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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

19 ejaculates obtained by EE were processed because the volume of the samples obtained with the AV did not allow subdivision into aliquots. Raw semen evaluation was performed at 37 °C, immediately after collection, and cooled semen was evaluated after 24 h of storage at 5 °C.

After raw semen evaluation, each ejaculate was divided into four aliquots and each one was diluted 1:1 with one of four different extenders: (I) 11% lactose (80%) with egg yolk (20%) (L–EY); (II) Tris–citric acid–fructose–egg yolk (T–F–EY); (III) llama blood serum (without complement) (40%)–PBS (60%) (S-PBS); (IV) Skim milk–glucose (Kenney's extender) (K).

Each aliquot of extended semen was incubated 10 min at 37 °C and, before cooling, sperm motility was again evaluated. Each sample was placed into a polypropylene bag, which was closed after removing the air. Each bag was then put into a conical 50 ml tube that was then closed and placed in a glass container with 150 ml of water at 37 °C. The container was then placed in a refrigerator at 5 °C for 24 h.

2.4.2. Experiment 2: artificial insemination using a fixed insemination dose (FID)

In Group A ($n = 8$ females; pre-ovulation AI with semen diluted at 37 °C), semen was diluted 1:1 with L–EY at 37 °C and inseminated at a dose of 12×10^6 live spermatozoa (FID), according to that reported by Bravo et al. (2000). Average sperm motility was $46.8 \pm 3.1\%$ (mean ± SD). Insemination was carried out between 22 and 24 h after buserelin administration (prior confirmation by rectal palpation and ultrasonic assessment that the female had not yet ovulated).

In Group B ($n = 8$ females pre-ovulation AI with cooled semen), semen was diluted 1:1 with L–EY, cooled to 5 °C and kept for 24 h. Average sperm motility was $41.9 \pm 21.6\%$ (mean ± SD). The same insemination protocol and FID as in Group A was used.

2.5. Experiment 3: pre- and post-ovulation AI using cooled whole ejaculates (variable insemination dose, VID)

According to the results of Experiment 2, where no pregnancies were obtained using cooled semen at a FID as opposed to raw semen, the whole ejaculate was inseminated (variable total number of sperm, hence variable insemination dose: VID). A total of 23 ejaculates were obtained from 8 males using EE or AV. Females ($n = 23$) were randomly distributed in two groups: C and D.

2.5.1. Group C (n = 10 females): pre-ovulation AI

The whole ejaculate [range of VID: $(57–133) \times 10^6$ live spermatozoa, motility: $53.3 \pm 15.6\%$ (mean ± SD)] was diluted 1:1 with L–EY, cooled to 5 °C, kept for 24 h, and inseminated between 22 and 24 h after buserelin administration (prior confirmation by rectal palpation and ultrasound that the female had not yet ovulated).

2.5.2. Group D (n = 13): post-ovulation AI

The whole ejaculate [range of VID: $(12–250) \times 10^6$ live spermatozoa; motility: $41.7 \pm 21.3\%$ (mean ± SD)] was diluted 1:1 with L–EY, cooled to 5 °C, kept for 24 h, and inseminated within 2 h after ovulation (confirmed by transrectal palpation and ultrasonography). This insemination time was chosen to shorten the interval between ovulation and insemination because cooling possibly decreases sperm survival in the female tract.

2.6. Reagents

The L–EY extender contained lactose (Carlo Erba, Milan, Italy), Mili Q deionized water and fresh egg yolk. The T–F–EY extender contained 3.63 g of Tris [(hydroxymethyl)-aminomethane] (Merck Química, Argentina), 1.99 g of citric acid (Mallinckrodt, NY, USA), 0.50 g of fructose (Merck Química, Argentina), 14 ml of fresh egg yolk and enough Mili Q deionized water for 100 ml.

