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by Kershaw, C.M., Evans, G., Rodney, R. and Maxwell, W.M.C.

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Papain and its inhibitor E-64 reduce camelid semen viscosity without impairing sperm function and improve post-thaw motility rates

C. M. Kershaw[a], G. Evans[b], R. Rodney[c], W.M.C. Maxwell[b]

[a] Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, Newport, Shropshire, UK

[b] Faculty of Veterinary Science, The University of Sydney, Camperdown, Sydney, NSW 2006 Australia

*Correspondence: Dr. C. Kershaw, Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, Newport, Shropshire, UK. Tel: +44 (0)1952815517; email: Ckershaw@harper-adams.ac.uk

Abridged Title: Cryopreservation of papain-treated alpaca semen

Abstract

In camelids, the development of assisted reproductive technologies is impaired by the viscous nature of the semen. The protease papain has shown promise in reducing viscosity, although its effect on sperm integrity is unknown. This study determined the optimal papain concentration and exposure time to reduce seminal plasma viscosity and investigated the effect of papain and its inhibitor E-64 on sperm function and cryopreservation in alpacas.

Papain (0.1mg/mL, 20 min, 37°C) eliminated alpaca semen viscosity whilst maintaining sperm motility, viability, acrosome integrity and DNA integrity. Furthermore E-64 (10µm, 5 min, 37°C) inhibited the papain without impairing sperm function.. Cryopreserved, papain-treatedalpaca spermatozoa, exhibited higher total motility rates after chilling and at 0 h and 1 h post-thaw compared to control (untreated) samples.

Papain and E-64 are effective at reducing alpaca seminal plasma viscosity without impairing sperm integrity and improve post-thaw motility rates of cryopreserved alpaca sperm. The use of papain and E-64 to eliminate the viscous component of camelid semen may aid the development of assisted reproductive technologies in camelids.

Introduction

The development of semen cryopreservation and other assisted reproductive technologies in camelids is hindered by the viscous nature of camelid seminal plasma. The highly viscous semen does not evenly homogenise with cryodiluents on mixing, preventing adequate contact between the cryoprotectants and sperm membrane during freezing. It is therefore necessary to reduce seminal plasma viscosity without impairing sperm function prior to freezing in order to improve the success and enhance the development of cryopreservation protocols in camelids.

In dromedary (Skidmore and Billah, 2006) and Bactrian (Niasari-Naslaji *et al.* 2007) camels the viscous seminal plasma partially liquefies within 20-30 min of ejaculation facilitating mixing of the diluent with the semen whereas the semen of new world camelids (alpaca, llama, vicuna and guanaco) is viscous for 18-24h after ejaculation (Garnica *et al.* 1993). The relatively rapid liquefaction of camel semen has enabled some success in sperm cryopreservation particularly in the Bactrian camel (Niasari-Naslaji *et al*, 2007) although pregnancy rates with frozen-thawed semen are still not commercially acceptable in the Dromedary (Deen *et al.* 2003). Conversely, in alpacas and llamas, cryopreservation of “non-liquefied” viscous semen is unsuccessful with low sperm motility obtained post-thaw (Adams *et al.* 2009).

The cause of the viscosity within seminal plasma is unknown. It has been postulated that glycosaminoglycans (GAGs) are responsible (Ali *et al.* 1976; Perk 1962). However, while GAGs are abundant in alpaca seminal plasma (Kershaw-Young *et al.* 2012), enzymes that degrade GAGs do not completely eliminate the viscosity of semen (Kershaw-Young *et al.* 2013) . Conversely, generic proteases including papain and proteinase K , trypsin, fibrinolysin, and collagenase (Bravo *et al.* 2000[a]; Bravo *et al.* 1999; Giuliano*, et al.* 2010; Morton *et al.* 2008) all reduce the viscosity of alpaca seminal plasma, suggesting that proteins, not GAGs, are the predominant cause of the viscosity. In Bactrian camels where seminal plasma viscosity is reportedly lower than dromedary or alpaca seminal plasma with little gelatinous material (Zhao, 2000) a reduction of viscosity via

mechanical stirring with a clip, aids the success of cryopreservation.(Niasari-Naslaji *et al.* 2007). Consequently, research on liquid and frozen storage of camelid semen has focussed on reducing the viscosity of the seminal plasma by mechanical and enzymatic methods (Bravo *et al.* 2000[a]; Bravo *et al.* 1999; Giuliano *et al.* 2010; Morton *et al.* 2008). Trypsin, fibrinolysin and proteinase K (Bravo *et al.* 2000[a]; Kershaw-Young *et al.* 2013) all have detrimental effects on sperm function and integrity. Some success has been achieved using collagenase (Conde *et al.* 2008; Giuliano *et al.* 2010) but other studies have reported deleterious effects of collagenase on sperm motility (Morton *et al.* 2008). Papain, the cysteine protease enzyme present in papaya (*Carica papaya*) has shown promise as a reducer of viscosity in seminal plasma however the acrosomes of alpaca spermatozoa were impaired when exposed to this enzyme over 10 min to 1 h at concentrations of 0.5 – 4 mg/ml (Morton *et al.* 2008). Conversely papain rapidly reduced seminal plasma viscosity with no effect on sperm motility, viability, DNA integrity or acrosome integrity when added to the viscous semen at a low final concentration of 0.1 mg/ml (Kershaw-Young *et al.* 2013).

