Theriogenology 125 (2019) 18–23

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Evaluation of the effect of mating, intrauterine deposition of raw seminal plasma or seminal plasma purified β-NGF on endometrial vascularization in llamas

Felipe Urra [a], Marcelo H. Ratto [b], Mauricio Silva [c, d, *]

[a] School of Graduate Studies, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile
[b] Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile
[c] School of Veterinary Medicine, Universidad Catolica de Temuco, Temuco, Chile
[d] Núcleo de Investigación en Producción Alimentaria, Universidad Católica de Temuco, Temuco, Chile

ARTICLE INFO

Article history:
Received 14 May 2018
Received in revised form
6 October 2018
Accepted 6 October 2018
Available online 9 October 2018

Keywords:
Llamas
Endometrial vascularization
Semen
Doppler ultrasonography

ABSTRACT

The aim of this study was to evaluate the endometrial vascularization area (EVA) of both uterine horns in llamas subjected to different intrauterine treatments resembling physiological conditions after a single mating. Llamas with a growing follicle (≥8 mm) were randomly assigned to: a) single mating with a fertile male (mating; positive control; $n = 6$); b) intramuscular administration of 50 μg of gonadorelin acetate plus an intrauterine infusion of 4 ml of PBS (GnRH; negative control; $n = 4$); c) intrauterine infusion of 4 ml of raw llama seminal plasma (SP; $n = 4$) or d) intrauterine infusion of 10 mg of β-NGF purified from llama semen diluted in 4 ml of PBS (spβ-NGF; $n = 6$). Females in GnRH, SP and NGF group received 50% of treatment volume into each horn by guiding an insemination pipet through the cervix. Ovaries were examined by ultrasonography every 12 h until Day2 (Day 0 = Day of treatment) to determine ovulation. Power-Doppler ultrasonography evaluation of EVA in a cross-section of the middle segment of each horn was conducted at 1 h before and 1, 3, 6, 12 and 24 h (intensive evaluation) and 2, 4, 6 and 8 days (long-term evaluation) after treatment administration. Serial EVA data was analyzed as a 2-by-2 factorial design for repeated measures using the MIXED procedure. The analysis included main effects of treatment (mating, SP, spβ-NGF or GnRH), uterine horn (left vs right), time, and their interactions. According to the 2 by 2 analysis there was no effect of uterine horn on EVA during the first 24 h and from Day 2 to Day 8 after treatment; therefore, data were grouped based on treatment type regardless of uterine horn for both periods of observation. Thus, EVA was affected by time ($P < 0.04$) and treatment by time interaction ($P < 0.02$) and tended ($P = 0.07$) to be influenced by type of treatment during the intensive evaluation period. Females on mating and spβ-NGF group showed a significant increase in EVA at 3 and 12 h after treatment compared to GnRH and SP groups. However, no effect of treatment, time or their interaction was observed during the long-term evaluation period. In spite of the limited number of animals used in this study, our results allow us to concluded that natural mating and intrauterine deposition of 10 mg of spβ-NGF induce a symmetrical increase in endometrial vascularization of both uterine horns during the first 24 h post treatment administration in llamas; however, this effect did not persist beyond that period.

1. Introduction

Seminal plasma is increasingly recognized as contributing to the reproductive process in roles different than just providing transport and nutritive support to sperm [1,2], such as eliciting molecular and cellular changes in female reproductive tissues after insemination. Indeed, seminal plasma contains several signaling molecules that are able to influence the female reproductive physiology improving the chances of conception and pregnancy success. In this regard, the most compelling information comes from rodents and shows that seminal fluid can influence sperm survival and competence, development of the pre-implantation embryo and the receptivity of

* Corresponding author. School of Veterinary Medicine, Universidad Catolica de Temuco, Temuco, Chile.
E-mail address: masilva@uct.cl (M. Silva).

https://doi.org/10.1016/j.theriogenology.2018.10.007
0093-691X/

the endometrium [2].