To obtain serum for the S-PBS extender (Dulbecco's PBS, Gibco BRL, New York, USA), blood was extracted from llamas without anticoagulant. The blood was left at room temperature for 2 h and then taken to the refrigerator 24 h. After this lapse of time, samples were centrifuged at 3000 rpm during 15 min and the supernatant was aspirated. To remove the complement, the serum was maintained at 56 °C for 30 min. The serum thus treated was divided into aliquots and frozen at −20 °C until use.

The K extender contained 2.4 g of powdered skim milk (Molico®, Argentina) and 4.9 g of glucose (Carlo Erba, Milan, Italy).

2.7. Statistical analysis

Statistical analysis was conducted using the R 2.2.1. Program. The Kolmorogov test was used to test normality of all variables.

2.7.1. Experiment 1

To compare sperm variables with the different extenders between raw and cooled semen, a mixed linear model was used considering the extenders as the fixed effect (five levels: raw semen, semen cooled with L–EY, T–F–EY, S-PBS and K) and the male as the random effect (eight levels, one for each of the eight males). As none of the variables studied showed a significant male-extender interaction, this was excluded from the model. Owing to the asymmetry shown by the variable of sperm motility, evaluated at 37 °C and at 24 h at 5 °C with the different extenders, Friedman's non parametric test was used instead of the mixed linear model.

To separately compare raw semen with each of the aliquots cooled with different extenders and to compare between extended aliquots, confidence intervals were made with a level of significance set at 95%.

2.7.2. Experiments 2 and 3

Descriptive statistics were performed. Mean insemination doses were compared using the *t* test.

3. Results

3.1. Experiment 1: semen extender selection

Results of the descriptive study are summarized in Table 1. No significant differences ($P > 0.05$) were observed between raw semen (control) and semen diluted with the different extenders at 37 °C. In contrast, significant differences ($P < 0.01$) were observed between semen diluted at 37 °C with the different extenders and cooled semen.

When assessed at the 95% confidence intervals, there were no significant differences between the percentages of sperm motility in raw semen and semen cooled in L–EY. In addition, the percentages of sperm motility in raw semen and L–EY cooled semen were greater ($P < 0.05$) than those obtained when cooling with the extenders: T–F–EY; S-PBS and K.

Differences ($P < 0.01$) were observed between raw semen (control) and semen cooled with the different extenders. When assessed at the 95% confidence intervals, no significant differences were observed between the percentages of spermatozoa with intact membranes in raw semen and those cooled with L–EY extender. The percentages of spermatozoa with intact membranes in semen cooled with L–EY, T–F–EY and K were greater ($P < 0.05$) than those obtained with semen cooled with S-PBS.

Differences ($P < 0.01$) were observed between raw semen (control) and semen cooled with the different extenders. When assessed at the 95% confidence intervals, no significant differences were observed between the percentages of sperm with functional membranes in raw semen, semen cooled in L–EY and semen cooled in K. Percentages of sperm with functional membranes were greater ($P < 0.05$) in semen cooled in L–EY and K than those cooled in T–F–EY and S-PBS.

3.2. Experiment 2: artificial insemination using a fixed insemination dose (FID)

The pregnancy rate in Group A (pre-ovulation AI with semen diluted at 37 °C and FID) was 75% (6/8) with a range in induction of ovulation (IO) and AI interval (IO–AI interval) of 22–24 h. The pregnancy rate in Group B (pre-ovulation AI with cooled semen and FID) was 0% (0/8) with a range in IO–AI interval of 22–24 h.

3.3. Experiment 3: pre- and post-ovulation AI using a variable insemination dose (VID) of cooled semen

The pregnancy rate obtained in Group C (pre-ovulation AI with cooled semen and VID) was 0% (0/10). The IO–AI interval ranged between 22 and 24 h. Average VID for this group was $72.7 \pm 60.44 \times 10^6$ live cooled spermatozoa (mean ± SD).

