



Australian Government

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Advancing Artificial Insemination in Camelids, Particularly the Alpaca

by Claire M Kershaw-Young, W.M. Chis Maxwell

January 2011

RIRDC Publication No 11/ (added by RIRDC)
RIRDC Project No R1882

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ISBN 1 74151 XXX X) (RIRDC to assign)
ISSN 1440-6845

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Researcher Contact Details

Dr. Claire M Kershaw-Young
Faculty of Veterinary Science,
University of Sydney, NSW, 2006

Phone: 02 9351 3463
Fax: 02 9351 3957
Email: claire.kershaw-young@sydney.edu.au

Professor WM Chis Maxwell
Faculty of Veterinary Science
University of Sydney, NSW, 2006

Phone: 02 9351 34864
Fax: 02 9351 3957
Email: chis.maxwell@sydney.edu.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON ACT 2600

PO Box 4776
KINGSTON ACT 2604

Phone: 02 6271 4100
Fax: 02 6271 4199
Email: rirdc@rirdc.gov.au
Web: <http://www.rirdc.gov.au>

Electronically published by RIRDC in xxxx 2011
Print-on-demand by Union Offset Printing, Canberra at www.rirdc.gov.au
or phone 1300 634 313

Foreword

Assisted reproductive technologies (ARTs) such as semen cryopreservation, artificial insemination (AI) and embryo transfer can have significant economic impacts on livestock industries. These technologies enhance the production of improved offspring via the introduction of superior genotypes, maximise the use of genetically superior males and control contagious diseases within flocks.

In camelids, which include alpacas, llamas, vicunas, guanacos, and bactrian and dromedary camels, the development of ARTs has been hindered by the unique reproductive physiology of this species. The alpaca industry is an emerging industry that is growing worldwide and aims to produce a soft lustrous fibre that is superior to that of sheep wool. Whilst advances have been made, the ability to utilise sperm cryopreservation and artificial insemination will advance the rapid dissemination of desirable genetic traits such as genetic diversity, fleece weight, fibre diameter and reproductive efficiency. Additionally, cryopreservation will enable long term sperm storage, and worldwide transportation of sperm eliminating the need for transportation of males and on farm matings.

The current major impediment to the development of sperm cryopreservation and AI in alpacas is the viscous nature of the seminal plasma which hinders assessment of sperm and prevents successful sperm cryopreservation. Previous attempts to reduce the viscosity with mechanical and enzymatic methods have been unsuccessful most likely due to a lack of understanding of the cause of viscosity, and its role in sperm function.

This project aimed to 1) identify the cause and source of the viscous seminal plasma 2) investigate ways to reduce the viscosity of the seminal plasma within impairing sperm integrity and function and 3) determine the role of seminal plasma on sperm function.

This project identified the protein Mucin 5B as the cause of viscosity in alpaca seminal plasma. The effect of various enzymes on seminal plasma viscosity and sperm function were investigated and the protease papain was found to be most effective. With further investigations of papain a protocol for the reduction of seminal plasma viscosity without impairing sperm function was developed. It is anticipated that this protocol will be utilised by the alpaca industry. The role of seminal plasma on sperm function, in particular the effect of semen dilution, was determined in order to aid the development of protocols for semen cryopreservation. The ability to cryopreserve viscosity-reduced semen was investigated and the advantage of reducing seminal plasma viscosity prior to semen cryopreservation was apparent. The research findings emphasise the need to reduce seminal plasma viscosity in order to advance the development of ARTs in camelids, and also highlights the requirement for further research into methods of semen cryopreservation and AI in alpacas.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Rare Natural Animal Fibres and New Animal Products R&D program, which aims to increase productivity by investment in reproduction and breeding research.

Most of RIRDC's publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation

Acknowledgments

The authors thank the following for their kind donation and loan of male alpacas for semen collection: Julienne Gelber (Bumble Hill Alpacas), Annalisa Tomich (Spitting Image, Tree Tops Park Alpacas), Dianna Rutter (Keiana Lodge Alpacas), Marilyn Matthews and Geoffrey Pearson (Daisy Bank Alpacas), Keith and Daphne Gregory (Chiverton Alpacas), Carl and Wendy Dowd (Halcyon Alpaca Stud) and Daniel and Diana McAusland (Artimis Alpacas).

Maureen Carey (Forest Glen Alpacas), Kate Bailey (Silly Oak Alpacas), Glynda Bluhm (Alpaca Magic), Karen and Trevor Scott (Vermillion Fire Alpacas), Sue Maynard (Gunnamatta Stud Alpacas) Lee Sadler (Fleur de Lys Alpacas) and Steve Ridout (Australian Game Processors) are thanked for the donation of testes.

The authors thank the following collaborators: Dr Jane Vaughan (CriaGenesis Australia) for her scientific input and contributions; Dr Lulu Skidmore (Camel Reproduction Centre Dubai) for her kind hospitality, use of facilities, advice and experimental contributions; Dr Xavier Druart (INRA, France) for his scientific input and experimental ideas and assistance specifically with protein analysis of alpaca semen and Dr Katherine Morton for donation of camel seminal plasma.

The following staff and students of The University of Sydney are thanked for their contributions: Byron Biffin and Keith Tribe for alpaca husbandry and handling; Kim Heasman for alpaca handling, blood sampling, blood hormone analysis, and excellent technical assistance and support; Associate Professor Peter Thomson for assistance with statistical analysis; Professor Gareth Evans for advice and support; Karen Barnes and Elaine Chew for histology; Dr Svetlana Mactier, Dr Phillipa Kohnke and Ben Crossett for assistance with proteomic analysis of alpaca seminal plasma; Dr Simon de Graaf and Dr Roslyn Bathgate for support; Jess Rickard, Jenna Lowe, Andrew Souter and Neil-Kershaw-Young for technical assistance and man power; and honours students Cassie Stuart, Sheri Young and Rachael Rodney for contributions to semen collection and laboratory experiments.

The authors also thank Dr Peter McInnes, Mrs June Murphy and Mrs Julie Bird and the Rare and Natural Fibres Committee for their continued support and enthusiasm.

Abbreviations

AI: Artificial insemination
ARTs: Assisted reproductive technologies
AV: Artificial vagina
CD14: Monocyte differentiation antigen CD14
CS: Chondroitin sulphate
DMMB: Dimethylmethylene blue
DNA: Deoxyribonucleic acid
DS: Dermatan sulphate
ELISA: Enzyme-linked immunosorbent assay
FITC PNA: Fluorescent isothiocyanate-conjugated lectin from *Arachis hypogaea*
FUCA1: Tissue alpha-L-fucosidase protein
x g: Gravity
GAG: Glycosaminoglycan
HA: Hyaluronan
HCl: Hydrochloric acid
HS: Heparin / heparin sulphate
IVF: In vitro fertilisation
KDa: Kilodaltons
Kg: Kilograms
KS: Keratan sulphate
M: Molar
mA: Milliamps
mg: Milligrams
mg/ml: Milligrams per millilitre
ml: Millilitres
mm: Millimetres
mM: Millimolar
mOsm: Milliosmoles
MUC 5B: Mucin 5B protein
µg: Micrograms
µl: Microlitres
µm: Micrometres
nm: Nanometres
PBS: Phosphate buffered saline
PEBP: Phosphatidylethanolamine-binding protein
PI: Propidium iodide
SDS: Sodium dodecyl sulphate
TEMED: N, N, N', N'-tetramethylethylenediamine
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling
U: Units
U/ml: Units per millilitre

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Executive Summary

What the report is about

This report describes the research findings of RIRDC project PRJ-54 in which the main objective was to advance the development of assisted reproductive technologies (ARTs) in camelids, particularly the alpaca. Assisted reproductive technologies such as sperm cryopreservation and artificial insemination in alpacas are not currently available due to an inability to reliably collect, assess and process sperm for cryopreservation as the seminal plasma component of alpaca semen is extremely viscous and hinders processing. The cause and source of the viscosity is unknown. Ascertaining the cause of this viscosity may help identify methods for viscosity reduction, thereby enabling sperm processing and advancing the development of cryopreservation protocols. In order to meet the main objective of the research project the following strategies were implemented. 1) identify the source and cause of the viscous seminal plasma, 2) investigate ways to alleviate the viscosity without impairing sperm function, and 3) determine the effect of viscosity reduction on the success of sperm cryopreservation in alpacas. This report describes, in individual research chapters, experiments and the findings of those experiments that have been conducted to achieve the aims and objectives of the project. In particular this report:

- outlines the major problems associated with the development of assisted reproductive technologies, particularly cryopreservation, via a review of the current literature and describes the research that must be conducted in order to aid the development of cryopreservation in alpacas
- provides a detailed explanation of the methods used throughout the project, in order to enable other scientists, breeders and veterinarians to repeat the techniques
- describes the expression of glycosaminoglycans (GAGs) in alpaca seminal plasma and the male alpaca reproductive tract to test the hypothesis that GAGs are the cause of viscosity in alpacas and reports the effect of GAG enzymes on alpaca seminal plasma viscosity.
- identifies the protein responsible for viscosity in alpaca seminal plasma and investigates ways to degrade this protein without affecting sperm function.
- describes a protocol for the reduction of alpaca seminal plasma viscosity that can be adopted by the camelid industry to aid the development of sperm cryopreservation in this species
- reports the effect of seminal plasma on sperm function and highlights the importance of optimising the concentration of seminal plasma for successful sperm storage
- investigates the effect of reducing seminal plasma viscosity on the success of cryopreservation
- highlights areas of further research that are integral to enable the development of sperm cryopreservation and artificial insemination in alpacas.

Who is the report targeted at?

The findings of this research project are aimed at camelid breeders, researchers including reproductive physiologists and camelid scientists, artificial breeding centres and veterinarians. It is anticipated that this report will assist scientists to undertake further research and thereby advance the development of protocols for sperm cryopreservation and AI in camelids. For camelid breeders the findings of this research will provide a sound understanding of the unique reproductive physiology of camelids and will enable them to thoroughly appreciate the obstacles that hinder rapid progression of ARTs for this species and the measures that are being taken to overcome these problems. For veterinarians and

breeding centres, this report provides a stepping stone on which to gain an understanding of alpaca reproductive physiology and protocols that will hopefully be adopted by the camelid industry (i.e. reduction of seminal plasma viscosity) for future breeding programmes.

Where are the relevant industries located in Australia?

Globally there are 3.5million alpacas and within Australia there are approximately 2500 stud farms with over 100,000 registered stud alpacas, the largest registered herd of alpacas worldwide. As a leader in alpaca farming, Australia has improved the genetics of the alpaca herd to produce a high quality fibre that is in considerable demand worldwide along with other luxurious products such as silk, angora and cashmere. Whilst recent global economic conditions have hurt the luxury end of the market, the quality and durability of alpaca products has maintained its relative market position, and thus there is still strong demand for alpaca products within Australia and internationally.

Alpaca studs are located throughout New South Wales (NSW), Victoria (VIC), South Australia (SA), Western Australia (WA), Queensland (QLD) and Tasmania (TAS) but not the Northern Territory due to the dry arid climate and wet summers. The majority of registered breeders are located in NSW and VIC.

New South Wales is divided into 5 alpaca breeding regions; Central coast and Hunter region, Western NSW, The Blue Mountains, Southern NSW and Sydney. The Southern region of NSW produces some of the nation's finest fibre and contains the state's largest number of registered breeders at approximately 150. The Central coast and Hunter region is also a thriving area for alpaca breeding with over 90 breeders, the majority of which have an average herd size of 25 to 40 alpacas although there are several studs with in excess of 80 alpacas. Although Sydney is the birthplace of the alpaca industry limited land prevents large herd sizes and consequently there are 40 breeders each with small herds. The Blue Mountains and Western NSW regions have 68 and 46 registered breeders respectively, the majority of which have relatively small herds.

Victoria contains almost 25% of all registered alpaca studs in Australia. It is divided in 3 alpaca breeding regions; Central VIC, Eastern VIC and Western VIC. Central VIC has over 300 registered alpaca studs making up more than 10% of all Australian breeders. The Eastern region is a geographically diverse region comprising many Australian terrains including rich farming country to vineyards. It has over 300 registered breeders some hobby farmers with just 5-6 animals and others established breeders with over 300 animals. Western VIC produces some of the finest alpaca fleece in Australia. It has just 90 breeders, but their animals represent more than 5% of the national herd.

South Australia has a broad range of soil types and climates and the most suitable region for alpaca breeding is the southern half of the state where the climate is cooler than the desert region. There are over 100 registered breeders in SA, and the numbers continue to grow. Whilst some of these breeders have small herds of 25 animals, there are a large number of breeders with large herds and one of the largest single herds of alpacas is found in SA.

In QLD, alpaca breeding is concentrated in the southern region where the climate is cooler and less sub-tropical. There are just 60 registered breeders in QLD, the majority of which have small herds, although some have in excess of 80 alpacas. The number of registered breeders is similar in TAS reaching just 50. In WA alpaca breeding is concentrated in the coastal plains north and south of Perth, and there are no breeders in the northern part of the state due to the hotter, drier climate. There are over 200 registered breeders in WA though most are small hobby farms with small herd sizes.

The findings of this research project will advance the development of ARTs in camelids. The camelid industry has the potential to significantly improve the genetics of desirable traits such as alpaca fleece quality, however, the rate of genetic gain is severely limited by the lack of assisted reproductive technologies in these species. The development of reliable, effective sperm cryopreservation and AI protocols will facilitate the rapid dissemination of superior genotypes. At the industry level producers will benefit from the higher prices gained by high quality low diameter fleece and higher fleece

weights. The cryopreservation of sperm will enable breeders to disseminate the genetics of their superior males or inseminate their females both domestically and internationally without the need to transport live animals, thereby reducing shipping costs. Additionally, domestic and international demand for cryopreserved semen of genetically superior males will enable breeders to benefit from increased stud fees. The increase in fibre quality would increase Australia's reputation as a producer of high quality alpaca fibre, thereby increasing our industry competitiveness in both the domestic and international fibre markets. Additionally the Australian industry and community will be at the forefront of the development of ARTs in camelids and will benefit from the increased reputation associated with being a world leader in this area.

Background

In order for the camelid industry to increase its rate of genetic gain for desirable traits, sperm cryopreservation and artificial insemination (AI) are essential.

Sperm cryopreservation in camelids has been an area of research focus worldwide, especially in the last 10 years (Bravo et al. 2000; Vaughan et al. 2003; Morton et al. 2008). Although some progress has been made the cryopreservation of camelid semen still remains problematic. Following cryopreservation sperm motility is low (approximately 20%) (Santiani et al. 2005; Niasari-Naslaji et al. 2006; Morton et al. 2007; Niasari-Naslaji et al. 2007; Morton et al. 2010) and is not commercially viable. Consequently commercially viable protocols for assisted reproductive technologies in camelids have not been developed.

Camelid semen is characterised as a highly viscous ejaculate with low sperm numbers that exhibit limited motility (Bravo et al. 2000; Deen et al. 2003) It is the viscous nature of camelid seminal plasma that prevents successful cryopreservation due to an inability of the cryoprotectants within the semen extender to interact with or permeate the sperm cell membrane during cooling and freezing. In order to develop effective, reliable methods of sperm cryopreservation in camelids, it is essential to reduce or eliminate the viscous component of the seminal plasma prior to cryopreservation.

The reduction of seminal plasma viscosity whilst maintaining sperm function and integrity has therefore been an area of focus for many research groups worldwide. Researchers have attempted to reduce or remove this viscosity by mechanical or enzymatic means (Bravo et al. 1999; Bravo et al. 2000; Vaughan et al. 2003; Giuliano et al. 2010) however, they have failed to determine the underlying cause of the viscosity. Consequently a randomised approach has been utilised by which a range of proteases and enzymes have been tested. Some success has been achieved using collagenase in which viscosity was eliminated and sperm function maintained in llamas (Giuliano et al. 2010) and alpacas (Bravo et al. 1999) however other researchers have documented a detrimental effect of collagenase in alpacas (Morton et al. 2008) and the ability to cryopreserve the collagenase treated sperm has not been assessed.

If the industry is to achieve practical application of sperm cryopreservation and a viable system of artificial insemination in camelids, particularly llamas and alpacas, it is necessary to determine the cause and source of the viscous seminal plasma and investigate methods to eliminate this viscosity without impairing sperm function. It has been postulated, but never proven that the viscosity is caused by glycosaminoglycans (previously referred to as mucopolysaccharides) (Perk 1962) although the ability of proteases such as trypsin and papain (Bravo et al. 2000; Morton et al. 2008) to reduce seminal plasma viscosity suggest that it may be caused by a protein. Understanding the cause of the viscosity will enable the identification of enzymes that specifically degrade the viscosity-causing component, thus reducing viscosity whilst potentially having minimal effect on sperm function, integrity and fertility.

Aims/objectives

The overall aim of this research is to advance the development of assisted reproductive technologies including sperm cryopreservation and artificial insemination in camelids, particularly alpacas. The

development of ARTs will help advance the rapid dissemination of desirable genetic traits such as genetic diversity, fleece weight, fibre diameter and reproductive efficiency. Additionally, cryopreservation will enable the long term storage of sperm, enabling genes from genetically superior males to be preserved indefinitely and the worldwide transportation of sperm from stud males eliminating the need for transportation of males and on farm matings.

The current major impediment to the development of sperm cryopreservation in camelids is the viscous nature of the seminal plasma which prevents cryopreservation of sperm without detrimental effects on sperm function, integrity and fertility. This project aims to:

- identify the source and constituent of the viscous seminal plasma
- investigate ways to reduce the viscosity of alpaca seminal plasma without impairing sperm function and integrity
- determine the role of seminal plasma on camelid sperm function
- investigate methods of sperm cryopreservation in alpacas and establish the effect of viscosity reduction on the success of sperm cryopreservation.

This research will benefit the camelid industry, in that it will advance the development of ARTs in order to aid the rapid dissemination of superior genetics worldwide. Additionally, veterinarians and artificial breeding centres will have access to up-to-date research that describes that advances that are being made enabling them to have a sound understanding of ARTs in camelids once the method is commercially available. Finally research scientists, including camelid scientists, reproductive physiologists and andrologists will benefit from the scientific findings of this research as new techniques for sperm assessment, analysis of semen components and viscosity reduction of camelid semen are described and can be utilised.

Methods used

Semen collection from alpacas was performed using a mannequin fitted with an artificial vagina using previously described methods (Morton et al. 2008). Semen collection was performed in the field with basic laboratory facilities, which enabled basic sperm assessments such as motility, concentration and viscosity to be performed utilising previously published methods (Morton et al. 2008). Being in a field setting it was not possible to perform more sophisticated analyses of sperm function at the time of semen collection. Consequently in order to fully determine the fertilising potential of alpaca sperm, it was necessary to develop techniques for the assessment of sperm viability, acrosome integrity and DNA integrity using sperm that were fixed or frozen at the time of collection. Extensive review of the literature was used to find methods used for other species including ram, stallion, mouse, human and monkey. These described methods were then adapted and validated for alpaca sperm.

Fluorescent microscopy or flow cytometry proved successful in assessing sperm viability using the fluorescent stain Propidium iodide (PI) as used for ram and stallion sperm. Propidium iodide binds to the DNA of the sperm, but is only able to bind if it can enter the cell membrane, which is only possible if the cell membrane is impaired. Consequently sperm that stain with PI are considered non-viable as their cell membrane is not intact.

Based on previously described methods for the assessment of alpaca sperm acrosome integrity (Morton et al. 2008) we were able to validate a method for fixed alpaca sperm using fluorescent microscopy or flow cytometry and the stain Fluorescent isothiocyanate-conjugated lectin from *Arachis hypogaea* (FITC-PNA). The FITC-PNA stain binds to β -D galactosidase residues in the acrosome of the sperm. When the acrosome is impaired FITC-PNA binds to these residues causing the sperm head to fluoresce, and consequently any fluorescent sperm are considered acrosome not-intact.

A technique for the assessment of DNA integrity was established using snap-frozen sperm and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) which was purchased as a kit (Roche Applied Science, Mannheim, Germany). Sperm DNA integrity was assessed according to the manufacturer's protocol with some minor amendments including permeabilising the sperm membrane with methanol prior to labelling with TUNEL in order to enhance the staining procedure. The TUNEL stain binds to fragmented DNA, consequently any sperm that stain positive with TUNEL have DNA that is impaired and these sperm can be considered damaged.

For the assessment of seminal plasma components semen was collected from male alpacas, centrifuged, and the seminal plasma decanted and stored until use. To determine the source of the components, alpaca reproductive tracts were collected from an abattoir and either fixed or frozen until use. The expression of glycosaminoglycans (GAGs) and proteins in the seminal plasma were assessed using ELISA and GAG assays (GAGs) or gel electrophoresis, mass spectrometry and western blot (proteins). The expression of GAGs in tissues was performed using histology with Alcian blue stain, and the expression of protein in the reproductive tract was analysed by western blot.

To investigate the potential of enzymes to reduce seminal plasma viscosity, semen samples were collected from male alpacas and incubated with enzyme for up to 2h. Semen viscosity and sperm motility, acrosome integrity, viability and DNA integrity were assessed during the incubation period.

To determine the role of seminal plasma on epididymal and ejaculated sperm function, sperm were incubated with varying concentration of seminal plasma over 6h and sperm motility, viability, acrosome integrity and DNA integrity were assessed throughout the incubation period. Epididymal sperm were collected utilising previously published protocols for the collection of epididymal alpaca sperm (Morton et al. 2008).

