Effect of enzyme treatment and mechanical removal of alpaca (*Vicugna pacos*) seminal plasma on sperm functional integrity

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Abstract

The highly viscous nature of camelid semen presents a major challenge to the development of AI technology. To facilitate the development of this technology, reduction of alpaca semen viscosity was investigated using a variety of mechanical and enzymatic treatments. Centrifugation recovered 62.7 ± 33.4% of sperm without affecting sperm motility (50.0 ± 5.8% vs. 66.7 ± 6.7%) or acrosome integrity (87.6 ± 3.1% vs. 78.9 ± 4.7%; $P > 0.05$). Processing through a 45:22.5 % PureSperm® gradient (compared with a 90:45 % gradient), recovered more sperm (70.2 ± 14.1% and 68.5 ± 9.8% vs. 17.3 ± 5.5% and 19.1 ± 4.9 %, 0.5 and 1.0 mL layers, respectively) with a higher motility (38.2 ± 6.5% and 34.0 ± 5.5% vs. 19.1 ± 4.7% and 28.2 ± 6.5%). Enzyme treatment (collagenase, papain and trypsin; 0.5, 1.0, 2.0 and 4.0 mg/mL for 10-60 min) reduced semen viscosity ($P < 0.05$) but was faster for papain (all concentrations) and 4.0 mg/mL collagenase and trypsin (< 10 min; $P < 0.05$). All concentrations of collagenase reduced sperm motility ($P < 0.05$), while all concentrations of papain reduced sperm acrosome integrity ($P < 0.05$). Collection of semen into Tris diluent plus papain reduced semen viscosity (1.3 ± 1.0 mm) compared with Tris with chymotrypsin (11.3 ± 4.9 mm) or Tris diluent alone (10.8 ± 3.0 mm; $P < 0.05$) without affecting sperm motility (Tris+papain: 47.5 ± 7.1%; Tris+chymotrypsin: 44.5 ± 6.4%; Tris diluent alone: 40.5 ± 6.2%; $P > 0.05$). Sperm acrosome integrity was reduced when semen was collected into Tris+papain (41.6 ± 14.3%) compared Tris+chymotrypsin (74.0 ± 12.3%) and Tris diluent (85.0 ± 2.6%; $P < 0.05$). Papain efficiently reduced alpaca semen viscosity but detrimental effects on sperm integrity were observed suggesting that further research is required to develop at efficient method to reduce alpaca semen viscosity.

Keywords: Artificial insemination, Camelid, semen, viscosity

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Introduction

Camelid semen is characterissed by a low volume, high viscosity with low numbers of sperm that display oscillatory rather than forward progressive motility (reviewed by Morton et al., 2008). Many researchers cite the viscous nature of camelid semen as the major hindrance that prevents the development of semen preservation and artificial insemination (AI) technology (reviewed by Bravo et al., 2000b).

Viscous semen is not unique to camelids, in fact it is common amongst mammals including non-human primates, humans and rodents (Robert and Gagnon, 1999). However, most animals which have viscous semen, such as pigs and horses, have a 'gel fraction' that acts as a vaginal plug. The vaginal plus is believed to be essential in preventing outflow of semen from the female's vagina (rodents, primates) or cervix (pig, horses). However, the physiological significance of coagulating semen is still under discussion in the literature (Robert and Gagnon, 1999) although it is thought to primarily exist to prevent subsequent insemination of sperm my non-dominant males. Vaginal plugs may also represent a reservoir and contribute to the gradual release of sperm in species where the female does not have a long cervix or certical mucus, and semen is deposited directly in to the uterus (Robery and Gagnon, 1999).

Human semen spontaneously coagulates into a semi-solid gelatinous mass, following ejaculation and also spontaneously liquefies, owing to the presence of chymotrypsin (Cohen and Aafjes, 1982), trypsin (Mortimer, 1984) and a chymotrypsin-like protease secreted from the prostate, known as prostate-specific antigen (PSA; Robert and Gagnon, 1999). However, a portion of human semen samples are hyper viscous (do not spontaneously liquefy; Mortimer, 1984) similar to camelid semen which can take up to 24 hours to liquefy when incubated at 37°C (Garnica et al., 1993).

Hyper viscous human semen samples must be liquefied prior to preservation using either mechanical or enzymatic methods (Mortimer, 1984). Mechanical methods include needling (the passing of semen back and forth through a needle), mixing viscous semen with culture medium, or gradient density centrifugation. Mixing of the culture medium with the semen is achieved by swirling the container, non-vigorous agitation or gentle pipetting through a wide-bore serological or Pasteur pipette (Mortimer, 1984). Enzymatic methods of liquefying viscous human semen include treatment with hydrolytic enzymes such as bromelain (Tucker et al., 1990), chymotrypsin (Tucker et al., 1990; Bollendorf et al., 1994), papain (Pattinson et al., 1990a), subtilisin (Pattinson et al., 1990a) and trypsin (Cohen and Aafjes, 1982).

Enzymatic methods have been used to liquefy camelid semen (Callo et al., 1999; Bravo et al., 1999; 2000a) with few reported detrimental effects after the use of collagenase, fibrinolysin, hyaluronidase and trypsin (Bravo et al., 2000ab) although their use within the scientific community remains limited.

Mechanical methods of semen liquefaction have not been used extensively with camelid semen. Santiani et al. (2005) used needling prior to the cryopreservation of alpaca semen but the authors did not quantify the effects on semen viscosity, sperm motility or sperm functional integrity. Needling has, however, been successfully used to partially liquefaction of human semen, although it is not recommended for samples where sperm will be assessed for functionality or integrity (Mortimer, 1984) as the high shear forces experienced by sperm are deleterious and result in a marked deterioration in sperm motility (Knuth et al., 1986). In addition, Vaughan et al. (2003) observed that vortexing was highly detrimental to alpaca sperm and did not reduce semen viscosity. However, Morton et al. (2008) observed that gentle pipetting of semen in a diluent was effective in reducing semen viscosity without compromising sperm motility or viability. While this is a simple and fairly effective method, it does not completely eliminate semen viscosity and further research is required to develop a suitable procedure.