The pregnancy rate obtained in Group D (post-ovulation AI with cooled semen and VID) was 23% (3/13). The IO–AI interval ranged between 26 and 30 h (average 28.0 ± 1.3 h) (mean ± SD). Average VID for this group was $51.52 \pm 49.93 \times 10^6$ live cooled spermatozoa (mean ± SD). The range in insemination dose with which pregnancy was obtained was between 76 and 210×10^6 spermatozoa. The

Table 1
Variations in sperm motility, membrane integrity (live spermatozoa) and membrane function (swelling) in raw semen, semen extended at 37 °C and semen cooled to 5 °C for 24 h ($n = 8$, 19 llama ejaculates collected using EE).

Variables	Raw semen	L–EY		T–F–EY		S-PBS		K	
	37 °C	37 °C	5 °C	37 °C	5 °C	37 °C	5 °C	37 °C	5 °C
Sperm motility (%)	$36.6 \pm 25.6^{a,b}$	40.7 ± 22.4^{a}	25.2 ± 10.2^{b}	40.1 ± 25.0^{a}	9.3 ± 8.5^{c}	24.0 ± 17.9^{a}	0.0 ± 0.1^{d}	23.7 ± 14.6^{a}	2.3 ± 5.3^{e}
Live spermatozoa (%)	62.6 ± 11.4^{a}	–	48.4 ± 15.3^{a}	–	33.0 ± 11.8^{a}	–	10.1 ± 6.2^{c}	–	36.1 ± 17.5^{d}
Swelling (%)	35.5 ± 11.1^{a}	–	44.5 ± 17.5^{a}	–	26.6 ± 10.4^{b}	–	15.8 ± 9.3^{c}	–	35.6 ± 14.2^{a}

Data are mean ± standard deviation.
L–EY: semen diluted 1:1 in 11% lactose (80%)–egg yolk (20%); T–F–EY: semen diluted 1:1 in Tris–citric acid–fructose–egg yolk; S-PBS: semen diluted 1:1 in llama blood serum (without the complement) (40%)–PBS (60%); K: semen diluted 1:1 in skim milk–glucose (Kenney's extender); 37 °C: semen at 37 °C; 5 °C: semen cooled to 5 °C for 24 h.
Different letters in rows indicate differences ($P \leq 0.05$).

Table 2
Interval between induction of ovulation (IO) and artificial insemination (AI), insemination dose (mean ± standard deviation) and pregnancy rates with semen diluted with L–EY at 37 °C and semen cooled to 5 °C for 24 h in llamas.

Group	Number of females inseminated	Temperature of the sample (°C)	Insemination dose (10^6)	IO–AI interval range (h)	Pregnancy rate (%)
A	8	37	12.0[a]	22–24	75 (6/8)
B	8	5	12.0[a]	22–24	0 (0/8)
C	10	5	72.7 ± 60.4[b]	22–24	0 (0/10)
D	13	5	51.5 ± 49.9[b]	26–30	23 (3/13)

IO–AI: interval between induction of ovulation and artificial insemination; A: pre-ovulation AI with semen diluted at 37 °C and FID; B: pre-ovulation AI with cooled semen and FID; C: pre-ovulation AI with cooled semen and VID; D: post-ovulation AI with cooled semen and VID; Insemination dose: the insemination dose was based on the number of live spermatozoa.
Different letters in columns indicate differences ($P \leq 0.05$).

results obtained in the different groups with the different AI protocols are summarized in Table 2.

4. Discussion

Given the importance of implementing AI programs with cooled *Lama glama* semen, it was necessary to study the protective capacity of different extenders on sperm motility and functional integrity and obtain an appropriate interval between induction of ovulation and insemination, such that pregnancy could be obtained in this species. As previous reports of pregnancy rates are 0% (Vaughan et al., 2003), the present study reports the first AI pregnancies in SAC using cooled semen.