Results/key findings

Identify the source and constituent of the viscous seminal plasma and investigate ways to reduce the viscosity of alpaca seminal plasma without impairing sperm function and integrity

Analysis of seminal plasma and alpaca reproductive tracts confirmed that GAGs are abundant in alpaca seminal plasma at concentrations 5-15 times higher than those found in ram and human, and that the source of these GAGs is the bulbourethral gland. The abundance of one particular GAG, keratan sulphate, was profound and was associated with viscosity. This suggested that GAGs, in particular keratan sulphate was the cause of viscosity in alpaca seminal plasma.

Next enzymes that specifically degrade GAGs, as well as the generic proteases papain and proteinase K were investigated for their potential to degrade seminal plasma viscosity without impairing sperm function. Whilst the GAG enzymes did reduce seminal plasma viscosity, the viscosity was not completely eliminated after 2h incubation, and the enzyme keratanase, which specifically degrades keratan sulphate, had a minimal effect. Conversely papain and proteinase K completely eliminated viscosity within 30min of treatment. This implied that proteins not GAGs are the cause of viscosity in alpaca seminal plasma

To identify the protein responsible for viscosity, semen was collected from males with high or low viscosity seminal plasma. The seminal plasma was analysed using mass spectrometry with iTRAQ in order to identify differences in the abundance of proteins between the two sample types. Four proteins were identified that differed significantly in abundance between high viscosity and low viscosity samples. Of these proteins, one; Mucin 5B, was identified as a large gel-forming protein. Review of the literature confirmed that this protein is also present in human seminal plasma (Russo et al. 2006) and the gel fraction of boar semen (Boursnell et al. 1970). We identified the source of this protein as the bulbourethral gland, as has been reported in human (Piludu et al. 2009). The research findings and the review of the literature strongly suggest that the protein Mucin 5B is the cause of viscosity in alpaca seminal plasma.

Whilst there are no commercially available enzymes that specifically degrade Mucin 5B, some suitable generic proteases were identified. Of these, papain was found to be most suitable based on a review of the available literature. Papain is a serine protease that reduces seminal plasma viscosity in alpacas (Morton et al. 2008), although at high concentration (>0.5mg/ml) and when sperm are exposed to papain over prolonged periods, the papain is detrimental to sperm function, in particular the integrity of the acrosome.

The potential for papain to reduce seminal plasma viscosity was investigated and the optimal papain concentration and time of exposure was determined. The use of the papain inhibitor E-64 to halt digestion of seminal plasma proteins with papain was also investigated. It was determined that 0.1mg/ml papain for 20min at 37°C followed by incubation with 10µM E-64 at 37°C for 5min reduced seminal plasma viscosity without impairing sperm motility, viability, acrosome integrity and DNA integrity.

This study has led to the development of a reliable, cost-effective protocol for the reduction of alpaca seminal plasma viscosity that can be implemented by the camelid industry to reduce viscosity prior to cryopreservation.

Determine the role of seminal plasma on camelid sperm function

When incubated in varying concentrations of seminal plasma the motility of ejaculated and epididymal alpaca sperm (incubated for 6h at 37 °C) was prolonged in the presence of 10% seminal plasma compared to 0, 25, 50, or 100% seminal plasma. Moreover, 10% seminal plasma maintained the acrosome integrity of ejaculated and epididymal sperm and also reduced the proportion of non-viable ejaculated sperm.

This study highlighted the need to retain 10% seminal plasma during sperm cryopreservation, or alternatively to ensure that upon thawing 10% seminal plasma remains in the sample. This could be achieved by adding 10% seminal plasma back to the sperm upon thawing (if sperm are removed from the seminal plasma for cryopreservation) or by maintaining at least 10% seminal plasma prior to freezing then diluting to a final seminal plasma concentration of 10% upon thawing

This finding will aid the rapid development of sperm cryopreservation protocols in camelids as it will enable other research scientist to optimise the seminal plasma concentration when developing semen extenders for camelids.

Investigate methods of sperm cryopreservation in alpacas and establish the effect of viscosity reduction on the success of sperm cryopreservation

The cryopreservation of papain and E-64 treated viscosity-reduced alpaca semen was investigated. The reduction of alpaca seminal plasma viscosity prior to cooling and cryopreservation improved the motility of frozen-thawed alpaca sperm. 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. Whilst this study indicated that papain and E-64 treatment improve the success of sperm cryopreservation, post-thaw motility rates were still not commercially acceptable (up to 25%) and thus research should now focus on using papain-treated viscosity reduced semen to develop an industry standard semen extender for alpaca sperm cryopreservation.

In order to benefit from the optimised method for seminal plasma viscosity reduction 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.

Implications for relevant stakeholders

The practical outcomes arising from this research are 1) the development of sophisticated in vitro methods for the assessment of sperm function including acrosome integrity, viability and DNA

integrity 2) identification of the cause of viscosity and the development of a protocol for the reduction of seminal plasma viscosity that can be adopted by the camelid industry and research scientists and 3) the recommendation that a final seminal plasma concentration of 10% is maintained prior to cryopreservation or post-thaw in frozen-thawed alpaca sperm.

Whilst these deliverables do not provide answers to the perfect method for sperm cryopreservation and AI in camelids, they enhance the development of such technologies.

The validation of methods for the sophisticated analysis of sperm integrity in camelids will enable other camelid research scientists to fully elucidate the function of camelid semen in order to predict its fertilising ability. This is important, as the ability to perform additional analyses of sperm function and integrity will provide greater data on the effect of cryodiluents, freeze thaw rates and storage methods (pellets or straws), thereby aiding the rapid progression of the further research that is required to establish ARTs in camelids.

The protocol described for the reduction of seminal plasma viscosity will directly benefit the camelid industry as well as benefit camelid researchers. The major impediment to the development of sperm cryopreservation in camelids was the viscous seminal plasma. Whilst there had been attempts to alleviate the viscosity, a lack of understanding of the cause of viscosity prevented identification of a suitable method to reduce the viscous component of the seminal plasma without impairing sperm function. In identifying the protein Mucin 5B as the protein responsible for viscosity, we were able to develop a protocol that degraded this protein, thereby reducing viscosity, without impairing sperm function and integrity. We have thereby overcome the current major problem associated with the development of cryopreservation in alpacas. In doing so, other camelid researchers can now make substantial progress in the development of camelid ARTs by using this protocol prior to cryopreservation in order to improve post-thaw sperm function and fertility. In order to fully benefit from this research outcome, considerable progress must be made in 1) determining the optimal cryodiluent for camelid sperm and 2) optimising the protocol for AI in camelids.

The finding that the presence of 10% seminal plasma is advantageous to sperm motility, viability, and acrosome integrity provides future researchers with a starting point for the development of storage protocols for camelid sperm. Whilst progress in sperm storage for alpacas has been made, the longevity of the sperm during liquid freezing and the post-thaw motility of frozen-thawed sperm are not commercially viable. In using a final seminal plasma concentration of 10% it is likely that greater sperm function will be achieved and hence, this research finding will advance the development of sperm storage protocols in camelids.

In conclusion, the practical outcomes of this research project will advance the development of cryopreservation and AI in camelids facilitating the rapid dissemination of superior genotypes thereby increasing the rate of gain of favourable characteristics such as a high fleece density and low fibre diameter. Producers will benefit from the higher prices obtained for high quality low diameter fleece and higher fleece weights. Cryopreservation will enable the long term storage of sperm and the worldwide transportation of sperm enabling genes from genetically superior males to be preserved indefinitely and breeders to disseminate the genetics of their superior males or inseminate their females both domestically and internationally without the need to transport live animals, thereby reducing shipping costs. Additionally domestic and international demand for cryopreserved semen of genetically superior males will enable breeders to benefit from increased stud fees. The increase in fibre quality gained by the use of these assisted reproductive technologies will increase Australia's reputation as a producer of high quality alpaca fibre, thereby increasing the industry competitiveness in both the domestic and international fibre markets. Additionally the Australian camelid industry will be at the forefront of the development of ARTs in camelids and will benefit from the increased reputation associated with being a world leader in this area.

Recommendations

Based on the findings of the current research the following recommendations are made to the camelid industry, breeding centres and research scientists:

- Camelid semen should be diluted 1:1 in a suitable semen extender such as tris-citrate-fructose then treated with 0.1mg/ml papain (final concentration) for 20min at 37°C to completely eliminate viscosity of semen. The effect of papain should then be halted by the addition of 10µM E-64 (final concentration) at 37°C for 5min. The viscosity-reduced semen can then be utilised for sperm cryopreservation procedures.
- A concentration of 10% seminal plasma (dilution rate of 1:9) should be used to preserve the longevity of alpaca sperm function. In particular frozen-thawed sperm should be either diluted to a final seminal plasma concentration of 10% or 10% seminal plasma should be added back to the sperm after thawing. Using a dilution ratio of 1:9 (semen: extender) will help improve the motility of cryopreserved sperm during freezing and post-thaw.

To fully benefit from the key findings of this research project, further research is essential. In determining the cause and source of the viscous seminal plasma in alpaca semen and establishing a protocol that reduces viscosity without impairing sperm function we have overcome the current major impediment to cryopreservation. These findings provide a platform on which to develop a successful cryopreservation and subsequent artificial insemination protocol which can be used commercially within the alpaca industry

The cryopreservation of camelids semen remains problematic. Despite numerous investigations into the optimal cryodiluent components, post-thaw motility remains low (approximately 20%) and is not commercially viable. In these studies, the viscous component of the seminal plasma was not removed or degraded prior to cryopreservation. Consequently in order to develop a cryopreservation protocol in camelids, it is necessary to optimise the cryopreservation of viscosity-reduced semen. This requires a systematic approach to 1) identify the most effective cryodiluent components for camelid sperm including buffer, energy source and cryoprotectants, 2) investigations on the effect of sperm storage (pellets or straws) on sperm function during cryopreservation and 3) determine the optimal freeze/thaw rates that maintain sperm function and integrity.

Of interest is also the role of seminal plasma in sperm function. In numerous species, seminal plasma is routinely diluted or removed during processing of semen for cryopreservation and this can induce either positive or negative effects on the function and fertility of sperm. Camelid semen requires the presence of 10% seminal plasma in order to maintain sperm function and integrity. In the absence of seminal plasma, sperm become acrosome impaired and therefore not fertile. It is therefore important to investigate the addition of seminal plasma proteins either pre-freeze or post-thaw on the fertilising ability of camelid sperm as this may aid the development of artificial insemination through enhanced fertility rates.

In order to fully benefit from the ability to cryopreserve sperm, the AI of females with frozen-thawed sperm must be reliable and effective in achieving pregnancy rates of over 60%. The development of successful AI protocols in camelids is hindered by a lack of understanding of reproductive physiology and knowledge of the optimal methods for fertilisation, in particular the timing of ovulation in female camelids and the optimal time of AI relative to ovulation as well as the sperm numbers required to achieve pregnancy and the optimal site of sperm deposition.

Whilst AI in camelids has been successful using fresh and frozen-thawed semen pregnancy rates are not sufficient for application within the camelid industry. The effect of sperm numbers and site of deposition of semen during AI implies that transcervical insemination is required to achieve reasonable pregnancy rates however the effect of deeper inseminations (i.e. at the uterine horn) is conflicting with some researchers reporting greater fertility and others reporting no difference. Despite attempts to develop AI protocols in camelids, the optimal sperm numbers, site of deposition and timing of

insemination have not yet been identified and thus pregnancy rates following AI in camelids do not reach the requirement for commercialisation (at least over 60%). In order to fully commercialise AI in camelids, these questions must be addressed.

In conclusion, in order for the industry to practically apply sperm cryopreservation and artificial insemination, the following must be determined:

- the optimal cryodiluent components including buffer, energy source and cryoprotectants to maintain sperm integrity and function of viscosity-reduced, frozen-thawed alpaca sperm
- the optimal storage method (pellets or straws) for viscosity-reduced, frozen-thawed alpaca sperm
- the optimal free-thaw rates for viscosity-reduced, frozen-thawed alpaca sperm
- the fertilising ability of viscosity-reduced frozen-thawed alpaca sperm in vitro and in vivo.
- the effect of seminal plasma pre-freeze or post-thaw on the fertilising ability of sperm
- the optimal ovulation synchronisation protocol prior to artificial insemination in alpacas
- the optimal sperm dose, site of deposition and timing of artificial insemination for viscosity-reduced frozen-thawed alpaca sperm

In addressing these challenges reliable, effective protocols for sperm cryopreservation and artificial insemination for use within the camelid industry can be established.

1. Introduction

The Australian Alpaca Industry

Globally there are 3.5million alpacas and within Australia there are approximately 2500 stud farms with over 100,000 registered stud alpacas, the largest registered herd of alpacas worldwide. As a leader in alpaca farming, Australia has already improved the genetics of the alpaca herd to produce a high quality fibre, however, due to a lack of understanding of camelid reproductive physiology, the rate of genetic gain of desirable characteristics is slow and consequently the proportion of animals producing a premium low micron, high density fleece is low. In order for the camelid industry to increase its rate of genetic gain for desirable traits including genetic diversity, fleece weight, fibre diameter and reproductive efficiency, assisted reproductive technologies (ARTs) including sperm cryopreservation and artificial insemination (AI) are essential.

Assisted Reproductive Technologies

Artificial insemination is used in other livestock species to 1) increase the rate of genetic gain by maximising the use of superior males 2) produce improved offspring by the introduction of superior genotypes 3) contain contagious disease within flocks by limiting the need for direct male-female matings and transport of animals between farms and 4) enable the use of incapacitated males, for example males with an injury that prevents natural mating can be used for semen collection and subsequent AI enabling his continued use for breeding. When use in conjunction with cryopreserved sperm, AI offers many other advantages. In particular sperm cryopreservation enables the long term storage of sperm, enabling genes from genetically superior males to be preserved indefinitely. The Australian alpaca industry is heavily based on large numbers of farms with small herd sizes of 20-40 alpacas. Consequently, many breeders do not own a male that is suitable for breeding superior genetics into the herd and thus genetically superior males are transported between farms for on-site matings. Cryopreserved semen is easily transported enabling the worldwide transportation of sperm from stud males. This reduces the need to maintain males on small properties and also eliminates the need for transportation of males and on farm matings hence reducing costs to the breeder, limiting the spread of disease and improving overall welfare.

In order for an AI programme to be successful, it is necessary to have reliable, effective, economically viable, commercially available standardised protocols for semen collection, semen and sperm assessment, sperm storage i.e. cryopreservation and AI. In camelids, establishing these protocols have been met with challenges that are unique to the species, in particular the unique reproductive physiology and the highly viscous nature of the semen.

Semen Collection

Semen collection in camelids has faced challenges due to the position of mating, prolonged mating time, the ejaculatory process of male alpacas and intrauterine deposition of semen. Males mate in sternal recumbancy for on average 20-25min with a range of 5-60min (Brown 2000; Vaughan et al. 2003; Morton et al. 2008; Adams et al. 2009). During copulation, males ejaculate consistently copulation with distinct urethral pulses that vary in frequency and length throughout copulation (Lichtenwalner et al. 1996), and semen is deposited into the uterus.

Numerous methods for semen collection have been investigated including intravaginal condoms, vaginal sponges, fistulation of the urethra, post-coital vaginal aspiration, electroejaculation and artificial vagina (AV) (Adams et al. 2009). Condoms, sponges, fistulation of the urethra and post-coital vaginal aspiration also produced sub-standard ejaculates with some ejaculates containing no sperm and others contaminated with blood. Electroejaculation and AV provide high ejaculation rates

(85-100%), however electroejaculation requires heavy sedation or general anaesthetic and resulted in contamination of the ejaculate with urine as well as a large variation in sperm concentration between ejaculates (Brown 2000; Giuliano et al. 2008). Consequently, owing to the disadvantages of the other techniques, an AV used with either a receptive female or alpaca mannequin has been adopted as the accepted method of semen collection in camelids. Although this requires training of the males for semen collection, the success of ejaculation of higher than other methods and the resulting ejaculate is representative of an ejaculate during natural mating. Attempts to improve the quality of the ejaculate include the addition of a cervix-like structure in the AV, the presence of females during collection and the inclusion of a semen extender in the collection vessel (Morton et al. 2009). Whilst the addition of a semen extender improved sperm motility, no other positive effects on sperm quality were noted. Although semen collection with an AV has some disadvantages, it is superior to the other available techniques and produces ejaculates of good quality that can be used for downstream applications such as sperm storage and AI. Consequently, whilst semen collection procedure has halted the development of ARTs in camelids, a standardised protocol is now available, and used routinely in the camelid scientific community.

Characteristics of Alpaca Semen

Semen is comprised of sperm cells derived from the testes and seminal plasma derived from testes, prostate gland and bulbourethral gland secretions. Camelids do not have a vesicular gland unlike other livestock species including the ram and bull. In alpacas approximately 85% of the ejaculate is seminal plasma and the remaining 15% sperm cells (Garnica et al. 1993; Bravo et al. 1997). The biochemical composition of alpaca seminal plasma has been characterised and contains, amongst other things citric acid, chloride, calcium, protein, lipids, fructose and glucose at concentrations similar to those observed in other farm species, with the exception of citric acid and fructose which were relatively low, most likely due to the absence of the vesicular gland in camelids (Garnica et al. 1993; Bravo et al. 2000). The protein composition of camelid semen has not been elucidated. Identifying the proteins present may aid the understanding of the role of seminal plasma on sperm function in this species. Additionally, characterisation of the proteins present may identify proteins that are integral to the fertilising capacity of the sperm or reproductive function.

Camelid semen is described as highly viscous, low volume with low sperm concentration that exhibit limited motility (Bravo et al. 1997; Bravo et al. 2002; Deen et al. 2003). The high viscosity associated with camelid semen makes the assessment of sperm motility, concentration and morphology difficult, however techniques used routinely for other domestic species such as ram and bull have been utilised. Alpaca ejaculates are clear to creamy-white in colour depending on sperm concentration with an average volume of 1-2ml (Garnica et al. 1993; Bravo et al. 1997; Vaughan et al. 2003). Sperm concentration ranges from 30,000 to 150 million sperm/ml and varies widely between males, ejaculates and semen collection procedure (Vaughan et al. 2003). Camelid sperm exhibit low mass motility, which move in an oscillatory manner with limited progressive motility (Garnica et al. 1993; Deen et al. 2003). It is believed that this limited motility is caused by the highly viscous nature of the semen which traps the sperm within the seminal plasma (fig 1.1)

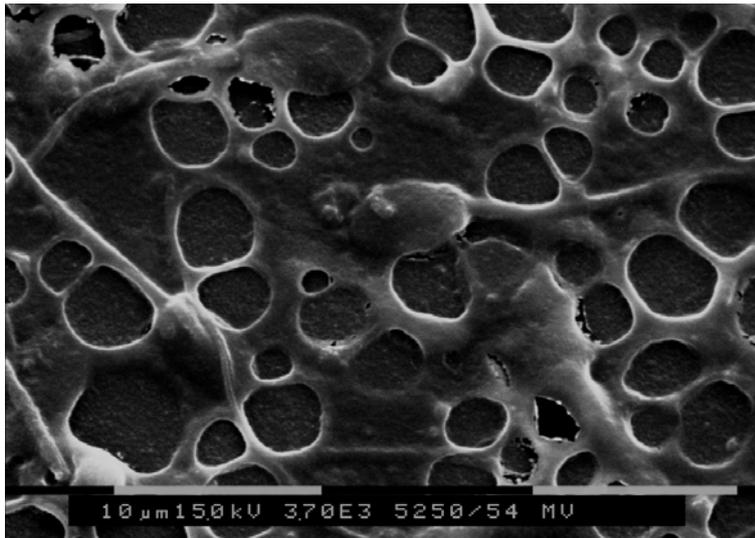


Figure 1.1 Scanning electron microscopy of glutaraldehyde-fixed alpaca sperm entrapped in viscous seminal plasma. Scale bar represents 10 μ M

Viscosity and Liquefaction of Camelid Seminal Plasma

Characteristic of Seminal Plasma Viscosity

The highly viscous nature of camelid semen makes semen assessment difficult and has also hindered the development of ARTs in this species. It is presumed that the viscosity prevents loss of semen from the reproductive tract after mating by reducing back flow to the vagina and vulva (Deen et al. 2003). Given the low sperm numbers and low ejaculate volume observed in camelids the sperm reservoir created by the viscous semen is likely to increase the chance of fertilisation. Additionally, female camelids are induced ovulators. An unknown factor present in the semen, termed ovulation inducing factor, induces ovulation approximately 30h after mating (Adams et al. 2005). It is postulated that the viscous semen enables the slow release of sperm from the ejaculate, prolonging the life of the sperm and ensuring that functional sperm are present when the female ovulates, thereby increasing the likelihood of successful fertilisation. However, despite these advantages for natural mating, the highly viscous seminal plasma is currently the major impediment to the development of sperm cryopreservation in camelids.