Following enzymatic degradation of viscosity, the downstream application of cryopreservation often entails prolonged chilling of the viscosity-reduced semen over a two hour period prior to freezing, resulting in prolonged exposure of the spermatozoa to any enzymes present in the "liquefaction" diluent. Consequently, in order to overcome the negative effects of prolonged exposure to papain on the acrosome integrity of alpaca spermatozoa, it would be advantageous to inhibit the papain following liquefaction. Trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) is a protease inhibitor that binds to the active thiol group of cysteine proteases, including papain, collagenase and trypsin, substantially reducing their function (Barrett *et al.* 1982; Barrett *et al.* 1981; Tamai *et al.* 1981). The specific nature and low toxicity of this inhibitor make it a promising option for inhibiting papain and reducing the potential impacts of long term exposure on spermatozoa.

As the viscous seminal plasma is currently the major impediment to the success of cryopreservation in camelids, a reduction in seminal plasma viscosity whilst maintaining sperm function could aid freezing and thawing. Consequently the potential of papain and its inhibitor E-64 to reduce viscosity and improve motility rates after cryopreservation merits investigation.

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In order to determine the potential use of papain as a viscosity reducing enzyme in camelid semen, we investigated (1) the effect of papain concentration and time, and the inhibitor E-64, on alpaca seminal plasma viscosity and sperm function,) and (2)the effect of papain treatment of semen on the viscosity of semen and motility of alpaca sperm during and after cryopreservation.

Materials and Methods

Animals

All experiments were performed using male alpacas under authorization from the University of Sydney animal ethics committee. Animals were housed in paddocks on natural pasture with water provided *ad libitum* and their diets supplemented with Lucerne hay. All males were > 3 y, had a body condition score >3 and had testes more than 3 cm long (Tibary and Vaughan, 2006).

Experimental Design

Three experiments were conducted. Experiments 1 and 2 determined the effect of concentration and time of exposure to papain (exp 1), and the papain inhibitor E-64 (exp 2), on the viscosity of alpaca seminal plasma and sperm function. Experiment 3 investigated the effect of treatment of spermatozoa with papain (Sigma-Aldrich, St Louis, MO, USA) and E-64 (Sigma-Aldrich, St Louis, MO, USA) on the total motility of alpaca sperm during chilling, freezing and post-thaw in order to investigate the effect of enzyme reduction in viscosity on the success of alpaca sperm

cryopreservation.

Experiment 1: Optimisation of Papain Concentration and Time

Semen was collected from six male alpacas (≥2 ejaculates/male, n = 15) using an artificial vagina fitted inside a mannequin (Morton *et al.* 2010[a]). Within 5 min of collection, semen was assessed for volume, viscosity, and total motility and concentration of spermatozoa as described below. Only samples with a volume >1mL, viscosity ≥ 15mm, total motility ≥ 50% and concentration ≥ 10 x 10^6 spermatozoa/ mL were used. Following collection, 1 mL of semen was diluted 1:1 in pre-warmed Tris-citrate-fructose buffer (300 mM Tris, 94.7 mM citric acid, 27.8 mM fructose) (Evans and Maxwell, 1987) and pipetted up and down six times to ensure even mixing. The diluted semen was allocated to four treatment groups: (1) 390µl diluted semen plus 10µl 0.02M PBS (control), (2) 390µl diluted semen plus 10µl 0.04 mg/mL papain (final concentration 0.001 mg/ml), (3) 390µl diluted semen plus 10µl 0.4 mg/mL papain (final concentration 0.01 mg/ml), (4) 390µl diluted semen plus 10µl 4.0 mg/mL papain (final concentration 0.1 mg/ml).Samples were incubated for 30 min at 37°C in a water bath. Semen viscosity, and total motility and acrosome integrity of spermatozoa were assessed immediately after dilution (time 0) and at 5, 10, 20 and 30 min after treatment.

Experiment 2: Inhibition of Papain with E-64

Semen was collected from six male alpacas (≥2 ejaculates/male, n = 15) and assessed and selected as for experiment 1. Semen was then diluted 1:1 in pre-warmed Tris-citrate-fructose buffer (Evans and Maxwell, 1987). In a preliminary experiment we determined that 0.1mg/mL papain incubated with 10µM N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64) at 37°C for 5 min then incubated with alpaca semen for 20min at 37°C was ineffective at reducing viscosity, indicating that 10µM E-64 for 5 min at 37°C inhibits papain as described previously (Barrett *et al*. 1982).

Consequently, 10µM E-64 for 5 min at 37°C was used in the present study.

Diluted semen samples were allocated to two treatment groups, (1) 792µl diluted semen plus 8µl 0.02 M PBS (control; treatment 1) and (2) 792µl diluted semen plus 8µl 10.0 mg/mL papain (final concentration 0.1 mg/mL; treatment 2), and incubated at 37°C for 20 min in a water bath. Each aliquot was then divided further into two treatment groups, (1) 297µl semen plus 3µl 0.02M PBS (control; treatment A) and (2) 297µl semen plus 3µl 1mM E-64 (final concentration 10µM; treatment B), and incubated at 37°C for 5 min in a water bath. This resulted in four samples for assessment: 1A (no papain, no E-64), 1B (no papain, E-64 treatment,) 2A (papain treatment, no E-64), 2B (papain treatment, E-64 treatment). Semen viscosity and total motility, acrosome integrity, viability and DNA integrity of spermatozoa were assessed immediately after dilution (0 min), after papain or PBS but prior to E-64 treatment (20 min), and after E-64 or PBS treatment (25 min).