The aims of the present study were therefore to investigate mechanical methods of separating sperm from the viscous seminal plasma (centrifugation and density gradient centrifugation) and enzymatic methods of liquefying alpaca semen (the addition of enzymes to neat semen and the collection of semen into Tris diluent supplemented with enzymes).

Materials and methods

Procedures described herein were approved by The University of Sydney's Animal Ethics Committee and performed in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

Animals

Male alpacas (*Vicugna pacos,* n=16; 7.03 ± 0.6 years old; range: 5.0 – 11.8 years) weighing 72.2 ± 0.9 kg (range: 64.0 – 79.0 kg) were maintained under field conditions at The University of Sydney's Animal Reproduction Unit, Cobbitty, NSW, Australia (latitude 34°01'S, longitude 150°40'E) for the duration of the experimental period (March and November 2007) were used for semen collection. Male aplacas were housed in two group of eight in half-acre paddocks. Alpacas were exposed to natural day length and ambient temperatures between, maintained on native pastures and supplemented with Lucerne hay (*Medicago sativa*), commercially formulated alpaca

pellets and had *ad libitum* access to water.

Reagents and media

Analytical grade, and where possible, cell culture tested chemicals were used. Unless otherwise stated, media components were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and media were prepared using tissue-culture grade water (Millipore, North Ryde, Australia), filtered (0.22 μm; Millipore) and frozen at –20 °C until use (within 8 wk).

Semen collection

Semen was collected using a previously published method (Morton et al., 2008; 2010). Briefly, semen was collected using an artificial vagina (AV) fitted inside a mannequin. The AV consisted of a rubber sheep AV (IMV Technologies, L'Aigle, France) with a silicone liner (constructed in house; Morton et al., 2008, 2010) and a camel collecting glass (IMV Technologies). An electric heating pad (Bodyzone™ Heat Pad HP700; Breville, Botany, NSW, Australia) was wrapped around the AV and mounted inside the wooden mannequin.

Semen evaluation and assessment of sperm characteristics

Semen was retrieved from the AV by repeated, sharp downward thrusts to dislodge the viscous semen from the AV liner and force it into the collecting glass. Semen was held at ambient temperature (26 – 28 °C) until assessment (within 10 min of collection). Ejaculate volume, semen viscosity, sperm concentration, sperm motility, acrosomal status and morphology were then determined.

Ejaculate volume was determined using the graduations on the collecting glass, and semen viscosity was determined using the thread formation technique (Morton et al., 2008; 2010). Briefly, 50 μL neat semen was removed from the ejaculate using a micropipette and approximately 25 μL was pipetted on to a glass slide. The pipette was slowly raised to form a thread of semen until the thread of semen broke. The distance between the pipette and the slide at the time the thread broke was measured using a ruler and recorded as the viscosity.

Sperm motility patterns were classified as either oscillatory or forward progressive. Sperm displaying forward progressive motility when the distance traveled was greater than one body length per second, and sperm that did not display forward motility were considered oscillatory. Sperm motility was estimated subjectively to the nearest 5% by examining five fields of the neat semen sample (10 μL) placed on a pre-warmed slide using phase contrast microscopy (X 100, Evans and Maxwell, 1987). Sperm concentration was determined using a haemocytometer (Evans and Maxwell, 1987).

Acrosome integrity was assessed by fluorescent isothiocyanate-conjugated peanut agglutin (FITC-PNA; Morton et al., 2007, 2008). Briefly, an aliquot of sperm was

smeared on a slide, air dried and fixed for 30 sec in 96% (v:v) ethanol. Slides were then stored at 4°C for 2 - 4 weeks. Immediately before assessment, slides were stained with 100 µg/mL FITC-PNA (Sigma) in phosphate buffered saline (Sigma) for 30 min in a humid 37 °C atmosphere. Slides were then rinsed with PBS to remove excess stain and placed in the dark at 37°C to dry. Fade retardant consisting of 90% (v:v) glycerol (Sigma), 10% (v:v) PBS (Sigma) and 0.1% (w:v) *p*-phenylenediamine (Sigma) was then placed on the stained slide and the area covered with a cover slip. Sperm were considered acrosome intact if the acrosome stained green while those with no staining or a single band of green staining at the equatorial segment were considered as having non-intact acrosomes.

Sperm were assessed for gross structural abnormalities after gluteraldehyde-fixation. Briefly, 50 µL neat semen was placed in a light-proof graduated microtube (Scientific Specialties Inc., Lodi, CA, USA), diluted 1:1 with 8% (v:v) gluteraldehyde in PBS (Sigma), mixed thoroughly by vortexing (10-20s) and stored at 4°C for 1-2 weeks. For examination, 10 µL of fixed sperm suspension was placed on a clean glass slide and examined (X 400 or X 1000). One hundred sperm per sample were examined and divided into five categories: normal morphology, abnormal heads, abnormal midpieces, abnormal tails, and cytoplasmic droplets (Evans and Maxwell, 1987).

Experimental design

Experiment 1: centrifugation

Semen collected from males (n=3; 3 ejaculates per male) was assessed for quality parameters, diluted 1:4 (v/v) with Androhep® and centrifuged (600 *g* for 7 min). The supernatant was removed and the pellet resuspended in 500 µL Androhep®. Sperm motility, concentration and acrosomal integrity were then assessed.