Camelid semen liquefies naturally when left at room temperature. Alpaca semen liquefies on average 23h after ejaculation, ranging from 8-48h (Garnica et al. 1993), whereas camel semen tends to liquefy quicker, with partial liquefaction within 20-30min (Skidmore and Billah 2006; Niasari-Naslaji et al. 2007) and complete elimination of viscosity after 1.5h of ejaculation (Wani et al. 2008). Natural liquefaction is observed in other species with viscous seminal plasma such as human (Lilja and Laurell 1984) and baboon (Amboka and Mwethera 2003) however in these species the coagulum generally liquefies with 15min of ejaculation enabling rapid use of the ejaculate for downstream applications such as sperm cryopreservation, AI and in vitro fertilisation (IVF). The prolonged liquefaction time in camelids prevents the processing of semen for ARTs as sperm integrity and function declines following ejaculation, and is not maintained during the liquefaction process. Consequently in order to apply technologies such as cryopreservation and AI in camelids, it is necessary to 1) determine the cause of the viscous seminal plasma and 2) determine ways to eliminate the viscosity without impairing sperm function and integrity.

The cause of the viscosity is unknown, however it has been postulated, but never proven that glycosaminoglycans (GAGs; previously termed mucopolysaccharides) derived from the bulbourethral gland may be responsible (Perk 1962; Mann 1964; Ali et al. 1976). Glycosaminoglycans are large

carbohydrate chains of repeating disaccharides, and are present in the seminal plasma of human (Binette et al. 1996). Fluids that contain GAGs create a ferning pattern when smeared onto a glass slide (Menarguez et al. 2003), and this phenomenon is observed in alpaca seminal plasma (fig 1.2) suggesting that GAGs may be the cause of viscosity. It is necessary to identify the cause of the viscosity in order to advance the development of ARTs in camelids.

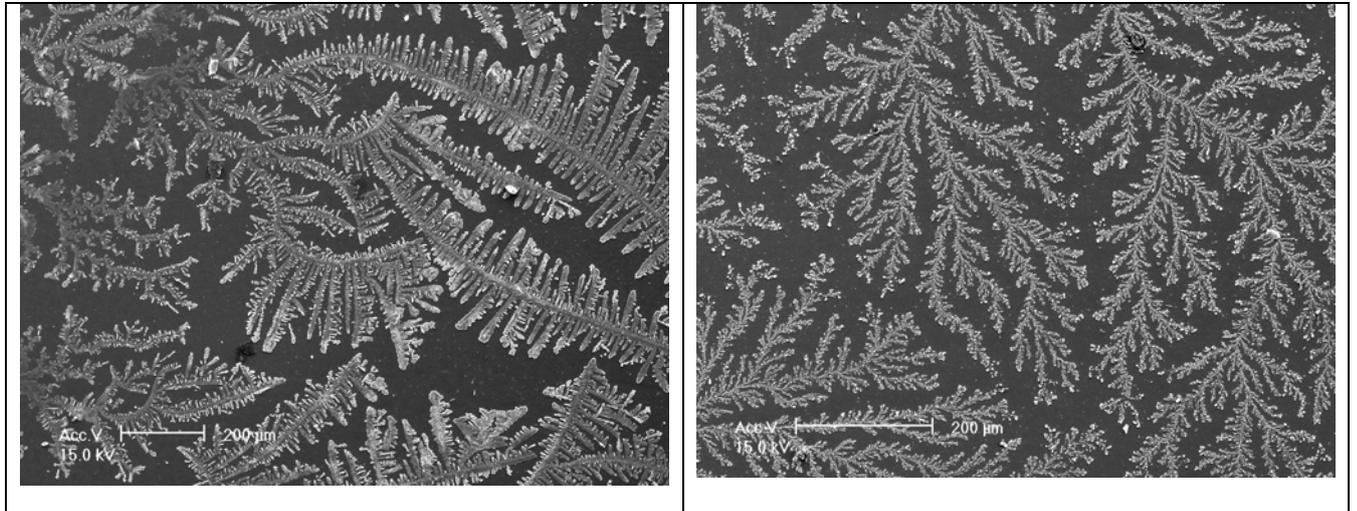


Figure 1.2 Scanning electron microscopy of ferning patterns in alpaca seminal plasma. Semen was centrifuged at 10,000 x g for 30min, the seminal plasma decanted and smeared onto a glass slide then left to dry at room temperature prior to scanning electron microscopy. Scale bars represent 200µm.

Liquefaction of Seminal Plasma

As the viscous component of the semen is the major impediment to the processing of camelid sperm for ARTs, the reduction of seminal plasma viscosity has been an area of research focus. Numerous methods have been investigated including mechanical and enzymatic. Protocols utilised for semen liquefaction in other species such as human have also been adapted and investigated for camelid semen.

Mechanical Methods

Mechanical approaches include centrifugation (Vaughan et al. 2003; Morton et al. 2008), vortexing (Vaughan et al. 2003), stirring (Mosaferi et al. 2005; Niasari-Naslaji et al. 2006), gradient centrifugation using PureSperm (Morton et al. 2008), needling (Santiani et al. 2005; Morton et al. 2008) and pipetting (Morton et al. 2008).

Attempting to separate camelid sperm from the viscous seminal plasma via centrifugation of the ejaculate did not impair sperm motility when used at low centrifugal forces (600 x g), however only 62% of sperm was recovered from the ejaculate (Morton et al. 2008). Additionally decanting the viscous seminal plasma from the centrifuged sample regularly caused disruption of the sperm pellet, more viscous samples required higher centrifugal forces and uncentrifuged sperm appeared to exhibit higher viability than centrifuge sperm (Vaughan et al. 2003).

Vortexing alpaca semen for 30min in an attempt to reduce viscosity resulted in death of all sperm and thus was not considered a suitable approach (Vaughan et al. 2003).

In bactrian camels stirring the ejaculate in a beaker containing a steel clip using a magnetic stirrer causes liquefaction of semen within 5min (Mosaferi et al. 2005; Niasari-Naslaji et al. 2006), and this approach has permitted the use of low viscosity samples for the development of semen cryopreservation protocols. However, personal investigations found this approach unsuitable for

dromedary and alpaca semen, perhaps owing to the higher viscosity semen observed in these species compared to the bactrian camel (Zhao 2000).

Gradient centrifugation of alpaca semen using PureSperm was investigated as a method of extracting sperm from the viscous seminal plasma as opposed to mechanically reducing seminal plasma viscosity. PureSperm gradient was successful at recovering 70% of sperm from the viscous seminal plasma without significantly reducing sperm motility or acrosome integrity, however, the method requires technical ability, is costly and given the low sperm concentrations in alpaca ejaculates a 30% reduction in sperm numbers was considered significant (Morton et al. 2008). Consequently this technique was not considered a viable option for adoption in the camelid industry.

The passing of alpaca semen through a small-gauge needle has been used to reduce viscosity prior to cryopreservation (Santiani et al. 2005) although the resulting effect on viscosity and sperm parameters were not reported. Morton et al. (2008) reported that needling was not effective in eliminating alpaca semen viscosity and that it also impaired sperm function.

The pipetting of alpaca semen using a 1ml pipette tip following dilution with semen extender reduced seminal plasma viscosity and improved sperm motility (Morton et al. 2008), however it is possible that the reduction in viscosity was attenuated by the semen extender as the effect of pipetting without dilution was not investigated. Although viscosity was reduced, pipetting did not completely eliminate viscosity, and thus other methods of viscosity reduction are required.

Enzymatic Methods

Whilst some mechanical approaches have shown promise, they have not been successful in completely eliminating the viscous component of the seminal plasma, consequently enzymatic methods of viscosity reduction have been attempted. To determine suitable enzymes for the reduction of viscosity, numerous researchers have employed techniques used for the liquefaction of human semen.

In humans, semen forms a coagulum immediately upon ejaculation which liquefies naturally within 15min. The viscosity is caused by the protein semenogelin which is secreted by the vesicular glands (Lilja and Laurell 1984; Lilja et al. 1987) and semenogelin is degraded by prostate-specific antigen that is secreted by the prostate gland (Lilja et al. 1987). This cause of viscosity and method of liquefaction are unlikely to be the cause of viscosity in camelid semen due to the lack of vesicular glands in camelids, however the presence of an enzyme occurring naturally in seminal plasma in order to cause reduction of viscosity suggests that enzymes may be successful in reducing camelid viscosity without impairing sperm function. In pathological conditions, human seminal plasma can be hyperviscous reducing the fertilising capacity of sperm and preventing its use for ARTs such as AI and IVF, therefore enzymatic means of reducing the viscosity have been investigated. Prostate specific antigen has been characterised as a serine protease (Watt et al. 1986), whilst other enzymes including trypsin (Pattinson et al. 1990; Mendeluk et al. 2000), α -amylase (Mendeluk et al. 2000), and chymotrypsin, subtilisin and papain (Pattinson et al. 1990) all reduce human seminal plasma viscosity. The effect of these enzymes on sperm function and integrity varies, but at the concentrations used in the studies they all (with the exception of α -amylase which was not studied) either reduced sperm motility or vitality.

The enzymatic reduction of human seminal plasma viscosity shows promise and therefore, the effect of similar enzymes on the viscosity of camelid semen has been investigated. Collagenase, fibrinolysin, hyaluronidase and trypsin all reduced, but did not eliminate llama and alpaca seminal plasma viscosity within 5min of treatment, however all but collagenase reduced sperm motility and viability (Bravo et al. 2000). Collagenase did not affect sperm motility or viability in alpaca but caused a 4% reduction in sperm motility in llama (Bravo et al. 2000). Collagenase-treated fresh llama sperm has been used successfully in intra-cytoplasmic sperm injection (ICSI) and in vitro fertilisation (IVF) (Conde et al. 2008) and fresh semen treated with 5mg/ml collagenase has been used for AI in alpacas generating pregnancy rates of 58-67% (Bravo et al. 1999) suggesting that collagenase-treated sperm retain their

fertilising ability. Conversely, collagenase concentrations of 0.5-4.0mg/ml have been reported to severely reduce alpaca sperm motility, with all sperm being non-motile within 20min of treatment (Morton et al. 2008). The apparent difference may be caused by the prolonged exposure time of collagenase in the latter study (10-60min compared to 2-5min) suggesting that it may be necessary to remove or inhibit the collagenase enzyme following liquefaction of seminal plasma in order to maintain sperm function. Whilst collagenase reduced seminal plasma viscosity, it did not completely eliminate it and thus it may be necessary to use higher concentrations of collagenase or longer incubation times in order to completely remove the viscous component of the seminal plasma. This may introduce negative effects on sperm function and integrity, although this requires further investigation. More positive effects of viscosity reduction have been observed using papain (Morton et al. 2008) in which 0.5-4mg/ml papain completely reduced alpaca seminal plasma viscosity within 20-40min of treatment without affecting sperm motility, although a decrease in acrosome integrity was observed within 10min at 4mg/ml papain and within 20min at 0.5-2mg/ml. As for collagenase prolonged exposure of sperm to papain and additionally high concentrations of papain appear to be detrimental to sperm function and integrity. It is evident that a balance between enzyme concentration and exposure time and the need to eliminate viscosity is established for enzymatic reduction of seminal plasma viscosity in order to maintain sperm function and integrity.

Although enzymatic reduction of camelid seminal plasma viscosity has had some success (i.e. pregnancies from collagenase-treated sperm, and elimination of viscosity with papain) there are still no standardised protocols for the reduction of viscosity and the effect of viscosity reduction on the success of sperm cryopreservation has not been thoroughly assessed. Enzymes that have been tested have been based on findings in humans, despite the high probability that the cause of seminal plasma viscosity in humans is caused by a different factor than that in camelid semen. Whilst this shot-gun approach to viscosity reduction in camelids has produced some interesting and promising findings, it is necessary to determine the cause and source of the viscous seminal plasma in order to identify enzymes that will degrade the viscous component of the seminal plasma without affecting sperm function.

Storage of Camelid Sperm

Attempts to store camelid sperm have been met with difficulties owing to a lack of understanding of the constituents of the seminal plasma as well the role of the seminal plasma on sperm function. Studies have been predominantly based on methods used for other species and have failed to systematically investigate the many factors associated with successful liquid storage or cryopreservation.

Liquid storage of semen at temperatures of 15 or 5°C and cryopreservation prolong sperm function though a reduction in the metabolic activity of the sperm. Liquid storage generally permits the storage of semen for up to 48h in other livestock species however cryopreserved sperm can be stored in liquid nitrogen indefinitely enabling the long-term storage of superior genetics as well as the ease of sperm distribution worldwide.

For successful sperm storage it is necessary to optimise the type and concentrations of constituents within the semen extender including buffer (used to maintain pH and tonicity), energy source i.e. the sugar for such as the type of sugar that can be metabolised by the sperm, cooling agent such as egg yolk or skimmed milk to protect the sperm cell membrane during cooling to 5°C, and cryoprotectants such as glycerol to protect the sperm against membrane damage during freezing. The storage method of the sperm (sperm pellets or straws) and the dilution, cooling, freezing and thawing rates of the sperm also affect the success of liquid storage and cryopreservation. With so many factors to consider and optimise it is not surprising that an optimal semen extender and liquid storage or cryopreservation protocol has not yet been established for camelids.

Liquid Storage

Numerous extenders including, lactose-based, sucrose-based, citrate-based, and fructose-based buffers in addition to the commercially available extenders manufactured for other livestock species such as Green buffer, Biladyl, Androhep and Triladyl have been investigated for their ability to liquid store camelid sperm. In bactrian camels a tris-based extender at a pH of 6.9 and osmolarity of 330mOsm was able to maintain sperm motility of up to 55% after 24h storage at 4°C. The tris-based extender was superior to Green buffer, lactose-based and sucrose-based extenders (Niasari-Naslaji et al. 2006). In dromedary camels, tris-tes-based, lactose-based and sucrose-based extenders were superior to fructose-based and citrate-based extenders in preserving sperm motility when stored at both room temperature and 5°C. Whilst sperm motility was maintained above 40% after 24h storage at both temperatures when using the optimal extenders, motility was less than 20% in all extenders after 48h incubation at either temperature. Additionally the acrosome integrity of sperm stored at 4°C decreased significantly within 12h of incubation (Wani et al. 2008). In alpacas, sperm motility rates of 50% and 45% were achieved at 24 and 48h of incubation respectively using the commercial semen extender Triladyl for liquid storage at 4°C (Vaughan et al. 2003). Biladyl was also successful in maintaining sperm motility rates of 45% and 35% at 24 and 48h of storage respectively, when stored at 4°C (Vaughan et al. 2003). In another study, motility rates of only 33% and 12% at 24 and 48h were observed for alpaca semen stored at 4°C using Triladyl, although Triladyl was superior to Androhep, lactose and tris-based buffers in maintaining sperm motility (Morton et al. 2009). The optimal extender was identified as Biladyl, and a ratio of 1 part semen to 4 parts Biladyl was found to be optimal for liquid storage of epididymal alpaca sperm at 4°C (Morton et al. 2008).

Whilst some studies have shown promising motility rates after 48h of sperm storage (Vaughan et al. 2003) others have not been in successful in maintaining over 40% motility, which given the low sperm numbers in camelid semen, represents a significant loss of sperm, suggesting that these samples would not be suitable for AI. It has been reported that liquid storage of llama semen was unsuccessful without prior elimination of viscosity, but that after mechanical reduction of viscosity, motility rates improved (reviewed in Adams et al. 2009). The generally lower viscosity of bactrian camel semen (Zhao 2000) compared to other camelids permits the reduction of viscosity using a steel clip and magnetic stirrer (Niasari-Naslaji et al. 2006), and it may be that the removal of the viscous seminal plasma component enabled more successful liquid storage of sperm compared to dromedary camel, alpaca and llama.

Cryopreservation

Numerous studies on the cryopreservation of camelid sperm, particularly dromedary and bactrian camels, have been conducted and extensively reviewed previously (Morton et al. 2008). In bactrian camels post-thaw motility rates of 35% have been reported using tris-based extenders (Niasari-Naslaji et al. 2007) whereas other researchers have described immediately post-thaw motility rates as high as 65% (reviewed in Morton et al. 2008). In dromedary camels, only 50% of ejaculates exhibited post-thaw motility rates of greater than 20% when using a tris-citrate-fructose-based buffer supplemented with egg yolk and glycerol (Deen et al. 2003) whereas El-Bahrawy et al (2010) reported post-thaw motility rates ranging from 20-38%. In llamas the motility rates of cryopreserved semen post-thaw range from 0-40% (reviewed in Morton et al. 2008). Up to 28% post-thaw motility has been observed in llama semen cryopreserved in a tris-based semen extender (von Baer and Hellemann 1999) and post-thaw motility rates of 30-40% were reported using a citrate-based extender (Bravo et al. 2000). In the latter study, llama semen viscosity was reduced with 1mg/ml collagenase prior to cryopreservation, and this cryopreserved sperm, when used for AI was successful in producing pregnancy rates of 26% (5/19 alpacas gave birth to live cria). The cryopreservation of alpaca sperm has been equally as unsuccessful with mean post-thaw motility rates of 4-20% when using tris-based or skimmed milk-fructose-based extenders (Santiani et al. 2005). Using the commercially available extenders Biladyl and Green buffer, post thaw motility rates averaged 21% and 17% respectively (range 0-40%) although attempts at using other commercial extenders, including Triladyl which was successful for

liquid storage of alpaca sperm, gave post-thaw motility rates of 0-10% (Vaughan et al. 2003). Post-thaw motility rates of 27% were achieved when cryopreserving epididymal alpaca sperm using a lactose-based extender a final glycerol concentration of 3 or 4% and freezing in pellets (Morton et al. 2007; Morton et al. 2010).

As indicated by the poor post-thaw motility rates, further research is required in order to optimise cryopreservation in camelids. The highest post-thaw motility rates were observed in bactrian camels, viscosity-reduced llama semen and epididymal alpaca sperm. Bactrian camel semen is generally less viscous than other camelids (Zhao 2000) and consequently the viscous component of the semen can be removed by mechanical methods prior to processing for storage (Niasari-Naslaji et al. 2007). Epididymal sperm are extracted from the epididymides of castrated males and thus have not come into contact with the seminal plasma secretions from the bulbourethral and prostate glands, and have therefore not been exposed to the viscous seminal plasma. Consequently, epididymal sperm are easier to assess for basic sperm parameters such as motility and concentration, and exhibit forward progressive motility as opposed to the oscillatory motility that is observed in ejaculated semen (Morton et al. 2007). The greatest success in the cryopreservation of llama sperm was observed when the viscous component of the seminal plasma was reduced by the enzyme collagenase, presumably because the semen was able to homogenise fully with the cryodiluent. The findings from these three studies highlight the detrimental effect of seminal plasma viscosity on the cryopreservation of camelid sperm. When the viscosity was removed either by mechanical (bactrian camel) or enzymatic (llama) means, or alternatively was completely eliminated by the use of epididymal sperm (alpaca), post-thaw motility rates improved.

Concluding Remarks on Storage of Camelid Sperm

Whilst some progress has been made in determining the optimal buffer, energy source, and cryoprotectant (glycerol) concentration for liquid storage and cryopreservation of camelid semen, much more research is required. Studies that utilised techniques to overcome the viscous nature of the camelid seminal plasma had greater success rates, highlighting the detrimental effect of seminal plasma viscosity on the development of sperm storage protocols and subsequent ARTs. During cooling, lipids within the egg yolk protect the sperm membrane against damage and cryoprotectants such as glycerol permeate the sperm cell membrane to reduce the formation of intracellular ice crystals. It is therefore essential that the sperm cells are able to interact with the semen extender and cryoprotective compounds in order to protect the sperm during cryopreservation. The viscous nature of camelid semen impedes the homogenous mixing of semen with extender, thereby limiting contact between the sperm cell membranes and cryoprotectants. In order to develop effective, reliable methods of sperm cryopreservation in camelids, it is essential to reduced or eliminate the viscous component of the seminal plasma prior to cryopreservation.

Artificial Insemination in Camelids

Due to the limited motility of liquid-stored sperm and the poor post-thaw motility rates of cryopreserved camelid sperm, the development of protocols for AI in camelids has been mainly based on the use of fresh or diluted semen.

In dromedary camels, the insemination 150×10^6 fresh motile sperm into the uterine body 24h after induced ovulation resulted in conception rates of 53% and deeper inseminations did not improve conception rates (Skidmore and Billah 2006). In llamas and alpacas, fertility rates of 2-75% have been reported using fresh or diluted semen (reviewed in Bravo et al. 2000), although the sperm dose was not reported in the majority of studies. Bravo et al. (1999) and Quispe (cited in Morton et al. 2008) reported pregnancy rates of 67% of 57% respectively when inseminating alpacas with 8×10^6 sperm, suggesting that 8 million sperm may be suitable for AI with fresh semen in alpacas, which is much lower than the dose required for camels.

Using frozen-thawed semen, the success rates of AI vary widely amongst studies. In particular, in bactrian camels, pregnancy rates of 95-100% were reported when using sperm doses ranging from 2.9×10^6 to 1.48×10^9 and either single or double inseminations. These pregnancy rates are higher than the 60-65% pregnancy rates observed for natural mating. Although remarkable fertility rates have been reported in bactrian camels, the success rates in dromedary camels, alpacas and llamas are much lower. In dromedary, only 1/13 camels were diagnosed pregnant following insemination with frozen-thawed sperm (Deen et al. 2003), in llamas pregnancy rates of 8% were obtained using frozen-thawed sperm (Aller et al. 2003 cited in Miragaya et al. 2006), and in alpacas no pregnancies were obtained using AI with frozen-thawed sperm (Vaughan et al. 2003). The greatest success using frozen-thawed sperm in llamas and alpacas was achieved using viscosity-reduced frozen-thawed semen. When the viscosity of llama and alpaca sperm was reduced using collagenase then cryopreserved and used for AI of alpacas, 5 of 19 females (26%) gave birth to live cria (Bravo et al. 2000). The findings of Bravo et al (2000) highlight the advantage of reducing seminal plasma viscosity prior to cryopreservation on the development of ARTs in camelids.

