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Extenders for alpaca epididymal spermatozoa: Comparison of INRA96 and andromed

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ABSTRACT

Artificial insemination would be a useful technique for alpaca breeders to use as an aid to breeding to increase fleece quality. The technique, however, is not well developed in alpacas, partly because of the viscous nature of their seminal plasma. Castration conducted for husbandry purposes can provide a source of epididymal spermatozoa to test semen extenders or handling regimens, thus circumventing the problem of the viscous ejaculate. In this experiment, two semen extenders (Andromed and INRA96) developed for other species (bovine and equine, respectively) were tested with alpaca spermatozoa derived from the cauda epididymis. Sperm total motility (mean ± SEM A: 29.1 ± 4.8 % compared with I: 35.4 ± 4.8 %; *NS*), membrane integrity (A: 58 ± 9% compared with I: 56 ± 9%; *NS*) and acrosome integrity (A: 65 ± 7% compared with I: 54 ± 7%; *NS*) were not different between the two extenders. Progressive motility with use of INRA96 was greater after incubating for 30 min than after incubating for 10 min (35 ± 4% vs. 12 ± 4%, respectively; $P = 0.03$). In conclusion, viable epididymal spermatozoa could be extracted from the castrated organs after overnight transport. There were no differences in sperm quality between the two extenders; therefore, it appears that either extender could be used for alpaca spermatozoa. These results could help in the development of a technique for artificial insemination in alpacas.

1. Introduction

Breeding animals by artificial insemination (AI) rather than by natural mating has many advantages, as reviewed previously (e.g., Morrell, 2011). One example is that AI helps prevent the spread of infectious or contagious diseases, as animals do not come into contact during mating. Furthermore, AI also allows males of superior genetic merit to produce offspring from a large number of females, thus increasing the rate of genetic improvement and production traits. Other advantages include being able to overcome geographical or chronological barriers, including being able to breed from the male after his demise. It is a powerful technique when used in conjunction with other reproductive biotechnologies such as sperm cryopreservation (Santiani et al., 2016).

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Even though there are these advantages, AI is not practiced widely in alpacas for several reasons, one being the difficulty of working with the viscous ejaculate that is characteristic of camelids (Santiani et al., 2005). The spermatozoa are trapped within the viscous seminal plasma and move slowly with an oscillatory movement and limited progression (Garnica et al., 1993; Deen et al., 2003). Viscous seminal plasma also inhibits the use of stains to evaluate sperm characteristics, or the penetration of cryoprotectants when attempting to freeze the samples (Kershaw-Young and Maxwell, 2012). There are, therefore, several challenges to overcome when developing protocols for semen handling, including selecting the optimal extenders to use. It has been recommended that enzymes be used to break down the viscous ejaculate (e.g., Tibary and Anouassi, 1997; Bravo et al., 2000; El-Bahrawy and El-Hassanein., 2009; Giuliano et al., 2020) with results from recent studies indicating papain as the most effective treatment (Kershaw-Young et al., 2013; Kershaw et al., 2016). There are, however, other reports of sperm damage with loss of functional capacity following enzyme treatment (Morton et al., 2012).

A first step in the development of a protocol for semen handling could be to use epididymal spermatozoa to test semen extenders, thus avoiding the problem of how to deal with the viscous ejaculate until a later stage. In Sweden, alpacas that are not required for breeding purposes are castrated to facilitate animal management and avoid indiscriminate breeding. In theory, epididymal spermatozoa can be collected from the discarded organs. In previous studies, extenders developed for other species have been used for spermatozoa or semen from South American camelids, mostly containing either egg yolk or skimmed milk (reviewed by Bravo et al., 2013) or bovine serum albumin (Santa Cruz et al., 2016). The problem with including egg yolk or skimmed milk is that these ingredients are not standardized, varying in composition according to the diet of the animal from which they were derived, and pose a biosecurity risk because these constituents may contain microorganisms (reviewed by Lima Verde et al., 2017). Commercial extenders that do not contain products of animal origin have been developed for various species, although no specific extenders are available for alpacas. The aim of the present study, therefore, was to compare two commercial semen extenders that do not contain material of animal origin for their suitability for alpaca epididymal spermatozoa, using material from castrations. Two commercial semen extenders, Andromed (A; Minitüb; Tiefenbach, Germany) for bull spermatozoa and INRA96 (I; IMV Technologies, L'Aigle, France) for stallion spermatozoa, were chosen for this study.

