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Biological activity of the seminal plasma of alpacas: stimulus for the production of LH by pituitary cells

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Abstract

South American camelids are induced ovulators and require a stimulus to trigger the LH surge responsible for the ovulation. Seminal plasma (SP) of fertile alpacas (*Lama pacos*) was tested using a bioassay of pituitary cells to study the effect of seminal plasma on LH release. Plates containing rat pituitary cells (2×10^5 cells/90–95% viability) were cultured adding: (A) whole SP (WSP) treated with charcoal-dextran, or 1:2 or 1:4 proportions diluted in culture medium (DMEM/HEPES + antibiotics), or (B) 1:2 SP + anti-GnRH rabbit serum (inhibitory potency 10^{-5} M), or (C) 1:2 SP + anti-GnRH + 100 nM synthetic GnRH (buserelin acetate) or (D) 100 nM, 50 nM, 10 nM, and 1 nM synthetic GnRH. Concentration (ng/ml) of LH secreted (Sec) and contained (Con) was analyzed using RIA ^{125}I and the percentage of Sec and Con in each experiment was determined. The results of LH Sec for the cells treated with 50, 10, and 1 nM GnRH were 39, 13, and 1.5%, respectively ($r^2 = 98.41\%$, $r = 0.9920$) but cells treated with 100 nM GnRH secreted 10% of LH. With WSP, 1:2, or 1:4 SP the LH Sec was of 44.5% (3.25 ng/ml), 27% (1.9 ng/ml), and 18% (1.2 ng/ml), respectively. The exposure of cells to 1:2 SP + anti-GnRH, or to 1:2 SP + anti-GnRH/100 nM GnRH produced 31% (2.20 ng/ml) and 30% (1.8 ng/ml) of LH Sec, respectively. These results suggest that the SP of alpacas could have some factor(s) different from GnRH that would contribute to the mechanisms of LH secretion and to the induced ovulation in the female alpaca.

Keywords: Alpaca; Seminal plasma; In vitro culture adenohypophysis; LH

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1. Introduction

South American camelids (SAC) as well as the camelids of the Old World have induced ovulation, whose response depends on the stimulus to which the female is put through. Alpacas (*Lama pacos*) and llamas (*Lama glama*), SAC that inhabit the Andes, ovulate in response to simple or multiple copulation with a fertile and/or vasectomized male (Fernández Baca et al., 1970; Sumar, 1991). The administration of synthetic GnRH or of hCG induced a surge of LH and ovulation in llamas and alpacas (Bravo et al., 1992; Bravo et al., 1997). Although the mechanisms that govern ovulation in camelids are not sufficiently known, it is possible that neuroendocrine and sensorial stimuli act together to determine the ovulatory response. Some experiments conducted on camels (Chen et al., 1985) and alpacas (Ríos et al., 1985) depositing intravaginal homologous semen, produced ovulatory responses in an important percentage of the females used. These results suggest the presence of some factor in the semen of these species that contributes to the ovulatory response. There is scarce information about the reproductive physiology of male SAC, domestic and wild, and because of the difficulties in obtaining semen only some seminal and biochemical morphological characteristics of the seminal plasma have been described (Sumar, 1991; Garnica et al., 1993). More recently, there has been information about the collection and artificial insemination in alpacas (Bravo et al., 1997). However, aspects associated with factors present in the seminal plasma and related to ovulation remain to be proved in systems controlled in vitro. This pilot experiment was undertaken to study the effect of seminal plasma of alpacas on the secretory response of LH using a bioassay of adenohypophysis cells (Scott et al., 1980) with modifications.

2. Material and methods

2.1. Collection and evaluation of semen

Semen from complete ejaculations of adult male alpacas of the Huacaya race and of known fertility was used. Samples were collected during a 20-min mating with a receptive female and obtained through an artificial vagina designed for the species, maintaining a temperature of 38°C and using polyethylene sheaths as described by Urquieta et al. (1997). Semen samples were evaluated as normal according to parameters previously obtained for the species and to rules of the World Health Organization (WHO, 1989) for routine spermiograms. Ultrastructural morphological analyses by scanning and transmission electron microscopy of spermatozoa separated from the seminal plasma of alpacas (Bustos-Obregón et al., 1995) were also used.