Summary

Camelid semen is characterised by a highly viscous low volume ejaculate with low sperm numbers and low sperm motility. The viscous nature of the seminal plasma is currently the major impediment to the development of ARTs in camelids, preventing successful liquid-storage and cryopreservation of sperm due to an inability of the protective agents in the semen extender to interact with the sperm cell membrane. The cause of the viscosity is unknown, and although numerous methods including mechanical techniques and enzymatic reduction of viscosity have been investigated they have been unsuccessful in that they either 1) do not completely eliminate viscosity or 2) they are detrimental sperm function and integrity. In order to develop ARTs in camelids it is essential to identify the cause, source and role of the viscous component of camelid semen and investigate ways to reduce the viscosity without impairing sperm function, integrity and fertility. The effect of viscosity reduction on the ability to cryopreserve camelid sperm must also be determined. Once reliable, efficient methods for viscosity reduction and cryopreservation have been established the focus should turn to developing a successful AI protocol for use within the camelid industry.

2. Objectives

Although considerable progress has been made with regards to semen collection, assessment and processing in camelids (RIRDC projects US-138A and AAA-1A) the cryopreservation of camelid semen is not implemented in the alpaca industry due to unreliable and inefficient sperm function after freezing and thawing. The major impediment to the development of sperm storage protocols in camelids is the viscous seminal plasma. If the industry is to achieve the practical application of liquid and frozen sperm storage, and a viable system of artificial insemination in camelids, particularly llamas and alpacas, it is necessary to determine: 1) The sources and constituents of the viscous seminal plasma and 2) The basic protein content, structure and function of camelid seminal plasma. Analysing the source and constituents responsible for semen viscosity is critical to developing methods to overcome or circumvent the deleterious effects of the viscous seminal plasma on sperm cryopreservation.

The main objectives of this project were therefore to:

- identify the source and constituent of the viscous seminal plasma
- investigate ways to reduce the viscosity of alpaca seminal plasma without impairing sperm function and integrity
- determine the role of camelid seminal plasma on sperm function
- investigate methods for the cryopreservation of alpaca sperm.

The expected outcomes of this project were:

- identification of the source, constituents, basic protein structure and function of the viscous camelid seminal plasma
- an understanding of the effect of seminal plasma on the function and integrity of ejaculated and epididymal sperm
- development of a method to reduce the viscosity of camelid seminal plasma to ameliorate the deleterious effects of the viscous seminal plasma on cryopreservation and the integrity of sperm
- optimisation of protocols for cryopreservation of camelid sperm.

These outcomes will aid the development of assisted reproductive technologies including sperm cryopreservation and artificial insemination in camelids and thereby facilitate the widespread use of sires of high genetic value (both Australian and International) increasing the rate at which superior genetics are disseminated.

Semen Assessment

Immediately following collection, the AV was removed from the mannequin and the AV was swung at arm's length to encourage the semen within the AV liner to flow into the camel collection glass. The collecting glass was detached from the AV, covered with parafilm, taken to the laboratory and maintained at 35-37°C in a water bath during assessment.

Semen Volume

Semen volume was determined using the measure on the camel collection glass which measured from 0-15ml in 0.1ml graduations (fig 3.2). When foam was present in the ejaculate, the ejaculate was allowed to settle for 5 min from the end of mating prior to assessing volume. When the foam did not settle it was not included in the final semen volume.



Figure 3.2 Assessment of semen volume using graduations on the camel collecting glass

Semen Viscosity

Viscosity was assessed using the thread test as described previously (Bravo *et al.* 2000). Fifty μ l of semen was drawn into a pipette, 25 μ l was pipetted onto a warm glass slide and the pipette was moved vertically from the glass slide forming a thread of semen. The length of the thread was measured using a ruler to the nearest mm and the length at which the thread of semen snapped was recorded as the measurement of viscosity (fig 3.3).



Figure 3.3 Viscous alpaca semen creating a thread when lifted vertically from slide with a pipette.

Sperm Assessment

Sperm Motility

Sperm motility was assessed subjectively at X 100 magnification under phase contrast microscopy (Olympus, Tokyo, Japan) by placing 10 μ l of neat semen on a warm slide and covering with a warm 22 x 22 mm coverslip as described previously (Evans and Maxwell 1987). At least 4 fields of view were assessed for motility which was measured in 5% increments. Unless otherwise stated in individual research chapters, all motile sperm, whether oscillatory or progressive were considered motile and used to generate a value for total motility.

Sperm Concentration

Sperm concentration was assessed using a haemocytometer as described previously (Evans and Maxwell 1987). Semen (10 μ l) was diluted 1:9 in 90 μ l 3% sodium chloride (Sigma) and mixed by vortexing to render the sperm immotile. Next 10 μ l of diluted semen was pipetted onto each side of a haemocytometer and allowed to settle for 5 min in a humidified chamber at room temperature then the sperm was observed at X 400 magnification under phase contrast microscopy (Olympus, Tokyo, Japan) and the number of sperm in each grid counted. The average from both sides and the dilution factor were used to calculate the total number of sperm per ml of ejaculate. Where sperm counts in each side of the haemocytometer differed by more than 10%, another concentration assessment was made.

Sperm Acrosome Integrity

To determine whether sperm acrosomes were intact or not-intact sperm were stained with fluorescent isothiocyanate-conjugated lectin from *Arachis hypogaea* (FITC-PNA). Two methods were used, one which required manual assessment under fluorescent microscopy and the second which utilised flow cytometry to count the number of stained sperm. The methods used are identified in each individual results chapter and are described below.

Acrosome Integrity by Microscopy

Acrosome integrity was assessed using a method validated in our laboratory for formalin fixed alpaca sperm based on methods by Morton et al. (Morton et al. 2007) and Leahy et al. (Leahy et al. 2010). Samples (20 μ l aliquots) were fixed in 0.1% (final concentration) neutral buffered formalin and stored at 4 °C for 24 to 48 h until analysis. To remove seminal plasma and reduce background staining, samples were resuspended in 200 μ L 0.02M PBS, centrifuged at 1000 x g for 10 min at room temperature, the supernatant discarded and the pellet resuspended in 0.02M PBS to 10 x 10⁶ sperm/ml. Twenty μ L of resuspended sperm was mixed with 4 μ L FITC-PNA (working concentration 40 μ g/ml; Sigma) and incubated at 37 °C for 15 min. Twenty μ L of sample was then placed onto a glass slide and covered with a 22 x 50 mm coverslip then allowed to settle for 5 min. A minimum of 200 sperm were observed under phase contrast at X 400 magnification using the Olympus BX51 fluorescent microscope with the U-MWIB filter (excitation filter 460-495nm, emission filter 510-550nm, 505nm 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 by Flow Cytometry

Acrosome integrity was assessed based on previously described methods (Leahy et al. 2010). Semen was diluted in 1ml pre-warmed (37°C) 0.02 M PBS (Sigma) to a final concentration of 1 x 10⁶ sperm/ml then incubated with 10 μ l FITC-PNA (working concentration 40 μ g/ml) at 37°C for 15 min. The samples were fixed with 10 μ l 10% neutral buffered formalin (final concentration 0.1%) and stored at 4°C for a maximum of 24h. Fluorescence was detected using a FACScan flow cytometer

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

Sperm Viability

To determine whether sperm were viable (membrane intact) or non-viable (membrane not intact) samples were diluted and fixed in 1ml 0.1% neutral buffered formalin in 0.02M PBS at a final sperm concentration of 1×10^6 /ml and stored at 4°C overnight. Next day the samples were stained with Propidium iodide (PI, Molecular Probes, Eugene, OR, USA) and Syto® 16 (Molecular Probes, Eugene, OR, USA) as follows: 10 µL of Syto-16 was added to 1ml diluted sperm (working concentration 10 µM) then incubated at room temperature for 20 min prior to the addition of 10µL PI (working concentration 240µM) and incubation at room temperature for a further 10 min. Viability of sperm was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), equipped with an argon ion laser (488nm, 15mW) for excitation (Leahy et al. 2010). Acquisitions were made using CellQuest 3.3 software (Becton Dickinson, San Jose, CA) and were stopped after recording 10,000 gated events. Sperm that stained positive for Syto-16 and negative for PI were deemed viable (membrane intact), and cells that stained negative for Syto-16 and positive for PI were deemed non-viable (membrane impaired).

Sperm Viability and Acrosome Integrity

Using dual staining with FITC-PNA and PI, it is possible to stain sperm for assessment of acrosome integrity and viability simultaneously. The advantage of this dual staining technique is the ability to determine the number of sperm in a sample that are both acrosome intact and viable, and therefore are likely to have fertilising ability.

Acrosome integrity and viability were assessed using a method validated in our laboratory for formalin-fixed alpaca sperm. Sample (30µl) was fixed in 0.1% (final concentration) neutral buffered formalin and stored at 4°C for 24 – 48 h until analysis. To remove seminal plasma and reduce background staining, samples were re-suspended in 200 µL 0.02M PBS, centrifuged at 300 x g for 10 min at room temperature, the supernatant discarded, and the pellet resuspended in 0.02M PBS to 10×10^6 sperm/ml. Thirty µL of resuspended sperm was incubated with 6µL fluorescent isothiocyanate-conjugated lectin from *Arachis hypogaea* (working concentration 40 µg/ml; FITC-PNA; Sigma) at 37°C for 10 min. Next 0.5 µL Propidium iodide (working concentration 0.6mM; PI, Molecular Probes, Eugene, OR, USA) was added to the sample and incubated at 37°C for 5 min. Twenty µl of stained sperm was placed onto a glass slide, covered with a 22 x 50 mm coverslip and allowed to settle for 5 min prior to analysis. A minimum of 200 sperm were observed under phase contrast at X 400 magnification using the Olympus BX51 fluorescent microscope with the U-MWIB filter (excitation filter 460-495nm, emission filter 510-550 nm, 505 nm dichromatic mirror). Sperm were considered non-viable (membrane permeabilised) if they stained red with PI, and acrosomes were considered not intact if the acrosome stained green with FITC-PNA. Sperm that did not stain were considered viable and acrosome intact and sperm that stained red and green were considered non-viable, acrosome not intact.

Sperm DNA Integrity

Deoxyribonucleic acid (DNA) integrity was assessed using a method validated in our laboratory for alpaca sperm based on the manufacturers protocol. Samples (20 µL) were snap frozen in liquid nitrogen (Chohan et al. 2006) and stored at -20 °C for 2 to 4 weeks until analysis (Gorczyca et al. 1993). To remove seminal plasma and reduce background staining, samples were resuspended in 200 µL 0.02M PBS (Sigma), centrifuged at 1000 x g for 10 min at room temperature, the supernatant discarded and the pellet resuspended in 0.02M PBS to 10×10^6 sperm/ml. Twenty µL of resuspended

sperm was smeared onto a glass slide and air-dried, then fixed in 100% ice cold methanol for 3 min at -20 °C. Slides were then washed 3 x 5 min in 0.02M PBS (Sigma) and air-dried. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction mixture (TUNEL enzyme plus TUNEL label; Roche Applied Science, Mannheim, Germany) was made according to the manufacturer's instructions with the exception that TUNEL enzyme was diluted 1:1 with 0.02M PBS prior to use. Twenty-five µL of TUNEL reaction mixture was placed onto each slide and covered with a 22 x 22mm coverslip. Slides were then incubated in the dark in a humidified chamber at 37 °C for 1 h. Next, slides were washed 3 x 5 min in 0.02M PBS, left to air-dry slightly, counterstained with DAPI (Vector Laboratories, CA, USA) and covered with a 22 x 50 mm coverslip. A minimum of 200 sperm was assessed with the Olympus BX51 fluorescent microscope with the U-MWIB filter (excitation filter 460-495nm, emission filter 510-550 nm, 505 nm dichromatic mirror). Sperm DNA was considered non-fragmented if there was no fluorescence, and fragmented if the sperm head stained green. Controls were included at each assessment. For negative controls, sample were processed as described above with the exception that instead of TUNEL reaction mixture, 25µl TUNEL label (no TUNEL enzyme) was added to the slide prior to incubation in a humidified chamber. Under these circumstances the TUNEL label was not able to bind to non-fragmented DNA and therefore no staining was observed. For positive controls, 20µl resuspended sperm were incubated with 1000U/ml DNase 1 for 30 min at 37°C to induce DNA strand breaks prior to smearing on glass slides. Under these circumstances all sperm DNA became fragmented and therefore all fluoresced green following incubation with TUNEL reaction mixture.

4. Glycosaminoglycans in Seminal Plasma and the Reproductive Tract

Introduction

The viscous nature of alpaca seminal plasma prevents the cryopreservation of alpaca spermatozoa limiting its use in artificial insemination and other assisted reproductive technologies. Numerous studies have attempted to eliminate the viscous component of alpaca seminal plasma with varying success. Enzymes including fibrinolysin, trypsin and hyaluronidase reduce the viscosity of camelid semen but have detrimental effects of sperm function and integrity (Bravo *et al.* 1999; Bravo *et al.* 2000). Some success has been achieved using collagenase (Conde *et al.* 2008; Giuliano *et al.* 2010) however other studies have reported deleterious effects of collagenase on sperm motility (Morton *et al.* 2008). In order to identify enzymes that could be used to eliminate the viscous component of alpaca semen whilst maintaining sperm integrity, it is fundamental to determine the constituents and the source of the viscous seminal plasma.

It has been postulated that the viscous nature of camelid seminal plasma is caused by glycosaminoglycans (GAGs) previously known as mucopolysaccharides although this has never been proven. This hypothesis is based on the presence of GAGs in camel accessory sex glands (Perk 1962; Ali *et al.* 1976) and the viscous effect of GAGs in other fluids such as cervical mucus. There are five known classes of GAGs; chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate and/or heparin (HS), and keratan sulphate (KS), which are all sulphated, and hyaluronan (HA) which is non-sulphated. All GAGs except keratan sulphate which was not studied, were present in human seminal plasma (Binette *et al.* 1996) however the presence and composition of GAGs in the seminal plasma of other vertebrate species, including the alpaca, has not been documented. The source of seminal plasma GAGs is also unknown. Histological studies have identified GAGs in the bulbourethral gland (Perk 1962; Ali *et al.* 1976) but not the prostate gland (Ali *et al.* 1976) of the camel inferring that the bulbourethral gland may be the predominant source of GAGs, although the presence of GAGs in the testes of camelids has not been investigated.

A greater understanding of the concentration and composition of GAGs in alpaca seminal plasma may enhance the development of protocols for the reduction of alpaca seminal plasma viscosity and thereby aid the development of cryopreservation in alpaca and other camelid species. Additionally, investigating the source of the GAGs may identify target cells or organs to reduce alpaca seminal plasma viscosity *in vivo*. There are currently no reports describing the presence of GAGs in alpaca seminal plasma or the reproductive tract.

This study investigated the concentration and composition of GAGs in alpaca seminal plasma, bulbourethral gland, prostate gland and testis and localised the expression of GAGs in the accessory sex glands and testis to examine the hypothesis that GAGs are abundant in alpaca seminal plasma and associated with semen viscosity and that the predominant source of GAGs is the bulbourethral gland. To enable comparisons with other species, the concentration and composition of GAGs in ram seminal plasma (which is non-viscous), testis, bulbourethral gland, vesicular gland and prostate gland were also investigated.

Materials and Methods

Tissues

Whole reproductive tracts were collected from five male alpacas and five rams following death by captive bolt and exsanguination. The testes were removed from the tunica vaginalis and two 1cm³ sections were cut from the testes using a sharp sterile scalpel then either fixed in 10% neutral buffered formalin for 24h and stored in 70% ethanol (1 section) or snap frozen in liquid nitrogen and stored at -

80°C until further analysis (1 section). The bulbourethral glands were cleared of the bulbocavernosus muscle and one gland was fixed and the other frozen as described for the testes. The alpaca prostate gland was removed from the reproductive tract then divided transversely into two equal sections prior to fixing (1 section) or snap freezing (1 section). In the ram, two 0.5cm long sections of the proximal urethra; the region where the prostate gland is located (Yarim *et al.* 2006) were taken and fixed (1 section) or snap frozen (1 section) as described for the testis. Then, using a dissecting microscope, the snap frozen ram prostate gland was identified and cleared of the proximal urethra and other unwanted tissues. The vesicular gland was also collected from the ram reproductive tract and cleared of all unwanted tissue and divided transversely into two equal sections then fixed (1 section) or snap frozen (1 section).

Seminal Plasma

Semen samples were collected from five males alpacas (3 samples/male; n = 15) using an artificial vagina fitted inside a mannequin as described in chapter 3. Immediately following collection, the sample was assessed for viscosity using the thread test as described in chapter 3. Briefly, 50µl of semen was drawn into a pipette, 25µl was pipetted onto a warm glass slide then moved vertically from the glass slide and a thread of semen formed. The length of the thread was measured using a ruler and the length at which the thread of semen snapped was recorded as the measurement of viscosity. Semen samples were collected from 3 rams (3 samples/ram; n = 9) using an artificial vagina (Evans and Maxwell 1987). Within 3 min of collection, alpaca and ram semen samples were centrifuged for 30 min at 10,000 x g, the seminal plasma decanted and the sperm pellet discarded, then centrifuged again at 10,000 x g for 30 min to ensure all sperm were removed from the ejaculate. Seminal plasma was stored at -80°C until further analysis.

Concentration and Composition of GAGs

Sample Preparation

To extract the GAGs from the tissues and prevent interference by protein or glycoproteins, ram and alpaca testes and accessory sex glands were digested in 0.1 M sodium acetate buffer pH 5.8 containing 0.25mg/ml papain (Roche Applied Science, Mannheim, Germany) at 60°C for 18 h, as described previously (Pitsillides and Blake 1992; Kershaw-Young *et al.* 2009). Alpaca and ram seminal plasma samples (200µl) were digested in 200µl 0.1 M sodium acetate buffer pH 5.8 containing 0.25mg/ml papain (Roche Applied Science, Mannheim, Germany) at 60°C for 2 h. The digestion was halted by the addition of 5mM iodoacetic acid at 37°C for 30 min and the sample centrifuged at 10,000 x g for 10 min to remove particulate matter. The supernatant was decanted and stored at -80°C until analysis.

Sulphated GAG Concentration

To determine the composition of sulphated GAGs, the digested tissue and seminal plasma supernatant was dispensed into five tubes (each containing less than 50µg of GAG) and treated with 1) water (no enzyme) for 2 h at 37°C, 2) 0.05U/ml Chondroitinase AC from *Flavobacterium heparinum* (Sigma-Aldrich, St Louis, MO, USA EC 4.2.2.5) for 2 h at 37°C, 3) 0.05U/ml Chondroitinase ABC from *Proteus vulgaris* (Sigma, EC 4.2.2.4) for 2 h at 37°C, 4) 10U/ml Heparinase II from *Flavobacterium heparinum* (Sigma) for 22 h at 37°C, 5) 10U/ml Heparinase II from *Flavobacterium heparinum* (Sigma) for 20 h at 37°C then 0.05U/ml Chondroitinase ABC from *Proteus vulgaris* (Sigma EC 4.2.2.4) for 2 h at 37°C (total heparinase II incubation for sample 5 was 22 h). These concentrations and times were based on previously published methods (Farndale *et al.* 1986) and preliminary observations in our laboratory. Following incubation, the samples were centrifuged at 10,000 x g for 10 min to remove particulate matter and the supernatant stored at -80°C until assayed. Chondroitinase ABC digests chondroitin and dermatan sulphates, chondroitinase AC digests chondroitin sulphate, and heparinase II digests heparan sulphate and heparin. The loss of reactivity with DMMB in samples

treated with enzymes compared to untreated or differently treated enzyme samples can be used to determine the concentration of a specific sulphated GAG (Table 4.1).

Table 4.1. Determination of GAG composition following enzyme treatment

Enzyme treatment	GAG digested	Calculation	GAG determined
Untreated (water)	none		Total sulphated GAG
Chondroitinase AC	chondroitin sulphate	untreated – chondroitinase AC treated	Chondroitin sulphate
Chondroitinase ABC	chondroitin sulphate and dermatan sulphate	chondroitinase ABC treated – chondroitin AC treated	Dermatan sulphate
Heparinase II	heparan sulphate and heparin	untreated – heparinase II treated	Heparan sulphate and heparin
Chondroitinase ABC and Heparinase II	chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin	Untreated - chondroitinase ABC and heparinase II treated	Keratan sulphate

DMMB Assay

Sulphated GAG concentrations were analysed by the dimethylmethylene blue (DMMB) assay based on a method by Fardale *et al.* (1986). Briefly, 16mg DMMB (Sigma) was dissolved in 2ml methanol then diluted in 1l water containing 3.04g glycine, 2.37g NaCl and 9.6ml 1M HCl. Next 10 μ l of sample or standard (range 0-200 μ g/ml) was added in duplicate to the wells of a 96 well plate and mixed with 250 μ l of DMMB solution. The optical density was read at OD₅₂₅ within 15seconds of adding the DMMB solution. The inter and intra-assay coefficients of variation were 8.8% and 9.1% respectively.

Hyaluronan Concentration

Hyaluronan concentration in the digested tissue supernatant and seminal plasma was assayed in duplicate by ELISA based on a method by Fosang *et al.* (1990) as described previously (Kershaw-Young *et al.* 2009). The range of the assay was 0.01 to 1.25 μ g/ml. The inter and intra-assay coefficients of variation were 9.4% and 6.6% respectively.