Experiment 3: Cryopreservation of Papain-treated Semen

Semen was collected from four male alpacas (≥2 ejaculates/male, n = 10) using an artificial vagina (Morton *et al.* 2010[a]) and assessed for volume, viscosity, and total motility and concentration of spermatozoa as described below. Only samples with a volume >1mL, viscosity ≥ 15mm, total motility ≥ 50% and concentration ≥ 40 x 10^6 spermatozoa/ mL were used. Following collection, semen was divided into 2 aliquots and diluted 1:1 in either pre-warmed Tris-citrate-fructose (fructose) extender (300 mM Tris, 94.7 mM citric acid, 27.8 mM fructose, pH 6.9) (Evans and Maxwell, 1987) or 11% lactose extender (11% lactose w/v, pH 6.9 (Morton *et al*. 2007) as used previously for camelid spermatozoa (Morton *et al*. 2007; Niasari-Naslaji *et al.* 2006) and pipetted up and down six times to ensure even mixing. Diluted semen samples were allocated to two treatment groups (1) 0.1mg/ml papain (final concentration) and (2) PBS (control) for 20min at 37°C. Papain-treated samples were then incubated with 10µM E-64 (final concentration) and

control samples with PBS for 5min at 37°C. Next, fructose-diluted samples were re-extended (1:1) with pre-warmed (37°C) tris-citrate-fructose freezing extender (300 mM Tris, 94.7 mM citric acid, 27.8 mM fructose, 20% egg yolk, 12% glycerol) and lactose-diluted samples were re-extended (1:1) with pre-warmed lactose freezing extender (11% lactose, 20% egg yolk, 12% glycerol). Final egg yolk and glycerol concentrations were 10% and 6%, respectively. Samples were chilled to 4°C over 2h then frozen as 200µl pellets on dry ice as described previously (Evans and Maxwell, 1987), then stored in liquid nitrogen. Total motility of spermatozoa and semen viscosity were assessed prior to dilution (pre-dilution) immediately after dilution (post-dilution), following papain and E-64 treatment (post-treatment) and after chilling to 4°C but before freezing (post-chill).

After 4 weeks storage in liquid nitrogen, the frozen pellets were thawed in glass tubes by vigorous shaking in a water bath at 37°C. Samples were then diluted with either pre-warmed fructose extender (samples cryopreserved in fructose extender) or 11% lactose extender (samples cryopreserved in lactose extender) to a final seminal plasma concentration of 10% as this concentration is optimal to prolong motility, preserve acrosome integrity and maintain viability of alpaca spermatozoa (Kershaw-Young and Maxwell, 2011) and total sperm motility was assessed at 0, 1 and 3h post-thaw.

Analysis of semen viscosity and sperm parameters

Viscosity of semen and concentration and motility of spermatozoa

Samples (10µl) were diluted (1:9) in 90µl 3% sodium chloride (Sigma) and the concentration of spermatozoa was assessed using a haemocytometer (Evans and Maxwell, 1987). Viscosity was assessed using the thread test (Bravo *et al.* 2000[a]). Briefly, 50µl of semen or sample was drawn into a pipette, 25µl was pipetted onto a warm glass slide and the pipette was lifted vertically forming a thread of sample. The length at which the thread snapped was recorded as the measurement of viscosity. As the viscosity of seminal plasma varies between males, the initial

viscosity measurement (mm) was taken as 100% viscosity. Subsequent measurements were recorded in mm then converted to a percentage value of the initial measurement for data analysis. Total motility of spermatozoa was assessed subjectively at X 100 magnification under phase contrast microscopy (Olympus, Tokyo, Japan) by placing 10 µL of semen or sample on a warm slide and covering with a warm coverslip (Evans and Maxwell, 1987). All motile sperm, whether oscillatory or progressive, were considered motile and used to generate a value for total motility.

Acrosome integrity of spermatozoa, experiment 1

Acrosome integrity of spermatozoa was assessed as described previously (Kershaw-Young and Maxwell, 2011). Briefly, 20µl of sample was fixed in 0.1% neutral buffered formalin and stored at 4°C until analysis. Seminal plasma was removed by centrifugation and the spermatozoa resuspended in 0.02M PBS to 10 x 10^6/mL. Twenty µL of resuspended spermatozoa was mixed with 4 µL fluorescent isothiocyanate-conjugated lectin from *Arachis hypogaea* (working concentration 40 µg/mL; FITC-PNA; Sigma) and incubated at 37 °C for 15 min, then pipetted onto a glass slide and covered with a 22 x 50 mm coverslip. A minimum of 200 spermatozoa were observed under phase contrast at X 400 magnification using the Olympus BX51 fluorescence microscope with the U-MWIB filter (excitation filter 460-495nm, emission filter 510-550 nm, 505 nm dichromatic mirror). Acrosomes were considered not intact if the acrosome stained green, and considered intact if there was no staining or if the equatorial segment was stained green.

Acrosome integrity of spermatozoa, experiment 2

Acrosome integrity was assessed based on previously described methods (Leahy *et al.* 2010). Semen was diluted in 1mL 0.02 M PBS to a final concentration of 1 x 10^6 spermatozoa/mL then incubated with 10µl FITC-PNA (working concentration 40 µg/mL) at 37°C for 15 min,. The samples were fixed with 10µl 10% neutral buffered formalin (final concentration 0.1%). Fluorescence was

208 detected using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), equipped with an
209 argon ion laser (488 nm, 15 mW) for excitation and acquisitions were made using CellQuest 3.3
210 software (Becton Dickinson, San Jose, CA). A minimum of 5,000 gated events were recorded.
211 Acrosomes were considered not intact if the acrosome stained green, and considered intact if
212 there was no staining.