2. Materials and methods

2.1. Epididymal spermatozoa

Scrotal contents were obtained from castration of males for husbandry purposes, performed on the farms by private veterinarians. The results reported in this manuscript are derived only from the organs that contained spermatozoa (i.e., from ten animals). The animals had not been used for breeding; their ages varied from approximately 2–10 years, and they were all healthy. After surgery, the organs were placed in a plastic bag containing phosphate buffered saline and were transported overnight to the laboratory at the Swedish University of Agricultural Sciences (SLU) in a Styrofoam box with a cold pack. This type of packaging is used to transport stallion semen and maintains the temperature at approximately 6 °C overnight. The tunica vaginalis, connective tissues and blood vessels were removed; after isolating the cauda epididymis from the testis, it was placed in warm (37 °C) semen extender. From each animal, one cauda epididymis was placed in Andromed (A; Minitübe International, Tiefenbach, Germany) and the other in INRA96 (I; IMV Technologies, L'Aigle, France). Several cuts were made in the epididymis to allow the contents to flow out (Fig. 1). Evaluation of the samples was conducted after incubation at 37 °C for up to 30 min.

Ethical approval is not required for this procedure because the castration was conducted for management purposes at the request of the animal's owner. The discarded organs were used for this study after obtaining the approval of the animal's owner. All animals were maintained using procedures consistent with national and international regulations on the housing and care of animals.

2.2. Sperm evaluation

2.2.1. Computer assisted sperm analysis

Sperm motility was evaluated using the computer assisted sperm analysis (CASA) system utilizing the SpermVision version 3.8 (Minitüb; Tiefenbach, Germany) after incubation for 10 min and 30 min. A 5 μL aliquot of each sample was placed on the heated stage (38 °C) of an Olympus BX 51 microscope (Olympus, Tokyo, Japan); approximately 200 spermatozoa were evaluated in each of eight

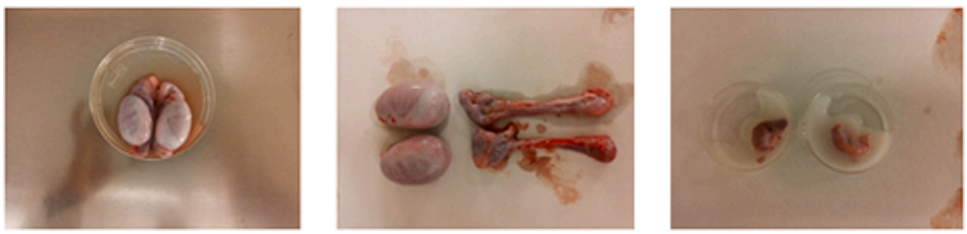

Fig. 1. Preparation of alpaca epididymal sperm samples.
Left: scrotal contents after transport to the laboratory; middle: dissection of the epididymis away from the testis; right: cut epididymis in medium to allow spermatozoa to swim out of the tissue.

fields per sample. The values for the following variables were recorded: total motility (%), progressive motility (%), average path velocity (VAP; μm/s), curvilinear velocity (VCL; μm/s), straight line velocity (VSL; μm/s), straightness (STR; VSL/VAP), linearity (LIN; VSL/VCL), wobble (WOB; VAP/VCL), amplitude of lateral head deviation (ALH; μm) and beat cross frequency (BCF; Hz). Particles of size 16−121 μm were considered to be spermatozoa and were motile if VAP > 20 μm/s.

2.2.2. Plasma membrane integrity

Plasma membrane integrity (MI) was assessed after staining with SYBR14 and propidium iodide (PI). Briefly, working solutions of SYBR14 (0.38 μM) and PI (340 μM) were prepared from the stock solutions in the Live-Dead Sperm Viability KIT (KIT L-7011; Invitrogen, Eugene, OR, USA). An aliquot (5 μL) of each sperm sample was mixed with 1 μL each SYBR-14 and PI working solutions and the mixture was incubated at 37 °C in the dark for 30 min. A 5 μL drop of each sample was placed on a glass slide, covered and 200 spermatozoa per sample were evaluated by fluorescence microscopy. Spermatozoa staining green were considered to have an intact membrane whereas those staining red had a damaged membrane.

