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Uterine endometrial vascularization during ovarian follicular growth in llamas: The effect of estradiol plasma concentration

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ABSTRACT

The aim of this study was to evaluated changes in endometrial vascularization area (EVA) between the left and right uterine horn: **a)** during the ovarian follicular growth in intact llamas, and **b)** after exogenous estradiol administration of estradiol benzoate in ovariectomized (OVX) llamas. In **experiment 1** follicle wave emergence was synchronized (n = 5 llamas) by follicle ablation (Day 0). Females were examined every other day from Day 1 to Day 27, using B mode ultrasonography to evaluate dominant follicle growth profile. Also, EVA was evaluated in each horn using Color-Power Doppler. Blood samples were taken every other day from Day 1 to Day 27 to measure estradiol (E2) plasma concentration by RIA. In **experiment 2** OVX llamas (n = 4 llamas/group) were given a single im administration of: **a)** 1 mg of estradiol benzoate (EB) or **b)** 1 mL of saline. Females were subjected to ultrasound examinations every 48 h from Day −4 until treatment (Day 0), every 12 h from Day 0 to Day 4, and again every 48 h from Day 5 to Day 11. Evaluation of EVA in both uterine horns was performed as described for experiment 1. Blood sampling for the measurement of E2 was carried out at the same time points indicated for the ultrasound examinations. Serial data were analyzed by one way ANOVA for repeated measures using the MIXED Procedure in SAS. Also, Pearson's correlation was used to determine the relationship between variables. In intact llamas there was an effect of day on the dominant follicle growing profile ($P < 0.01$) and estradiol plasma concentration ($P < 0.05$). Dominant follicle diameter positively correlated ($r = 0.4$; $P < 0.017$) with estradiol plasma concentration. Also, EVA of right and left uterine horn did not differ ($P = 0.89$) during the evaluation period; however, it was affected by time ($P < 0.05$). In ovariectomized llamas estradiol concentration was significantly ($P < 0.001$) affected by treatment, time and their interaction. Accordingly, treatment with EB ($P < 0.0001$), time ($P < 0.05$) and their interaction ($P < 0.01$) affected EVA of both uterine horns; however, this variable did not differ between horns ($P = 0.98$). In conclusion, circulating concentrations of estradiol determined an increase in uterine vascularization, during the phase of follicular growth in intact llamas and after the exogenous administration of EB to ovariectomized females; however, no differential effect in endometrial vascularization area between right and left uterine horn was observed.

1. Introduction

The establishment of pregnancies almost exclusively in the left uterine horn, regardless of laterality of ovulation, is one of the most intriguing reproductive features in llamas and alpacas. Early studies [1–3] have documented that more than 95% of gestations are carried out in the left horn and approximately 50% of those pregnancies are supported by the corpora lutea located on the right ovary. This evidence suggests that embryos originated from the right-ovary ovulations must migrate to the contralateral uterine horn in order to establish a viable implantation process. The causes for this particular pattern of embryo implantation have not yet been elucidated.

Moreover, post-mortem anatomical studies of uterine characteristics in llamas and alpacas [4,5] and in situ ultrasonographic

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examinations [6] have determined that the left uterine horn is slightly larger than its right counterpart; a difference not caused by gestation, since it is also observed in nulliparous females and fetuses [4,5]. Del Campo et al. [4] suggested that this distinct anatomical asymmetry is the result of differences on the arteriovenous arrangement that irrigates and drainages both uterine horns. The presence of a prominent cross-over arterial branch extending from the right uterine artery to the left uterine horn indicates that this uterine side is supplied with a potential greater blood flow, and this may well reflect and evolutionary provision for the greater size and function of the left uterine horn in these species [4]. These particular anatomical features provide a rationale for the hypothesis that a differential basal blood flow between uterine horns in llamas may determine an asymmetrical endometrial irrigation between the left and right horn.

Interestingly, besides the size and vascular asymmetry, no other differences have been described at the ultrasonographic [6], histological [5,7] and/or sub-cellular level [8,9] between uterine horns, which could explain the particular pattern of embryo implantation and consequently the high incidence of left horn gestations observed in these species. Endometrial glands development was similar between both uterine horns in llama fetuses at an early age (from 34.5 cm crown-rump length onward) or during adulthood [5]. Additionally, the endometrial expression of both estrogen receptors (ERα and ERβ) was not different between horns, in both pregnant and non-pregnant female llamas [8]. Accordingly, Bianchi et al. [9] did not observe any differences in the expression of ERα, progesterone receptor (PR) and COX-2 between both horns, only describing a higher expression of oxytocin receptor in the right uterine horn.