Even though the exposure of the female genital tract to seminal components is not mandatory for the initiation and maintenance of a successful pregnancy, evidence in livestock species [3,4] supports the notion that the success and quality of pregnancy could be partially compromised if the reproductive tract was not previously expose to seminal fluid. In this regard, semen or seminal plasma deposition in the female genital tract facilitates implantation and pregnancy in various domestic species [4,5] and experimental mammal models [6,7]. Interestingly, exposing the uterus of llamas to homologous seminal plasma before artificial insemination and embryo transfer seems to enhance subsequent fertility of females [8].

Successful implantation in mammalian species requires a receptive endometrium, a functional embryo at the blastocyst stage and a synchronous dialogue between maternal and embryonic tissues [9]. Accordingly, it is recognized that an adequate endometrial blood supply is essential for a successful embryo implantation [10,11]. On the other, new evidence generated in different animal species [12–14] has demonstrated that the exposure of the endometrium to seminal plasma components can generate changes in vascular perfusion, some of which occur very rapidly through inflammation related processes, while others develop after several days due to angio-architecture modifications [5].

In the mare and sow the intrauterine deposition of semen or seminal plasma influences uterine/endometrial vascularization. In the mare intrauterine infusion of raw semen or seminal plasma induces changes in uterine perfusion which are related to an endometrial inflammatory response and to the presence of vasodilatory components in these fluids [12]. Similarly, gilts mated with a vasectomized male developed an acute transient inflammatory response in the endometrium resulting in marked changes in the presence and distribution of leukocytes and extensive proliferation of the endometrial glands [13]. Even after artificial insemination (AI) in heifers, where small volumes of diluted semen are involved, there was an increase in uterine vascularization 4 h after the AI procedure as a result of an inflammatory response [14]. Surprisingly, along with early inflammatory vascular changes, the induction of vascular bed development has been reported as a longer-term modification of the uterus after intrauterine deposition of seminal plasma in pigs [5]. These changes in angio-architecture have been described to occur shortly before the time of maternal recognition of pregnancy in sows, highlighting the importance of seminal components in evoking long-lived effects that could influence early embryo-maternal interactions [5].

South American camelids have several unique reproductive features, among which the high rate (>90%) of gestations established in the left uterine horn is one of the most striking [15]. It has been proposed that this unique pattern of early embryo migration and implantation is influenced by an anatomical and vascular asymmetry favoring the size of the left horn and the arteries that irrigate it [16–18]. On the other hand, llama and alpaca seminal plasma presents a great concentration of β-Nerve Growth Factor (β-NGF) accounting for 30–50% of total protein content of an ejaculate [25,44]. Interestingly, besides its ovulation-inducing properties in llamas [19–21], it has been demonstrated that NGF is a potent angiogenic factor increasing de expression of Vascular Endothelial Growth Factor (VEGF) and promoting the proliferation of vascular cells in female reproductive organs [22,23]. The above evidence supports the hypothesis that molecules present in the seminal plasma of llamas (e.g.β-NGF) may affect uterine vascularization differentially between left and right horn due to the particular vascular asymmetry observed in the uterus of this species.

Therefore, the aim of the present study was to evaluate the effects of natural mating, intrauterine infusion of raw seminal plasma or β-NGF purified from llama seminal plasma (spβ-NGF) on endometrial vascularization of both uterine horns in llamas.

2. Material and methods

The present study was conducted during August–November 2015 at the Universidad Austral, Valdivia, Chile (39° 48′ S - 73° 14′ W and 14 m above sea level). All procedures were reviewed and approved by the Universidad Austral de Chile 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 ($n = 20$; aged 5–8 y; weight: 147.5 ± 14.1 Kg; Body Condition Score: 3 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).

2.2. Seminal plasma collection and protein purification

Semen from 5 adult male llamas was collected twice per week for three months prior to the start of the study. Semen was collected with the use of an artificial vagina designed for sheep that was fitted into a phantom mount built of wood and covered with a llama hide [24]. An average of 20 ejaculates were collected from each male.