When comparing sperm motility at 37 °C between raw semen and semen diluted 1:1 with the different extenders, no significant differences were observed between the samples. Nevertheless, only the samples with extenders having egg yolk (L–EY and T–F–EY) showed a similar mean motility to raw semen samples and a greater motility than the samples extended with S-PBS and K (Table 1). These results would seem to indicate that the presence of egg yolk in the extender at 37 °C would maintain sperm motility in this species. To be able to explain the effect of the egg yolk in the extender on sperm motility, Miceli et al. (1998) examined the effect of preserving human semen in the TEST buffer with egg yolk (TYB). These authors observed that specific surface proteins on spermatozoa were phosphorylated to a greater extent in sperm incubated with TYB than those incubated in other media. Results of this previous study also indicated that TYB proteins could also be phosphorylated by the spermatozoa during the incubation and attached to the sperm membranes. Taking into account that initiation and maintenance of progressive sperm motility seem to be regulated by phosphoproteins, it would be necessary to conduct studies in llama semen to confirm if the observations made by these authors in human semen are also valid in llamas.

When studying the influence of the extenders on sperm variables after 24 h of cooling, samples diluted with L–EY were the only ones that maintained sperm motility after cooling and that this motility was greater than that obtained with the rest of the extenders. Huanca and Gauly (2001) diluted llama semen 1:1 in BSA–glucose, cooled the samples to 5 °C and obtained a 50% decrease in the initial sperm motility 12 h after cooling. Vaughan et al. (2003) obtained a sperm motility of 50% after cooling alpaca ejaculates for 24 h in a commercial extender with egg yolk (Triladyl®). Santiani et al. (2005), however, extended samples with either: (1) Tris, glucose, egg yolk (20%) and cryoprotectants such as glycerol or ethylene glycol or (2) cow skim milk, egg yolk (5%), glucose and glycerol or ethylene glycol. They kept the samples 30 min at 5 °C and obtained similar percentages of motility to those of the raw semen in all groups except the samples extended with milk, egg yolk and glycerol. Morton et al. (2007), after extending and cooling alpaca epididymal sperm to 4 °C, also reported that the percentage of sperm motility was higher extending 1:1 with 11% lactose (80%)–egg yolk (20%) than extending 1:1 or 1:2 with Tris–citric acid–glucose and egg yolk (20%). In addition, Morton et al. (2010) observed that alpaca epididymal sperm motility was similar after cooling to 4 °C with 11% lactose (80%)–egg yolk (20%) to after harvesting the sperm. These results would confirm the beneficial effect egg yolk has on sperm motility in SAC as it is present in the commercial extender used by Vaughan et al. (2003) and in the extender used by Santiani et al. (2005) in the same proportion (20%) as we used in this study.

With regard to sperm membrane integrity (viability), it is interesting to note that only samples cooled with the L–EY extender did not differ in viability from raw semen. It is also noteworthy that although the K extender maintained the percentage of sperm with membrane function (swelling), this was not similar for motility; and that only L–EY extender was able to maintain both sperm motility and membrane function.

The amount of egg yolk used in this study (20%) was similar to that used by other authors: 5–20% in alpaca (Santiani et al., 2005); 20% in epididymal alpaca sperm (Morton et al., 2007, 2010); 15–30% in bovines (Vishwanath and Shannon, 2000); 20% in buffalo (Sansone et al., 2000) and 10–20% in camels and dromedaries (Tibary and Anouassi, 1997). The mechanisms by which egg yolk protects sperm from cold shock are not completely clear. There is some evidence the protective effect of egg yolk is due to its ability to interact with the membrane lipid bilayer (Foulkes, 1977; Watson, 1995), and thus prevent phase transition events in the membrane lipids (Drobnis et al., 1993; Watson, 1995). Nevertheless, inconsistencies in results exist concerning the stability of this association between egg yolk and the plasma membrane (Watson, 1975; Foulkes, 1977). Bergeron and Manjunath (2006) have suggested that the protective effect of egg yolk consists in competing with the harmful factors present in the seminal plasma. In this previous study, it was determined that in bull seminal plasma there is a family of proteins that bind to the

lipids (BSP proteins) that would have an adverse effect on sperm conservation by inducing cholesterol and phospholipid removal from the sperm membrane. It was, therefore, suggested that the main mechanism of protection consists in the low density lipoproteins (LDL) of the egg yolk confiscating this family of BSP proteins.