2.2.3. Acrosome status

The procedure of Axnér et al. (2004), modified from Cheng et al. (1996) was followed. An air-dried smear was prepared from 5 μL sperm sample. The spermatozoa were permeabilized in 96 % ethanol for 30 s and the slide was air-dried again. A working solution of 100 μg/mL FITC-conjugated peanut agglutinin (FITC-PNA; Sigma, St. Louis, USA) was prepared in phosphate buffered saline with added PI (340 μM); 20 μL of this mixture was spread over the slide. After incubating in a moist chamber at 6 °C for 30 min, the slide was rinsed with cold distilled water. After drying, the slide was mounted with 10 μL antifade, sealed with nail polish and evaluated by fluorescence microscopy under oil, x1000. Spermatozoa were classified as follows: i) acrosome intact: spermatozoa with bright green fluorescence of the acrosomal cap; ii) damaged acrosome: patch-like fluorescence of acrosomal cap; iii) lost acrosome: spermatozoa with a fluorescent band at the equatorial segment or no fluorescence. All spermatozoa are stained red with PI because membranes have been permeabilized.

2.3. Statistical analysis

Means were compared by mixed model using the SAS® software, version 9.3 (SAS® Cary, NC, USA). Data were first tested for normality with Shapiro-Wiks test and the homogeneity of variances was evaluated with Levenes test. Tukey's *post-hoc* adjustment was applied. The statistical model included the fixed effects of extenders (A and I) and the time (10 and 30), in addition to extenders*time interaction, and the random effects of animals ($n = 10$). Statistical significance was set at a P value ≤ 0.05. Pearson correlation was conducted on membrane integrity and motility using the statistical package contained in Microsoft Excel software (2016 version).

3. Results

Values for sperm kinematics after 10 and 30 min incubation are provided in Table 1. There were no differences in values for sperm kinematics with use of the different extenders. For sperm samples in A, progressive motility was not different between the two incubation times (11 ± 4% at 10 min and 17 ± 4% at 30 min) whereas in I, progressive motility increased from 12 ± 4% after 10 min incubation to 25 ± 4% after 30 min ($P < 0.05$).

Sperm membrane integrity was not different between the two extenders (58 ± 9% vs. 56 ± 9% for A and I, respectively; *NS*). There was no correlation between motility and membrane integrity for either extender (A: $r = 0.19$; I: $r = 0.04$; *NS*)

Alpaca spermatozoa stained with FITC-PNA are depicted in Fig. 2, where the majority of spermatozoa have intact acrosomes as indicated by green fluorescence over the head region. There was no difference in the proportion of sperm with intact acrosomes

Table 1
Kinematics of alpaca epididymal spermatozoa in Andromed or INRA96 (LSMeans ± SEM) after 10 or 30 min incubation at 37 °C ($n = 10$).

	After 10 min		After 30 min	
Kinematics	Andromed	INRA96	Andromed	INRA96
MOT (%)	18.88 ± 4.75	21.06 ± 4.75	29.10 ± 4.75	35.43 ± 4.75
PM (%)	10.89 ± 4.21	12.13 ± 4.21*	17.01 ± 4.21	24.67 ± 4.21*
VAP (μm/s)	40.95 ± 5.19	40.96 ± 5.19	48.88 ± 5.19	46.37 ± 5.19
VCL (μm/s)	57.56 ± 8.85	64.14 ± 8.85	71.87 ± 8.85	76.08 ± 8.85
VSL (μm/s)	24.89 ± 2.83	24.09 ± 2.83	24.25 ± 2.83	26.78 ± 2.83
STR	0.57 ± 0.06	0.55 ± 0.06	0.49 ± 0.06	0.53 ± 0.06
LIN	0.43 ± 0.05	0.36 ± 0.05	0.34 ± 0.05	0.33 ± 0.05
WOB	0.66 ± 0.06	0.58 ± 0.06	0.69 ± 0.06	0.56 ± 0.06
ALH (μm)	2.74 ± 0.37	3.02 ± 0.37	2.84 ± 0.37	3.18 ± 0.37
BCF (Hz)	14.89 ± 3.34	21.69 ± 3.34	23.56 ± 3.34	21.56 ± 3.34

Note: MOT = total motility; PM = progressive motility; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity; STR (VSL/VAP) = straightness; LIN (VSL/VCL) = linearity; WOB VAP/VCL) = wobble; ALH = amplitude of lateral head deviation; BCF = beat cross frequency.

