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Development of an artificial insemination protocol in llamas using cooled semen

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ABSTRACT

The objective of this study was to design an AI protocol using cooled semen to obtain pregnancies in the llama. Each raw ejaculate was subdivided into four aliquots which were extended 1:1 with: (1) 11% lactose–egg yolk (L–EY), (2) Tris–citrate-fructose–egg yolk (T–F–EY), (3) PBS-llama serum (S-PBS) and (4) skim milk-glucose (K). Each sample reached 5 °C in 2.5 h and remained at that temperature during 24 h. Percentages of the semen variables (motility, live spermatozoa) in ejaculates and samples cooled with L–EY were significantly greater than those obtained when cooling with the other extenders; therefore this extender was used (1:1) for all inseminations. Females were randomly divided into four groups (A–D) according to insemination protocol. Group A: females were inseminated with a fixed dose of 12×10^6 live spermatozoa kept at 37 °C. Group B: females were inseminated with a fixed dose of 12×10^6 live spermatozoa, cooled to 5 °C and kept for 24 h. Group C: females were inseminated with the whole ejaculate (variable doses), cooled to 5 °C and kept for 24 h. These groups (A–C) were inseminated between 22 and 24 h after induction of ovulation. Group D: females were inseminated with the whole ejaculate (variable doses), cooled to 5 °C, kept for 24 h and AI was carried out within 2 h after ovulation. Pregnancy rates were 75%, 0%, 0% and 23% for groups A, B, C and D respectively. These results indicate that it is possible to obtain llama pregnancies with AI using cooled semen and that the success of the technique would depend on the proximity to ovulation.

1. Introduction

A complete knowledge of the reproductive characteristics and the implementation of artificial insemination (AI) programs and of assisted reproductive technology (ART) are of utmost importance for increasing fertility in herds and for obtaining genetic improvement. With regard to the development of AI in South American Camelids (SAC), this technology is limited to the use of raw semen, with a maximum pregnancy rate of 77% in experimental insemination centers and no more that 50% in private establishments (Huanca et al., 2007). Although AI with cooled semen has shown very good results in allowing genetic improvement in different species, this has not been the case for SAC. The only report of AI with cooled alpaca semen is from Vaughan et al. (2003) with no pregnancies obtained. These authors inseminated female alpacas prior to ovulation

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with semen extended 4:1 with Triladyl® and cooled to 4 °C during 24 h. The insemination dose was 125–170 × 10^6 spermatozoa with approximately 45% motility. The lack of success of these results is not only due to the problems in semen collection and evaluation, but also because the semen extender most adequate for conserving fertilizing ability in cooled camelid sperm is currently unknown. Another unknown variable in these species is the optimal interval between induction of ovulation and insemination when using cooled semen (Adams et al., 2009). Pregnancy rates greater than 40% have been reported when raw semen or semen extended at 37 °C was inseminated, either immediately or 24 h after inducing ovulation (Aller et al., 1997; Bravo et al., 1997) and fertilization rates of 52% have been reported using raw semen (Calderon et al., 1968). Nevertheless, when this same interval (24 h) was used to inseminate cooled semen, no pregnancies were obtained (Vaughan et al., 2003). Lack of information has restricted cooled semen AI implementation in SAC, limiting this technology to use with raw semen (Huanca et al., 2007). Development of an AI protocol with cooled semen would permit conservation and transport of genetic material from greater quality animals.

The objective of this study was therefore to design an AI protocol using cooled semen to obtain pregnancies in the *Lama glama* species.

2. Materials and methods

2.1. Location and animals

The study was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires, in Buenos Aires, Argentina. The city is situated at sea level, latitude 34°36' and longitude 58°26'. A total of 8 llama males, between 5 and 10 years old and an average weight of 148 ± 14.40 kg (mean ± SD) and 39 llama females, between 6 and 10 years old and an average weight of 100 ± 25 kg (mean ± SD) were used. Animals were kept out at pasture in pens and supplemented with bales of alfalfa; they also had free access to fresh water and shade throughout the study. This study was approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2010/24).

2.2. Semen collection and evaluation

Ejaculates were obtained using an artificial vagina (AV), according to the technique described by Giuliano et al. (2008) and using electroejaculation (EE) according to the technique described by Director et al. (2007).

Macroscopic and microscopic raw semen characteristics evaluated were: semen pH and volume, sperm concentration, motility, sperm showing swelling, live spermatozoa and sperm morphology. The pH was measured using a universal indicator (Merck, Germany) with a range of 6.4–8. Volume was measured in a graduated collection tube. Sperm concentration was calculated using a Neubauer chamber. Sperm motility was evaluated on a warm stage (37 °C) using a phase contrast microscope. Unless otherwise specified, percentage of motility is total, i.e. progressive and non-progressive. The percentages of sperm with membrane swelling were obtained according to Giuliano et al. (2008). Briefly, semen was incubated (37 °C) 20 min in a hypoosmotic stock solution of fructose–sodium citrate, adjusted to 50 mOsm. The reaction was stopped by adding a hypoosmotic formaldehyde solution. A minimum of 200 spermatozoa were evaluated using a phase contrast microscope (400×). Percentage of live spermatozoa was evaluated using a supravital stain with the fluorochromes: 6-Carboxifluorescein diacetate (CFDA) and Propidium iodide (PI) according to Giuliano et al. (2008). Briefly, samples of semen were incubated at 37 °C in staining medium for 20 min. This medium contained 10 μl of a stock solution of CFDA and 10 μl of a stock solution of PI in 500 μl of saline medium. A Leica® model DMLS microscope was used with the corresponding filters and 200 cells per sample were evaluated. Sperm morphology was evaluated using phase contrast microscopy, obtaining the percentages of normal and abnormal sperm after evaluating 200 cells per sample. No fixative was used to evaluate morphology because the seminal plasma of this species tends to coagulate in the presence of saline formaldehyde solutions.

2.3. Management of the female llamas

Ovarian dynamics was monitored daily by rectal palpation and ultrasonography, using a Berger® LC 2010 plus ultrasound (Buenos Aires, Argentina) with a 5 MHz linear transducer. When a dominant follicle (≥7 mm) in the growth phase was detected, ovulation was induced by intravenously administering 8 μg of buserelin (Receptal®, Laboratorio Hoescht, Buenos Aires, Argentina).

The AI maneuvres were conducted with the female either standing or in sternal recumbency. The animal was restrained in stocks, the tail was wrapped and the rectum was emptied of faeces. The perineum was then scrubbed using a 2% iodine solution, rinsed carefully with clean water and then dried. A lubricated gloved hand was placed in the rectum to hold the cervix while an assistant separated the vulva labia and an AI pipette, covered with a sterile sheath, was inserted into the vagina. Cervical threading was performed through transrectal manipulation and the semen was deposited as close as possible to the uterotubal junction of the uterine horn ipsilateral to the ovary with the dominant follicle.

Pregnancy diagnosis was performed by transrectal ultrasound visualization of the embryonic vesicle 21 days after inducing ovulation and 3 days later, embryo viability was confirmed by observing the heartbeat.

2.4. Experimental design

2.4.1. Experiment 1: semen extender selection

A split-plot experimental design was used to study the protective capacity of different extenders on motility and functional integrity of cooled spermatozoa. A total of 30 ejaculates were collected from 8 llama males using either AV (11/30) or EE (19/30). Nevertheless, only the