2.2. Treatment of semen and of seminal plasma

Semen obtained was centrifuged ($2500 \times g$ for 20 min) and the supernatant without spermatozoa was considered as seminal plasma. The seminal plasma was treated with a 1.25% suspension of charcoal-dextran (Charcoal) in a buffer, pH 7.4, stirring during 60

min at 0°C to separate the steroidal fraction. After centrifugation at 2500 × *g* for 20 min the supernatant was recovered. One milliliter aliquots were stored at −20°C until required.

2.3. Preparation of the cellular culture

Adenohypophysis of 15 Sprague–Dawley adult male rats were excised, cut, and homogenized during 60 min at 160 oscillations/min and at 37°C in 10 ml of culture medium supplemented with enzymes (DMEM, 2.2 g/l sodium bicarbonate, 5.958 g/l HEPES, 1 g/l BSA (V), 10 ml amphotericin B, 1 mg/ml collagenase, 0.5 mg/ml hyaluronidase, 5 μg/ml trypsin) and subsequently 1 mg/ml of DNAase was added during 20 additional min. The cellular suspension was washed with 10 ml of DMEM free of enzymes and was centrifuged (500 × *g* for 15 min) four times, the first one with 1 mg/ml of trypsin inhibitor. The sediment was re-suspended with DMEM plus 10% bovine fetal serum and the concentration and cellular viability were estimated (0.5% trypan blue in 0.1 M PBS, pH 7.2). Finally, the cellular suspension (90–95% of viability) was placed in culture dishes of 24 wells and the concentration was adjusted to 200 000 cells/well in 0.5 ml of culture medium, at 37°C in incubation with 5% CO_2 and 95% air. Cells were cultured during 48 h for adherence to the dish.

2.4. Bioassay of adenohypophysial cells

Cells were incubated with the different treatments according to the following scheme:

1. 100 nM, 50 nM, 10 nM, and 1 nM synthetic GnRH, buserelin acetate, (Conceptal, Hoescht).
2. Whole seminal plasma (WSP) or seminal plasma diluted with culture medium (1:2 and 1:4 SP).
3. 1:2 seminal plasma + anti-GnRH rabbit serum with inhibitory potency up to 10^{-5} M of synthetic GnRH (Bustos-Obregón et al., 1992).
4. 1:2 seminal plasma + anti-GnRH + 100 nM GnRH.

In each well 0.2 ml of test material plus 0.2 ml of culture medium were placed for 72 h. Subsequently, cells were washed in 0.3 ml of medium free of serum and again were exposed to the same test substances during six additional hours, placing in each well 0.15 ml of the test material and 0.15 ml of medium.

Moreover, wells containing cells cultured only with culture medium were used as non-stimulated controls. Once incubation was finished, supernatants were recovered and frozen at −20°C whereas cells were washed with 0.3 ml of medium and were lysed overnight at 4°C with 0.5 ml of PBS supplemented with 0.1% Tritón X-100.

2.5. LH assay

The concentrations (ng/ml) of LH secreted (Sec) and contained (Con) by the adenohypophysial cells were analyzed by means of radioimmunoassays of solid phase (RIA-^{125}I) (NHPP, Rockville, MD, USA). The highly purified antigen (NIDDK-rLH-I-9) was iodinated with 0.5 mCi of ^{125}I by the iodogenic method, thus obtaining a specific

activity of 11 705 cpm/ng of hormone. The first antibody produced in rabbit (NIDDK-anti-rLH-S-11) was used at a final dilution of 1:700 000, whereas the second antibody goat-anti IgG of rabbit (Immunobead, Bio-Rad Lab, CA, USA) was used in excess. Results were expressed in reference to the standard (NIDDK-rLH-RP-3). Inter and intraassay variation coefficients using male rat normal serum were of 15.9% and 4.9%, respectively. Then, the percentages corresponding to Sec and Con in each experiment (in triplicate) were determined.

2.6. Analysis of the results

RIA curves were analyzed using a logit–log model. Comparison between treatments was made by the Mann–Whitney Test and the dose-dependent effects were analyzed by Pearson correlation (r) using ANOVA for its validation. Statistical differences were established with 5% of significance.