Alcian Blue Staining

To determine the localisation of GAGs in bulbourethral gland, prostate, testes and vesicular gland, fixed tissue samples were wax embedded, sectioned at 5 μ m and mounted onto glass slides. Paraffin embedded sections (n = 4 per tissue per animal) were de-waxed, rehydrated and stained with 1% alcian blue in 3% glacial acetic at pH 2.5 (n = 2 per tissue per animal) or 1% Alcian Blue in 0.1M HCl at pH 1.0 (n = 2 per tissue per animal) for 1h at 37°C. Sections were then washed, dehydrated and mounted in DePeX. At pH 1.0, alcian blue stains sulphated GAGs including CS, DS, HS and KS (not HA) and at pH 2.5, alcian blue stains, in addition, HA and sialoproteins.

Statistical Analysis

Data were analysed using a linear mixed model regression in GENSTAT (Version 11, VSN International, Hemel Hempstead, UK) with post-hoc comparisons using the least significant difference (LSD) test where appropriate. Tests for normality of data were performed using residual plots for each regression analysis and skewness where appropriate. To determine the composition of GAGs in seminal plasma, ram and alpaca were analysed independently. GAG type was specified as a fixed

factor, and animal ID and replicate nested within animal ID were specified as random effects. To determine the composition of GAGs within tissues, alpaca and ram were analysed independently. GAG type, tissue type and their interaction were specified as fixed factors and animal ID was included as a random factor. To compare GAG concentrations in seminal plasma between species, species was specified as a fixed factor and animal ID and replicate nested within animal ID were specified as random effects. To compare GAG concentrations in tissue between species and tissues, species, tissue and their interaction were specified as fixed factors and animal ID and species were included as random effects. To determine if GAG concentrations and seminal plasma viscosity were correlated, data were analysed using a general linear regression in GENSTAT and R^2 values were calculated using the formula $R^2 = 1.0 - (\text{sum squares regression} / \text{sum squares total})$.

Results

Concentration of GAGs in seminal plasma

Keratan sulphate was the major GAG in alpaca seminal plasma and CS and HS were intermediate ($P < 0.001$; Table 4.2). A similar composition of GAGs was seen in ram seminal plasma, whereby the concentration of KS was greater than all other GAGs ($P = 0.002$; Table 4.2). The concentration of total GAG, ($P < 0.001$), HS ($P < 0.05$) and KS ($P < 0.001$) was greater in alpaca than ram seminal plasma, however the concentration of CS, DS and HA did not differ between species (Table 4.2).

Table 4.2. Glycosaminoglycan concentration (mean \pm SEM) in alpaca and ram seminal plasma.

Glycosaminoglycan	Alpaca ($\mu\text{g/ml}$)	Ram ($\mu\text{g/ml}$)
Chondroitin sulphate	21.7 \pm 7.96 ^a _x	3.3 \pm 1.44 ^a _x
Dermatan sulphate	11.2 \pm 3.23 ^{ab} _x	3.4 \pm 1.42 ^a _x
Hyaluronan	3.4 \pm 1.14 ^b _x	2.3 \pm 0.72 ^a _x
Heparan sulphate/heparin	18.6 \pm 5.62 ^a _x	2.1 \pm 0.83 ^a _y
Keratan sulphate	324.9 \pm 57.40 ^c _x	14.0 \pm 4.40 ^b _y
Total GAGs	379.8 \pm 63.70 _x	24.1 \pm 6.51 _y

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

_{x,y} Within a row, means without a common subscript differed ($P < 0.05$).

Alpaca seminal plasma viscosity is correlated with GAG concentration

Alpaca seminal plasma viscosity was positively correlated with KS concentration ($p = 0.05$; $R^2 = 0.2635$) and tended to be positively correlated with total GAG concentration ($p = 0.053$, $R^2 = 0.2587$). The concentration of CS, DS, HS and HA were not correlated with alpaca seminal plasma viscosity ($P > 0.05$).

Concentration of GAGs in reproductive tissues

In the alpaca, the bulbourethral gland contained more total GAG ($P < 0.001$), CS ($P < 0.001$), HS ($P = 0.005$) and KS ($P = 0.002$) than the prostate, and more total GAG ($P < 0.001$), HA ($P < 0.001$) and KS ($P = 0.002$) than the testes (Fig. 4.1). Conversely in the ram, the prostate was the major source of GAG and contained more CS ($P < 0.001$) and HA ($P < 0.001$) than other tissues. The predominant source of KS was the bulbourethral gland ($P = 0.002$; Fig. 4.2).

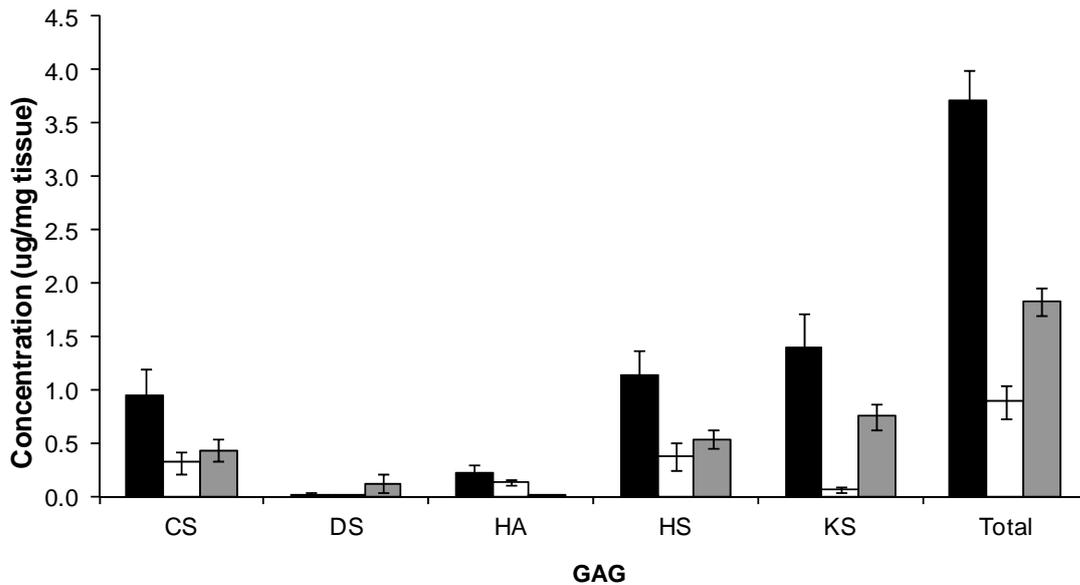


Figure 4.1. Mean GAG concentration ($\mu\text{g GAG/mg tissue}$) in the alpaca bulbourethral gland (black bars), prostate gland (white bars) and testes (grey bars). CS: chondroitin sulphate, DS: dermatan sulphate, HA: hyaluronan, HS: heparan sulphate/heparin, KS: keratan sulphate, Total: total GAGs.

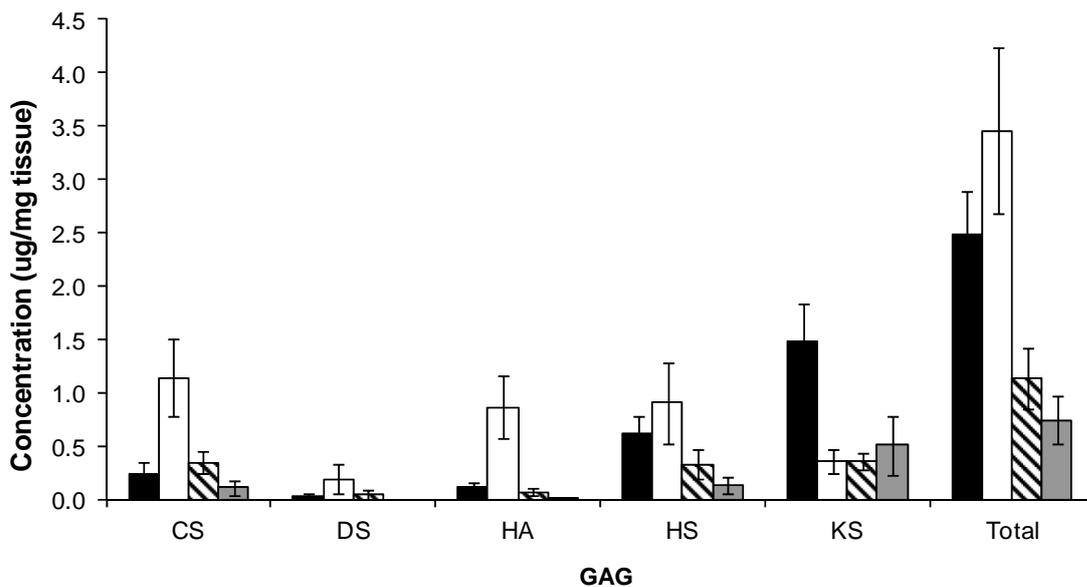


Figure 4.2. Mean GAG concentration ($\mu\text{g GAG/mg tissue}$) in the ram bulbourethral gland (black bars), prostate gland (white bars) vesicular gland (black and white bars) and testes (grey bars). CS: Chondroitin sulphate, DS: dermatan sulphate, HA: hyaluronan, HS: heparan sulphate/heparin, KS: keratan sulphate, Total: total GAGs.

Composition of GAGs in reproductive tissues

The composition of GAGs in the alpaca reproductive tract differed between tissues ($P < 0.001$). In the bulbourethral gland KS was the major GAG, HS and CS were intermediate and DS and HA were lower than all other GAGs ($P < 0.001$). A similar pattern of expression was observed in the alpaca testes whereby KS and HS were the major GAGs, CS was intermediate, and HA and DS were lowest ($P < 0.001$). The concentrations of GAGs in the alpaca prostate were not different from each other.

The composition of GAGs in the ram reproductive tract differed between tissues ($P < 0.001$). In the bulbourethral gland KS was higher than all other GAGs, followed by HS, CS, HA and DS. In the ram prostate CS and HS were the major GAGs, HA was intermediate and the concentration of DS and KS was minimal. In the ram testes and vesicular gland the concentrations of GAGs did not differ from each other.

The alpaca bulbourethral gland contained more total GAG ($P < 0.001$) and CS ($P < 0.001$) than the ram bulbourethral gland, however there were no differences in HS, DS, KS or HA. The alpaca testes contained more total GAG ($P < 0.001$), CS ($P < 0.001$) and HS ($P = 0.005$) than ram testes, however KS, DS and HA did not differ between species. Conversely the ram prostate contained more total GAG ($P < 0.001$), CS ($P < 0.001$) and HA ($P < 0.001$) than the alpaca prostate but DS, KS and HS did not differ between species.

Expression of GAGs in reproductive tissues

The localisation of GAGs in alpaca bulbourethral gland, prostate, and testes are shown in figure 4.3. In the alpaca bulbourethral gland, intense staining was observed in the epithelium at pH 2.5 (fig. 4.3a) and sulphated GAGs were expressed in the epithelium and connective tissue stroma as indicated by Alcian blue staining at pH 1.0 (fig. 4.3b). In the alpaca prostate GAGs were expressed in the luminal epithelium and smooth muscle, although the intensity of staining at pH 2.5 (fig. 4.3c) and 1.0 (fig. 4.3d) was low suggesting that GAG concentrations are low as determined by DMMB assay and HA ELISA. In the alpaca testes, Alcian blue staining was observed predominantly in the interstitial tissue (fig 4.3e and 4.3f).

The localisation of GAGs in ram bulbourethral gland, prostate, testes and vesicular gland are shown in figure 4.4. In the bulbourethral gland GAGs were expressed in the epithelium and surrounding connective tissue stroma (fig. 4.4a and 4.4b). In the prostate, GAGs were expressed in the epithelium and smooth muscle (fig 4.4c and 4.4d). In the ram testes minimal staining was observed in the interstitial tissue (fig 4.4e and 4.4f). In the vesicular glands GAGs were observed predominantly in the smooth muscle surrounding the tubules (fig. 4.4g and 4.4h) and there was minimal GAG expression in the luminal epithelium.

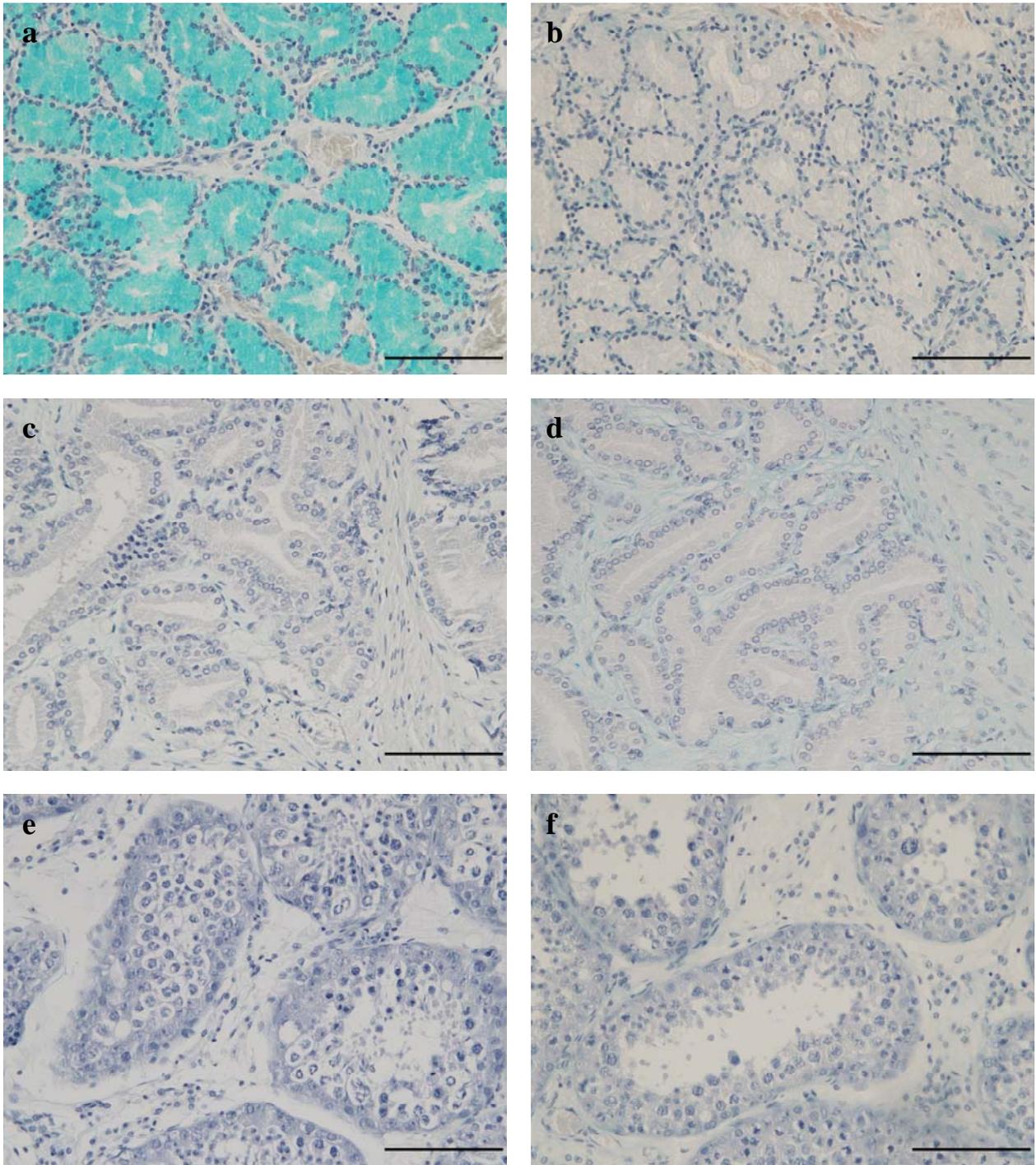


Figure 4.3. Staining of GAG with Alcian Blue at pH 2.5 (a, c, e) and 1.0 (b, d, f) in alpaca bulbourethral gland (a, b), prostate (c, d) and testes (e, f). Scale bar is 100 μ m

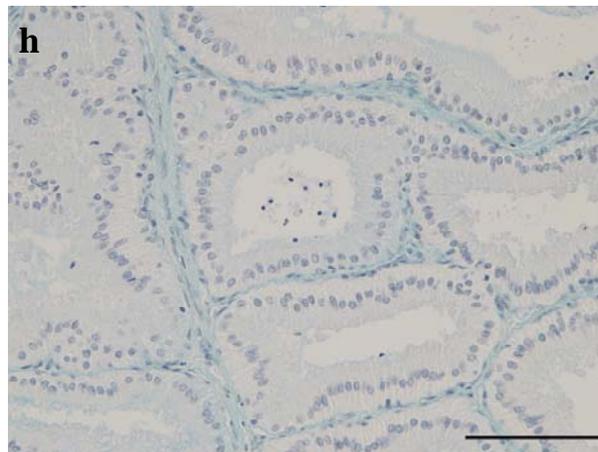
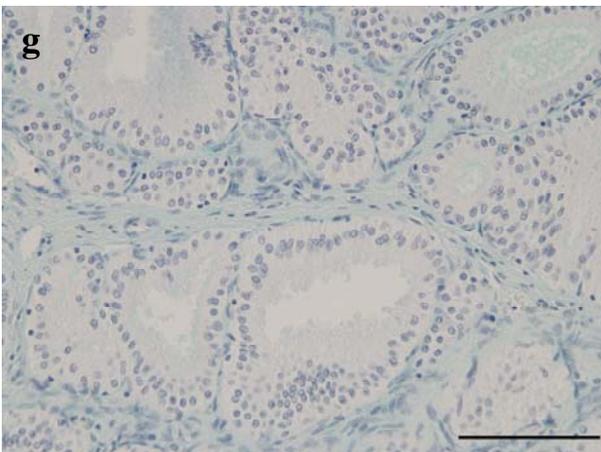
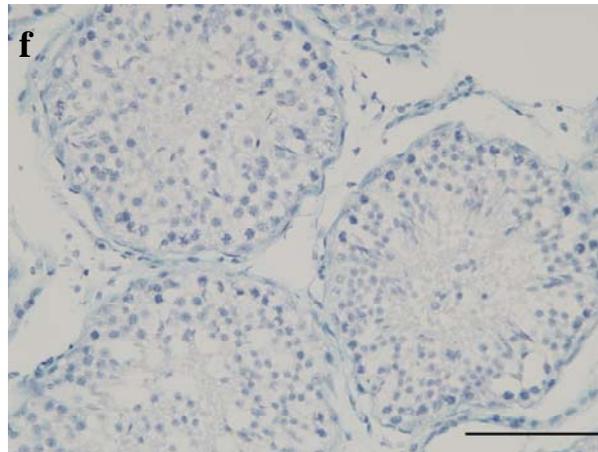
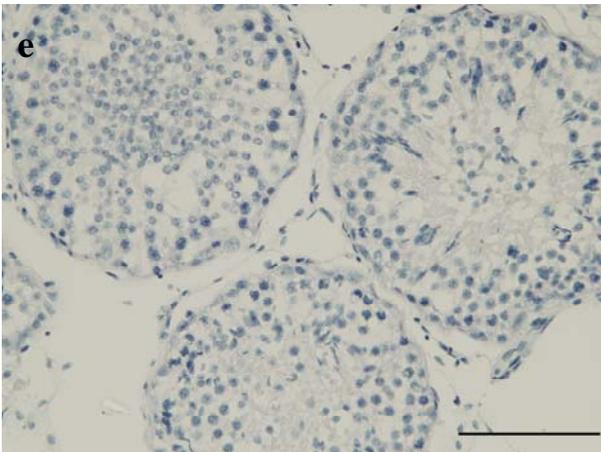
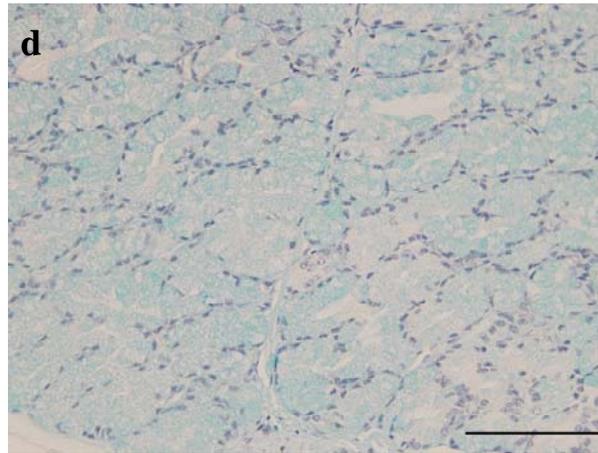
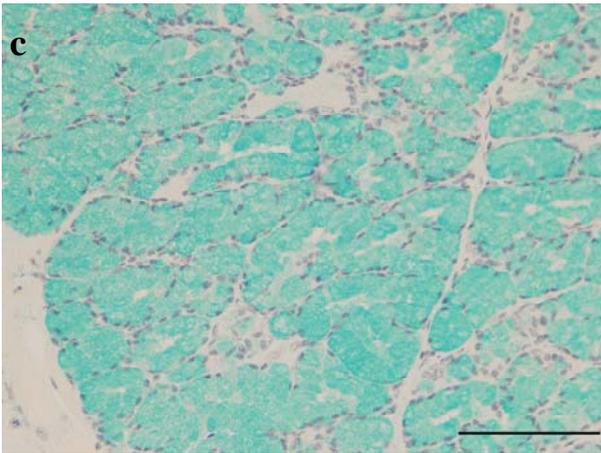
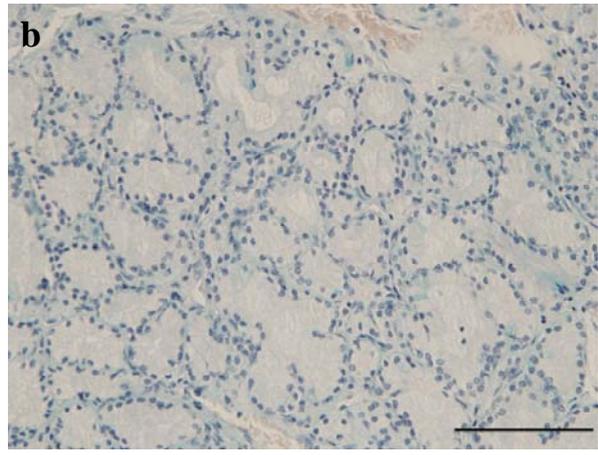
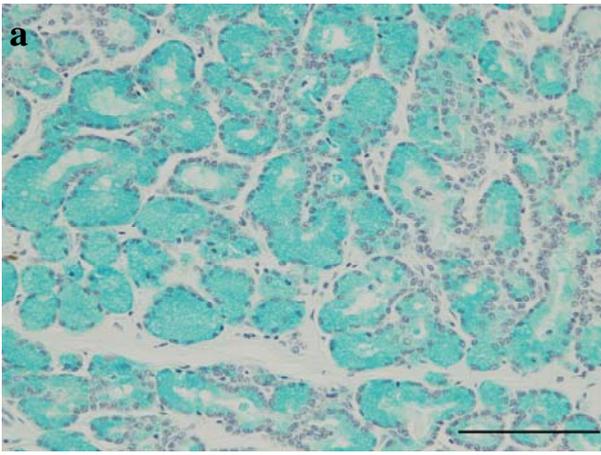