Llama semen processing and purification of β-NGF has been described elsewhere [20,25]. In brief, semen was diluted with phosphate buffered saline (PBS, Gibco, Grand Island, NY, USA) and centrifuged for 30 min at 1.500 × g at room temperature. The supernatant was decanted to remove sperm and sperm-free seminal plasma was stored at −80 °C. Upon thawing, the diluted seminal plasma was pooled, sonicated to reduce viscosity and centrifuged at 10,000 × g for 20 min to remove particulate matter.

Purification of spβ-NGF was performed in a 2-step procedure as previously described [20,25]. Llama seminal plasma was loaded into a Type 1 macro-prep ceramic hydroxylapatite column (1 cm × 10 cm, 20 μm, BIO-RAD laboratories, Hercules, CA, USA) previously equilibrated with 10 mM sodium phosphate at a pH of 6.8. Elution was carried out at room temperature using a lineal gradient with 350 mM sodium phosphate, pH 6.8, and a flow rate of 0.5 ml/min. An eluted fraction showing a major protein on SDS-PAGE, was concentrated in phosphate buffered saline (PBS, pH 7.4) using a 5 kDA cut-off membrane filter device (Vivaspin, Sartorius, Goettingen, Germany) and subsequently loaded onto a gel filtration column (SEC, Hi Prep™ 26/60 Sepahacryl™ S-100, Amersham Laboratories, Piscataway, NJ, USA). The purification procedure was carried out at room temperature at a flow rate of 0.5 mL/min using fast protein liquid chromatography (FPLC, Amersham Laboratories, Piscataway, NJ, USA). Elution was performed isocratically using PBS at pH 7.4. The bioactive fraction after gel filtration was identified and defined as purified llama spβ-NGF.

2.3. Experimental design

Llamas were examined once daily by transrectal ultrasonography using a mode B scanner with a 7.5 MHz linear-array probe (Aloka SSD 500, Aloka Co., Tokyo, Japan). Females with a follicle ≥8 mm in diameter that had grown for three consecutive days were randomly assigned to one of the following groups to receive: a) a single mating with a fertile male (mating; positive control; $n = 6$),

b) an intramuscular administration of 50 μg of GnRH analogue gonadorelin acetate plus and intrauterine infusion of 4 ml of PBS (Phosphate Buffer Saline) (GnRH; negative control; $n = 4$); c) intrauterine infusion of 4 ml of raw llama seminal plasma (SP; $n = 4$); or d) intrauterine infusion of 10 mg of spβ-NGF contained in 4 ml of PBS (spβ-NGF; $n = 6$). Intrauterine infusions were performed by guiding an insemination pipet through the cervix via transrectal palpation, and infusing 2 ml into each uterine horn. Selected doses of seminal plasma and spβ-NGF for intrauterine infusion resemble physiological conditions after a single natural mating [25,26]. Indeed, the intrauterine infusion of 10 mg of spβ-NGF per female corresponds to the minimal dose respect of the total amount present in an average llama ejaculate. In previous studies [26–28] the average volume of collected llama ejaculates ranged from 2 to 4.5 ml, with a total protein concentration ranging from 15 to 16 mg/ml, out of which 30–50% corresponded to spβ-NGF [26].

2.4. Ultrasonographic examinations

Uterine horns were examined by Colour/Power Doppler ultrasonography (Esaote, MyLab 5, Netherlands) using a 7.5 MHz linear-array transrectal transducer. Fixed pre-installed Doppler system settings (PRF: 1.4 KHz; Frequency: 7.5 MHz; Doppler Gain: 70%; Depth: 8 cm) were used throughout of the entire experiment to avoid variations in recording. Examinations were performed at 0 (pre-treatment), 1, 3, 6, 12 and 24 h (intensive evaluation period) and 2, 4, 6, 8 days (long-term evaluation period) after treatment administration to determine endometrial vascularization area (EVA). In brief, the transducer was placed over the middle segment of each uterine horn to obtain the images as described by several authors in mares and heifers [14,29–31]. Endometrial vascularization area (EVA) was objectively assessed by off-line measurements of the number of colored pixels as an indicator of blood flow area. Three still images from cross-sections of each uterine horn were used for the determination of the number of colored pixels and the average was used in the analyses. Power-Doppler images were recorded, edited and analyzed using the ImageJ software (NIH open access, USA) through a blinded process. Additionally, ovaries were examined, by B-mode ultrasonography, every 12 h until Day 2 (Day of treatment = Day 0) to determine ovulation. Ovulation was defined as the sudden disappearance of a large follicle ≥8 mm detected on previous examination and it was confirmed by subsequent formation of a functional CL.