3. Results

The primary characteristics of the ejaculations collected by artificial vagina were similar to those previously described (Sumar and Leiva, 1981; Garnica et al., 1993;

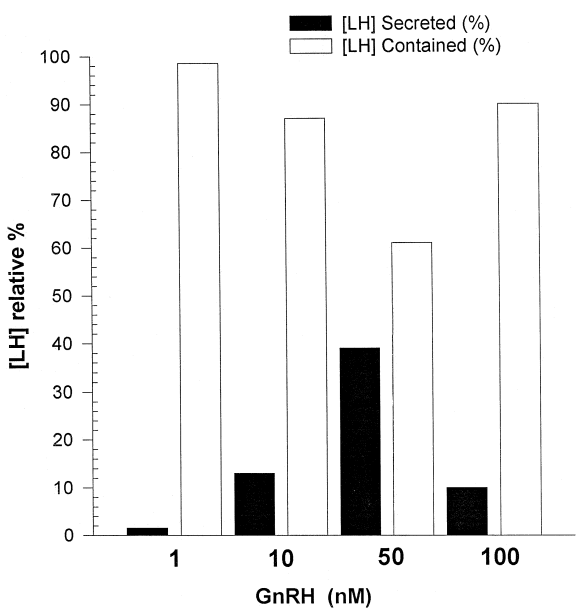

Fig. 1. The effect of different concentrations of synthetic GnRH (1–100 nM) on LH release by 2×10^5 pituitary cells.

Bravo et al., 1997). The duration of the collection with the artificial vagina in each mating was of 20 min and consequently the semen obtained was considered similar to that ejaculated by males during natural mating, whose average duration is of 21 min (Bravo et al., 1997).

Cellular response of LH Sec observed after the treatment with synthetic GnRH to the doses of 50, 10, and 1 nM was of 39%, 13%, and 1.5%, respectively ($r^2 = 98.41\%$; $r = 0.9920$; ANOVA $p > 0.05$). However, with GnRH doses of 100 nM the LH Sec was significantly lower (10%)and did not increase progressively as expected (Fig. 1).

The exposure of the cells to decreasing amount of SP caused a progressive decrease in the LH Sec, with values of 3.25 ng/ml (44.5%) for the WSP, being significantly different ($p < 0.03$) from 1.9 ng/ml (27%) for 1:2 SP, and 1.2 ng/ml (18%) for 1:4 SP. The non-stimulated cells (control) secreted during the same period 1.28 ng/ml, a value that did not differ significantly from the secretion of LH detected in the wells containing cells stimulated with 1:2 and 1:4 SP. The exposure of the cells to treatments 1:2 SP + anti-GnRH and 1:2 SP + anti-GnRH/synthetic GnRH produced a secretory response of 2.2 ng/ml (31%) and of 1.8 ng/ml (30%) of LH Sec, respectively (Fig. 2), similar to the response shown by treatment with 1:2 SP. These results show that the exposure of the cells to SP with anti-GnRH did not modify the LH Sec.

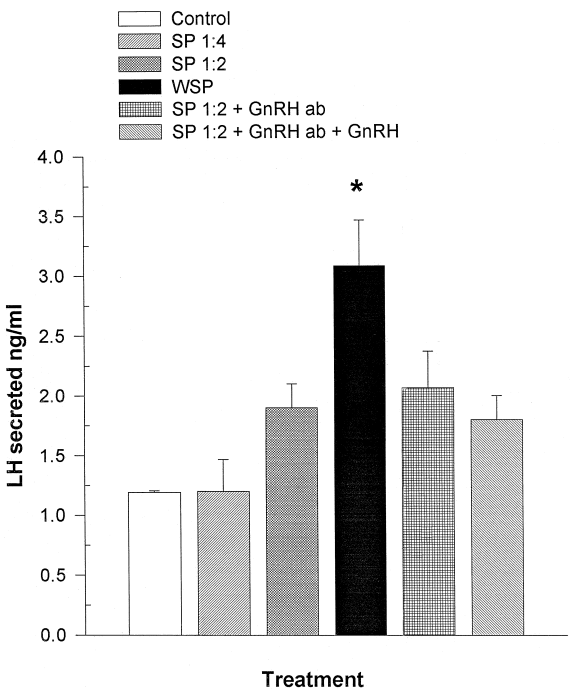

Fig. 2. The effect of different treatments of alpaca seminal plasma (SP 1:4; SP 1:2; WSP) or seminal plasma and anti-GnRH (SP 1:2 + GnRH ab) or seminal plasma and anti-GnRH and GnRH (SP 1:2 + GnRH ab + GnRH) on LH release by 2×10^5 pituitary cells. Each data point represents mean ± SEM of three separate wells in two different replicates. * Significantly different at 3%.