213

214 ***Viability of spermatozoa, experiment 2***

215 Viability, measured as spermatozoa with non-impaired membranes, was assessed as described
216 previously (Kershaw-Young and Maxwell, 2011). Briefly, samples were fixed in 1 mL 0.1% neutral
217 buffered formalin in 0.02M PBS at a final concentration of 1 x 10^6 spermatozoa/mL and stored at
218 4°C overnight. Next day, samples were incubated with 10µl Syto-16 (Molecular Probes, Eugene,
219 OR, USA; working concentration 10 µM) at room temperature for 20 min, then 10 µL Propidium
220 iodide (PI, Molecular Probes, Eugene, OR, USA, working concentration 240 µM) at room
221 temperature for a further 10 min. Viability of spermatozoa was determined using a FACScan flow
222 cytometer as described above. Spermatozoa that stained positive for Syto-16 and negative for PI
223 were deemed viable, and those that stained negative for Syto-16 and positive for PI were deemed
224 non-viable.

225

226 ***DNA Integrity of spermatozoa***

227 The integrity of sperm DNA was assessed as described previously (Kershaw-Young and Maxwell,
228 2011). Briefly, samples were snap frozen in liquid nitrogen and stored at -20°C until analysis.
229 Samples were resuspended to a concentration of 10 x 10^6 spermatozoa/mL, smeared onto a glass
230 slide and fixed in 100% ice cold methanol. Next, slides were incubated with Terminal
231 deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction mixture (Roche Applied
232 Science, Mannheim, Germany) in a humidified chamber at 37°C for 1 h, then counterstained with
233 DAPI (Vector Laboratories, CA, USA). A minimum of 200 spermatozoa was assessed with the BX51

fluorescence microscope, as described for acrosome integrity. Sperm DNA was considered non-fragmented if there was no fluorescence, and fragmented if the sperm head stained green.

Statistical Analysis

Data were analysed using Genstat version 16 (VSN International, Hemel Hempstead, UK).

For experiment 1, viscosity of semen, and total motility and acrosome integrity of spermatozoa were analysed using a REML linear mixed model where papain concentration, incubation time and their interaction were specified as the fixed effect in the model.

In experiment 2, viscosity of semen, and total motility, acrosome integrity, viability and DNA integrity of spermatozoa were analysed using a REML linear mixed model. Male, replicate and papain treatment were used as random effects while the individual treatment was used as the fixed effect in the model. Observations with residuals more than three standard deviations from the mean were considered statistical outliers and were removed prior to analysis. In all cases statistical significance was defined as $P < 0.05$.

In experiment 3, viscosity of sample and total motility of sperm were analysed using a REML linear mixed model where treatment, time and their interaction were specified as the fixed effects and male, replicate and treatment were used as the random effects.

Results

Experiment 1: Optimisation of Papain Concentration and Time

Papain treatment significantly reduced the viscosity of alpaca seminal plasma ($P < 0.001$; Fig. 1). At 5, 10 and 20 min after treatment viscosity was less in 0.1mg/mL papain-treated samples compared to other treatment groups. Viscosity was completely eliminated in samples containing 0.1mg/mL papain within 20min of treatment, and with 0.01mg/mL papain within 30min. Viscosity was not completely eliminated within 30min in 0.001mg/mL and 0 mg/mL (control) papain-

259 treated samples. However, after 30 min incubation, all papain-treated samples had less viscosity
260 than control samples. Viscosity reduced significantly over time in all treatment groups, although
261 the reduction was most rapid for samples treated with 0.1mg/mL papain ($p < 0.001$; Fig. 1).

262 Motility of spermatozoa differed between treatments at each time point ($P = 0.01$; Table 1). A
263 decrease in motility of spermatozoa was observed from 0 to 30 min post treatment in all groups
264 ($P = 0.01$; Table 1).In samples treated with 0.1mg/mL papain, the decline in total motility was
265 slower than other treatment groups and consequently at 10 and 20 min after treatment, motility
266 was higher in samples treated with 0.1mg/mL papain than control samples, and at 30 min motility
267 was higher in samples treated with 0.1mg/mL papain than in all other treatment groups.

268 The percentage of spermatozoa with intact acrosomes differed between concentrations of papain
269 ($p < 0.001$) and between time points ($p = 0.007$) although there was no interaction. Due to lack of
270 interaction, comparisons of concentration were made using data pooled across all time points,
271 and comparisons of time were made using data pooled across all concentrations of papain. The
272 percentage of spermatozoa with intact acrosomes (mean ± sem) was higher in samples treated
273 with 0.1mg/mL papain (53.9 ± 0.50) compared to those containing 0 (51.7 ± 0.58), 0.001 (51.9 ±
274 0.59) and 0.01 mg/mL papain (52.3 ± 0.59). Acrosome integrity decreased significantly over time
275 and was greater at 0 (52.7 ± 0.51), 5 (53.48 ± 0.60) and 10min (52.6 ± 0.71) compared to 30min
276 (51.4 ± 0.71 %) after treatment. Acrosome integrity at 20min post-treatment (52.1 ± 0.63 %) did
277 not differ from the other time points.

278

279 **Experiment 2: Inhibition of Papain with E-64**

280 As observed in experiment 1, the viscosity of seminal plasma was completely eliminated within 20
281 min of treatment with 0.1mg/mL papain. Viscosity (mean mm ± sem) was significantly lower in
282 papain-treated samples compared with the control ($P < 0.001$) at both 20min (pre-E64; papain-

283 treated 0.0 ± 0.0 vs. control 78.7 ± 5.41) and 25min (post-E64; papain-treated 0.0 ± 0.0 vs. control
284 66.5 ± 3.29) of treatment. The papain inhibitor E64 did not affect viscosity (P = 0.734).