2.5. Blood collection and hormone assays

Blood samples (5 mL) were taken by jugular venipuncture on Day 0 and 8 (Day of treatment = Day 0) to confirm ovulation and CL function. Blood was collected into heparinized tubes (Vacutainer Systems, Becton Dickinson, USA), centrifuged at 1800 rpm for 10 min and plasma was stored at −20 °C. Plasma progesterone concentration was determined using a commercial, solid-phase radioimmunoassay kit (Coat-a-Count total progesterone, DPC, CA, USA) as indicated previously [19,21,32]. The intra-assay coefficients of variation were 3.7, 2.9 and 3.5% for low, medium and high-reference plasma (means: 1.8, 3.5 and 16.5 ng/mL). The sensitivity of the assay was 0.1 ng/mL.

2.6. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System software package (SAS Learning Edition, version 4.1, SAS Institute Inc., Cary, NC, USA, 2006). Ovulation rates were compared among groups by Chi-square including all females. Then, only in ovulated females single point data (i.e. follicle diameter at the time of treatment, interval from treatment to ovulation; CL diameter at Day 8 and plasma progesterone concentration on Day 0 and 8) were compared among groups using one-way analysis of variance (ANOVA). Serial data (i.e. endometrial vascularization area for the left and right uterine horn), for intensive and long-term evaluation period, was analyzed as a 2-by-2 factorial design for repeated measures using the MIXED procedure. The analysis included main effects of treatment (mating, SP, spβ-NGF or GnRH), uterine horn (left vs right), time, and their interactions. If significant ($P \leq 0.05$) main effects or interactions were detected, Tukey's post-hoc test for multiple comparisons was used to locate differences. Unless otherwise stated, all values are expressed as mean ± SEM, and significance was declared at $P \leq 0.05$.

3. Results

The pre-ovulatory follicle diameter at the time of treatment, ovulation rate, interval from treatment to ovulation, CL diameter at Day 8 and plasma progesterone concentration at Day 0 and Day 8 did not differ among groups (Table 1). Based on the 2 by 2 analysis there was no effect of uterine horn on EVA neither during the first 24 h nor from Day 2 to Day 8 after treatment administration. Therefore, data were grouped based on the effect of treatment regardless of uterine horn for both periods of observation. Thus, endometrial vascularization area was affected by time and treatment by time interaction ($P < 0.05$) and tended ($P = 0.07$) to be influenced by type of treatment during the first 24 h after treatment administration (Fig. 1). The first significant ($P < 0.001$) increase and maximum level ($P < 0.001$) of EVA was observed at 3 and 12 h respectively, after mating or intrauterine deposition of spβ-NGF; however, no changes were observed on females of PS or GnRH group (Fig. 1). In females from mating group endometrial vascularization area displayed its first significant decrease by 6 h after copulation (Fig. 1). On the contrary, no significant reduction was observed in spβ-NGF treated llamas (Fig. 1).

Table 1
Mean (±SEM) follicle diameter at treatment, ovulatory response, CL size, and plasma progesterone concentration on Day 0 and 8 in llamas mated with a fertile male, treated with an i.m. GnRH dose, with an intrauterine infusion of 4 mL of homologous raw seminal plasma or 10 mg of β-NGF (Day 0 = Day of treatment).