4. Discussion

Our results show that the SP of adult alpacas could have some biologically active factor or factors that can induce rat hypophysial gonadotrophic cells to secrete LH, as was demonstrated by the significant increase in the LH Sec when stimulated with WSP. However, the secretion of LH was not significantly increased when the SP was diluted (1:2 and 1:4) since the LH Sec was similar to that detected in the control, non-stimulated cells. On the other hand, the addition of anti-GnRH antibodies did not modify the response of the cells to the stimulus of SP, suggesting the stimulating effect could be mediated by a factor(s) chemically different from a GnRH.

We could also prove that cells in culture responded progressively and dose-dependently to the stimulation with synthetic GnRH, but that could not be related to the response of the cells when exposed to WSP and the relative secretion of LH (44.5%) was similar to that observed when a 50-nM dose of synthetic GnRH (39%) was applied. The reason for the depression produced in the secretion of LH when the cells were exposed to 100 nM synthetic GnRH is not known, although a down-regulation effect by saturation of the GnRH cellular receptors could have conditioned this lower response in the secretion of LH (10%). The treatment of SP with charcoal supposes that the stimulating effects on the secretion of LH detected in the cellular cultures have not been mediated by steroidal factors.

Other work using SP of pigs (Waberski et al., 1995) showed that there is an additive effect among non-steroidal factors present in the fluid and oestradiol artificially added to it to provoke ovulation in female pigs, despite the fact that these factors present in the SP were more effective than the oestradiol itself to provoke the effect cited. In experiments with Bactrian camels, a species phylogenetically related to SAC, the presence in the SP of a factor expressing GnRH-like biological activity (Zhao et al., 1992) and that can stimulate the secretion of LH in adenohypophysial cells in culture, has been detected. In this experiment, SP stimulated the release of both LH and FSH from the culture pituitary tissue of mice, guinea pigs and rabbit in a dose-dependent manner, but it began to inhibit FSH release when added at a rate of 0.3 ml or more per culture well. Seminal plasma had a synergistic effect on gonadotrophin release induced by GnRH and the factor responsible for this action could be extracted from SP by acetic acid/ethanol (Zhao et al., 1992). In turn, it has been demonstrated that intramuscular injection and insemination with SP of camels induce ovulation with increases of LH and FSH in plasma similar to the endocrine events that occur when ovulation is produced by natural mating in this species (Pan et al., 1992). Moreover, it has been proved that rectal palpation or cervical or vaginal stimulation of female camels do not provoke ovulation. This shows that the neural stimulus is not similar to that reported for rabbits or cats, both induced ovulating species, and that the effect in camelids could be also mediated by factors present in the SP (Marie and Anouassi, 1987).

Seminal plasma is composed of secretions from the testes and from accessory glands of the male reproduction system and in its composition numerous peptide hormones, interleukins, inhibin, and activin have been detected. The study of such factors present in human SP has identified and characterized in normospermic men GnRH-immunoreactive factors with important biological activity, in quantities equivalent to 0.23 ng/ml.

But these factors differ chemically and immunologically from native GnRH (Chan and Tang, 1983; Sokol et al., 1985). Recently, Waberski et al. (1995) determined in the SP of pigs a fraction of 1–10 kDa from non-steroid proteic origin that when transcervically administered to female pigs shortened significantly the interval between the start of oestrus and ovulation, showing a local effect of this component of the SP on the induction of ovulation.

Results obtained in vitro in this experiment are not conclusive evidence for the presence of a GnRH-like factor, but suggest that some factor(s) in the SP of alpacas could contribute to some extent to the secretion of LH and, consequently to the induction of ovulation in receptive females of this species. The native SP of alpacas has been used to measure the inductive effect on ovulation in receptive females of the species (Paolicchi, 1995). Although in this work, the percentage of animals that had an ovulatory response was low, the dynamics in the secretion of LH and the plasma concentrations of oestradiol and progesterone coincided with the hypophysial and ovarian endocrine events detected after natural copulation in adult alpacas. Experiments using more animals and the chemical-functional characterization of a putative ovulation-inducing factor in the SP should be done to determine its true biological significance in the ovulatory process of SAC.

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