285 The total motility of spermatozoa did not differ between treatments (p = 0.505), nor was there
286 any treatment x time interaction. Total motility (mean % ± sem) was not different between the
287 control (50.7 ± 1.16), E64 only (46.7 ± 2.05), papain only (49.2 ± 1.40), and papain with E64 (47.3 ±
288 2.12) treatment groups. Total motility declined significantly (p < 0.001) over time (% mean ± sem)
289 from 0 (54.7 ± 1.50), 20 (50.3 ± 1.92) and 25 min (46.8 ± 1.47), although this was similar for all
290 treatments.
291 The percentage of spermatozoa with intact acrosomes was higher in papain-treated samples (43.8
292 ± 2.71) compared to samples that were not treated with papain (36.1 ± 2.28 %; p < 0.01) but was
293 not affected by E64 treatment or time (P > 0.05).
294 The percentage of viable spermatozoa was not affected by papain or E64 treatment (p > 0.05) and
295 did not differ over time (P > 0.05). Viability (mean ± sem) was similar in the control (76.0 ± 2.36),
296 E64 only (76.4 ± 3.68), papain only (76.6 ± 2.72) and papain with E64 (77.7 ± 3.75) treatment
297 groups.
298 The percentage of spermatozoa with intact DNA (mean ± sem) was not different between control
299 (97.5 ± 0.22), E64 only (97.7 ± 0.39), papain only (97.6 ± 0.25) and papain with E64 (97.9 ± 0.38)
300 treated samples, and did not change over time (P > 0.05).
301
302 **Experiment 3: Cryopreservation of papain-treated semen**
303 Papain treatment significantly reduced seminal plasma viscosity (P < 0.001). Viscosity (mean mm
304 ± sem) did not differ between treatments prior to dilution (56.3 ± 9.11) and following dilution (33.
305 4 ± 3.02) but was significantly lower in samples treated with fructose-papain (0 ± 0.0) and lactose-
306 papain (0 ± 0.0) compared to the fructose control post-treatment (24.9 ± 5.81) and post-chill (16.6
307 ± 3.72) and the lactose control post-treatment (26.5 ± 6.13) and post-chill (15.1 ± 3.63).
308 The total motility of spermatozoa differed between treatment groups at each time point (p =

0.03; Table 2). Prior to, and following dilution, there were no differences between treatments. However, total motility was significantly lower in lactose control samples, both post-treatment and post-chill, compared to all other treatment groups. Additionally immediately post-thaw (0 h) total motility was significantly lower in lactose control samples than fructose-papain and lactose-papain treated samples whereas fructose control spermatozoa exhibited intermediate total motility. At 1h post-thaw, total motility of fructose-papain treated spermatozoa was significantly higher than fructose-control samples and lactose control samples contained significantly less motile spermatozoa than all other treatments. At 3h post-thaw there were no significant differences in the motility of spermatozoa between treatment groups. Total motility also differed between time points in each treatment group (Table 2). Generally, total motility of spermatozoa increased after dilution compared to pre-dilution, remained high post-treatment (except in lactose-control samples) then declined post-chill to intermediate levels, and declined further at 0h and 1h post-thaw. Motility was significantly less at 3h post-thaw in all treatment groups compared to all other time points ($P < 0.001$).

Discussion

This study investigated: the effect of papain concentration and time, and the inhibitor E-64, on alpaca seminal plasma viscosity and sperm function, and the effect of papain treatment of semen on the success of cryopreservation in alpaca spermatozoa.

Alpaca seminal plasma viscosity was completely eliminated within 20 min of treatment using 0.1mg/mL papain and within 30 min of treatment using 0.01mg/mL papain. The reduction of seminal plasma viscosity for use within the Camelid industry must be rapid, reliable, effective and have no detrimental effect on sperm function and integrity. Previously ~~studies have suggested that~~ generic proteases including trypsin, fibrinolysin, and collagenase and papain ~~are~~ were detrimental to sperm motility, viability and acrosome integrity in alpacas and llamas (Bravo *et al.* 2000[a]; Morton *et al.* 2008). In the present study, ~~papain concentrations of~~ 0.1 to 0.001mg/ml

papain ~~were~~ was not detrimental to sperm motility and acrosome integrity within 30 min of treatment, ~~suggesting~~ indicating that the lower concentrations of papain ~~used~~ were ~~low enough to~~ effective in reducinge viscosity without causing sperm damage. Furthermore all ~~seminal plasma~~semen samples exhibited 0 mm viscosity within 20 min of treatment when treated with 0.1 mg/mL papain indicating that this protocol is reliable and effective in 100% of samples tested. It is also worth noting that ejaculates used through the study ranged from 49.5 to 272 x 10^6 spermatozoa/mL (average 84.9 x 10^6/mL), and therefore this protocol did not appear to impair sperm function irrespective of sperm concentration.

~~A reduction in the~~The acrosome integrity of alpaca spermatozoa ~~is observed~~declines when ~~following 10 to 60 min exposure~~ exposed to 0.5 - 0 4 mg/mL papain for 10 to 60 min, and ~~whilst~~ despite attempts ~~were made~~ to remove the papain using PureSperm gradient~~, this was ineffective in preventing damage to the~~ acrosome damage was observed (Morton *et al.* 2008). As the cryopreservation of semen often involves chilling over a 2 h period prior to freezing, it is necessary to inhibit the papain following liquefaction in order to overcome any negative effects of prolonged papain exposure. Treatment with E-64 did not affect sperm motility, acrosome integrity, viability and DNA integrity suggesting that this inhibitor is not toxic to alpaca sperm. The specific nature and low toxicity of E-64 make it a suitable option for inhibiting papain in order to reduce any potential impacts of long term exposure on sperm, in particular the effect of prolonged papain exposure on acrosome integrity.