	Groups			
	Mating (n = 6)	β-NGF (n = 6)	S. Plasma (n = 4)	GnRH (n = 4)
Follicle diameter (mm)[a]	12.0 ± 1.0	10.7 ± 0.7	10.2 ± 0.8	9.9 ± 1.5
Ovulation rate (%)[b]	83 (5/6)	83 (5/6)	100 (4/4)	100 (4/4)
Interval to ovulation (h)[a]	29.5 ± 3.7	29.7 ± 3.7	30.2 ± 2.8	30.1 ± 2.4
CL diameter Day8 (mm)[a]	13.2 ± 0.8	13.6 ± 0.7	14.5 ± 0.4	12.8 ± 1.0
Progesterone Day 0 (ng/ml)[a]	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
Progesterone Day8 (ng/ml)[a]	4.1 ± 1.2	3.9 ± 0.6	3.2 ± 0.8	3.1 ± 1.0

[a] Only ovulated females were included in this analysis.
[b] All females were included in this analysis.

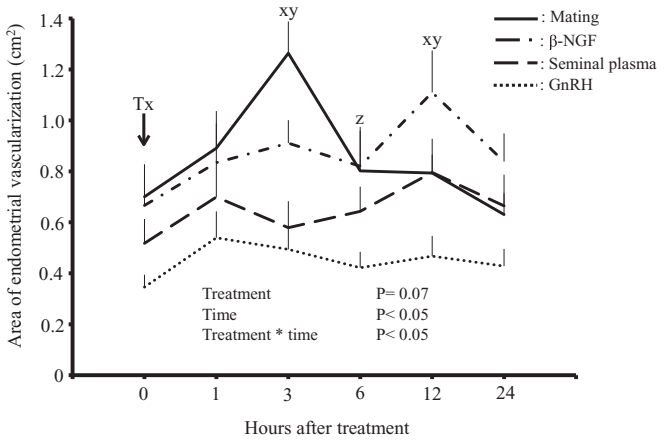

Fig. 1. Endometrial vascularization area (mean ± SEM) in llamas mated with a fertile male (n = 5), treated with an i.m. GnRH dose (n = 4), with an intrauterine infusion of 4 mL of homologous raw seminal plasma (n = 4) or 10 mg of β-NGF (n = 5) before (0 h pre-treatment) and 1, 3, 6, 12 and 24 h after treatment. [x]: Within group, the first significant increase from pretreatment concentration (P < 0.001). [y]: Within group, the maximum concentration (P < 0.001). [z]: Within group, the first significant decrease from maximum concentration (P < 0.001). Tx: Treatment administration.

On the other hand, no effect of treatment, time or their interaction was observed on endometrial vascularization area during the long-term period of observation (from Day 2 to Day 8 after treatment; Fig. 2).

4. Discussion

The results of this study demonstrate that mating and intrauterine infusion de spβ-NGF induce a symmetrical increase of endometrial vascularization of both uterine horns in llamas during the first 24 h post-treatment. Mating induced the greatest and most rapid increase of endometrial vascularization compared to the other intrauterine treatments. On the other hand, no effect of mating or any intrauterine treatment (seminal plasma or spβ-NGF) was observed on endometrial vascularization during the long-term period of observation (from Day 2 to Day 8) after treatment administration.

In llamas and alpacas, the establishment of gestation take place almost exclusively in the left uterine horn, regardless of laterality of ovulation. Indeed, early studies reviewed by Vaughan and Tibary [15] determined that above 95% of gestations are carried out in the left uterine horn, with approximately 50% of those pregnancies being supported by corpora lutea located in the right ovary. Indeed, llamas and alpaca embryos must be located into the left uterine horn before Day 10 in order to avoid luteolysis [33]. The reasons for these particular patterns of embryo migration, early embryo development and implantation have not yet been completely elucidated, but new scientific evidence generated in humans highlights the role that endometrial vascularization has on the success of embryo implantation and survival [10,34,35], suggesting that differences in endometrial receptivity between uterine horns may play a role in the development of these reproductive phenomena in llamas.

Nowadays, it is well recognized that seminal fluid besides being a transport media for spermatozoa can also act directly on the female reproductive tract after insemination affecting several reproductive events including tissue remodeling associated with endometrial receptivity [2,36,37]. The exposure of the female genital tract to seminal components is not mandatory for the initiation and maintenance of a successful pregnancy [3,4], but there is vast evidence that it improves pregnancy rate and the quality of gestation [4–7]. Interestingly, exposing the llama uterus to homologous seminal plasma before artificial insemination or embryo transfer seems to enhance female fertility [8].