Figure 4.4. Staining of GAG with Alcian Blue at pH 2.5 (a, c, e, g) and 1.0 (b, d, f, h) in ram bulbourethral gland (a, b) prostate (c, d) testes (e, f) and vesicular gland (g, h). Scale bar is 100µm

Discussion

The concentration of total GAGs in alpaca seminal plasma were 15 times higher than the ram (present study) and almost 3 times higher than that in human (Binette *et al.* 1996). The predominant GAG in alpaca seminal plasma was KS, and KS concentration was positively correlated with viscosity. Additionally the concentration of KS was significantly greater in viscous alpaca seminal plasma compared to non-viscous ram seminal plasma. The presence of KS in the seminal plasma of other species has not been documented, however it has been reported in cervical (Lee *et al.* 1986; Fischer *et al.* 2001) and airway (Monzon *et al.* 2006) mucus which exhibit viscous properties, and it has been suggested that KS may be responsible for the viscous nature of cervical mucus (Fischer *et al.* 2001). These findings suggest that KS may be the cause of viscosity in alpaca seminal plasma. Although KS was also the predominant GAG in ram seminal plasma, the concentration was 23 times lower than that in alpaca seminal plasma and represented only 58% of total GAGs as opposed to 85% in alpaca seminal plasma. Keratan sulphate exists as a proteoglycan attached to a core protein and interacts with other molecules forming cross-links within the extracellular matrix. It is possible that in the ram, KS plays a role in the structure of the seminal plasma extracellular matrix but that the relatively low concentrations do not cause viscosity.

The concentration of heparan sulphate and heparin was also greater in alpaca seminal plasma than ram suggesting a potential role of HS in viscosity. In human seminal plasma, the viscosity causing protein semenogelin is degraded by prostate specific antigen resulting in liquefaction (Lilja *et al.* 1987). Prostate specific antigen is inhibited by protein C inhibitor (Suzuki *et al.* 2007) which in turn is activated by binding of heparin and heparan sulphate (Pratt and Church 1992). This implies an indirect effect of seminal plasma HS on seminal plasma viscosity in humans; whether a similar mechanism occurs in the alpaca remains to be determined. Given the lack of vesicular glands in the alpaca, the presence of semenogelin in alpaca seminal plasma is extremely unlikely, however it is possible that GAGs in addition to proteins cause viscosity. This hypothesis is supported by evidence that proteases including trypsin (Bravo *et al.* 2000), chymotrypsin and papain (Morton *et al.* 2008) degrade camelid seminal plasma viscosity.

Hyaluronan accounted for less than 1% of total GAGs in alpaca seminal plasma and HA concentration did not differ between alpaca and ram suggesting that HA does not cause viscosity. Bravo *et al.* (2000) noted a 40% reduction in alpaca seminal plasma viscosity following treatment of semen with hyaluronidase. As we found limited concentrations of hyaluronan in alpaca seminal plasma, it is reasonable to suggest that the reduction in viscosity was not caused by degradation of HA. Hyaluronidase is not specific and degrades CS as well as HA, thus the reduction in viscosity may be caused by degradation of CS. Alternatively the reduction in viscosity may be an effect of time rather than enzyme as the viscosity of untreated samples was not reported.

Chondroitin sulphate and DS were detected in alpaca and ram seminal plasma but the concentration did not differ between species. Chondroitin sulphate is the most dominant GAG in human seminal plasma (Binette *et al.* 1996) and induces sperm capacitation in numerous species including bull (Therien *et al.* 2005), pig (Song *et al.* 2008) and dog (Kawakami *et al.* 2000). Dermatan sulphate is also present in human seminal plasma (Binette *et al.* 1996) and induces capacitation of bull sperm (Bergqvist *et al.* 2007). Chondroitin sulphate and DS may be involved in sperm capacitation in the alpaca, although this requires further investigation.

In the alpaca, the composition of GAGs in the bulbourethral gland and testes was similar to that observed in the seminal plasma with KS being the major GAG. It is likely that seminal plasma KS is derived predominantly from the bulbourethral gland because GAGs were mainly expressed in the interstitial tissue of the testes which do not contribute to the seminal plasma. Additionally in alpacas, sperm comprises just 11% of the total ejaculate (Garnica *et al.* 1993) illustrating the relatively low contribution of the testes to semen. Conversely, in the bulbourethral gland, Alcian blue staining was strong in the secretory epithelium as observed previously in the camel (Perk 1962) suggesting that GAGs are secreted by the bulbourethral gland. Although the concentration of seminal plasma KS was greater in alpaca than ram, there was no difference in the concentration of KS between alpaca and ram bulbourethral gland. The difference in KS concentration

observed in the seminal plasma may be caused by the relative contributions of the accessory sex glands in alpaca and ram. Specifically, the alpaca does not have vesicular glands whereas in the ram the largest proportion of the ejaculate originates from the vesicular gland (Maxwell *et al.* 2007).

The intense Alcian blue staining of the alpaca bulbourethral gland at pH 2.5 compared to pH 1.0 is similar to that observed in the boar (Badia *et al.* 2006) and goat (Tsukise and Yamada 1987). Hyaluronan ELISA determined that there were low concentrations of HA in the alpaca bulbourethral gland, and therefore this staining is most likely attributable to sialoproteins.

In the ram, the prostate and bulbourethral gland had the highest GAG concentrations and Alcian blue staining confirmed that GAGs were expressed predominantly in the epithelium of these tissues. Our findings suggest that that ram seminal plasma KS is derived from the bulbourethral gland and seminal plasma CS and HA are derived from the prostate.

In conclusion, alpaca seminal plasma contained high concentrations of GAG compared to ram seminal plasma. The major GAG was KS, which was correlated with viscosity and is most likely derived from the bulbourethral gland. Our findings suggest that KS may be the cause of viscosity in alpaca seminal plasma and therefore enzyme that degrade KS may help to reduced seminal plasma viscosity without impairing sperm function.

This study has highlighted the need to investigate the effect of GAG enzymes on alpaca seminal plasma viscosity and sperm function. If GAGs are the major cause of viscosity, GAG enzymes could specifically degrade the viscosity-causing GAG whilst maintaining sperm function thus aiding the development of protocols for seminal plasma viscosity reduction and subsequent cryopreservation.

5. Enzymatic Reduction of Viscosity

Introduction

In alpacas, the viscous nature of the seminal plasma prevents successful cryopreservation of sperm, and thereby limits its use in assisted reproductive technologies including artificial insemination. Attempts to cryopreserve camelid sperm have had limited success (Deen *et al.* 2003; Niasari-Naslaji *et al.* 2007) most likely due to the viscous seminal plasma components preventing association of the cryoprotectants with the sperm membrane. Consequently, in order to advance the development of semen cryopreservation it is necessary to reduce the viscosity of the seminal plasma prior to cryopreservation without impairing sperm function. Whilst numerous studies have reduced alpaca seminal plasma viscosity using enzymes including fibrinolysin, trypsin and hyaluronidase these enzymes have detrimental effects of sperm function and integrity (Bravo *et al.* 1999; Bravo *et al.* 2000). Some success has been achieved using collagenase (Conde *et al.* 2008; Giuliano *et al.* 2010) however other studies have reported deleterious effects of collagenase on sperm motility (Morton *et al.* 2008).

It is postulated that the viscosity is caused by seminal plasma glycosaminoglycans (GAGs) and/or proteoglycans secreted by the bulbourethral gland (Perk 1962). Glycosaminoglycans are large carbohydrate chains of repeating disaccharides that covalently link to a core protein forming proteoglycans. Glycosaminoglycans are abundant in alpaca seminal plasma (Kershaw-Young *et al.* 2011) and are also present in the seminal plasma of human (Binette *et al.* 1996) and ram (Kershaw-Young *et al.* 2011). All five classes of GAGS (hyaluronan, dermatan sulphate, chondroitin sulphate, heparan sulphate/heparin, and keratan sulphate) were present in alpaca seminal plasma although keratan sulphate, chondroitin sulphate and heparan sulphate were most abundant, and only keratan sulphate was correlated with viscosity (Kershaw-Young *et al.* 2011). If GAGS are the main cause of viscosity, enzymes that cleave GAG chains may reduce seminal plasma viscosity without impairing sperm function. Of the commercially available GAG enzymes hyaluronidase preferentially degrades hyaluronic acid but also chondroitin sulphate and dermatan sulphate, keratanase degrades keratan sulphate, chondroitinase ABC degrades chondroitin sulphate and dermatan sulphate, and heparinase degrades heparan sulphate. Heparinase is expensive and requires 18-24 h to degrade heparan sulphate (Kershaw-Young *et al.* 2011) and is therefore not a suitable enzyme for use within the alpaca industry, however the remaining enzymes initiate their effects within 1-2 h and may be suitable for reduction of seminal plasma viscosity.

As GAGs bind to proteins to form large proteoglycans, proteases which degrade the protein core of the proteoglycan, thereby unlinking the GAG chains but leaving the GAG chains intact, may also reduce seminal plasma viscosity. The enzymes papain (Kavanagh *et al.* 2002) and proteinase K

(Grande-Allen *et al.* 2004) degrade the protein core of proteoglycans without degrading the GAG chains and can be used to determine if GAGs or proteins are the cause of viscosity.

This study investigated the effect of hyaluronidase, chondroitinase ABC, keratanase, proteinase K and papain, on alpaca seminal plasma viscosity and sperm function. The reduction of viscosity with these enzymes will help identify the cause of viscosity. Additionally the identification of an enzyme that reduces semen viscosity in alpacas, and potentially other camelids, whilst maintaining sperm function may facilitate the development of sperm cryopreservation for these species.

Materials and Methods

Animals

This study was performed from February to November 2009 using five male alpacas under authorization from the University of Sydney animal ethics committee. All males were > 3 years, had a body condition score > 3 and had testes more than 3 cm long.

Experimental Design

This study was performed as two experiments with the same alpacas used in each study. Experiment 1 determined the effect of hyaluronidase, chondroitinase ABC and papain on seminal plasma viscosity and sperm function. Experiment 2 determined the effect of keratanase, proteinase K and papain on seminal plasma viscosity and sperm function. Papain was included in both experiments to act as an internal control and to confirm reproducibility of the findings from experiment 1.

In experiment 1 semen was collected from 4 male alpacas (≥ 3 ejaculates/male, $n = 15$) using an artificial vagina fitted inside a mannequin as described previously (Morton *et al.* 2009). Within 5 min of collection, semen was assessed for volume, viscosity, sperm motility and concentration as described in chapter 3 and briefly below.

Only samples with a volume > 1 ml, \geq viscosity 15 mm, motility $\geq 50\%$ and sperm concentration $\geq 10 \times 10^6$ sperm/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 homogenisation. The diluted semen was then divided into five treatment groups; 1) Control: 380 μ l diluted semen plus 20 μ l 0.01% BSA, 2) Hyaluronidase: 380 μ l diluted semen plus 20 μ l of 100 U/ml hyaluronidase (final concentration 5 U/ml), 3) Chondroitinase ABC: 380 μ l diluted semen plus 20 μ l 4 IU/ml chondroitinase ABC (final concentration 0.25 IU/ml), 4) Hyaluronidase and chondroitinase ABC: 380 μ l diluted semen plus 10 μ l 200 U/ml hyaluronidase (final concentration 5 U/ml) and 10 μ l 8 IU/ml Chondroitinase ABC (final concentration 0.25 IU/ml), 5) Papain: 380 μ l diluted semen plus 20 μ l 2 mg/ml papain (final concentration 0.1 mg/ml). Samples were incubated for 2 h at 37°C in a water bath and semen viscosity, sperm motility, viability and acrosome integrity were assessed following dilution (time 0) and at 30, 60 and 120 min following enzyme treatment. The DNA integrity of sperm was assessed at 0 and 60 min following enzyme treatment.

In experiment 2 semen was collected from 5 male alpacas (3 ejaculates/male, $n = 15$) as described above. Within 5 min of collection semen volume, viscosity, sperm motility and concentration were assessed and the same selection criteria were applied as experiment 1. Next, 1 ml semen was diluted 1:1 in tris-citrate-fructose buffer as described above and divided into 4 treatment groups; 1) Control 380 μ l diluted semen plus 20 μ l 0.01% BSA 2) Keratanase: 380 μ l diluted semen plus 20 μ l 10 U/ml keratanase (final concentration 0.5 U/ml), 3) Proteinase K: 380 μ l diluted semen plus 20 μ l 20 mg/ml proteinase K (final concentration 1 mg/ml), 4) Papain: 380 μ l diluted semen plus 20 μ l 2 mg/ml papain (final concentration 0.1 mg/ml). Samples were incubated for 2 h at 37°C in a water bath and semen viscosity, sperm motility, viability and acrosome integrity were assessed following dilution (time 0) and at 30, 60 and 120 min following enzyme treatment. The DNA integrity of sperm was assessed at 0 and 60 min following enzyme treatment.

Enzymes

Hyaluronidase type 1-S from bovine testes (Sigma-Aldrich, St Louis, MO, USA) was resuspended in 0.01% Bovine Serum Albumin Cohn fraction V (BSA; Sigma) to a stock concentration of 100 U/ml or 200 U/ml. Chondroitinase ABC from *Proteus vulgaris* (Sigma EC 4.2.2.4) was resuspended in 0.01% BSA to a stock concentration of 4 U/ml or 8 U/ml. Keratanase from *Pseudomonas Sp.* (Sigma, EC 3.2.1.103) was resuspended in 0.01% BSA to a stock concentration of 10 U/ml. Papain from *Carica papaya* (Sigma-Aldrich, St Louis, MO, USA #76216) was diluted in 0.02 M PBS to a stock concentration of 2 mg/ml. Proteinase K from *Engydotium album* (Sigma, EC 3.4.21.64) was resuspended in 0.02 M PBS to a stock concentration of 20 mg/ml.

Semen and Sperm Analysis

Viscosity, Concentration and Motility

Sample (10 μ l) was diluted (1:9) in 90 μ l 3% sodium chloride (Sigma) and the concentration of sperm was assessed using a haemocytometer (Evans and Maxwell 1987). Viscosity was assessed using the thread test in which 50 μ l of sample was drawn into a pipette, 25 μ l was pipetted onto a warm glass slide the pipette lifted vertically and the length at which the thread snapped recorded as the measurement of viscosity. Motility was assessed subjectively at X 100 magnification under phase contrast microscopy (Olympus, Tokyo, Japan) by

placing 10µl of semen or sample on a warm slide and covering with a warm coverslip (Evans and Maxwell 1987).

Viability and Acrosome Integrity

Acrosome integrity and viability were assessed using the dual staining method described in chapter 3. Briefly, for each treatment at each time point 30µl sample was fixed in 0.1% (final concentration) neutral buffered formalin, stored at 4°C until analysis then washed and resuspended in 0.02M PBS to 10×10^6 sperm/ml. Thirty µl of resuspended sperm was incubated with 6µl fluorescent isothiocyanate-conjugated lectin from *Arachis hypogaea* (working concentration 40 µg/ml; FITC-PNA; Sigma) at 37°C for 10min then 0.5µl Propidium iodide (working concentration 0.6mM; PI, Molecular Probes, Eugene, OR, USA) at 37°C for 5min. Twenty µl of stained sperm was placed onto a glass slide and covered with a 22 x 50 mm coverslip. A minimum of 200 sperm were observed under phase contrast at X 400 magnification using the Olympus BX51 fluorescent microscope with the U-MWIB filter (excitation filter 460-495nm, emission filter 510-550 nm, 505 nm dichromatic mirror). Sperm were considered non-viable (membrane permeabilised) if they stained red with PI, and acrosomes were considered not intact if the acrosome stained green with FITC-PNA. Sperm that did not stain were considered viable and acrosome intact and sperm that stained red and green were considered non-viable, acrosome not intact.

DNA Integrity

The integrity of sperm DNA was assessed at time 0h and 60 min as described in chapter 3. Briefly, samples (20µl) were snap frozen in liquid nitrogen and stored at -20°C until analysis. Samples were resuspended to 10×10^6 sperm/ml and smeared onto a glass slide then fixed in 100% ice cold methanol. Next 25µl of Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction mixture (Roche Applied Science, Mannheim, Germany) was placed onto each slide and covered with a 22 x 22mm coverslip. Slides were then incubated in the dark in a humidified chamber at 37°C for 1h prior to counterstaining with DAPI (Vector Laboratories, CA, USA). A minimum of 200 sperm was assessed with the BX51 fluorescent microscope. 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 a linear mixed model regression in GENSTAT (Version 11, VSN International, Hemel Hempstead, UK) with post-hoc comparisons using the least significant difference (LSD) test where appropriate. To accommodate the experimental design, the random effects in the model were alpaca ID, replicate nested within alpaca ID, and treatment nested within replicate. The latter term was included to accommodate the splitting of the replicate (ejaculate sample) into sub-samples, each allocated a different enzyme treatment. Time, treatment and their interaction were specified as fixed factors. For all analyses, $P < 0.05$ was considered significant. Data are reported as mean \pm SEM.

Results

Experiment 1

Percentage semen viscosity differed among enzyme treatments at each time point except 0h ($P < 0.001$ fig. 5.1). At 30, 60 and 120min, viscosity was significantly less in papain treated samples than all other samples. Additionally viscosity was less in hyaluronidase, chondroitinase, and hyaluronidase and chondroitinase treated samples than control samples at 30 and 60min (fig. 5.1). Viscosity decreased over time in all treatment groups although papain treated samples did not differ from 30 to 120min as viscosity was completely eliminated at 30min (fig. 5.1).

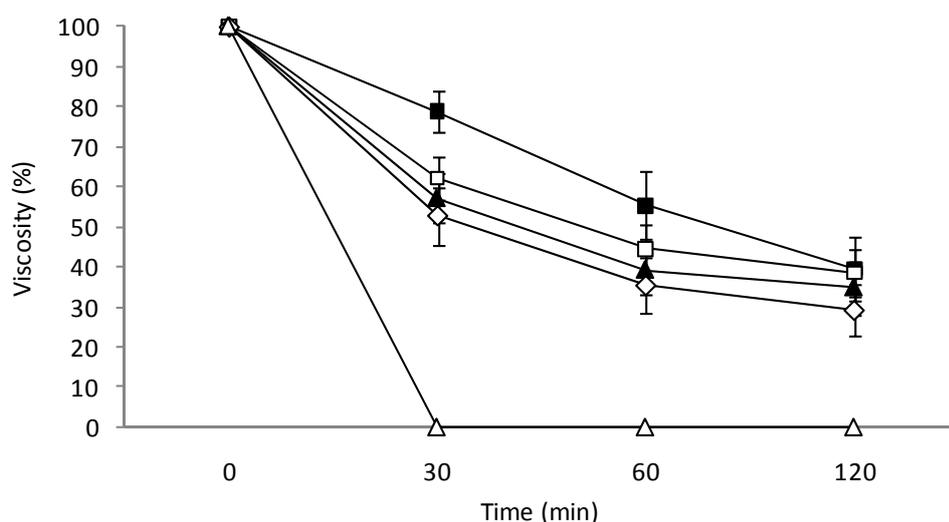


Figure 5.1. Percentage viscosity (mean \pm SEM) of alpaca semen treated with BSA (control, ■) hyaluronidase (□), chondroitinase ABC (▲), hyaluronidase plus chondroitinase ABC (◇), and papain (△) at 0, 30, 60 and 120 min after treatment.