Experiment 2: PureSperm® gradient centrifugation

Semen was collected from males (n=9; 4 ejaculates per male) into collecting glasses containing no diluent (control), Androhep® (AH; Minitüb, Tiefenbach, Germany), Tris, or Skim-milk diluent. Androhep® was prepared according to the manufacturers instructions (pH adjusted to 7.2). Tris diluent was modified from that described by Evans and Maxwell (1987) and consisted of 300 mM Tris (hydroxymethyl)aminomethane, 27.8 mM fructose, 94.7 mM citric acid, and 33.3 mM glucose supplemented with 0.3% (w:v) bovine serum albumin (BSA; Sigma A-9647) and ultra-heat treated (UHT) skim-milk was used as the skim-milk diluent (Evans and Maxwell, 1987). Diluents were warmed to 37°C, 500 µL placed into the collecting glass prior to the AV being fitted inside the mannequin. AVs containing diluents were randomLy allocated to pens and the

males allowed to mate with the mannequins. Mating duration was measured with a stopwatch. The male mounting the mannequin was considered the beginning of mating and when the male stood up from the mannequin for more than one minute and displayed no further interest in the mannequin, mating was considered to have ceased.

Immediately after the end of semen collection, semen quality parameters were assessed and semen (200-500 µL) was then layered onto 45:90% or 22.5:45% PureSperm® gradients constructed using layers of consisting of 0.5 or 1.0 mL. Samples were then centrifuged at 600 *g* for 20 min. The sperm pellet was then resuspended in 200 µL Androhep® and sperm motility, concentration and acrosome integrity assessed.

Experiment 3: enzyme addition post semen collection

Semen was collected from males (n=4; 6 ejaculates per male) and semen quality parameters were assessed as described above. Semen was then carefully and slowly divided in to 13 aliquots and diluted 1:1 (v/v) with Androhep® (control), or Androhep® supplemented with 1.0, 2.0, 4.0 or 8.0 mg/mL collagenase (Type IA, EC 3.4.24.3; C9722, Sigma), papain (EC 3.4.22.2; P5306 Sigma), or trypsin (EC 3.4.21.4, T9935, Sigma; final enzyme concentrations 0.5, 1.0, 2.0, or 4.0 mg/mL). Semen viscosity, sperm motility and acrosome integrity were assessed at 5, 10, 20 and 40 min after enzyme addition.

Experiment 4: collection of semen into Tris diluent supplemented with chymotrypsin and papain.

Semen was collected from males (n=11; 3 ejaculates per male) into collecting glasses containing Tris diluent (control) or Tris diluent supplemented with chymotrypsin (α-chymotrypsin type IV-S, EC 3.4.21.1; CHY5S-10VL, Sigma) or papain (Sigma). To ensure that ejaculates from different males were exposed to approximately the same concentration of enzymes (0.5 mg for chymotrypsin and papain), the initial concentration of the enzymes was adjusted based on the average of previous ejaculate volumes. Chymotrypsin and papain were diluted to 5.5 mg/mL and 10 mg/mL respectively with Tris diluent and stored at -20 °C until use. Immediately before use, the enzymes were diluted to the concentrations determined for each male based on average semen volume with Tris diluent and warmed to 37 °C. Warm diluent (500 µL) was placed in the collecting glass and the AV fitted inside the mannequin. AVs containing the different diluents were randomly allocated to pens and the males allowed to mate with the mannequins. Semen quality parameters were assessed immediately after semen collection. Semen was then layered onto a PureSperm® gradient (0.5 mL 22.5: 0.5mL 45 %) and centrifuged at 600 *g* for 20 min. The sperm pellet was then resuspended in 200 µL

Androhep® and sperm motility, concentration and acrosome integrity were assessed.

Experiment 5: effect of papain addition on the protein profiles of alpaca seminal plasma

Semen was collected from males (n=7; 3 ejaculates per male) and centrifuged (1000 *g* for 10 min) to remove sperm and cellular debris and stored at –20 °C. Samples were then thawed and centrifuged twice (4000 *g* for 25 min) to further remove cellular material and stored at –20 °C until analysis. Samples were then pooled by month (n=6 per month) and male (n=3 per male) and divided into two aliquots. Papain (0.5 mg/mL; Sigma) was then added to half of the aliquots. Samples (28 μL, final protein concentration 1 mg/mL) were then diluted in loading buffer (62.5mM Tris HCL, 10 % (v/v) glycerol, 5 %(v/v) 2-mercaptoethanol, 2 % (v/v) SDS, 0.02 % (w/v) bromophenol blue) before 1D separation on a 12 % acrylamide gel (Mini-protean II apparatus, 7 cm length, BIO-RAD, Regents Park, NSW, Australia). Proteins were separated for 75 min at a constant voltage of 125V. Proteins were visualized by overnight staining in Sypro Ruby (BIO-RAD, Regents Park, NSW, Australia).

Statistical analysis

Statistical differences were determined after log (base *e*) transformation of semen viscosity, sperm concentration and total number of sperm in the ejaculate, and logit transformation of sperm motility, acrosome integrity, and sperm morphology. Data were analysed using a linear mixed model (REML) procedure with fixed effects specified for the factor of interest (e.g. PureSperm® group, gradient size, enzyme, enzyme concentration, exposure time) and collection day (Experiment 4) and a random effect for male to account for repeated observations. Significance of the treatment effects was assessed by Wald Chi-square tests, and pairs of means compared using least significant differences (LSD) with $P < 0.05$ considered significant. GenStat (Release 10, Lawes Agricultural Trust, Rothamsted, UK) was used for the analysis. Data are presented as mean±SEM unless otherwise stated.

Results

Experiment 1: centrifugation

Sperm motility was similar before (50.0 ± 5.8 %) and after (66.7 ± 6.7 %) centrifugation and 62.73 ± 33.4 % of sperm were recovered. Sperm acrosome integrity was not affected by centrifugation (before: 87.6 ± 3.1 %; after: 78.9 ± 4.7%; $P > 0.05$). Removal of the seminal plasma supernatant was difficult as the semen did not liquefy, and attempts to remove the supernatant resulted in turbulence that disrupted the sperm pellet.