A successful implantation requires a receptive endometrium in mammals [9]. Accordingly, it is recognized that an adequate endometrial blood supply improves endometrium receptivity and it is and essential requirement for a successful embryo implantation [10,11].

Because of the high concentration of β-NGF, a potent angeogenic factor [22,23,45,46] in llama seminal plasma [25,44], and the uterine anatomical/vascular asymmetry previously described in this species [16–18], we hypothesized that intrauterine infusion of llama seminal plasma components would induce changes in endometrial blood perfusion, observed as an asymmetrical increase in endometrial vascularization area, favoring the left uterine horn

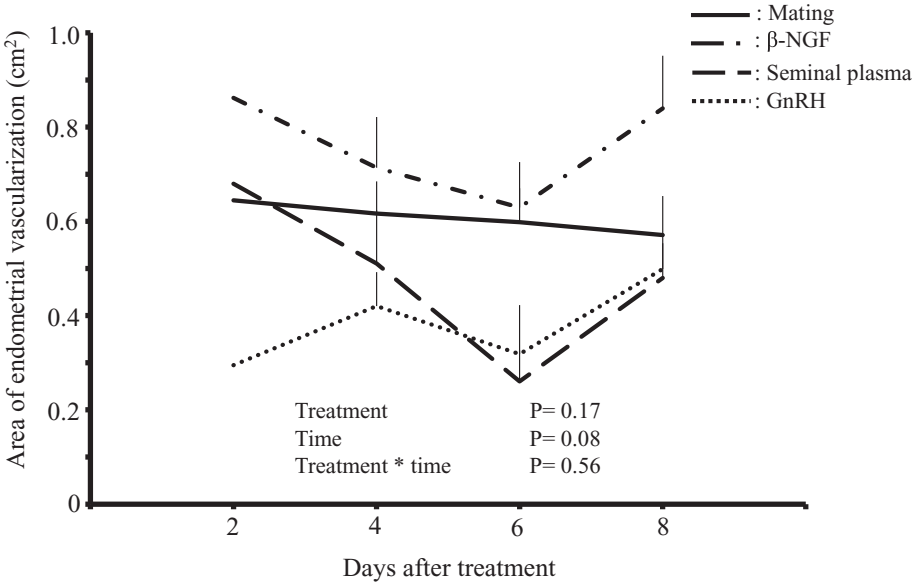

Fig. 2. Endometrial vascularization area (mean ± SEM) in llamas mated with a fertile male (n = 5), treated with an i.m. GnRH dose (n = 4), with an intrauterine infusion of 4 mL of homologous raw seminal plasma (n = 4) or 10 mg of β-NGF (n = 5) before (Day 0) and 2, 4, 6 and 8 days after treatment administration (Day 0 = Day of treatment).

in llamas.

In the present study, natural mating induced a rapid and transient increase of endometrial vascularization 3 h after copulation. In llamas and alpacas copulation length vary from 3 to 65 min and ejaculation appears to occur throughout the entire mating [38,39]. While copulating the male penetrates the cervix with his penis depositing semen deep into both uterine horns [40,41], during multiple ejaculations using mild thrusting pelvic movements [15]; therefore, an acute transient inflammation of the endometrium is generated as a consequence of repeated abrasion of the penis [40,41]. Acute inflammation is characterized by hyperemia of the affected organs; indeed, after and mild and transient vasoconstriction an active marked dilation of arterioles, capillaries and venules occurs generating a rapid increase in the blood flow of the affected tissues [42]. Thus, the particular characteristics of copulation in llamas could explain the greatest increase in endometrial vascularization observed rapidly after mating in both uterine horns. Accordingly, in mares [12] and gilts [13] a strong relationship between uterine perfusion and a transient inflammation has been established after copulation/artificial insemination or mating with a vasectomized male respectively. Although during ejaculation, contrary to llamas, the males of these last two species deposit their semen intracervically [43] without any direct mechanical effect on the endometrium.