Sperm motility was not affected by treatment but decreased significantly from 0 to 120min ($P < 0.001$, table 5.1). The DNA integrity of sperm did not differ between treatment groups or time points (table 5.1). The percentage of sperm (mean \pm SEM) that was both viable (membrane not permeabilised) and acrosome intact was greater in samples treated with papain (14.0 ± 1.21) compared to control (10.8 ± 0.63), hyaluronidase (11.3 ± 0.59), chondroitinase (11.5 ± 0.77) and hyaluronidase plus chondroitinase (11.5 ± 0.82) treated samples ($P = 0.02$). Viability and acrosome integrity also decreased over time ($P < 0.001$, table 4.1). The percentage of non-viable, acrosome not intact sperm did not differ between treatment groups or time points (table 5.1).

Table 5.1 Percentage of motile sperm (motility), DNA intact sperm (DNA intact), viable, acrosome intact sperm (viable intact) and non-viable, acrosome not intact sperm (non-viable not intact) at 0, 30 60 and 120min in control, hyaluronidase, chondroitinase, hyaluronidase and chondroitinase, and papain treated samples. Values are mean (\pm SEM) across treatment groups.

Time (min)	Motility (%)	DNA integrity (%)	Viable intact (%)	Non-viable, not intact (%)
0	61.8 ± 1.00^a	94.6 ± 3.93^a	14.0 ± 0.63^a	44.0 ± 1.54^a
30	57.8 ± 0.93^b		12.1 ± 0.76^b	48.1 ± 1.97^a
60	50.2 ± 1.13^c	94.5 ± 0.58^a	10.63 ± 0.70^{bc}	48.5 ± 1.56^a
120	33.0 ± 2.29^d		10.38 ± 0.81^c	48.5 ± 1.86^a

Within a column, means without a common superscript differed ($P < 0.05$)

Experiment 2

Percentage semen viscosity differed among treatments at each time point except 0h ($P < 0.001$). At 30, 60 and 120min following treatment, papain and proteinase K treated samples had lower viscosity than control and keratanase samples (fig. 5.2). Semen viscosity decreased significantly from 0 to 120min in control and keratanase samples, although there was no difference in viscosity from 30 to 120min in both papain and proteinase K treated samples due to a complete elimination of viscosity by 30min (fig 5.2).

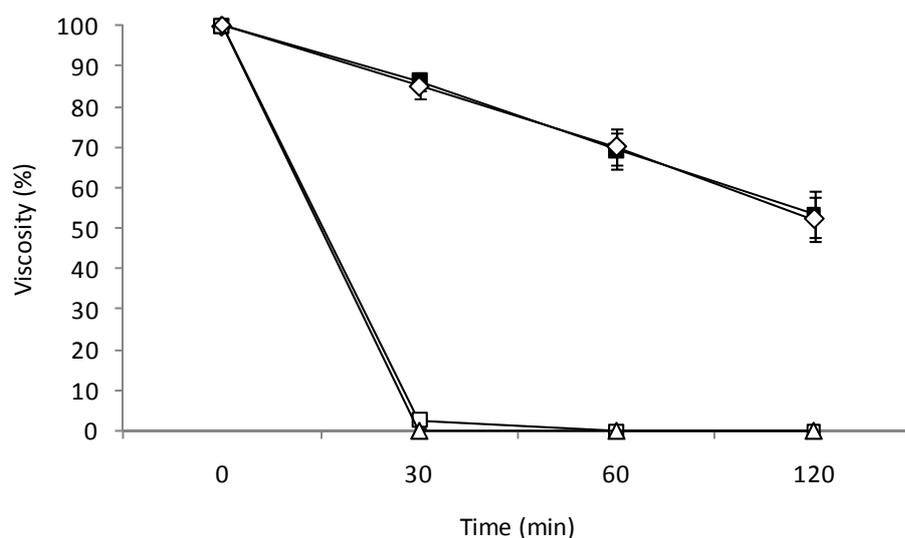


Figure 5.2 Percentage viscosity (mean \pm SEM) of alpaca semen treated with BSA (control, ■), keratanase (◇), proteinase K (□) and papain (△) at 0, 30, 60 and 120 min after treatment.

Sperm motility (mean \pm SEM) was not affected by enzyme treatment, although it decreased significantly over time ($P < 0.001$) from 56.3% at 0min to 32.3% at 120min (table 5.2). Sperm DNA integrity was not different among treatment groups or time points (table 5.2). The percentage of sperm (mean \pm SEM) that was viable and acrosome intact was greater in control (37.2 ± 1.20) and papain (37.3 ± 1.13) treated samples compared to those treated with keratanase (34.8 ± 1.32) or proteinase K (32.9 ± 1.30 , $P < 0.001$). Additionally, the percentage of viable acrosome intact sperm declined significantly from 0 to 120min ($P < 0.001$, table 5.2). The percentage of sperm that was non-viable acrosome not intact increased significantly over time ($P < 0.001$, table 5.2) but did not differ among treatment groups ($P = 0.162$).

Table 5.2 Percentage of motile sperm (motility), DNA intact sperm (DNA intact), viable, acrosome intact sperm (viable intact) and non-viable, acrosome not intact sperm (non-viable not intact) at 0, 30 60 and 120min in control, keratanase, proteinase K and papain treated samples. Values are mean (\pm SEM) across treatment groups.

Time (min)	Motility (%)	DNA integrity (%)	Viable intact (%)	Non-viable, not intact (%)
0	56.3 ± 1.18^a	95.5 ± 0.31^a	42.1 ± 0.94^a	12.4 ± 0.98^a
30	52.8 ± 1.53^b		36.6 ± 1.20^b	11.4 ± 0.84^a
60	44.8 ± 1.66^c	94.9 ± 0.30^a	32.6 ± 1.08^c	14.8 ± 0.96^b
120	32.3 ± 2.14^d		30.8 ± 1.24^c	17.3 ± 1.22^c

Within a column, means without a common superscript differed ($P < 0.05$)

Discussion

This study investigated the effect of the glycosaminoglycan enzymes hyaluronidase, chondroitinase ABC and keratanase, and the proteases papain and proteinase K on alpaca semen viscosity and sperm function. Glycosaminoglycan enzymes cleave GAG chains and dissociate GAGs from the protein core of proteoglycans whilst leaving the protein core intact. Consequently, if GAGs are the predominant cause of viscosity in alpacas, GAG enzymes should markedly reduce, or completely eliminate semen viscosity. Conversely both papain (Kavanagh *et al.* 2002) and proteinase K (Grande-Allen *et al.* 2004) degrade the protein core of proteoglycans leaving the GAG chains intact, therefore, if GAGs are the cause of viscosity, neither papain nor proteinase K should affect viscosity. If however, proteoglycans or other seminal plasma proteins cause viscosity, papain and proteinase K would reduce viscosity.

In the present study, hyaluronidase, chondroitinase ABC, hyaluronidase plus chondroitinase ABC and

keratanase reduced viscosity by 62%, 65%, 71% and 48% respectively over 2h. Glycosaminoglycans are abundant in alpaca seminal plasma and keratan sulphate was correlated with viscosity (Kershaw-Young *et al.* 2011), however in the present study seminal plasma viscosity following keratanase treatment was not different from control, suggesting that keratan sulphate does not cause viscosity. The limited response to hyaluronidase and chondroitinase also suggests that hyaluronan, chondroitin sulphate and dermatan sulphate are not the predominant cause of seminal plasma viscosity in alpaca. Hyaluronidase reduced seminal plasma viscosity in llamas and alpacas (Bravo *et al.* 2000) but, in contrast to the present study also reduced sperm motility and viability. The difference may be explained by the hyaluronidase concentration used; the present study used 5U/ml, whereas Bravo *et al.* (2000) used 0.25mg/ml which equates to 75-250U/ml.

Papain and proteinase K completely eliminated viscosity within 30 min and 60 min respectively indicating that semen viscosity in alpacas is caused by proteoglycans or other seminal plasma proteins. Proteases such as trypsin, chymotrypsin, papain, fibrinolysin and collagenase have been used previously to reduce the viscosity of camelid (Bravo *et al.* 1999; Bravo *et al.* 2000; Morton *et al.* 2008; Giuliano *et al.* 2010) monkey (Hoskins and Patterson 1967) and human (Cohen and Aafjes 1982) semen. Trypsin (Suzuki *et al.* 1992), chymotrypsin (Mathews 1971), papain (Kavanagh *et al.* 2002) and fibrinolysin (Mochan and Keler 1984) all degrade proteoglycans, however collagenase does not (Alexander and Werb 1991). Collagenase completely eliminates semen viscosity in llama and alpaca (Bravo *et al.* 2000; Morton *et al.* 2008). These findings and those of the present study imply that seminal plasma viscosity is caused by seminal plasma proteins and not proteoglycans. Identification of this protein requires investigation and may aid the development of assisted reproductive technologies in alpacas.

In the present study, proteinase K reduced viscosity but also increased the proportion of non-viable, acrosome not-intact sperm. Additionally, proteinase K treated sperm appeared hyperactivated and was agglutination at the sperm heads (data not reported). Consequently, proteinase K is not a suitable enzyme for the reduction of alpaca semen viscosity as it reduces the fertilising capacity of sperm. Papain was most effective of all the enzymes tested at reducing alpaca semen viscosity, and had no detrimental effect on sperm motility, DNA integrity, viability or acrosome integrity. Papain reduces seminal plasma viscosity in alpacas when used at 0.5 – 4mg/ml, but it also impairs acrosome integrity (Morton *et al.* 2008). It is likely that the lower concentration used in the present study (0.1mg/ml) was sufficient to reduce viscosity but low enough to prevent acrosome damage. Papain treatment therefore seems a suitable method to reduce alpaca semen viscosity without impairing sperm function. Further investigations that determine the optimal papain concentration and incubation time would ensure limited effects on sperm function and integrity, particularly as at higher papain concentrations, acrosome integrity decreases over time (Morton *et al.* 2008).

In conclusion, this study demonstrated that GAG enzymes are not a suitable approach for the reduction of alpaca seminal plasma viscosity. The elimination of viscosity when using the proteases papain and proteinase K suggest that alpaca seminal plasma viscosity is caused by a protein, not GAGs. It is essential to determine the protein responsible for viscosity in order to identify suitable enzymes for viscosity reduction without impairing sperm function. Although papain was effective at reducing viscosity and did not have detrimental effects on the sperm parameters assessed, determining the protein responsible for viscosity may identify other suitable enzymes for viscosity reduction. In particular, enzymes that target the protein of interest specifically, as opposed to generic proteases such as papain, may be available. It is likely that a specific enzyme will have minimal effect on sperm function, integrity and fertility as it will only degrade the viscosity causing protein and not other proteins within the seminal plasma that may protect the sperm against damage.

This study has highlighted the need to 1) identify the protein responsible for seminal plasma viscosity in alpacas and 2) investigate the potential of papain and/or other specific enzymes to reduce viscosity without impairing sperm function and to determine their effect on the success of sperm cryopreservation in alpacas.

6. Cause and Source of Viscosity

Introduction

The development of assisted reproductive technologies such as sperm cryopreservation and artificial insemination are hindered in camelids by the viscous nature of the seminal plasma. In order to successfully cryopreserve camelid sperm, it is necessary to degrade the component responsible for viscosity prior to cooling to enable protective agents present in the freezing diluent to coat the sperm head and prevent sperm damage during chilling and freezing.

Whilst it has been postulated that glycosaminoglycans (GAGs) are the cause of seminal plasma viscosity in camelids, we have determined that the GAG enzymes hyaluronidase, chondroitinase ABC and keratanase do not completely eliminate alpaca seminal plasma viscosity within 2 h of treatment (chapter 5). In addition, these enzymes reduced the integrity of alpaca sperm making them an unsuitable approach for the reduction of viscosity. Conversely the generic proteases papain and proteinase K completely eliminated viscosity within 30 and 60 min of treatment respectively, suggesting that a protein, not GAGs are the main cause of viscosity (chapter 5). Other proteases including collagenase (Conde et al. 2008; Giuliano et al. 2010) fibrinolysin and trypsin (Bravo et al. 1999; Bravo et al. 2000) also reduce alpaca and llama seminal plasma viscosity, supporting the hypothesis that proteins not GAGs are the major cause of viscosity. Although these generic proteases reduce viscosity they have all been shown to reduce sperm motility and/or acrosome integrity (Bravo et al. 2000; Morton et al. 2008). Some success has been achieved using collagenase (Conde et al. 2008; Giuliano et al. 2010) and papain (chapter 5) however, in order to identify enzymes that specifically degrade viscosity without impairing sperm function, it is essential to determine the protein that causes viscosity.

Whilst proteins present in the seminal plasma many species including the human (ref), bull (ref) and ram (ref) are well documented, there are currently no studies describing the proteins present in camelid seminal plasma. In addition, the source of the viscosity-causing protein is unknown. Identifying the source of the protein may identify target cells or organs to enable *in vivo* approaches to the reduction of viscosity to be investigated.

Commonly used methods for protein expression including 1-dimensional and 2-dimensional gel electrophoresis are not successful for alpaca seminal plasma due to the high abundance of proteoglycans and glycosaminoglycans which inhibit separation of the proteins. Consequently it is necessary to use other approaches such as mass spectrometry to identify the proteins present within the sample. Whilst mass spectrometry is able to identify the proteins present, it is not sufficient to quantify the amount of protein within a sample, therefore it is not able, when used alone, to identify differences in the abundance of proteins present in low viscosity and high viscosity seminal plasma. A relatively new approach termed iTRAQ uses labels of differing molecular weights to label different samples such as those that are highly viscous and those that have low viscosity in order to determine the relative abundance of different proteins within each sample via quantitative mass spectrometry. This approach can therefore be used to help identify proteins that may cause viscosity in alpaca seminal plasma.

This study aimed to identify the protein responsible for viscosity in alpaca seminal plasma using iTRAQ labelling and mass spectrometry and to determine the source of the protein in order to advance the development of sperm cryopreservation protocols for this species.

Materials and Methods

Seminal Plasma

Semen samples were collected from four male alpacas during October 2009 (3 samples/male) using an artificial vagina fitted inside a mannequin as described in chapter 3. Immediately following collection, the

sample was assessed for viscosity using the thread test as described in chapter 3 and categorised as high viscosity (> 20mm) or low viscosity (<5mm). Within 3 min of collection semen samples were centrifuged for 30min at 10,000xg at 4°C, the seminal plasma decanted and the sperm pellet discarded, then centrifuged again at 10,000xg for 30min at 4°C to ensure all sperm were removed from the ejaculate. Seminal plasma was stored at -80°C until further analysis by mass spectrometry or western blot.

Mass Spectrometry and iTRAQ Analysis of Seminal Plasma Proteins

Sample Preparation

Samples were thawed at 4°C then equal volumes of seminal plasma from each ejaculate were pooled for each male to generate a total of 4 samples (1 sample/male x 4 males).

The concentration of protein in each sample was determined using the Pierce BCA protein quantification assay (Pierce, Illinois, USA) according to the manufacturer's instructions. Briefly, 25µl of sample or standard was pipetted in duplicate into a microplate well and mixed with 200µl working reagent for 30 sec then incubated at 37°C for 30 min. The absorbance of each sample was read at 562nm using the BioRad plate reader and the absorbance of the standards used to generate a standard curve from which the protein concentration of the samples was determined.

Proteins were digested with trypsin and labelled with iTRAQ tags according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Proteins (20µg) were diluted in 50mM ammonium bicarbonate to a final protein concentration of 1µg/µl and reduced with 50mM tris-(2-carboxyethyl) phosphine (TCEP) at 60°C for 1h. Cysteine residues were then blocked with 200mM methylmethane thiosulfonate (MMTS) at room temperature for 30 min. Next the proteins were digested in 1µg sequencing grade modified trypsin (Promega, Madison, WI, USA)/50µg protein at 37°C overnight (12-16 h). To degrade complex carbohydrates and abundant glycoproteins, samples were diluted 1:1 with 100mM ammonium acetate (final pH > 8.0) and digested with 0.6U N-glycosidase F (Roche Applied Science)/µg protein at 37°C overnight. Samples were dried in a Vacuum Centrifugal Concentrator 5301 (Eppendorf, Hamburg, Germany) then resuspended in 5µl 1M TEAB pH 8.5. The iTRAQ reagents were dissolved in 50µl 100% ethanol and 25µl was added to each peptide sample to give a final ethanol concentration > 65% (v/v) then incubated at room temperature for 2 h. Samples were labelled with isotopic tags as follows: low viscous 1, 114; low viscous 2, 115; high viscous 1, 116; high viscous 2, 117. After labelling, low and high viscosity samples were combined to generate 1 sample.

Prior to mass spectrometry (MS) analysis, excess salts, sugars, enzyme and iTRAQ label was removed from the sample using a HLB column (Waters Oasis) as follows. The column was conditioned with 1ml 100% methanol followed by 1ml 100% acetonitrile then washed with 1ml 5% acetonitrile plus 0.1% formic acid (wash buffer). Next the sample was passed through the column and the column washed in 1ml wash buffer. The peptides were eluted in 0.5ml 50% acetonitrile plus 0.1% formic acid, then in 0.5ml 70% acetonitrile plus 0.1% formic acid. The eluted sample was dried in a Vacuum Centrifugal Concentrator 5301 (Eppendorf, Hamburg, Germany) then resuspended in 0.1% formic acid in distilled water to a final protein concentration of 0.1µg/µl.

Mass Spectrometry and Data Analysis

The sample was analysed using reverse phase LC MS/MS on a Q-STAR Elite mass spectrometer (Applied Biosystems). The peptides were separated on an Agilent 1100 HPLC system using a 2 h gradient of acetonitrile (%) in 0.1% formic acid as follows: 5 min 5%, 85 min 15%, 15 min 30%, 10 min 70%, 5min 5% and eluted peptides were analysed with Analyst QS 1.1 software (Applied Biosystems). The three most abundant peptides with +2 to +4 charge states were selected for tandem mass spectrometry and subjected to product ion analysis. The iTRAQ MS/MS data were analysed with ProteinPilot 3.0 software (Applied Biosystems) using the uniprot-taxonomy-mammalia database. Only proteins with 95% confidence and an unused protein score of >1.3 were reported. Relative peptide abundances were determined using the MS/MS scans of iTRAQ labelled peptides where the ratios of iTRAQ reporter ions reflect the relative abundances of the peptides and therefore the proteins in the samples. Relative abundances were determined against the

abundance of reporter ion 114 as this seminal plasma sample had the lowest viscosity (mean \pm sem; 0.0 ± 0.0).

Western Blot Analysis

Sample Preparation

Seminal Plasma

Seminal plasma was collected, pooled and stored from 4 male alpacas as described above and used for western blot analysis of mucin 5B. The protein concentration of each pooled seminal plasma samples was determined using the Pierce BCA protein assay (Pierce, Illinois, USA) as described above. Seminal plasma (50 μ g protein) was reduced in lammeli buffer (62.5mM Tris-HCl pH 6.8 (Sigma-Aldrich, St Louis, MO, USA), 10% (v/v) glycerol (Sigma), 2% (v/v) sodium dodecyl sulphate (SDS, Sigma), 5% β -mercaptoethanol (Sigma) and 0.2% bromophenol blue at 100°C for 15 min. To prevent reduced proteins from reforming, samples were alkylated in 25mM iodoacetic acid at room temperature (25°C) for 1 h in the dark as described previously (Rousseau et al. 2007)

Tissues

Whole reproductive tracts were collected from three male alpacas following death by captive bolt and exsanguination. The testes, bulbourethral gland and prostate gland were dissected from the reproductive tract then snap frozen in liquid nitrogen and stored at -80°C.

To extract proteins, tissue (100-200mg) was thawed and finely chopped using a sterile scalpel then homogenised on wet ice in 3ml cell lysis buffer (62.5mM Tris-HCl pH 6.8 (Sigma), 10% (v/v) glycerol (Sigma), 2% (v/v) SDS (Sigma) and 1% (v/v) protease inhibitor cocktail (Calbiochem). Lysates (1ml) were boiled at 100°C for 5 min then centrifuged at 5000 x g for 15 min and the supernatant decanted. The protein concentration of the lysates was determined using the Pierce BCA protein assay (Pierce, Illinois, USA) as described above. Following protein quantification, 5% β -mercaptoethanol (Sigma) and 0.2% bromophenol blue were added to the lysate. Samples were then alkylated in 25mM iodoacetic acid for 1 h at room temperature (25°C) in the dark as described previously (Rousseau et al. 2007)

Sodium Dodecyl Sulphate-Agarose-Polyacrylamide Electrophoresis (SDS-AgPAGE)

Due to the high molecular weight of mucin 5B (600KDa), it is necessary to use a low concentration SDS-PAGE gel strengthened with agarose to enable mucin to run onto a gel and be subsequently detected using western blot.

One dimensional SDS-AgPAGE gels (3% bis-acrylamide, 0.5% agarose, 0.5% ammonium persulphate, 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED)) were cast in a mini-Protean gel casting apparatus (BioRad, Hercules CA, USA) and left to polymerise at room temperature for 1 h then at 4°C for 1 h based on previously described methods (Schulz et al. 2002). Protein (seminal plasma, 100 μ g; tissue lysate, 50 μ g) was loaded onto each lane with one lane containing Novex sharp pre-stained marker (Invitrogen, Mulgrave, VIC, Australia) and ran for 2h at 90V until the dye front migrated out of the gel and the 150KDa marker was at the bottom of the gel.