Experiment 2: density gradient centrifugation using PureSperm®

Motility and acrosome integrity for sperm, prior to, and after centrifugation through 90:45% and 45:22.5% PureSperm® gradients

Table 1. Sperm motility and recovery rates after centrifugation through 90:45, and 45:22.5 % PureSperm® gradients made with 0.5 or 1.0 mL layers.

PureSperm® gradient (%)	Layer volume (mL)	Pre-PureSperm®		Post-PureSperm®		
		Sperm motility (%)	Acrosome integrity (%)	Sperm motility (%)	Acrosome integrity (%)	Sperm recovery rate (%[#])
90: 45	0.5	41.8 ± 6.5	86.2 ± 4.0	19.1 ± 4.7[a]	83.0 ± 3.5[a]	19.1 ± 4.9[a]
	1.0			28.2 ± 6.5[a]	82.9 ± 3.6[a]	17.3 ± 5.5[a]
45: 22.5	0.5			38.2 ± 6.5[b]	81.9 ± 2.2[a]	70.2 ± 14.1[b]
	1.0			34.0 ± 5.5[b]	88.8 ± 1.4[a]	68.5 ± 9.8[b]

[#]Sperm recovery rate was calculated by dividing the total number of sperm recovered from the pellet after centrifuging by the total number of sperm layered on top of the PureSperm® gradient. Values within a column with a different superscript are significantly different (P<0.05).

comprising of 0.5 and 1.0 mL layers are presented in Table 1. The volume of the PureSperm® layers (0.5 or 1.0 mL) did not affect sperm motility, acrosome integrity or sperm recovery rate (P > 0.05). However, sperm motility and sperm recovery rate were higher (P < 0.05) after processing through 45:22.5% PureSperm® gradients compared with 90:45% gradients (Table 1). Acrosome integrity was similar for sperm processed through either gradient (90:45% or 45:22.5%) and was similar before and after PureSperm® processing (P > 0.05).

Experiment 3: addition of collagenase, papain and trypsin to alpaca neat alpaca semen

Semen viscosity after dilution in Androhep® (control), or Androhep® supplemented with 0.5, 1.0, 2.0, or 4.0 mg/mL collagenase, papain and trypsin are presented in Table 2. Semen viscosity was reduced, compared with the control, ten minutes post-addition of 4.0 mg/mL collagenase, 4.0 mg/mL trypsin and any concentration of papain (P < 0.05). Similar patterns were observed 10 and 20 min post-treatment. By 60 min post-treatment 4.0 mg/mL collagenase, all concentrations of papain and 2.0 and 4.0 mg/mL trypsin had reduced semen viscosity as compared with the control (P < 0.05).

Semen viscosity was reduced after 40 min of incubation in the control group (diluted in Androhep®). However, semen did not completely liquefy within the 40 min period. For semen treated with 1.0-4.0 mg/mL collagenase, semen viscosity was reduced after 20 min. However, the viscosity of semen treated with 0.5 mg/mL collagenase was not reduced during the 60 min observation period (P > 0.05).

Treatment of semen with all concentrations of papain resulted in an immediate reduction of semen viscosity (P < 0.05; Table 2). For 0.5, 1.0 and 2.0 mg/mL trypsin treated semen, viscosity continued to be reduced until 40 min post-trypsin

Table 2. Semen viscosity (mm) after treatment of semen with 0.5, 1.0, 2.0 and 4.0 mg/mL collagenase, papain and trypsin.

Enzyme	Conc. (mg/mL)	Times (min) 5	10	20	40
Control		3.3 ± 0.8^{aA}	3.3 ± 0.7^{aA}	1.8 ± 0.5^{aB}	1.4 ± 0.5^{abB}
Collagenase	0.5	2.7 ± 0.8^{aA}	2.4 ± 0.8^{abA}	2.2 ± 0.7^{aA}	1.7 ± 0.7^{aA}
	1.0	2.9 ± 0.7^{aA}	1.9 ± 0.7^{bA}	1.7 ± 0.7^{abB}	1.4 ± 0.7^{abB}
	2.0	2.2 ± 0.6^{abA}	1.9 ± 0.6^{bA}	1.8 ± 0.7^{abAB}	0.8 ± 0.5^{abB}
	4.0	1.4 ± 0.6^{cAB}	2.1 ± 0.7^{abA}	0.8 ± 0.4^{bcB}	0.3 ± 0.2^{bcB}
Papain	0.5	0.5 ± 0.4^{cdA}	0.0 ± 0.0^{cA}	0.0 ± 0.0^{cA}	0.0 ± 0.0^{cA}
	1.0	0.5 ± 0.3^{cdA}	0.0 ± 0.0^{cA}	0.0 ± 0.0^{cA}	0.0 ± 0.0^{cA}
	2.0	0.4 ± 0.4^{dA}	0.3 ± 0.3^{cA}	0.5 ± 0.5^{cA}	0.6 ± 0.6^{bcA}
	4.0	0.0 ± 0.0^{dA}	0.7 ± 0.5^{cA}	0.0 ± 0.0^{cA}	0.0 ± 0.0^{cA}
Trypsin	0.5	3.2 ± 0.8^{aA}	2.8 ± 0.6^{abA}	2.0 ± 0.7^{aAB}	1.5 ± 0.5^{aB}
	1.0	3.7 ± 0.8^{aA}	3.1 ± 0.8^{aA}	2.5 ± 0.8^{aA}	1.2 ± 0.6^{abB}
	2.0	2.2 ± 0.6^{aA}	2.3 ± 0.7^{abA}	1.6 ± 0.6^{abA}	0.4 ± 0.3^{bcB}
	4.0	1.6 ± 0.3^{bA}	1.7 ± 0.5^{bA}	0.6 ± 0.3^{cB}	0.2 ± 0.2^{bcB}

Values within a row (A,B) or column (a,b) with a different superscript are significantly different ($P<0.05$).

addition compared with 4.0 mg/mL trypsin treated semen which was reduced at 20 min.