On the other hand, estrogen has a well-recognized and potent vasodilatory effect [10,11]. Several studies [12,15] have shown a positive correlation between blood flow in the uterine and ovarian arteries and the plasma estrogen concentration during estrus. Interestingly, regular variations of uterine blood flow (UBF) have been described in species with symmetric uterus (cows, sows, ewes [12,14] and mares [13,15]) during the estrous cycle, which are mainly produced by the cyclic changes in concentration of sex steroid hormones on systemic blood. Thus, a rapid increase in UBF occurs at or just prior to estrous followed by a gradual reduction during the luteal phase, a variation that is associated to the daily estrogen:progesterone ratio in blood plasma [16].

It is unknown whether or not changes in systemic estradiol concentration during the follicular phase affect basal uterine blood flow and endometrial vascular perfusion between right and left uterine horn in llamas. We propose to evaluate changes in endometrial vascularization during the follicular growth and to use an ovariectomized llama model to determine the effects systemic estradiol on endometrial vascularization in this species.

2. Materials and methods

The present study was conducted during March–May 2, 0015 at the Universidad Católica de Temuco, Temuco, Chile (38° 45′S - 72° 40′W and 122 m above sea level). All procedures were reviewed and approved by the University Bioethics Committee and were performed in accordance with the animal care protocols established by the same institution.

2.1. Animals

Non-pregnant, non-lactating llamas ($n = 13$; age: 5–8 y; weight: 127.5 ± 14.1 Kg; Body Condition Score: 3.5 out of 5; parity: 4 ± 2) were maintained on pasture supplemented with hay and water *ad libitum*. Llamas were housed indoors at night and offered 250 g/animal of a commercial diet supplement containing 140 g/kg crude protein and 150 g/kg crude fibre (Vaca14, Cisternas Nutrición Animal, Paine, Chile). A subset of llamas (n = 8) was bilaterally ovariectomized by ventromedial laparotomy 10 months before the beginning of the study.

2.2. Experimental design

2.2.1. Experiment 1: effect of follicular growth on endometrial vascularization in llamas

Adult, non-pregnant and non-lactating llamas (n = 5) were submitted to ultrasound guided follicle ablation of every follicle ≥4 mm in diameter using a 50 cm 18G needle fitted in a 5.0 MHz transvaginal sectorial-array probe coupled to an ultrasound monitor (Aloka SSD-500), to induce the synchronous emergence of a new follicular wave (Day 0 = day of follicle ablation) as previously described [17]. Afterwards, females were examined once daily by B-mode and Power-Doppler transrectal ultrasonography to evaluate follicular wave emergence, the diameter of dominant follicle and endometrial vascularization area respectively for a period of 27 days.

The evaluation of the endometrial vascular perfusion area, using Power-Doppler ultrasonography, was conducted every other day, from Day 1 (Day 0 = Day of follicle ablation) until Day 27, between 08:00 and 12:00, using a 5.0 MHz lineal array transducer coupled to an ultrasound monitor (Sonosite M-Turbo, USA) as described previously for other species [18–20]. In brief, the transducer was place over a cross section of the middle segment of each uterine horn where a 10 s video-clip was registered. Vascular perfusion of the endometrium was objectively assessed by off-line measurements of the number of colored pixels as an indicator of blood flow area. Three still images of each horn were used for the determination of the number of colored pixels, and the average value was used for the statistical analyses. Power-Doppler images were recorded, edited, and analyzed using the ImageJ software (NIH open access, USA).

Additionally, blood samples (5 mL) were taken, every other day during the entire 27 day evaluation period. Blood was collected into heparinized tubes (Vacutainer Systems, Becton Dickinson, USA) by jugular venipuncture, centrifuged at 1800 rpm for 10 min and plasma was stored at −20 °C. Plasma estradiol concentration was determined using a commercial solid-phase radioimmunoassay kit (E2-RIA-CT kit, DIASource ImmnoAssays S.A., Belgium) as previously reported [21].

2.2.2. Experiment 2: effect of estradiol on endometrial vascularization in an ovariectomized llama model

Ovariectomized llamas (n = 8) were randomly given an intramuscular administration of: **a)** 1 mg of estradiol benzoate (Laboratorios Syntex®, Buenos Aires, Argentina, n = 4) or **b)** 1 mL of saline solution (negative control group, n = 4). All females were subjected to daily ultrasound examinations for 16 consecutive days, beginning 4 days before treatment administration (Day of treatment = Day 0) and until Day 11. Ultrasonographic evaluations were performed every 48 h from Day −4 until treatment administration (Day 0), every 12 h from Day 0 to Day 4, and again every 48 h from Day 5 to Day 11. Evaluation of endometrial vascularization area in both uterine horns was performed as described for experiment 1.

Additionally, as described for experiment 21 blood samples (5 mL) were collected for the measurement of estradiol plasma concentration from all females every 48 h from Day −4 until treatment administration (Day 0), every 12 h from Day 0 to Day 4, and again every 24 h from Day 5 to Day 11. Blood processing and hormonal analyses were carried out as described for experiment 1.