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Different enzymatic antioxidative pathways operate within the sheep caruncular and intercaruncular endometrium throughout the estrous cycle and early pregnancy

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ABSTRACT

There has been a growing interest in the role played by antioxidant enzymes in the regulation of endometrial function in mammals. However, little is known about enzymatic antioxidative pathways involved in conditioning the cyclic and early pregnant endometrium for conceptus attachment and implantation in domestic ruminants. We aimed to investigate changes in activities of superoxide dismutase 1 and 2 (SOD1, SOD2), glutathione peroxidase (GPX), glutathione reductase (GR) and catalase (CAT) in sheep caruncles (CAR) and intercaruncles (ICAR) endometrial tissues of cyclic and early pregnant ewes. Irrespective of day of cycle or pregnancy, CAR demonstrated higher activities of SOD1 and SOD2 than in ICAR. On day 12 of the estrous cycle, ICAR demonstrated higher activity of GPX and GR than in CAR tissues. On days 12 and 16 the estrous cycle, ICAR demonstrated higher activity of CAT than in CAR. CAR demonstrated higher activity of GPX on day 18 than on days 4, 8, 12 and 16 of the estrous cycle. CAR demonstrated higher activity of CAT on day 18 than on days 4, 8, 12 and 16 of the estrous cycle. ICAR demonstrated higher activity of CAT on day 18 than on days 4, 8, and 16 of the estrous cycle. The activity of CAT in ICAR increased from days 4 and 8 to day 12 of the estrous cycle. The activity of SOD2 in CAR increased from day 16 to day 18 of pregnancy. On day 12 of pregnancy, CAR demonstrated higher activity of GPX than in ICAR. On day 16 of pregnancy, ICAR demonstrated higher activity of GPX than in CAR. The activity of GPX in ICAR increased from day 12 to day 16 of pregnancy. The activity of GPX in CAR increased from day 16 to day 18 of pregnancy. The activity of GR in CAR and ICAR increased from days 12 and 16 to day 18 of pregnancy. On days 16 and 18 of pregnancy, ICAR demonstrated higher activity of CAT than in CAR. The activity of CAT in CAR decreased from day 12 to days 16 and 18 of pregnancy. The activity of CAT in ICAR decreased from day 12 to day 16 of pregnancy and then increased from day 16 to day 18 of pregnancy. In conclusion, different antioxidant mechanisms operate within CAR and ICAR endometrium throughout the estrous cycle and during early pregnancy. This might be related to the different but important roles of CAR and ICAR endometrial tissues for the establishment of pregnancy.

1. Introduction

The control of reactive oxygen species (ROS) by several antioxidant enzymes plays important roles in folliculogenesis, oocyte maturation, endometrial function, implantation, embryogenesis, and prenatal development [1,2]. Establishment of pregnancy is a dynamic process associated with significant physiological changes in the enzymatic antioxidative pathways, which are important for sheep reproductive organ functions, such as the corpus luteum [3–5] and endometrium [6,7]. The endometrium provides nutrients, including glucose, amino acids, glutathione (GSH), calcium, and potassium for the unattached conceptus (embryo and associated extraembryonic membranes), as well as a vital biological surface for attachment of the extraembryonic membranes [8]. Worthy of note is that defective uterine environment is associated with inappropriate secretion of ovarian steroids and/or antioxidant

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enzyme activities, which contributes to early pregnancy failure [9]. There has been a growing interest in the role played by antioxidant enzymes in the regulation of rodent [10], guinea pig [11], human [12–15] and sheep [6,7,16] endometrial function aimed at favoring conceptus attachment and successful establishment of pregnancy. However, little is known about activities of antioxidant enzymes in ruminant endometrium throughout the estrous cycle and during early pregnancy.

The sheep is an appropriate small ruminant model to explore endometrial antioxidant machinery and its regulation [6,7,17]. The sheep uterus (see Fig. 1 A for supplementary information), like that of goat and cattle, has developed specialized endometrial tissues [18], namely caruncle (CAR) and intercaruncle (ICAR) areas (see Fig. 1 B for supplementary information). CAR and ICAR are histo-architecturally different playing important roles in the establishment of pregnancy [19]. CAR areas are glandless dense stromal protuberances and lack glands (see Fig. 1C & E for supplementary information). They represent specialized sites of initial attachment of the extraembryonic membrane. ICAR areas contain branched glands (see Fig. 1 D & F for supplementary information) that produce histotroph nutrition to support early conceptus development and survival [20]. Both CAR and ICAR are covered by a simple luminal epithelium (see Fig. 1E and F for supplementary information). Therefore, we aimed to investigate changes in activities of the key antioxidant enzymes, superoxide dismutase 1 and 2 (SOD1, SOD2), glutathione peroxidase (GPX), glutathione reductase (GR) and catalase (CAT), in sheep CAR and ICAR endometrial tissues collected on days 4, 8, 12, 16 and 18 of the estrous cycle (experiment 1) and on days 12, 16 and 18 of pregnancy, corresponding to conceptus pre-attachment, attachment and early post-attachment periods, respectively (experiment 2).