Lactose, the remaining component of the L–EY extender, and other non-permeable sugars such as fructose, glucose, galactose, sucroseand trehalose, have all been used for preserving bull sperm (Vishwanath and Shannon, 2000); rams (Aisen et al., 2000); buffalo (Sansone et al., 2000); alpacas (Morton et al., 2007, 2010), camels and, dromedaries (Tibary and Anouassi, 1997). The activity of lactose in preserving the osmotic pressure of the medium is reflected in the maintenance of the percentage of spermatozoa with intact plasma membranes.

The present and previous results generate new questions and new research possibilities, such as, which are the mechanisms the L–EY extender uses to maintain sperm motility and membrane function and integrity. With regard to the curves used for cooling, Vaughan et al. (2003), Santiani et al. (2005) and Morton et al. (2007, 2010) all reported similar curves (between 1.5 and 2 h) to those used in the present study.

In the present study, AI were performed using the same insemination route used in a majority of the previous research in llamas and alpacas (Fernández Baca and Novoa, 1968; Aller et al., 1997, 2003; Bravo et al., 1997, 1999, 2000; Vaughan et al., 2003), and also in camels and dromedaries (Skidmore and Billah, 2006) as it is the most physiological imitation of the placing of semen during ejaculation. The 75% pregnancy rate obtained inseminating with raw semen diluted 1:1 with L–EY (Group A) is greater than the AI pregnancy rates obtained by Leyva et al. (1977) and Aller et al. (1997, 1999) using raw llama semen both with and without dilution. Likewise, it is also greater than those obtained by Fernández Baca and Novoa (1968), Calderon et al. (1968), Leyva et al. (1977), Bravo et al. (1997, 1999) and Apaza et al. (2001) using raw alpaca semen. In addition, it is similar to the percentages reported by Huanca et al. (2007) for AI of raw or diluted semen in the Andean camelid producing communities of Peru.

With regard to the IO–AI interval, the references indicate that pregnancy rates are greater than 40% (Aller et al., 1997; Bravo et al., 1997) and fertilization rates greater than 52% (Calderon et al., 1968) when inseminating with raw semen (either diluted or undiluted) at 37 °C, both immediately and 24 h after having induced ovulation. When using this same IO–AI interval with cooled semen in the present study, pregnancies did not occur (Groups B and C), and neither were there pregnancies in alpacas when these techniques were used (Vaughan et al., 2003). If one considers that ovulation occurs 28.6 ± 0.36 h after exogenous administration of LH or GnRH analogues (Bourke et al., 1992) one could deduce that at the time of insemination (24 h), most females had not yet ovulated, a fact which we confirmed ultrasonographically (Groups A–C). It should be emphasized that when insemination using cooled semen was performed after ultrasononic confirmation of ovulation, more desirable results were obtained. This was reflected in the 23% (3/13) pregnancy rate seen in Group D. Calderon et al. (1968) also obtained more desirable results (greater fertilization rates, 75%) when they inseminated alpacas using an IO–AI interval of 35–45 h.

Due to the fact that in Groups C and D the whole ejaculate was inseminated, the VID range was very large ($(12–250) \times 10^6$ live spermatozoa). The insemination doses that achieved pregnancy (as from 76 million live spermatozoa) are greater than the doses used to attain pregnancies with raw semen: 12×10^6 live spermatozoa (FID) in our study, $28.9 \pm 18.7 \times 10^6$ total spermatozoa (Aller et al., 1997) and $(4–12) \times 10^6$ total spermatozoa (Bravo et al., 1999). In this previous study, results obtained were similar to results obtained with the cooled semen doses used by Vaughan et al. (2003), $(125–170) \times 10^6$ total sperm, where no pregnancies were obtained. Therefore to obtain pregnancies with cooled semen it seems to be necessary to inseminate soon after confirming ovulation and use a greater dose than when using raw semen. This is similar to what occurs in other species, where more sperm are needed to achieve an acceptable degree of fertilization when using preserved semen (Watson, 2000). The need to shorten the time between ovulation and insemination could indicate that cooling is perhaps inducing sperm capacitation or producing a capacitation-like process, as has been reported in other species (Watson, 1995; Neild et al., 2003; Silva and Gadella, 2006) making it necessary to shorten the interval between the meeting of the gametes or sperm will acrosome react before reaching the oocyte. This would be achieved by inseminating after confirming ovulation (not more than 2 h later).