This study compared the effect of viscosity reduction on the motility of alpaca sperm following cryopreservation. The total motility of papain-E-64 treated alpaca spermatozoa was significantly greater after chilling to 4°C and at 0 and 1 h post-thaw implying that a reduction in seminal plasma viscosity prior to sperm cryopreservation is advantageous to the sperm. During cryopreservation it is essential that cryoprotectants such as egg yolk and glycerol are able to interact with or permeate the sperm membrane in order to enhance their protective capacity and reduce sperm damage. It is likely that, in the present study, the reduction in viscosity enabled the

cryoprotectants to act accordingly as opposed to viscous semen in which the seminal plasma traps the sperm preventing contact of the sperm membrane with the cryoprotectants.

Sperm motility rates after chilling (32% to 51%) and immediately post thaw (13% to 25%) were similar to those reported previously for epididymal alpaca sperm of 5-25% (Morton *et al.* 2007; Morton *et al.* 2010[b]) and ejaculated alpaca sperm: 4 - 40% (Bravo *et al.* 2000[b]; Santiani *et al.* 2005) Recently, our protocol using papain and E-64 to reduce seminal plasma viscosity has been utilised to aid the cryopreservation of dromedary spermatozoa (Crichton *et al.* 2015). Papain treatment successfully reduced viscosity enabling removal of the seminal plasma and subsequent cryopreservation of cholesterol-supplemented spermatozoa obtained post-thaw motility rates of 44% (Crichton *et al*. 2015). This suggests that the viscosity reduction protocol developed in this study has application in the development of camelid assisted reproductive technologies.

~~Although there was no significant difference in motility between treatments at 3h post-thaw, fructose-papain treated samples tended to have higher motility at 9%. Furthermore, this is superior to epididymal alpaca sperm which exhibit motility rates of 0-3% at 3h post-thaw (Morton *et al.* 2007). Consequently the cryopreservation of viscosity-reduced ejaculated alpaca semen may be a more suitable method for sperm storage than using epididymal sperm from castrated or deceased males. Another advantage to using ejaculated sperm is that males of high genetic merit can be used for sperm collection and natural matings over prolonged periods as opposed to requiring castration which is unfavourable for breeders. Additionally, cryopreservation of ejaculated sperm will enable a larger number of ejaculates to be preserved from one individual, this increasing the potential for the spread of genetics within the industry as more females can be inseminated.~~

In the present study, the motility of ejaculated alpaca sperm was often significantly lower in lactose-control samples than fructose-control or fructose-papain treated spermatozoa. Whilst 11% lactose has been reported to be the optimal extender for liquid or frozen storage of camelid sperm (Morton *et al.* 2007; Wani *et al.* 2008) other studies report that tris-based extenders

containing fructose or glucose are superior (Deen *et al.* 2003; Niasari-Naslaji *et al.* 2006; Vaughan *et al.* 2003; Vyas *et al.* 1998). Numerous extenders have been used for the cryopreservation of camelid sperm, and the results are conflicting and difficult to interpret as successful cryopreservation of sperm requires many factors to be optimised, including the most suitable cryodiluent reagents (i.e. energy source, glycerol concentration, egg yolk concentration), the optimal cooling, freezing and thawing and dilution rates of the sperm, and the optimal storage method (pellet or straws). In the present study, the final egg yolk concentration was 10% as is used routinely for ram sperm (Evans and Maxwell, 1987) and has been used for alpaca sperm (Morton *et al.* 2010[b]; Santiani *et al.* 2005).The final glycerol concentration was 6% as this was found to be superior to 4% and 8% for cryopreservation of camel sperm (Niasari-Naslaji *et al.* 2007). ~~In order to~~To fully benefit from the optimised ~~method for seminal plasma~~ viscosity reduction protocol using papain and E-64 it is necessary to systematically and thoroughly investigate the effect of all semen extender components on the integrity and function of alpaca sperm during and after cryopreservation. Furthermore, it is integral that fertilising ability of viscosity-reduced camelid semen is investigated to determine the effect of treatment on pregnancy.

In conclusion, the treatment of alpaca semen with 0.1mg/mL papain for 20 min at 37°C followed by 10µm E-64 for 5 min at 37°C does not ~~affect~~ impair sperm function and integrity in alpacas~~.~~. Furthermore, the treatment of alpaca semen with papain and E64 is beneficial to spermatozoa motility after chilling and at 0h and 1h post-thaw. This is most likely due to the ability of cryoprotectants to interact with or permeate the sperm cell membrane in samples with reduced viscosity compared to those with high viscosity.

The success of papain and E-64 in reducing semen viscosity and improving post-thaw motility rates without negatively impacting sperm function and integrity make this a promising solution to semen viscosity and could significantly aid the development of assisted reproductive technologies in camelids.

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References

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

Ali, H. A., Moniem, K. A., and Tingari, M. D. (1976). Some histochemical studies on the prostate, urethral and bulbourethral glands of the one-humped camel (*Camelus dromedarius*). *Histochem. J*. **8**, 565-578.

Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M., and Hanada, K. (1982). L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* **201**, 189-198.

Barrett, A. J., Kembhavi, A. A., and Hanada, K. (1981). E-64 [L-trans-epoxysuccinyl-leucyl-amido(4-guanidino)butane] and related epoxides as inhibitors of cysteine proteinases. *Acta. Biol. Med. Ger*. **40**, 1513-1517.

Bravo, P. W., Ccallo, M., and Garnica, J. (2000[a]). The effect of enzymes on semen viscosity in Llamas and Alpacas. *Small Rumin. Res.* **38**, 91-95

Bravo, P. W., Pacheco, C., Quispe, G., Vilcapaza, L., and Ordonez, C. (1999). Degelification of alpaca semen and the effect of dilution rates on artificial insemination outcome. *Arch. Androl*. **43**, 239-246.

Bravo, P. W., Skidmore, J. A., and Zhao, X. X. (2000[b]). Reproductive aspects and storage of semen in Camelidae. *Anim. Reprod. Sci*. **62**, 173-193.