Sperm motility was not reduced by the addition of 0.5 - 4.0 mg/mL papain or trypsin at 5 or 10 min post-enzyme addition. However, sperm motility was reduced ($P < 0.05$) by the addition of collagenase (all concentrations) as early as 5 min post collagenase addition (Table 3). Addition of higher (2.0 and 4.0 mg/mL) collagenase reduced sperm motility compared with 0.5 and 1.0 mg/mL at 5 min, but there was no difference in sperm motility between collagenase concentrations at 10, 20 and 40 min ($P > 0.05$).

Within each time point, sperm motility was not affected by the concentration of papain or trypsin ($P > 0.05$). However, sperm motility was reduced by 20 min after papain addition (all concentrations; $P < 0.05$) and had dropped again by 40 min for 1.0 and 2.0 mg/mL papain ($P < 0.05$; Table 4). A similar difference was observed for trypsin.

Sperm acrosome integrity after the addition of collagenase, papain and trypsin is presented in Table 4. The addition of 0.5 - 4.0 mg/mL collagenase did not reduce sperm acrosome integrity at 5, 10, 20 or 40 min compared with the control ($P > 0.05$). The addition of 4.0 mg/mL trypsin reduced acrosome integrity at 20 and 40 min compared with the control, but 0.5 and 1.0 mg/mL trypsin did not affect sperm acrosome integrity at any time ($P > 0.05$).

Table 3. Sperm motility (%) after treatment of semen with 0.5, 1.0, 2.0 and 4.0 mg/mL collagenase, papain and trypsin.

Enzyme	Conc. (mg/mL)	Time (min) 5	10	20	40
Control		49.0 ± 3.6^{aA}	32.3 ± 4.1^{aAB}	16.9 ± 4.5^{aB}	8.7 ± 2.8^{aB}
Collagenase	0.5	3.8 ± 1.1^{bA}	0.0 ± 0.0^{bB}	0.0 ± 0.0^{bB}	0.0 ± 0.0^{bB}
	1.0	3.8 ± 1.2^{bA}	0.6 ± 0.6^{bB}	0.7 ± 0.7^{bB}	0.4 ± 0.4^{bB}
	2.0	1.4 ± 0.6^{c,A}	0.0 ± 0.0^{bB}	0.0 ± 0.0^{bB}	0.0 ± 0.0^{bB}
	4.0	0.4 ± 0.4^{dA}	0.0 ± 0.0^{bA}	0.0 ± 0.0^{bA}	0.0 ± 0.0^{bA}
Papain	0.5	48.3 ± 3.2^{aA}	30.4 ± 3.8^{aA}	16.5 ± 4.2^{aB}	7.4 ± 2.3^{aceB}
	1.0	42.7 ± 4.0^{aA}	27.3 ± 2.9^{aAB}	16.7 ± 4.4^{aBC}	8.8 ± 2.5^{aceC}
	2.0	42.5 ± 3.2^{aA}	25.8 ± 4.7^{aAB}	15.0 ± 4.3^{aB}	7.7 ± 3.0^{aceC}
	4.0	40.0 ± 4.1^{aA}	26.3 ± 4.7^{aAB}	15.4 ± 3.4^{aB}	10.2 ± 2.6^{aeB}
Trypsin	0.5	43.5 ± 2.8^{aA}	28.9 ± 4.0^{aAB}	15.8 ± 3.8^{aB}	8.3 ± 3.1^{cdC}
	1.0	43.6 ± 3.2^{aA}	25.0 ± 4.2^{aAB}	12.9 ± 3.2^{aB}	7.3 ± 2.7^{cdC}
	2.0	41.0 ± 3.0^{aA}	23.8 ± 3.2^{aAB}	14.2 ± 2.6^{aBC}	7.4 ± 1.4^{ceC}
	4.0	35.6 ± 2.9^{aA}	25.6 ± 4.2^{aAB}	17.3 ± 3.3^{aAB}	10.0 ± 1.7^{eB}

Values within a row (A,B) or column (a,b) with a different superscript are significantly different (P<0.05).

Table 4. Sperm acrosome integrity (%) after treatment of semen with 0.5, 1.0, 2.0 and 4.0 mg/mL collagenase, papain and trypsin.

Enzyme	Conc. (mg/mL)	Time (min) 5	10	20	40
Control		90.6 ± 1.9^{aA}	86.2 ± 2.4^{abA}	87.7 ± 3.5^{aA}	76.7 ± 6.7^{abB}
Collagenase	0.5	87.8 ± 2.0^{aA}	87.2 ± 2.7^{abA}	87.2 ± 3.5^{aA}	79.3 ± 6.7^{abA}
	1.0	88.1 ± 2.3^{aA}	89.7 ± 1.4^{aA}	75.9 ± 9.8^{abA}	75.7 ± 8.7^{abA}
	2.0	89.4 ± 2.6^{aA}	89.0 ± 1.8^{abA}	90.9 ± 2.5^{aA}	81.1 ± 6.1^{abA}
	4.0	89.8 ± 2.7^{aA}	85.0 ± 5.4^{abA}	88.2 ± 3.0^{aA}	90.1 ± 1.4^{aA}
Papain	0.5	55.1 ± 8.5^{abA}	36.2 ± 8.7^{bcA}	41.0 ± 9.0^{abA}	29.3 ± 9.9^{bA}
	1.0	54.1 ± 9.7^{abA}	40.7 ± 10.1^{cA}	37.3 ± 8.3^{bcA}	12.7 ± 5.4^{bB}
	2.0	43.6 ± 8.8^{abA}	15.9 ± 8.9^{dB}	25.4 ± 8.1^{cB}	16.3 ± 7.2^{bAB}
	4.0	29.6 ± 9.1^{bA}	18.0 ± 8.8^{dB}	20.6 ± 11.3^{dB}	5.6 ± 4.4^{cC}
Trypsin	0.5	86.2 ± 2.5^{aA}	85.3 ± 2.6^{abA}	75.4 ± 5.9^{aA}	74.6 ± 5.6^{abA}
	1.0	84.8 ± 3.7^{aA}	88.3 ± 1.5^{aA}	86.2 ± 4.0^{aA}	70.1 ± 9.2^{abA}
	2.0	74.2 ± 10.2^{abA}	78.9 ± 10.5^{abcA}	73.9 ± 10.2^{abA}	73.9 ± 10.6^{abA}
	4.0	65.9 ± 12.3^{abA}	63.2 ± 11.0^{abcA}	65.0 ± 13.0^{bA}	62.7 ± 12.3^{bA}

Values within a row (A,B) or column (a,b) with a different superscript are significantly different (P<0.05).

