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Nerve growth factor from seminal plasma origin (spβ-NGF) increases CL vascularization and level of mRNA expression of steroidogenic enzymes during the early stage of Corpus Luteum development in llamas

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ABSTRACT

The objectives of the study were to determine the effect of seminal plasma β-NGF on Corpus Luteum morphology and function and level of mRNA expression of steroidogenic enzymes. Llamas were assigned (n = 12/per group) to receive an intramuscular dose of: (a) 1 ml phosphate buffered saline (PBS), (b) 5 µg gonadorelin acetate (GnRH), or (c) 1.0 mg of purified llama spβ-NGF. Ovaries were examined by transrectal B-mode ultrasonography from treatment to ovulation (Day 0 = treatment). B mode/Power Doppler ultrasonography and blood samples collection were performed at Days 4, 8 and 10 (n = 3 llamas per treatment group/per time point) to determine CL diameter, vascularization and plasma progesterone concentration respectively. Plasma progesterone concentration was analyzed in all llamas at Day 0. Then females were submitted to ovariectomy at Days 4, 8 and 10 (n = 3 llamas/treatment/time), CL was removed to determine vascular area, proportion of luteal cells and *CYP11A1*/P450scc and STAR expression by RT-PCR. Ovulation was similar between llamas treated with GnRH or spβ-NGF and CL diameter did not differ between GnRH or spβ-NGF groups by Day 4, 8 or 10. Vascularization area of the CL was higher ($P < 0.01$) in llamas from the spβ-NGF than GnRH-treated group by Day 4 and 8. Plasma progesterone concentration was higher ($P < 0.05$) in llamas from the spβ-NGF compared to females of GnRH group by Day 4 and 8. The proportion of small and large luteal cells did not differ between GnRH or spβ-NGF groups by Day 8. *CYP11A1*/P450scc was upregulated 3 folds at day 4 and 10 by spβ-NGF compared to GnRH. STAR transcription was 3 folds higher at day 4 in females treated with spβ-NGF. In conclusion, the luteotrophic effect of spβ-NGF could be related to an increase of vascularization and up regulation of *CYP11A1*/P450scc and STAR transcripts enhancing progesterone secretion.

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1. Introduction

The ovulatory process in mammals is regulated by a complex endocrine, cellular and molecular interplay of events ending in the rupture of the ovulatory follicle and release of the oocyte. In induced ovulators species (e.g. rabbits, cats, camelids) the physical stimuli of copulation in the female reproductive tract induce hypothalamic GnRH secretion followed by the preovulatory release of LH and subsequently ovulation [1]. However, recent evidence suggest that mechanical stimulation may not be the only stimuli responsible of inducing ovulation in camelids. Studies conducted in both old and new world camelids have described that the presence of an ovulation inducing factor (OIF) in the seminal plasma has a pivotal role on inducing ovulation in these species [2–5]. The ovulation inducing-factor is a protein that has been isolated and purified from seminal plasma of llama [6] and recently characterized as beta nerve growth factor (β-NGF) in llamas and alpacas respectively [7,8]; referred herein as spβ-NGF for its seminal plasma origin. Although, spβ-NGF induce ovulation by stimulating direct or indirectly the GnRH neurons [9], the mechanism of action remains

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unknown. Moreover, spβ-NGF has been described not only as a potent ovulatory factor but also as a protein with a significant luteotrophic effect [3,6,9–11] as evidenced for a greater CL and higher progesterone secretion in spβ-NGF- than GnRH-treated llamas. The positive relationship between the magnitude of LH secretion and higher progesterone concentration in llamas treated with raw seminal plasma or purified spβ-NGF support the hypothesis that the luteotrophic effect of spβ-NGF may be mediated by LH. In a recent llama study [11] the use of Power Doppler ultrasonography demonstrated that the preovulatory follicle and the early CL had a greater vascularization in llamas treated by intramuscular administration of spβ-NGF compared to GnRH-treated females.

The preovulatory LH surge is the key signal for inducing not only ovulation but also may play an important role during the initial stage of luteinization and CL formation and function. The luteinization process is regulated by the intervention of luteotrophic hormones, growth factors and steroidogenic enzimes [12]. A great change in mitochondrial enzyme activity has been reported to be necessary to achieve the steroid production [13]. The synthesis of progesterone starts with the conversion of cholesterol to pregnenolone by cytochrome P450, family 11, subfamily A, polypeptide 1 (*CYP11A1*/P450scc [14]), followed by the conversion of pregnenolone to progesterone by hydroxy-delta-5-steroid dehydrogenase 3b (HSD3B) [15]. However, the rate-limiting step is the transport of cholesterol into the mitochondria by the steroidogenic acute regulatory protein (STAR) [16].

We hypothesize that the peculiar pattern of LH secretion induced by spβ-NGF produces an up regulation on steroidogenic enzymes resulting in the amplification of progesterone secretion.

