and remains to be elucidated. Taken together, our results indicate that oPL has no luteotropic action in sheep.

The characteristic cleavage of DNA into oligonucleosomes found in apoptosis has been shown to occur in the sheep CL at the time of luteolysis (McGuire *et al.* 1994) and after exogenous administration of PGF_{2α} during the midluteal phase (McGuire *et al.* 1994, Murdoch 1995). The generation of reactive oxygen species (ROS) within the CL has been suggested to be involved in the mechanism of PGF_{2α}-induced luteolysis in the rat (Riley & Behrman 1991, Sawada & Carlson 1991). An *in vitro* study demonstrated that PGF_{2α} induced apoptosis through the production of ROS by rat luteal cells (Tanaka *et al.* 2000). Recently, we showed that the activities of key antioxidant enzymes in the sheep CL are subject to major changes during the first trimester of pregnancy, suggesting that the CL of early pregnancy may be rescued from luteolysis through increasing activities of antioxidant enzymes and inhibition of apoptosis (Al-Gubory *et al.* 2004). In addition, *in vitro* (Sugino *et al.* 1998) and *in vivo* (Takiguchi *et al.* 2000) experiments demonstrated that rodent PL have stimulatory effects on SODs expression in the rat CL, suggesting that PL may play important roles in the maintenance of luteal function and structure by increasing the antioxidant capacities of the CL. Experiment 2 of the present study was designed to examine whether oPL could protect the CL from apoptosis by increasing the activity of key antioxidant enzymes. Treatments of ewes with roPL at a dose of 80 µg/kg live weight per day for 3 days, which began before the initiation of luteolysis, had no effect on the activity of the five main antioxidant enzymes, Cu, Zn-SOD, Mn-SOD, GPX, GSR and GST, and failed to prevent or reduce apoptosis. These results failed to support the hypothesis that oPL may play a role in the maintenance of sheep CL function either by inhibiting apoptosis or by increasing antioxidant status of luteal cells.

Two populations of steroidogenic cells are present within the sheep CL of the estrous cycle and pregnancy and are referred to as small and large luteal cells (O'Shea *et al.* 1979). Recent *in vitro* studies show that addition of roPL at a dose of 100 ng/ml to the culture medium increases basal progesterone secretion of ovine luteal cells isolated from CL of days 45 and 70 of pregnancy (Gregoraszczyk *et al.* 2000). However, addition of oPL at doses of 4.2 or 140 ng/ml had no effect on progesterone secretion from either small or large ovine luteal cells obtained from fully developed CL of the estrous cycle

(Rodgers *et al.* 1983). In the present study, the direct luteotropic effect of roPL was examined by using luteal explants from both cyclic and pregnant ewes. We used luteal explants instead of whole-cell preparations because the integrity of the luteal tissue ensures the cell-to-cell communication between different types of luteal steroidogenic cells (Rodgers 1990). We also used oLH as a positive control because it is a key endocrine regulator of CL maintenance during early pregnancy in sheep (Denamur 1968, Kaltenbach *et al.* 1968). Since maintenance of pregnancy in sheep (duration ≈ 150 days) depends on progesterone secretion from the CL at least until day 60 (Al-Gubory *et al.* 1999), we used CL of day-60 pregnancy in our study. We showed that roPL added to culture media at doses of 10, 100 or 1000 ng/ml had no significant effect on progesterone secretion during 24-h incubation of luteal explants obtained from CL of day 10 of the estrous cycle (experiment 3) or CL of day-60 pregnancy (experiment 4). In contrast, the secretion of progesterone by luteal explants obtained from both cyclic (experiment 3) and pregnant (experiment 4) ewes was significantly increased by the application of 100 and 1000 ng oLH/ml. The present *in vitro* experiment using ovine CL of day-60 pregnancy clearly did not support the possibility of a direct luteotropic role of oPL during early pregnancy in sheep raised by Gregoraszczyk *et al.* (2000).

In conclusion, these *in vivo* and *in vitro* studies failed to demonstrate luteotropic or luteoprotective actions of oPL in sheep.

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