The addition of 4.0 mg/mL papain reduced acrosome integrity ($P < 0.05$) at 5 min compared with the control. By 10 min, all concentrations of papain had reduced acrosome integrity ($P < 0.05$) compared with the control, and the higher concentrations of papain (2.0 and 4.0 mg/mL) resulted

in higher levels of damage ($P < 0.05$) than the lower concentrations (0.5 and 1.0 mg/mL). At 20 and 40 min, 4.0 mg/mL papain produced more damage to sperm acrosomes than 2.0, 1.0 or 0.5 mg/mL papain ($P < 0.05$).

Sperm acrosome integrity was not reduced by increasing exposure time for 0.5 – 4.0 mg/mL collagenase and trypsin ($P > 0.05$). Acrosome integrity was not affected by exposure time for sperm treated with 0.5 mg/mL papain but was reduced at 40 min for sperm treated with 1.0 mg/mL, and at 10 min for sperm treated with 2.0 and 4.0 mg/mL papain ($P < 0.05$).

Experiment 4: collection of semen into enzyme supplemented Tris diluent

Collection of semen into Tris diluent supplemented with papain significantly reduced semen viscosity and sperm acrosome integrity ($P < 0.05$) compared with semen collected into Tris diluent or Tris diluent supplemented with chymotrypsin ($P > 0.05$; Table 5). Mating length, semen volume, sperm concentration, total number of sperm in the ejaculate and sperm motility were similar ($P > 0.05$; Table 5) between treatments.

Collection of semen into Tris diluent supplemented with chymotrypsin or papain increased the proportion of morphologically normal sperm, and reduced the proportion of sperm with tail abnormalities ($P < 0.05$) compared with the control. The proportion of sperm displaying head or midpiece abnormalities were unaffected by collection of semen into Tris diluent, or Tris diluent supplemented with chymotrypsin or papain ($P > 0.05$).

Sperm recovery rate after PureSperm® processing was similar for sperm collected in Tris diluent, or Tris diluent supplemented with chymotrypsin or papain ($P > 0.05$; Table 6). Sperm motility was similar for all treatments and there was a trend for sperm motility to increase after PureSperm® processing ($P > 0.05$). After PureSperm® processing, sperm acrosome integrity was higher for semen collection in Tris diluent and Tris diluent supplemented with chymotrypsin compared with Tris diluent supplemented with papain ($P<0.05$).

Experiment 5: effect of papain addition on the protein profiles of alpaca seminal plasma

The protein profiles of pooled alpaca semen from March, April and May with or without papain treatment are presented in Figure 1. The results clearly demonstrate that papain has broken down the large molecular weight proteins into smaller molecular weight protein by-products with the 'new' protein band around 23kDa being most likely papain.

Table 5. Mating length and quality parameters of fresh alpaca semen after collection using an artificial vagina and collecting glass containing Tris diluent alone (control) or supplemented with chymotrypsin or papain.

Parameter	Control	Chymotrypsin	Papain
Mating length (min)	17.0 ± 2.2[a]	18.8 ± 3.4[a]	18.8 ± 4.2[a]
Semen volume (mL)	1.1 ± 0.4[a]	0.7 ± 0.2[a]	0.7 ± 0.1[a]
Semen viscosity (mm)	10.8 ± 3.0[a]	11.3 ± 4.9[a]	1.3 ± 1.0[b]
Sperm concentration (x10^6/mL)	382.2 ± 199.6[a]	107.7 ± 32.7[a]	125.2 ± 44.7[a]
Total no. sperm in ejaculate (x10^6)	99.6 ± 20.4[a]	77.4 ± 26.1[a]	58.2 ± 14.7[a]
Sperm motility (%)	40.5 ± 6.2[a]	44.5 ± 6.4[a]	47.5 ± 7.1[a]
Acrosome integrity (%)	85.0 ± 2.6[a]	74.0 ± 12.3[a]	41.6 ± 14.3[b]
Morphologically normal (%)	56.6 ± 3.4[a]	68.8 ± 5.3[b]	64.6 ± 5.5[b]
Head abnormalities (%)	2.4 ± 0.8[a]	2.2 ± 1.1[a]	2.2 ± 0.4[a]
Midpiece abnormalities (%)	5.6 ± 1.3[a]	5.0 ± 1.1[a]	5.9 ± 1.4[a]
Tail abnormalities (%)	14.6 ± 2.9[a]	8.2 ± 2.4[b]	7.3 ± 1.9[b]
Cytoplasmic droplets (%)	17.4 ± 2.7[a]	12.2 ± 1.8[a]	17.9 ± 3.3[a]

Values within a row with a different superscript are significantly different (P<0.05).

Table 6. Sperm motility, acrosome integrity and recovery rates after centrifugation of alpaca semen collected in Tris diluent supplemented with chymotrypsin or papain through 45: 22.5 % PureSperm® gradients

Treatment	Pre-PureSperm®		Post-PureSperm®		Sperm recovery rate (%#)
	Sperm motility (%)	Acrosome integrity (%)	Sperm motility (%)	Acrosome integrity (%)	
Control	38.3 ± 6.0[a]	95.0 ± 2.6[a]	48.3 ± 6.0[a]	83.2 ± 3.7[a]	46.0 ± 11.5[a]
Chymotrypsin	41.0 ± 7.6[a]	74.0 ± 12.3[a]	52.0 ± 8.0[a]	76.8 ± 6.2[a]	49.3 ± 12.4[a]
Papain	37.5 ± 4.8[a]	41.6 ± 14.3[b]	52.5 ± 1.4[a]	60.9 ± 4.7[b]	53.2 ± 10.6[a]

[#]Sperm recovery rate was calculated by dividing the total number of sperm recovered by the total number of sperm layered on top of the PureSperm® gradient.Values within a column with a different superscript are significantly different (P<0.05).