* $P = 0.03$.

between the two extenders (A: 65 ± 7%, I: 54 ± 7%, respectively; NS).

4. Discussion

The objective of the present study was to identify a suitable extender for alpaca spermatozoa that does not contain material of animal origin, using epididymal spermatozoa obtained from organs discarded after castration. Results from this study indicate viable spermatozoa could be collected from the organs after overnight transport to the laboratory (i.e., that cooled storage of the organs overnight does not impair sperm viability), and that both of the extenders evaluated could be suitable for the processing of spermatozoa of this species.

In previous studies, a variety of home-produced or commercially available extenders have been used for alpaca spermatozoa. Santiani et al. (2005) concluded that use of tris-citrate and skimmed milk diluents produced approximately equivalent results for alpaca epididymal spermatozoa. In contrast, following a comparison of citrate, tris- and lactose-based diluents, Morton et al. (2007) recommended a lactose-based diluent. Morton et al. (2009), however, reported that the use of the commercially available extender, Biladyl, resulted in maintenance of alpaca epididymal spermatozoa viability for 24 h. According to a review by Bravo et al. (2013), of the 15 extenders reported to be used for alpaca spermatozoa or semen, only $^{1}/_{3}$ resulted in sperm motility of more than 30 % after 24 h. In studies comparing a range of different extenders for ejaculated dromedary camel semen, a variety of extenders were evaluated and different extenders were considered to be optimal for storage in each study (Al-Bulushi et al., 2019; Swelum et al., 2019). It should be noted that the experimental conditions for the different studies were different from those in the present study. In ther present study, epididymal spermatozoa were extracted from the organs approximately 24 h after the donors were castrated. The relatively lesser motility (29 %–35 % after 30 min incubation) might be due to the prolonged period spent in unfavorable conditions before extraction. The method for transporting the organs was the same as the one used in the laboratory where the present study was conducted for transporting canine organs for studies on preservation of dog spermatozoa (Korochkina et al., 2014). It, however, was noticeable that mean membrane integrity was much greater than mean motility for these samples and the two sets of measurements were not correlated. It, therefore, might be beneficial to adjust the settings for the CASA instrument to provide a lesser threshold for immotile/motile spermatozoa than the one used in the preent study. Alternatively, the components needed for maximum motility in alpaca spermatozoa may not have been provided by the extenders used because these components were not originally developed for this species. This aspect warrants further study.

Results from the present study indicated that both of these extenders could be used to maintain alpaca epididymal spermatozoa during storage, although further research would be necessary to evaluate sperm functionality, either in an insemination study or at least with an oocyte binding assay. It was not possible to use a binding assay in the present study due to the unpredictability of the supply of organs. In Sweden, alpacas are castrated at a relatively young age, and <10 % of the organs utilized in the present study actually contained spermatozoa. The results from the present study are derived only from the organs that contained spermatozoa. Although material from castrations should theoretically have provided a useful source of epididymal spermatozoa with which to test various extenders, in practice it was not sufficiently reliable for extensive studies to be conducted. In countries where alpacas are slaughtered for meat, it should be possible to obtain a regular supply of organs from a slaughterhouse (e.g., Santiani et al., 2016) for such experiments.

5. Conclusion

Viable epididymal spermatozoa could be obtained from the material from castrations even when there is need for overnight transport. There were no differences in sperm quality between the two extenders; therefore, it appears that either extender could be used for alpaca spermatozoa. Spermatozoa motility, however, might be increased if a different extender were used; therefore, further research is needed in this species to ascertain whether there is a more effective extender for this purpose.

Author's contributions

EAE and CA extracted the epididymal spermatozoa and evaluated sperm quality, under the guidance of PK and EA. KdV and RB provided the contacts for the material and were part of the supervisory team. JMM conceived the idea, obtained the funding, designed

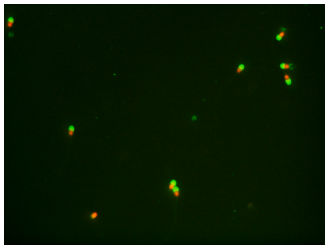

Fig. 2. Alpaca epididymal spermatozoa stained with Fitc-PNA.
Notes: The intact acrosomal cap fluoresces green in this preparation. One spermatozoon (bottom left) has lost its acrosome.

the experiment and drafted the manuscript. All authors read and approved the final version.

Declaration of Competing Interest

The authors have no conflicting interests to declare

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