Conde, P. A., Herrera, C., Trasorras, V. L., Giuliano, S. M., Director, A., Miragaya, M. H., Chaves, M. G., Sarchi, M. I., Stivale, D., Quintans, C., Aguero, A., Rutter, B., and Pasqualini, S. (2008). In vitro production of llama (*Lama glama*) embryos by IVF and ICSI with fresh semen. *Anim. Reprod. Sci*. **109**, 298-308.

Crichton, E. G., Pukazhenthi, B. S., Billah, M., and Skidmore, J. A. (2015). Cholesterol addition aids the cryopreservation of dromedary camel (*Camelus dromedarius*) spermatozoa. *Theriogenology*. **83**, 168-174.

Deen, A., Vyas, S., and Sahani, M. S. (2003). Semen collection, cryopreservation and artificial insemination in the dromedary camel. *Anim. Reprod. Sci*. **77**, 223-233.

Evans, G., and Maxwell, W. M. C. (1987). 'Salamon's Artificial Insemination of Sheep and Goats' (Butterworths: Sydney).

Garnica, J., Achata, R., and Bravo, P. W. (1993). Physical and biochemical characteristics of alpaca semen. *Anim. Reprod. Sci*. **32**, 85-90.

Giuliano, S., Carretero, M., Gambarotta, M., Neild, D., Trasorras, V., Pinto, M., and Miragaya, M. (2010). Improvement of llama (*Lama glam*a) seminal characteristics using collagenase. *Anim. Reprod. Sci*. **118**, 98-102.

Kershaw-Young, C. M., Evans, G., and Maxwell, W. M. (2012). Glycosaminoglycans in the accessory sex glands, testes and seminal plasma of alpaca and ram. *Reprod. Fertil. Dev.* **24**, 368-369.

Kershaw-Young, C. M., and Maxwell, W. M. C. (2011). The effect of seminal plasma on alpaca sperm function. *Theriogenology*. **76**, 1197-1206.

Kershaw-Young, C. M., Stuart, C., Evans, G., and Maxwell W. M. C. (2013). The effect of glycosaminoglycan enzymes and proteases on the viscosity of alpaca seminal plasma and sperm function. *Anim. Reprod. Sci.* **138**, 261-267.

462 Leahy, T., Celi, P., Bathgate, R., Evans, G., Maxwell, W. M., and Marti, J. I. (2010). Flow-sorted ram
463 spermatozoa are highly susceptible to hydrogen peroxide damage but are protected by seminal
464 plasma and catalase. *Reprod. Fertil. Dev.* **22**, 1131-1140.
465 Morton, K. M., Bathgate, R., Evans, G., and Maxwell, W. M. (2007). Cryopreservation of
466 epididymal alpaca (*Vicugna paco*s) sperm: a comparison of citrate-, Tris- and lactose-based
467 diluents and pellets and straws. *Reprod. Fertil. Dev*. **19**, 792-796.
468 Morton, K.M., Evans, G., and Maxwell, W. M. (2010[b]). Effect of glycerol concentration, Equex
469 STM® supplementation and liquid storage prior to freezing on the motility and acrosome integrity
470 of frozen-thawed epididymal alpaca (*Vicugna pacos*) sperm. *Theriogenology*. **74**, 311-316.
471 Morton, K. M., Thomson, P.C., Bailey, K., Evans, G., and Maxwell, W. M. (2010[a]). Quality
472 parameters for alpaca (*Vicugna pacos*) semen are affected by semen collection procedure. *Reprod*
473 *Domest Anim*. **45**, 637-643.
474 Morton, K. M., Vaughan, J., and Maxwell, W.M. (2008). 'Continued development of artificial
475 insemination technology in alpacas'. (Rural Industries Research and Development Corporation:
476 Kingston, Australia).
477 Niasari-Naslaji, A., Mosaferi, S., Bahmani, N., Gerami, A., Gharahdaghi, A.A., Abarghani, A., and
478 Ghanbari, A. (2007). Semen cryopreservation in Bactrian camel (*Camelus bactrianus*) using
479 SHOTOR diluent: effects of cooling rates and glycerol concentrations. *Theriogenology*. **68**, 618-
480 62.5
481 Niasari-Naslaji, A., Mosaferi, S., Bahmani, N., Gharahdaghi, AA., Abarghani, A., Ghanbari, A., and
482 Gerami, A. (2006). Effectiveness of a tris-based extender (SHOTOR diluent) for the preservation of
483 Bactrian camel (*Camelus bactrianus*) semen. *Cryobiology.* **51**, 12-21.
484 Perk, K. (1962). Seasonal changes in the glandula bulbo-urethralis of the camel. B*ull. Res. Counc.*
485 *Isr. Sect. E Exp. Med*. **10**, 37-44.

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

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

Tamai, M., Hanada, K., Adachi, T., Oguma, K., Kashiwagi, K., Omura, S., and Ohzeki, M. (1981). Papain inhibitions by optically active E-64 analogs. *J. Biochem*. **90**, 255-257.

Tibary, J., and Vaughan, J. (2006). Reproductive infertility and physiology in male South American camelids: A review and clinical observations. *Small Rum. Res*. **61**, 283-298.

Vaughan, J., Galloway, D., and Hopkins, D. (2003). 'Artificial Insemination in Alpacas (*Lama Pacos*).' (Rural Industries Research and Development Corporation: Kingston, Australia).

Vyas, S., Goswani, P., Rai, A. K., and Khanna, N. D. (1998). Use of Tris and lactose extenders in preservation of camel semen at refrigerated temperature. *Indian Vet J.* **75**, 810-812.