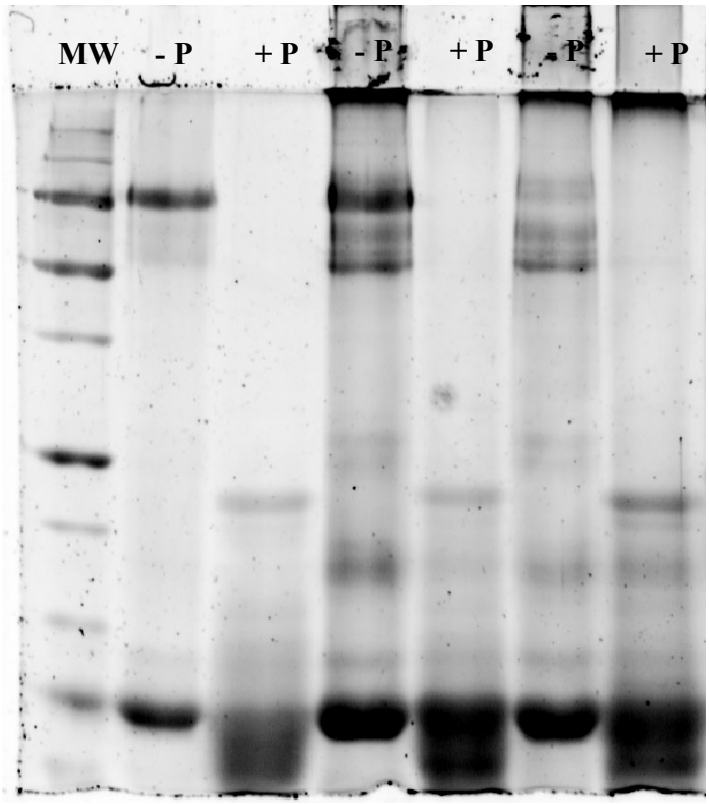

Figure 1. SDS-PAGE zymogram from pooled alpaca seminal plasma with (+P) or without (-P) papain addition.

Discussion

In the present study, various mechanical methods were trialled to separate the sperm and seminal plasma but were largely unsuccessful. Alpaca semen was successfully liquefied by enzymatic methods with the lowest concentration of papain effectively liquefying semen and causing the least damage to the sperm cells.

Centrifugation was not effective for separating sperm from the viscous seminal plasma as the turbulence created during sperm removal resulted in significant loss of cells and only an average of 60% of the sperm were recovered. Previous studies involving llama semen also observed that centrifugation had a highly deleterious effect on sperm motility immediately (not cent: 31.6%, cent: 26.6%) and 1 hr post-centrifugation (not cent: 25.0%: cent: 5.0%; Ferre et al., 2000). Furthermore, given the variation in viscosity between males and ejaculates (Vaughan et al., 2003; Morton et al., 2008), centrifugation would become a costly and time-consuming procedure in a commercial artificial breeding setting as different centrifugation times and speeds would be required for ejaculates of different viscosities. Additionally, Mortimer (1984) emphasizes that centrifugal pelleting of unselected sperm populations must be avoided under all circumstances where the sperm are to be tested for their function or used for therapeutic purposes.

Gradient centrifugation using 45:22.5% PureSperm® gradients was more effective in recovering sperm from the viscous seminal plasma than the 90:45% gradients, but was not affected by the volume of the gradient used. Recovery rates for alpaca sperm after PureSperm® gradient processing were comparable to camels (Morton et al., 2008) but higher than the 35.8% reported for cattle by Maxwell et al. (2007) using a 90:45 % gradient. Gradient centrifugation using a 45:22.5 % gradient resulted in similar sperm recovery rates compared with non-graident centrifugation, though neither were considered particularly effective for the separation of alpaca sperm from the viscous seminal plasma.

Collection of semen into Tris diluent containing the proteolytic enzyme papain significantly reduced semen viscosity but whilst chymotrypsin proved ineffective. Collection of semen into media containing an enzyme did not affect sperm motility but acrosome integrity was reduced for samples collected into Tris containing papain. Similarly, when papain was added to neat semen after collection semen, viscosity was eliminated almost immediately, sperm motility was not compromised but acrosome integrity was reduced. The reduction in semen viscosity and acrosome integrity occurred in a dose dependant manner, with less acrosome damage occurring when 0.5 mg/mL of papain was added to the semen. Compared with papain, collagenase

and trypsin did not cause as much damage to sperm acrosomes nor were they as effective in reducing semen viscosity.

Removal of papain after collection of semen into Tris diluent containing papain may prevent damage to the sperm acrosome but this was ineffective as the data showed that the damage had already occurred. These results suggest that collection of semen into diluent containing enzymes is not a suitable method for reducing semen viscosity in alpacas.

Removal of enzymes after a shorter exposure to papain, such as addition of papain in the laboratory, may be more effective. However, damage to sperm membrane integrity was observed after as little as 5 min exposure to the enzymes suggesting that the exposure period to papain must be less than this. Addition of papain to neat semen followed by immediate centrifugation and resuspension in diluent (without enzymes) might be an effective method of reducing semen viscosity but further studies are required to confirm this.