In contrast to observations made in mares [12] accumulation of intrauterine fluid was not observed after natural mating or any of the intrauterine treatments in female llamas. A rapid and significant increase in the amount of intrauterine fluid was observed in mares 1 h after infusion of raw semen, seminal plasma and even semen extender based on dried skim milk [12]. Compared to this previous study, our results may suggest that llama uterus is less prone to inflammation, since treated females did not develop such an acute inflammation process even through mating implies an aggressive mechanical abrasion of the endometrial surface by the penis [41].

On the other hand, intrauterine treatment with 10 mg of spβ-NGF but not with 4 mL of raw seminal plasma, increased endometrial vascularization area 12 h after treatment administration. The increase on vascular irrigation has been described as one of the earliest changes observed in the uterus after intrauterine deposition of seminal components [12]. Although in the mare the inflammatory process is considerable lower after intrauterine deposition of seminal plasma compared to raw semen, an increase of uterine blood flow is still detectable, suggesting that the seminal fluid contains vasodilatory properties that influence endometrial vascularization [12]. Interestingly, β-NGF is a highly concentrated protein of llama semen [25,44], that promotes angiogenesis and/or induces de expression of angiogenic molecules in several tissues, such as cornea [45], muscle [46] and ovary [21] among others. A recent llama study using Colour-Power Doppler ultrasonography demonstrated a significant increase in pre-ovulatory follicle and CL vascular irrigation after intramuscular administration of purified llama spβ-NGF [47]. The effect of repeated intramuscular administrations of llama spβ-NGF to female llamas, during the peri-ovulatory period, on follicle irrigation reinforce the previous observations [48]. Our results demonstrate that this seminal molecule has also a potent effect stimulating endometrial vascularization locally.

Intriguingly, females treated with seminal plasma did not developed a significant increase in endometrial vascularization after intrauterine treatment administration, in contrast to the NGF treated group. Contrary to spβ-NGF administration (10 mg per female) the exact amount of β-NGF present in the individual seminal plasma treatment (4 ml) given to females in that group was not measured, and since a pool of semen from different males was use, its concentration could have been lower than expected. In this regard, high individual variations in the amount of total protein in seminal plasma was reported by our research group in this same herd of males [28]. On the other hand, llama semen is a very viscous fluid [49], and its viscosity increases with handling, storing and processing. How this change in seminal plasma fluidity alters the diffusion of molecules present in this fluid is unknown, but it has been suggested that viscous semen degrades slowly once deposited in the female reproductive tract [49], so this could be also a factor that altered/retarded the exposure of endometrium to seminal components under the present experimental design.

No effect of treatment was observed after mating or any of the intrauterine treatments on EVA, during the long-term period of observation after treatment (from Day 2 to Day 8). However, as shown in Fig. 2, endometrial vascularization tended to decrease by day 6 after treatment administration. We believe that the long-term effect of these treatments could be blurred by the increasing concentration of progesterone produced by the induced corpus luteum during this period. Regular variations of uterine blood flow in cows, sows, ewes [50,51] and mares [52,53] during the estrous cycle have been associated to cyclic changes on systemic steroids hormones concentration. In contrast to estradiol, progesterone is thought to have a negative effect on uterine perfusion [54,55]. Although no effect of treatments on endometrial vascularization was observed in the present study during the 8 days evaluation period post-treatment administration, it has been described, that the expression of angiogenesis markers such as von Willendbrand Factor (VWF) and vascular endothelial growth factor isoforms (VGEF164 and VEGF120) are significantly modified after 10 days of endometrial exposure to seminal plasma in swine [5]. Interestingly, these angio-architecture changes occur around the time of maternal recognition of pregnancy in sows, highlighting the importance of seminal components in evoking long-lived effects, which could influence early embryo-maternal interactions [5].

In spite of the limited number of animals used in this study, our results allow us to concluded that natural mating and intrauterine deposition of 10 mg of spβ-NGF induce a symmetrical increase in endometrial vascularization of both uterine horns during the first 24 h post treatment administration in llamas; however, this effect did not persist beyond that period.

Acknowledgements

Research supported by the Chilean National Science and Technology Research Council, Grant Fondecyt de Iniciación 11140396 assigned to Mauricio Silva.

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