2. Materials and methods

2.1. Animals and management

The French Ministry of Agriculture approved all procedures relating to care and use of animals according to the French regulation for animal experimentation (authorization no° 78–34). This study used multiparous ewes of the Préalpes-du-Sud breed (18 months of age, n = 35 ewes). All the ewes were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronize oestrous. Ewes assigned to the pregnant group were mated twice at the time of the synchronized oestrus with fertile rams of the same breed at an interval of 12 h. Throughout the experiment, the ewes were housed under conditions of natural day-length and temperature, fed straw and 2 Kg of hay per day par animal, and had free access to mineral licks and water.

2.2. Blood sampling and progesterone assay

In the present study daily plasma progesterone (P4) was determined during one entire estrous cycle of 3 ewes. In addition, plasma P4 was determined on days 4, 8, 12, 16 and 18 of the estrous cycle (experiment 1), and on days 12, 16 and 18 of pregnancy (experiment 2). Blood samples were taken from the jugular veins into evacuated heparinized tubes. After centrifugation (3000 g, 4 °C) for 30 min, plasma was collected and stored at −20 °C until assayed for P4 concentrations. The concentrations of P4 were determined by radioimmunoassay (RIA) in unextracted plasma as described [21] and validated for sheep jugular venous plasma with slight modifications [22]. Tritiated P4 (1,2,6, 7-3H-P4, sp act 88 Ci/mmol) was obtained from Amersham (Bucks, UK), and a specific anti-progesterone antibody was obtained from the Pasteur Institute (Paris, France). The minimum detectable concentration of P4 was 0.1 ng/ml and the intra-assay coefficient of variation was less than 10%.

2.3. Tissue collection

All the ewes were slaughtered at a local abattoir in accordance with protocols approved by the local institutional animal use committee at the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). In experiment 1, ewes were randomly allocated for slaughter on days 4 (n = 4 ewes), 8 (n = 4 ewes), 12 (n = 4 ewes), 16 (n = 4 ewes) and 18 (n = 4 ewes) of the estrous cycle. In experiment 2, ewes were randomly allocated for slaughter on days 12 (n = 4 ewes), 16 (n = 4 ewes) and 18 (n = 4 ewes) of pregnancy corresponding to conceptus pre-attachment, attachment and early post-implantation period, respectively. The stages of pregnancy were confirmed by the presence of one or two conceptus in uterine flushing [20]. The reproductive tract of each ewe was collected within 10 min of death, placed on crushed ice and transported to the laboratory. All subsequent manipulation of the tissue was performed at 4 °C. The uterine horns were opened and all CAR and ICAR were separately dissected from the entire two uterine horns of each ewe, snap-frozen in liquid nitrogen and then stored at −80 °C until processed for activities of the superoxide ($^{\bullet}O_2^-$) scavenging antioxidant enzymes, SOD1 and SOD2, and the hydrogen peroxide (H_2O_2) scavenging antioxidant enzymes, GPX, GR and CAT. For morphological analysis, endometrial tissues were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and then processed for routine histology or histochemistry.

2.4. Conventional histology

Endometrial tissue was washed in PBS, dehydrated through a series of increasing concentrations of ethanol, cleared with butanol:ethanol (V:V), butanol, embedded in paraffin wax, and sectioned at 7 μm. Sections were deparaffinised in toluene, hydrated through decreasing concentrations of ethanol, washed in distilled water and stained with hematoxylin and eosin.

2.5. Histochemistry

Endometrium sections (7 μm) were treated for permeabilization and coloration in 1.25 μg/ml FITC-Phalloidin (Sigma, St. Quentin Fallavier, France) and 0.05% saponin in PBS for 40 min. After washing in PBS, the sections were then incubated 10 min with 1 μg/ml DAPI (Sigma) in PBS for nucleus localization. After washing in PBS, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK). Confocal microscopy observations were performed with a Zeiss confocal microscope.

2.6. Antioxidant enzyme activity assays

The CAR or ICAR were homogenized in cold phosphate buffer (50 mM, pH 7.4) and then the homogenates were centrifuged at 15000 × g for 30 min, 4 °C. The resulting supernatant was used for determination of protein concentration [23]. A standard SOD assay [24] that had been validated for different sheep reproductive tissues [3,6,17,25] was used. Total SOD activity was measured using the pyrogallol assay based on the competition between pyrogallol oxidation by $^{\bullet}O_2^-$ and $^{\bullet}O_2^-$ dismutation by SOD. Enzymatic activity of manganese-SOD (SOD2) was determined by assaying for SOD activity in the presence of sodium cyanide, which selectively inhibits copper/zinc-SOD (SOD1) but not SOD2 [26]. SOD1 activity was calculated by subtracting SOD2 activity from total SOD activity. The