The results of the present experiments examining the effect of enzymes on alpaca semen viscosity contradict those of Bravo et al. (1999; 2000a) which utilised trypsin (0.3, 0.6, 1.25 and 2.5 % solutions; Bravo et al., 1999), collagenase (1, 5, 10 and 20 mg/mL; Bravo et al., 1999, and 0.25 mg/mL; Bravo et al., 2000a), fibrinolysin (Bravo et al., 2000a) and hyaluronidase (Bravo et al., 2000a) to liquefy alpaca and/or llama semen. Bravo et al. (2000a) reported that all concentration of collagenase and trypsin reduced semen viscosity and Bravo et al. (1999) reported that once semen was in contact with the enzymes, viscosity was eliminated. There are a number of factors which could account for the differences obseved between the present study and those of Bravo et al. (1999; 2000a) the most likely of which is the source, purity and type of enzymes used. However, sufficient detail is not available to draw definitive conclusions.

Bravo et al. (2000a) reported that, despite reduction in semen viscosity, sperm motility remained oscillatory after enzyme addition and did not display progressive motility. The results of the present study, indicated that sperm motility became more progressive as a consequence of enzyme treatment. This could be explained by the presence of a sperm motility inhibitor in alpaca semen, similar to that present in human semen (Robert and Gagnon 1995). This 52 kDa seminal plasma sperm motility inhibitor (SMI) is present at ejaculation and rapidly transformed into smaller masses during liquefaction (Robert and Gagnon, 1994). The presence of a SMI in alpaca semen has yet to be confirmed, although it could have resulted from an evolutionary need to maintain the sperm in a quiescent state from ejaculation until ovulation takes place.

Other studies examining the effects of enzymes have reported a number of interesting, and often contradictory findings. Deen et al. (2003) added α-chymotrypsin to camel semen and reported that motility was not increased but failed to quantify the effects on seminal plasma viscosity or sperm ultrastructure. Hyper viscous human semen samples are routinely treated with chymotrypsin and no differences have been observed in sperm motility, membrane integrity or morphology between samples liquefied by dilution and pipetting or treatment with chymotrypsin (Zavos et al., 1997). Shinohara et al. (1985) demonstrated that chymotrypsin treatment of hamster sperm accelerated the sperm acrosome reaction and chymotrypsin treatment of human semen reduced sperm penetration of zona free hamster oocytes (Pattinson et al., 1990a). Other authors, observed that chymotrypsin treatment of human semen did not reduce the pregnancy rate after intrauterine insemination (IUI; Tucker et al., 1990) and increased the *in vitro* fertilizing ability of viscous human semen (Cohen and Aafjes, 1982). Unfortunately, chymotrypsin was not effective in reducing semen viscosity in the concentrations examined in the present study.

In agreement with the findings of the present study, treatment of Capuchin monkey semen with trypsin and hyaluronidase did not cause any adverse effects on sperm motility, vigour and acrosome integrity, but enzymes only partially liquefied the semen (Rodrigues de Paz et al., 2006). Treatment of sperm with trypsin adversely affected the motility of bovine sperm (Wheeler and Seidel, 1989; Silva et al., 1999) and accelerated the acrosome reaction of hamster sperm (Shinohara et al., 1985), but did not reduce the penetration of zona free hamster oocytes (Pattinson et al., 1990a).

The effects of papain on sperm in the present study were dose dependent, in agreement with Pattinson et al. (1990a) who observed a dose dependent reduction in sperm vitality was observed after incubation with papain. Concentrations over 25 U/mL reduced sperm motility and papain treatment altered cell membranes (measured by eosin exclusion with a nigrosin background stain; Pattinson et al., 1990a) and sperm treated with 50 U/mL of papain displayed impaired penetration of zona-free hamster oocytes (Pattinson et al., 1990a). Of the enzymes tested by Pattinson et al. (1990ab), trypsin exhibited the most restricted specificity of the enzymes, being directed towards lysyl and arginyl residues, while chymotrypsin was also quite specific in hydrolysing phenylalanyl tyrosyl and tryptophanyl residues. The activity of papain is broader than either trypsin or chymotrypsin, being capable of cleaving the peptide bonds adjacent to most amino acids. This may explain the strong efficacy of papain to liquefy semen in the present study, and to

release sperm motility from the "bonds" of its containment by the viscous gel, and to also deleteriously affect the sperm acrosomal membrane.

One dimensional gel electrophoresis clearly demonstrated the breaking down of the large molecular weight proteins into small molecular weight protein by-products by papain. Given the effectiveness of papain to reduce semen viscosity at concentrations of 0.5 mg/mL, further reducing the concentration of papain may be beneficial as damage to sperm functional integrity could be eliminated but further studies are required to investigate this.

The favorable outcomes demonstrated by the use of papain, both by observation of semen viscosity and 1-D gel analysis of the seminal plasma, also proved that the viscous component of seminal plasma was highly susceptible to liquefaction by proteolytic enzymes, thus proving that it has a major protein component. It has been reported in the literature that the viscous semen is composed of sulphated mucopolysaccharides secreted by the bulbourethral gland (El-Nagger and Abdel-Raouf, 1977). Based on the lack of evidence for this claim, and in addition to studies determining the optimal use of proteolytic enzymes such as papain, studies are required to confirm the findings of El-Nagger and Abdel-Raouf (1977).

The results in the present study demonstrate that mechanical methods, such as centrifugation and gradient centrifugation are not effective in removing viscous seminal plasma, and that while enzymatic methods are effective at reducing semen viscosity there are detrimental effects on sperm structure and viability. Procedures for the treatment of camelid semen with enzymes require considerable optimization with respect to enzyme exposure time, enzyme concentration and choice of enzyme. Moreover, further studies are also required to identify the components responsible for the viscosity before effective, efficient and commercially viable methods for the liquefaction of alpaca semen can be developed.

Acknowledgements

This research was supported by the Rural Industries Research and Development Corporation (RIRDC) and the Australian Alpaca Association. The authors wish to thank Ms. K.J. Heasman, K.J. Bailey and S.J. Wilson for their excellent technical assistance and Mr. B. Biffin and K. Tribe for their on-farm assistance.

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