The CL is a dynamic gland in which profound cellular differentiation and vascular changes take place during its early and middle stages of development; indeed, the blood flow supply of the mammalian CL is greater than that of any other tissue or organ on a per unit of tissue base [17]. The luteal vascular system supplies hormonal substrates, circulating hormones and nutrients to luteal cells that are indispensable to support progesterone secretion [18]. A close relationship between luteal blood flow and progesterone secretion has been observed in horses [19] and cows [20].

The bovine CL is composed of four distinct types of luteal cells: small and large steroidogenic cells, capillary endothelial cells, and fibroblasts [21]. The large steroidogenic cells represent approximately 40% of the volume of the CL, although they constitute only approximately 10% of the total cell number. The small steroidogenic cells represent approximately 20% of the CL volume and 25% of the cells. In turn, the capillary endothelial cells constitute approximately 10% of the CL volume [21]. It has been documented the ratio of small and large luteal cells can be altered in sheep after LH treatment [22–24]. Previous reports of the luteotrophic effects of spβ-NGF give support to the hypothesis that this molecule could induce changes in the number or ratio of small and large luteal cells.

The objective of the present study was to test the hypothesis that purified spβ-NGF exerts a luteotrophic effect throughout changes in the ratio of luteal cells, CL vascularization and up regulation the expression of steroidogenic enzymes driven progesterone secretion.

2. Materials and methods

Experimental 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. Semen collection and protein purification

Semen was collected at the Animal Reproduction Laboratory, Universidad Austral de Chile, Valdivia (39° 38′S - 73° 5′W and 19 m above sea level) from five mature male llamas, twice per week for 2 months before the start of the experiment. Semen was collected using a sheep artificial vagina adapted for use in llamas that was fitted into a phantom mount built of wood and covered with a llama hide [3]. An average of 15 ejaculates was collected from each male. Each ejaculate was diluted 1:1 (vol/vol) with phosphate buffered saline (PBS, GIBCO, Grand Island, NY, USA) and centrifuged for 30 min at 1500 g at room temperature. The sperm-free seminal plasma was stored at −20 °C. Purification of OIF/NGF was performed in a two-step procedure, as previously described [6,7]. In brief, llama seminal plasma was loaded into a type 1 macro-prep ceramic hydroxylapatite column (1 cm × 10 cm, 40 μm, Bio-Rad Laboratories, Hercules, CA, USA) previously equilibrated with 10 mM sodium phosphate, pH 6.8, and flow rate of 0.5 mL/min. An eluted fraction showing a major protein on SDS-PAGE was concentrated in PBS (pH 7.4) using a 5 kDa cut-off membrane filter device (Vivaspin, Sartorius, Göttingen, Germany) and subsequently loaded onto a gel filtration column (SEC, hi Prep 26/60 Sephacryl 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 using an *in vivo* llama ovulation bioassay (Ratto et al., 2011) and was defined according to previous studies [25,26] as spβ-NGF.

2.2. Treatment groups

Non-pregnant, non-lactating female llamas (n = 36) ≥4 years of age and weighing 90–120 kg, were used during February to April at the Quimsachata Research Station in the Department of Puno, Peru (15°S, 71°W, and 4500 m above sea level). Llamas were maintained in separate pens and had access to natural pasture supplemented with hay and water *ad libitum*. The ovaries of the llamas were examined daily by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka, SSD-500, International Clinics, Santiago, Chile). When a growing follicle ≥8 mm in diameter was detected, llamas were assigned randomly to one of three treatments (n = 12/per group) and given an intramuscular dose of: (a) 50 μg gonadorelin acetate (GnRH, positive control group; Ovalyse, Pfizer Chile SA, Santiago, Chile), (b) 1.0 mg of purified llama spβ-NGF, or (c) 1 ml phosphate buffered saline (PBS, negative control group). Intramuscular injections were given in the semimembranosus or semitendinosus muscle using a 21-gauge 40 mm-long needle (injection sites were monitored after treatment to determine signs of inflammation). The ovaries were examined by transrectal B-mode ultrasonography from treatment to ovulation (Day 0 = Day of treatment, n = 12/per group). Ovulation was defined as the sudden disappearance of a large follicle ≥8 mm that was detected during the previous examination.

2.3. Power Doppler ultrasonography and image analysis

Only Ovulated llamas from GnRH (n = 12) and spβ-NGF (n = 12) groups were examined by Power Doppler mode ultrasonography (7.5 MHz linear array-transducer; Mylab Five, Esaote, Maastricht, Netherlands) at days 4, 8 and 10 respectively after treatment (n = 4 llamas per time point for each treatment). Cineloops (10 s in length) of the CL vascularization were recorded during Power Doppler imaging and downloaded into VLC media player (www.videolan.

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