In conclusion, when using cooled llama semen it would be important to carry out artificial insemination within 2 h after detecting ovulation and use insemination doses greater than 76 million total live spermatozoa. The present study provided evidence that is possible to implement cooled semen protocols for AI, thus allowing the transportation of semen from genetically superior animals.

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References

Adams, G.P., Ratto, M.H., Collins, C.W., Bergfelt, D.R., 2009. Artificial insemination in South American camelids and wild equids. Theriogenology 71, 166–175.

Aisen, E.G., Alvarez, H.L., Venturino, A., Garde, J.J., 2000. Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology 53, 1053–1061.

Aller, J., Ferre, L., Rebuffi, G.E., Alberio, R.H., 1997. Inseminación artificial en llama (*Lama glama*) primera comunicación en Argentina. Veterinaria Argentina XIV (136), 394–400.

Aller, J., Cancino, A.K., Rebuffi, G.E., Alberio, R.H., 1999. Inseminación artificial en llamas en la Puna. In: Actas II Congreso Mundial sobre Camélidos, Cusco, Perú, p. 72.

Aller, J.F., Rebuffi, G.E., Cancino, A.K., Alberio, R.H., 2003. Influencia de la criopreservación sobre la motilidad, viabilidad y fertilidad de espermatozoides de llama (*Lama glama*). Arch. Zootec. 52, 15–23.

Apaza, N., Sapana, R., Huanta, T., Huanca, W., 2001. Inseminación artificial en alpacas con semen fresco en comunidades campesinas. Rev. Invest. Vet. Suppl. 1, 435–438.

Bergeron, A., Manjunath, P., 2006. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Mol. Reprod. Dev. 73, 1338–1344 (Review).

Bourke, D.A., Adam, C.L., Kyle, C.E., McEvoy, T.G., Young, P., 1992. Ovulation, superovulation and embryo recovery in llamas. In: Proc. 12th Int. Cong. Anim. Reprod, pp. 1193–1195.

Bravo, P.W., Flores, U., Garnica, J., Ordoñez, C., 1997. Collection of semen and artificial insemination of alpacas. Theriogenology 47, 619–626.

Bravo, P.W., Pacheco, C., Quispe, G., Vilcapaza, L., Ordoñez, C., 1999. Degelification of alpaca semen and the effect of dilution rates on artificial insemination outcome. Arch. Andrology 43, 239–246.

Bravo, P.W., Skidmore, J.A., Zhao, X.X., 2000. Reproductive aspects and storage of semen in Camelidae. Anim. Reprod. Sci. 62, 173–193.

Calderon, W., Sumar, J., Franco, E., 1968. Avances de la inseminación artificial de las alpacas (*Lama pacos*). Rev. Fac. Med. Vet. (Lima) 22, 19–35.

Director, A., Giuliano, S., Carretero, M., Pinto, M., Trasorras, V., Miragaya, M., 2007. Electroejaculation and seminal characteristics in llama (*Lama glama*). J. Camel Prac. Resch. 14, 203–206.

Drobnis, E.Z., Crowe, L.M., Berger, T., Anchordoguy, T.J., Overstreet, J.W., Crowe, J.H., 1993. Cold shock damage is due to lipid phase transitions in cell membrane: a demonstration using sperm as a model. J. Exp. Zool. 265, 432–437.

Fernández Baca, S., Novoa, C., 1968. Primer ensayo de inseminación artificial (*Lama paco*) con semen de vicuña (*Vicugna vicugna*). Rev. Fac. Med. Vet. (Lima) 22, 9–18.

