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Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas

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The hypothesis that ovulation-inducing factor/nerve growth factor (OIF/NGF) isolated from llama seminal plasma exerts a luteotrophic effect was tested by examining changes in circulating concentrations of LH and progesterone, and the vascular perfusion of the ovulatory follicle and developing CL. Female llamas with a growing follicle of 8 mm or greater in diameter were assigned randomly to one of three groups ($n = 10$ llamas per group) and given a single intramuscular dose of PBS (1 mL), GnRH (50 μg), or purified OIF/NGF (1.0 mg). Cineloops of ultrasonographic images of the ovary containing the dominant follicle were recorded in brightness and power Doppler modalities. Llamas were examined every 4 hours from the day of treatment (Day 0) until ovulation, and every other day thereafter to Day 16. Still frames were extracted from cineloops for computer-assisted analysis of the vascular area of the preovulatory follicle from treatment to ovulation and of the growing and regressing phases of subsequent CL development. Blood samples were collected for the measurement of plasma LH and progesterone concentrations. The diameter of the dominant follicle at the time of treatment did not differ among groups ($P = 0.48$). No ovulations were detected in the PBS group but were detected in all llamas given GnRH or OIF/NGF (0/10, 10/10, and 10/10, respectively; $P < 0.0001$). No difference was detected between the GnRH and OIF/NGF groups in the interval from treatment to ovulation (32.0 ± 1.9 and 30.4 ± 5.7 hours, respectively; $P = 0.41$) or in maximum CL diameter (13.1 ± 0.4 and 13.5 ± 0.3 mm, respectively; $P = 0.44$). The preovulatory follicle of llamas treated with OIF/NGF had a greater vascular area at 4 hours after treatment than that of the GnRH group ($P < 0.001$). Similarly, the luteal tissue of llamas treated with purified OIF/NGF had a greater vascular area than that of the GnRH group on Day 6 after treatment ($P < 0.001$). The preovulatory surge in plasma LH concentration began, and peaked 1 to 2 hours later in the OIF/NGF group than in the GnRH group ($P < 0.05$). Plasma progesterone concentration was higher on Day 6 in the OIF/NGF group than in the GnRH group ($P < 0.001$). Results support the hypothesis that OIF/NGF exerts a luteotrophic effect by altering the secretion pattern of LH and enhancing tissue vascularization during the periovulatory period and early stages of CL development.

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1. Introduction

Ovulation has been considered an inflammatory response involving cellular changes in the ovarian surface epithelium, tunica albuginea, and layers of the

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preovulatory follicle associated with changes in the steroid and eicosanoid pathways [1] These changes are associated with the recruitment or synthesis of growth factors, interleukins, cytokines, and matrix metalloproteinases which are involved in the degradation of the extracellular matrix found in collagenous connective tissues present in the follicular wall and ovarian stroma [1–3]. In spontaneous ovulators, ovulations occur at regular intervals because of the stimulatory effect of high circulating concentrations of estradiol on the LH surge when circulating progesterone concentrations are low [4,5]. In contrast, a copulatory stimulus triggers LH release and elicits ovulation in camelids [6–8]. The copulatory stimulus in camelids (induced ovulators) has been attributed to an ovulation-inducing factor (OIF) in the seminal plasma of this species [9]. This protein elicits ovulation, CL formation, and progesterone secretion in a dose-dependent manner through an effect on LH release [9–12].

The preovulatory LH surge is the key signal for inducing not only ovulation but also for initiating the early stages of luteinization and CL formation. Plasma LH concentration triggered by the administration of whole seminal plasma [9] or purified OIF [12,13] remained elevated above basal levels for longer periods than that observed in GnRH-treated females llamas [9]. The secretion pattern of LH induced by OIF was associated with higher plasma progesterone concentration than in GnRH-treated llamas [9,14]. Results of three subsequent studies using OIF isolated and purified from the seminal plasma of llamas confirmed the LH-releasing effects of OIF in seminal plasma, and were consistent with the notion that OIF is luteotrophic [12,13,15]. Recently, OIF has been identified as the neurotrophin, nerve growth factor (β-NGF; [16]), and it appears to be an abundant component of the seminal plasma of camelids [17,18]. Although β-NGF has been characterized by its role in promoting neuron survival and growth [19], it has become increasingly evident that this protein is involved in ovarian function at several levels. For the purposes of this report, OIF/NGF will be used to denote β-NGF of seminal plasma origin.

In addition, the preovulatory surge of LH initiates a cascade of events that includes local changes in blood flow, proteolytic degradation of the follicular wall, oocyte maturation, and luteinization of granulosa and theca cells [20–22]. Changes in ovarian blood flow have been correlated with changes in steroidogenesis in both the preovulatory follicle and the mature CL [23]. Angiogenesis plays an important role during CL formation; the CL receives more blood per unit of tissue than any other organ of the body [24]. The preovulatory LH surge is the main signal for upregulating vascular endothelial growth factor (VEGF) that induces angiogenesis by stimulating the proliferation of endothelial cells of preexisting capillaries [25]. High levels of VEGF were expressed in granulosa cells of the preovulatory follicle in cattle, and maximum expression was observed during CL formation [26]. Intrafollicular administration of VEGF antagonist into the preovulatory follicle in monkeys inhibited ovulation and CL formation, suggesting that VEGF plays a pivotal role in ovulation and luteinization in mammals [27].

Color Doppler ultrasonography has been used to evaluate ovarian vascular flow in the preovulatory follicle and CL in cattle [28]. Recently, changes in the area of blood flow in the CL in cattle were assessed by color Doppler imaging, and were correlated with function [29].

The objectives of the present study were to test the hypothesis that OIF/NGF isolated from llama seminal plasma exerts a luteotrophic effect by changing the secretion pattern of LH, and to determine if the effect is reflected in an increase in vascular perfusion of the ovulatory follicle and developing CL.

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 Universidad Austral de Chile.

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 six mature 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 [9]. An average of 12 ejaculates was collected from each male. Each ejaculate was diluted 1:1 (vol/vol) with PBS (GIBCO, Grand Island, NY, USA), and centrifuged for 30 minutes at 1500× *g* at room temperature. The supernatant was decanted to remove sperm, and a drop was evaluated by microscopy to confirm the absence of cells. If sperm were still observed, the sample was centrifuged again in a similar manner. The sperm-free seminal plasma was stored at −20 °C. Upon thawing, the diluted seminal plasma was pooled and sonicated to reduce viscosity, as previously described [13]. After sonication, seminal plasma was centrifuged at 2500× *g* for 20 minutes to remove particulate matter.

Purification of OIF/NGF was performed in a two-step procedure, as previously described [13,16]. In brief, llama seminal plasma was loaded into a type 1 Macro-Prep Ceramic hydroxyapatite 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 5kDA cutoff membrane filter device (Vivaspin; Sartorius, Göttingen, Germany), and subsequently loaded onto a gel filtration column (HiPrep 26/60 Sephacryl S-100, Amersham Laboratories, Piscataway, NJ, USA). The purification procedure was carried out under room temperature at a flow rate of 0.5 mL/min using fast protein liquid chromatography (Amersham Laboratories). 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 [13], and was defined as OIF/NGF.

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