Wani, N. A., Billah, M., and Skidmore, J. A. (2008), Studies on liquefaction and storage of ejaculated dromedary camel (*Camelus dromedarius*) semen. *Anim. Reprod. Sci*. **109**, 309-318.

Zhao, X. X. (2000), Semen characteristics and artificial insemination in the Bactrian camel. In: Skidmore J. A. and Adams, G. P. eds. *Recent Advances In Camelid Reproduction*. International Veterinary Information Service.

Table 1.

Percentage motility (mean ± SEM) of alpaca sperm treated with 0, 0.001, 0.01 and 0.1 mg/mL papain at 0, 5, 10, 20 and 30 min of treatment.

Time (min)	0.0 (mg/mL)	0.001 (mg/mL)	0.01 (mg/mL)	0.1 (mg/mL)
0	$56.0 \pm 2.30^{a}_{x}$	$56.0 \pm 2.30^{a}_{w}$	$56.0 \pm 2.30^{a}_{x}$	$56.0 \pm 2.30^{a}_{x}$
5	$54.0 \pm 2.59^{a}_{x}$	$54.3 \pm 2.33^{a}_{wx}$	$56.5 \pm 2.85^{a}_{x}$	$55.7 \pm 2.12^{a}_{x}$
10	$51.3 \pm 2.60^{a}_{y}$	$53.0 \pm 2.53^{ab}_{xy}$	$53.1 \pm 3.18^{ab}_{y}$	$54.33 \pm 2.53^{b}_{xy}$
20	$48.7 \pm 2.56^{a}_{z}$	$51.7 \pm 2.57^{bc}_{y}$	$51.5 \pm 3.02^{b}_{yz}$	$53.7 \pm 2.41^{c}_{y}$
30	$47.1 \pm 2.61^{a}_{z}$	$47.7 \pm 2.88^{a}_{z}$	$50.4 \pm 3.37^{b}_{z}$	$51.3 \pm 4.27^{c}_{y}$

^{a,b,c} Within a row, means without a common superscript differed ($P < 0.05$)

$_{w,x,y,z}$ Within a column, means without a common subscript differed ($P < 0.05$)

Table 2.

Percentage motility (mean ± SEM) of ejaculated alpaca sperm pre-dilution (Pre-D), post-dilution (PD), post-treatment (PT), post-chill (PC), and 0 (0h), 1 (1h) and 3 (3h) hours post-thaw when diluted then cryopreserved using fructose, fructose with papain, lactose, and lactose with papain extenders.

Time (min)	Fructose	Fructose with papain	Lactose	Lactose with papain
Pre-D	$54.5 \pm 2.41^{a}_{u,v}$	$54.5 \pm 2.41^{a}_{x}$	$54.5 \pm 2.41^{a}_{v}$	$54.5 \pm 2.41^{a}_{w,x}$
PD	$65.5 \pm 3.29^{a}_{w,x}$	$65.5 \pm 3.29^{a}_{w}$	$61.0 \pm 2.67^{a}_{v}$	$61.0 \pm 2.67^{a}_{x}$
PT	$61.5 \pm 2.89^{a,b}_{v,x}$	$63.5 \pm 3.25^{a}_{w}$	$42.5 \pm 5.69^{c}_{w}$	$54.5 \pm 2.41^{b}_{w,x}$
PC	$47.0 \pm 4.29^{a}_{u}$	$51.5 \pm 2.69^{a}_{x}$	$32.5 \pm 6.75^{b}_{x}$	$48.1 \pm 5.08^{a}_{w}$
0h	$19.0 \pm 2.69^{a,b}_{y}$	$25.5 \pm 2.63^{a}_{y}$	$13.0 \pm 4.16^{b}_{y}$	$24.0 \pm 4.00^{a}_{y}$
1h	$16.5 \pm 3.25^{a}_{y}$	$26.0 \pm 3.06^{b}_{y}$	$7.2 \pm 2.86^{c}_{yz}$	$21.0 \pm 3.40^{a,b}_{y}$
3h	$1.1 \pm 0.66^{a}_{z}$	$9.0 \pm 2.08^{a}_{z}$	$0.7 \pm 8.52^{a}_{z}$	$4.0 \pm 1.80^{a}_{z}$

^{a,b,c} Within a row, means without a common superscript differed ($P < 0.05$)

$_{u,v,w,x,y,z}$ Within a column, means without a common subscript differed ($P < 0.05$)

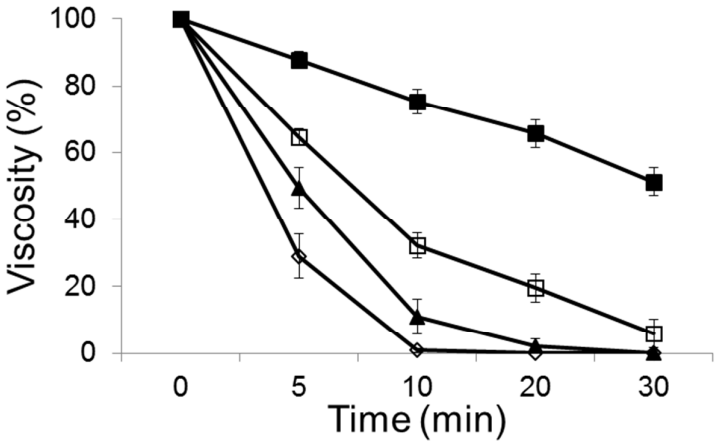

Fig. 1 Percentage viscosity (mean ± SEM) of alpaca semen treated with 0 (control, ■), 0.001 (□), 0.01 (▲) and 0.1 (◇) mg/mL papain at 0, 5, 10, 20 and 30 min after treatment.