Foulkes, J.A., 1977. The separation of lipoproteins from egg yolk and their effect on the motility and integrity of bovine spermatozoa. J. Reprod. Fertil. 49, 277–284.

Giuliano, S., Director, A., Gambarotta, M., Trasorras, V., Miragaya, M., 2008. Collection method, season and individual variation on seminal characteristics in the llama (*Lama glama*). Anim. Reprod. Sci. 104, 359–369.

Huanca, W., Gauly, M., 2001. Conservación de semen refrigerado de llamas. Rev. Inv. Vet. Peru 1, 460–461.

Huanca, W., Cordero, A., Huanca, T., Adams, G., 2007. Biotecnologías reproductivas en camélidos sudamericanos domésticos: avances y perspectivas. Arch. Latinoam. Prod. Anim. 15, 195–201.

Leyva, V., Franco, E., Sumar, J., 1977. Inseminación artificial en camélidos sudamericanos. In: Proceedings, I Reunión Científica Anual de la Asociación Peruana de Producción Animal (APPA) Lima, p. 21.

Miceli, D.C., Llanos, R., Jimenez Díaz, M., Peralta, L., 1998. Phosphorylation of endogenous and TEST-yolk buffer proteins by intact human sperm. Zygote 6, 203–212.

Morton, K.M., Roslyn Bathgate, A.B., Evans, G., Maxwell, W.M., 2007. Cryopreservation of epididymal alpaca (Vicugna pacos) sperm: a comparison of citrate-, Tris- and lactose-based diluents and pellets and straws. Reprod. Fertil. and Dev. 19 (7), 792–796.

Morton, K.M., Evans, G., Maxwell, W.M., 2010. Effect of glycerol concentration, Equex STM supplementation and liquid storage prior to freezing on the motility and acrosome integrity of frozen-thawed epididymal alpaca (Vicugna pacos) sperm. Theriogenology 74 (2), 311–316.

Neild, D.M., Bart, M., Gadella, M., Chaves, G., Miragaya, M.H., Colenbrander, B., Agüero, A., 2003. Membrane changes during different stages of a freeze–thaw protocol for equine semen cryopreservation. Theriogenology 59, 1693–1705.

R 2.2.1. Program. Copyright, 2005. The R Foundation for Statistical Computing Version 2.2.1 (2005-12-20 r36812) ISBN 3-900051-07-0.

Sansone, G., Nastri, M.J., Fabbrocini, A., 2000. Storage of buffalo (*Bubalus bubalis*) semen. Anim. Reprod. Sci. 62, 55–76.

Santiani, A., Huanca, W., Sapana, R., Huanca, T., Sepúlveda, N., Sánchez, N., 2005. Effects on the quality of frozen-thawed alpaca (Lama pacos) semen using two different cryoprotectants and extenders. Asian J. of Androl. 7 (3), 303–309.

Silva, P.F.N., Gadella, B.M., 2006. Detection of damage in mammalian sperm cells. Theriogenology 65, 958–978.

Skidmore, J.A., Billah, M., 2006. Comparison of pregnancy rates in dromedary camels (Camelus dromedarius) after deep intra-uterine versus cervical insemination. Theriogenology 66, 292–296.

Tibary, A., Anouassi, A., 1997. Theriogenology in Camelidae, Anatomy, Physiology, Pathology and Artificial Breeding, 1st ed. Actes Editions, Rabat, Maroc, pp. 413–452.

Vaughan, J., Galloway, D., Hopkins, D., 2003. Artificial insemination in alpacas (*Lama pacos*). RIRDC Rural Industries Research and Development Corporation, Pub. No. 03/104, Kingston, Australia, pp. 74–77.

Vishwanath, R., Shannon, P., 2000. Storage of bovine semen in liquid and frozen state. Anim. Reprod. Sci. 62, 23–53.

Watson, P.F., 1975. The interaction of egg yolk and ram spermatozoa studied with a fluorescent probe. J. Reprod Fertil. 42, 105–111.

Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 4, 871–891 (Review).

Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60, 481–492.