Following electrophoresis, the gel was washed in Towbin buffer (50mM Tris, 192mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS) for 15 min. Polyvinylidene fluoride (PVDF) membrane (BioRad) was cut to the size of the gel, saturated in methanol for 20 sec then rehydrated in Towbin buffer for 15 min. Two pieces of filter paper were also cut to the size of the gel and soaked in Towbin buffer for 15 min, and two fibre pads were soaked in Towbin for 15 min. The gel, PVDF membrane, fibre pads and filter paper were arranged on the white side of the blotting cassette (BioRad) blotting apparatus as follows: fibre pad, filter paper, PVDF membrane, 1D SDS-Ag-PAGE gel, filter paper, fibre pad. Proteins were transferred to the PVDF membrane at 300mA for 90 min at 4°C. Next the membrane was blocked in 5% skimmed milk powder diluted in TBS-Tween (50mM tris, 150mM sodium chloride, 0.1% (v/v) Tween-20 pH 7.4) for 2 h at room temperature then washed 3 x 10 min in TBS-Tween. The membrane was incubated overnight at 4°C with primary antibody

(Mucin 5B H-300 rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in TBS-Tween. The membrane was then washed 3 x 10 min in TBS-Tween, incubated with secondary antibody (goat anti-rabbit alkaline phosphatase (AP) conjugated, Sigma) diluted 1:10,000 in TBS-Tween for 2 h at room temperature, then washed 3 x 5 min in TBS-Tween.

The proteins were detected using Attophos Reagent (Promega) which detects alkaline phosphatase. The membrane was washed 1 x 5 min in 0.02M PBS (Sigma) incubated with Attophos reagent for 2 min at room temperature (1ml Attophos reagent/5cm² membrane) then visualised using the Typhoon scanner (BioRad).

To determine the relative amount of mucin 5B protein in each sample the bands were quantified using PD Quest density software. The resulting area and volume of each band were used to calculate a density score (volume/area) and the density score of the blank (an area of the membrane in which there was no staining) was subtracted from the band density score to provide a final density score for each protein.

Statistical Analysis

For differences in protein expression between low viscous and high viscous samples data were analysed in Microsoft Excel using a 2-tailed student's t-test with equal variances. The average ratio of low and high viscosity samples (compared to reporter ion 114 which was assigned a value of 1 for all proteins) was calculated and used to determine significant differences in protein expression between high and low viscosity samples.

The difference between density scores in low viscosity and high viscosity samples as determined by western blot were analysed in Microsoft Excel using a 2-tailed student's t-test with equal variances.

The difference between density scores in alpaca bulbourethral gland, prostate gland and testis was analysed a one-way ANOVA in GENSTAT (Version 11, VSN International, Hemel Hempstead, UK) with post-hoc comparisons using the least significant difference (LSD) test where appropriate.

For all analyses, P<0.05 was considered significant.

Results

iTRAQ Mass Spectrometry of Alpaca Seminal Plasma

Using a protein cut off of 95% confidence, a total of 48 proteins were identified in alpaca seminal plasma including lactotransferrin, beta-nerve growth factor, mucin 5AC, mucin 5B, sulfhydryl oxidase 1, epididymal secretory protein and nucleobindin (see appendix 1 for full list of proteins). Of these 48 proteins, 4 differed significantly in their expression between viscous and non-viscous samples (table 6.1)

Table 6.1 Relative abundance of proteins detected by iTRAQ and LC MS/MS that differ between low viscosity and high viscosity alpaca seminal plasma samples.

Protein	Low viscous (mean ± sem)	High viscous (mean ± sem)	Ratio ^a	P-Value
Mucin 5B	1.29 ± 0.29	7.91 ± 1.20	5.84	0.04
Monocyte differentiation antigen CD14	0.96 ± 0.04	0.46 ± 0.02	0.48	0.01
Phosphatidylethanolamine-binding protein	0.95 ± 0.05	0.62 ± 0.03	0.65	0.03
Tissue alpha-L-fucosidase	1.03 ± 0.03	0.68 ± 0.00	0.66	0.007

^a average abundance in high viscous divided by protein in low viscous

When comparing the abundance in high viscosity to low viscosity samples, one protein, mucin 5B, was more abundant in high-viscosity samples with over 5 times more protein present. The remaining 3 proteins were less abundant in high viscosity samples (ratio < 1) with approximately half the abundance observed.

Monocyte differentiation antigen CD14 (CD14) is a protein receptor that, upon activation from pathogens, triggers an innate immune response. It is present in human seminal plasma and on the sperm membrane (Harris et al. 2001; Malm et al. 2005) and is thought to help prevent infection of the male genital tract. It is unlikely to be the cause of viscosity in alpaca seminal plasma.

Phosphatidylethanolamine-binding protein (PEBP) is a protein receptor that binds phospholipids and is thought to have an inhibitory effect on sperm capacitation (Nixon et al. 2006; Moffit et al. 2007). Phosphatidylethanolamine-binding protein is expressed on the membrane of bull (D'Amours et al. 2010) and mice (Nixon et al. 2006) sperm although this is the first report in camelid seminal plasma. As PEBP has not been associated with viscosity in any biological fluids its can be assumed that it is not the cause of viscosity in alpaca seminal plasma.

Tissue alpha-L-fucosidase (FUCA1) is an enzyme glycoprotein previously reported in the seminal plasma of bull (Jauhiainen and Vanha-Perttula 1986) and human (Venditti et al. 2007) which may be involved in sperm oocyte binding. It is unlikely to be the cause of viscosity in alpaca seminal plasma.

Mucin 5B is a large gel-forming protein that is present in the seminal plasma of human (Russo et al. 2006) and the gel fraction of boar semen (Boursnell et al. 1970). The increased abundance of mucin 5B in viscous seminal plasma is interesting as, due to its gel forming capacities, it is likely that mucin 5B is the cause of viscosity in alpaca seminal plasma. In order to confirm these findings, the expression of mucin 5B in alpaca seminal plasma, and the source of this protein were determined by western blot analysis.

Western Blot

Seminal Plasma

The expression of mucin 5B in alpaca seminal plasma was confirmed by western blot (fig 6.1)

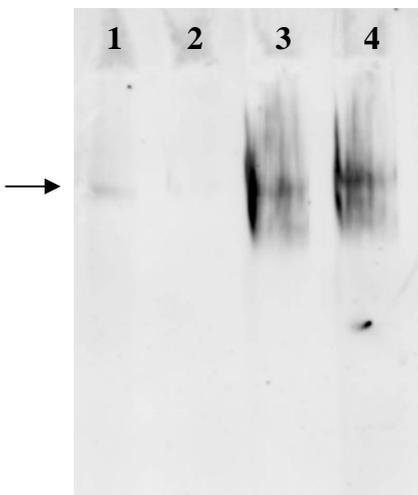


Figure 6.1 Western blot of Mucin 5B expression in alpaca seminal plasma. Lanes 1 and 2: low viscous samples, Lanes 3 and 4: high viscous samples. As commonly observed for mucin 5B, the protein appears as a streaked band on the SDS-AgPAGE gel. Arrow indicates mucin 5B band.

Densitometry analysis (mean \pm sem) of the bands indicated that the expression of mucin 5B is significantly greater in alpaca seminal plasma samples with high viscosity (12586.6 ± 103.75) compared to those with low viscosity (1199.8 ± 650.87 ; $P = 0.003$). This data confirms the findings from the iTRAQ MS data in which mucin 5B protein expression is significantly greater in viscous seminal plasma, suggesting that mucin 5B is the cause of viscosity.

Tissues

Mucin 5B protein was predominantly expressed in alpaca bulbourethral gland, intermediate expression was observed in the prostate and there was no or minimal expression in the testes as determined by western blot (figure 6.2). Densitometry analysis indicated that the expression of mucin 5B differed significantly between reproductive tissues ($P < 0.001$) and was higher in the bulbourethral gland than prostate or testis. Mucin 5B protein expression was intermediate in the prostate, and was lower in the testis than both the bulbourethral gland and prostate.

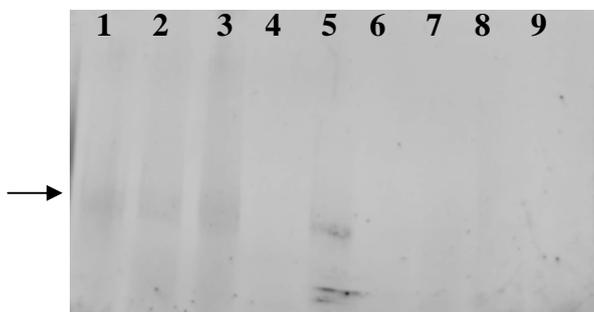


Figure 6.2 Western blot of Mucin 5B expression in alpaca reproductive tract. Lanes 1-3 bulbourethral gland, lanes 4-6 prostate gland, lanes 7-9 testis. Arrow indicates mucin 5B band.

Discussion

This is the first study to characterise the proteome of alpaca seminal plasma. Whilst in other species such as ram and human up to 300 proteins have been identified, only 48 proteins were identified in the seminal plasma of the alpaca. This is most likely due to the limited number of sequenced genes and proteins in camelids which prevent matching of species-specific peptide sequences to the protein rather than a lack of proteins in the seminal plasma. Of the 48 proteins identified, many have been characterised previously in seminal plasma, indicating that 2D LC MS/MS was successful in identifying seminal plasma proteins. Other proteomic techniques such as 2-dimensional gel electrophoresis were not successful in characterising the proteins of alpaca seminal plasma, mainly because the viscous nature and the high concentration of glycoproteins within the sample prevented adequate separation of proteins based on charge and size. Additionally, very large proteins, for example mucin, do not migrate onto the gel and can therefore only be detected using techniques such as mass spectrometry. Consequently, the technique adopted for this study suited the needs and requirements and was able to identify a relatively large number of proteins present in alpaca seminal plasma. Although more proteins may be identified with sequencing of the alpaca genome and proteome, the 48 proteins identified provide a starting ground on which research can be based, and may help identify proteins involved in sperm function or fertilisation.

The use of the relatively new iTRAQ technique enabled differences in protein abundance to be determined between viscous and non-viscous samples and identified four proteins that differed between seminal plasma samples of low and high viscosity. These were monocyte differentiation antigen CD14 (CD14), phosphatidylethanolamine-binding protein (PEBP), tissue alpha-L-fucosidase (FUCA1), and mucin 5B. All four proteins are expressed in the seminal plasma and/or sperm of other species however this is the first report of their presence in camelid seminal plasma.

Monocyte differentiation antigen CD14 (CD14) is a protein receptor that is involved in the innate immune response. In the presence of bacteria and pathogens, CD14 is activated and triggers a cascade of events that enable the cells to act accordingly eliciting an immune response to resist the pathogen. CD14 is present in human seminal plasma and on the sperm membrane and is produced by the epididymis (Harris et al. 2001; Malm et al. 2005). It is postulated that the presence of CD14 in semen helps prevent infection of the male genital tract and may also protect sperm when deposited in the female reproductive tract. It is unlikely, due to its role in the innate immune response, to be the cause of viscosity in alpaca seminal plasma. The reason for a reduction in CD14 expression in viscous samples is unknown but may be caused by the relative

contribution of the different reproductive glands to the seminal plasma. Perhaps more viscous semen contains a higher proportion of seminal plasma from the prostate or bulbourethral gland and therefore less protein from the epididymis although this requires further investigation.

Phosphatidylethanolamine-binding protein (PEBP, also termed raf kinase inhibitor protein or RKIP) is a protein receptor that binds phospholipids. Its role in sperm function is unknown, however recent research suggests that PEBP may have an inhibitory effect on sperm capacitation (Nixon et al. 2006) or that PEBP is a decapacitation factor (Moffit et al. 2007). Capacitation is an event that occurs following ejaculation to enable the sperm to undergo the acrosome reaction and subsequently penetrate the oocyte for fertilisation. It is a reversible process in that decapacitation factors can bind to capacitated sperm making them uncapacitated. This is advantageous to sperm as it ensures that the sperm do not exhibit fertilising ability prior to ovulation of the oocyte and therefore increases the longevity of the sperm and its fertilising potential. Phosphatidylethanolamine-binding protein is expressed on the membrane of bull (D'Amours et al. 2010) and mice (Nixon et al. 2006) sperm although this is the first report in camelid seminal plasma. The expression of PEBP is higher in cryopreserved bull sperm that exhibit high fertilising ability compared to those with low fertility (D'Amours et al. 2010) suggesting that higher concentrations of PEBP reduce the number of sperm that become capacitated during cryopreservation. The lower concentrations of PEBP in viscous alpaca seminal plasma may reflect a lower fertilising ability of cryopreserved sperm from viscous semen, however this requires further investigation.

Tissue alpha-L-fucosidase (FUCA1) is an enzyme glycoprotein which has been reported in the seminal plasma of bull (Jauhiainen and Vanha-Perttula 1986) and human (Venditti et al. 2007), though its presence in other species has not been reported. Its function is not fully understood although it is postulated to be involved in sperm oocyte binding. It is unlikely to be the cause of viscosity in alpaca seminal plasma however its role in fertilisation may suggest that sperm in low viscosity seminal plasma exhibit higher fertilising ability than sperm contained within highly viscous seminal plasma, although this requires further investigation.

Mucin 5B (MUC5B, also termed MG1) is a member of the mucin protein family which contains, to date, 17 genes including MUC 1-4, 5AC, 5B, 6-13, 15-17, 19 and 20. There are two classes of mucins, those that are membrane associated (MUC 1, 3, 4, 11, 12, 13, 15, 16, 17 and 20) and the remainder of which are secreted. The secreted mucins are further classified into large gel forming mucins (MUC 2, 5AC, 5B, 6 and 19) and small soluble mucins (MUC 7 and 9) (Russo et al. 2006). Consequently, mucin 5B is defined as a large gel-forming protein that is secreted by epithelial cells of glands. It was first identified in human saliva (Loomis et al. 1987) but has since been described in the mucus lining of the respiratory tract (Wickstrom et al. 1998) as well as human cervical mucus (Andersch-Björkman et al. 2007), illustrating its presence in viscous biological fluids.

The increased abundance of mucin 5B in viscous seminal plasma is interesting as due to its gel forming capacities it is highly likely that mucin 5B is the cause of viscosity in alpaca seminal plasma. Mucin 5B was 5 times higher in highly viscous seminal plasma compared to low viscosity alpaca seminal plasma, and this difference in protein abundance was confirmed by western blot analysis. Although not previously reported in camelid seminal plasma, mucin 5B is present in human seminal plasma (Russo et al. 2006) and the gel fraction of boar semen (Bournsnel et al. 1970). It is not known to play a specific role in sperm function, and therefore acts predominantly as a gel-forming protein. In boars, the ejaculate contains a sperm-rich fraction followed by a gel fraction. High concentrations of mucin 5B will enable a strong gel fraction to be deposited in the female tract following deposition of sperm during mating and thus helps prevent sperm loss. It is likely that mucin 5B plays a similar role in alpacas, in that the viscous semen remains in the female reproductive tract following mating prevents sperm loss. This is extremely beneficial to the species given the relatively low sperm concentrations in each ejaculate (around 300×10^6 sperm/ml). Additionally, the viscous semen probably degrades slowly once deposited in the female reproductive tract allowing small numbers of sperm to be released over time, increasing the likelihood of fertile sperm being present following the induction of ovulation by the ovulation inducing protein that is present in alpaca semen.

Western blot analysis of the alpaca bulbourethral gland, prostate gland and testis indicated that mucin 5B was predominantly expressed by the bulbourethral gland. In human, despite being present in seminal plasma,

mucin 5B protein expression was not detected in the testis, vas deferens, epididymis, vesicular gland or prostate gland (Russo et al. 2006) but was detected in the bulbourethral gland (Piludu et al. 2009). Mucin 5B is a large 600kDa glycoprotein consisting of a serine and threonine-rich protein core that also contains cysteine-rich domains. Linked to the protein core are O-linked carbohydrate chains and it is these chains that contribute to the large mass of the molecule. The structure of mucin 5B is therefore similar to that of glycosaminoglycans (GAGs), except that it is much larger. It is therefore not surprising that it was postulated that GAGs are the cause of viscosity in camelid seminal plasma. Mucin 5B, like GAGs binds Alcian Blue, and this explains the strong AB8GX staining observed in the alpaca bulbourethral gland at pH 2.5 despite the lack of hyaluronan (fig. 4.3, chapter 4). Strong Alcian blue staining was also observed in the camel bulbourethral gland (Perk 1962; Ali et al. 1976) and it is possible that this is attributable to mucin 5B as opposed to GAGs as previously thought. Mucins have also been detected in the boar bulbourethral gland epithelium (Badia et al. 2006) and are present in boar seminal plasma (Boursnell et al. 1970) supporting our findings that seminal plasma mucin 5B originates in the bulbourethral gland.

The identification of the protein responsible for viscosity in camelid seminal plasma will aid the development of protocols for the reduction of viscosity and thereby enhance methods of cryopreservation for camelid sperm. Mucin 5B is a very large glycoprotein and thus removal of this protein from the seminal plasma using techniques such as centrifugation or chromatography will be difficult and are likely to cause damage to the sperm. It is therefore more suitable to degrade the protein to reduce viscosity. The protein core of Mucin 5B does not have a repeating protein sequence that is unique to mucin, instead it is comprised of many protein sequences including serine, threonine and cysteine-rich D domains (Perez-Vilar and Hill 1999). Serine and threonine provide binding sites for the large carbohydrate chains which are O-linked and the cysteine-rich D domains provide sites for homo-multimerization of gel-forming mucin molecules and it is these large homo-multimers that create the viscous properties of mucin 5B. Understanding the structure of mucin 5B helps identify potential enzymes for degradation of the protein. Enzymes such as O-glycosidase F degrade the O-links and thereby will dissociate the large carbohydrate chains from the protein core in a similar fashion to GAG enzymes (chapter 4). However these enzymes tend to be slow-acting, require controlled conditions such as a specific PH and temperature and are relatively expensive compared to generic proteases such as trypsin and papain. As the mucin 5B protein core is rich in serine and threonine, proteases that specifically target serine and or threonine may degrade mucin 5B. Serine proteases include trypsin and chymotrypsin which have both been used successfully to reduce camelid seminal plasma viscosity (Bravo et al. 2000; Morton et al. 2008), although both have detrimental effects on sperm function and fertility, suggesting that serine proteases may not be a suitable approach. Proteases that specifically degrade threonine are uncommon, found only in the proteosome of the nucleus of eukaryotic cells consequently these enzymes are unlikely to be suitable for viscosity degradation.

Mucin 5B also contains cysteine-rich domains and it is at these domains that the gel forming mucin molecules are attached. Therefore, cysteine proteases may be a suitable option and may have a more profound effect on viscosity reduction than other proteases as they are targeting the site of the gel-forming molecules. Cysteine proteases include; caspases, calpains, cathepsins and papain. Caspases are involved in the activation and implementation of apoptosis (programmed cell death) and are therefore likely to cause damage or death to the sperm. Calpains cleave intracellular proteins involved in cell motility and adhesion and therefore although they may reduce viscosity, they are likely to permeate the sperm membrane and affect fertility either via a reduction in sperm motility, sperm cell death or a degradation of proteins required for successful fertilisation of the oocyte. Cathepsins are a large family of proteases, the majority of which are active at low pH. Alpaca semen has a pH ranging from 6.5-8.5, and the majority of extenders used for liquid or frozen storage of sperm have a pH of 7-8 which is not within the range required for cathepsin activation. Therefore cathepsins are not a suitable option for the degradation of alpaca seminal plasma viscosity as lowering the pH to enable cathepsins activation will cause damage to the sperm. Papain is a plant cysteine protease and degrades alpaca seminal plasma viscosity whilst having minimal effect on sperm motility, viability, acrosome integrity and DNA integrity (chapter 5). It can degrade tissues enabling extraction of cells for cell culture whilst leaving the cells intact and viable (Huettner and Baughman 1986; Kinoshita et al. 2003) suggesting it has limited effects on the cells. Additionally, papain degrades mucin 5B (Moncada et al. 2003). Given its relatively specific action, the promising effect on seminal plasma viscosity and the low toxicity to cells, papain seems the most suitable enzyme for degradation of mucin 5B in alpaca semen whilst maintaining sperm function. Further investigations that determine the optimal papain concentration and

incubation time would ensure limited effects on sperm function and integrity and therefore further investigation on the use of papain for reduction of alpaca seminal plasma are warranted.

In conclusion, this study determined that mucin 5B is five times higher in high viscosity alpaca seminal plasma compared to low viscosity seminal plasma, and that mucin 5B is therefore the likely cause of viscosity. The predominant source of mucin 5B is the bulbourethral gland and there is limited mucin 5B protein expression in the prostate and no expression in the testes. As mucin 5B is a large glycoprotein, enzymes that degrade the protein core should be investigated as a method of viscosity reduction. Of the available proteases that degrade mucin 5B, papain shows the most promise having limited effect on cell viability and also previously being investigated as a method for viscosity reduction in alpaca seminal plasma (chapter 5, (Morton et al. 2008)).

This study has highlighted the need to investigate the potential of papain to reduce viscosity without impairing sperm function and to determine its effect on the success of sperm cryopreservation in alpacas.

7. Papain Treatment of Alpaca Semen

Introduction

The development of semen cryopreservation and 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 for this species (Niasari-Naslaji et al. 2007) although pregnancy rates with frozen-thawed semen are still not commercially acceptable (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). Consequently, research on liquid and frozen storage of semen has focussed on reducing the viscosity of the seminal plasma by mechanical and enzymatic methods (Bravo et al. 1999; Bravo et al. 2000; Morton et al. 2008; Giuliano et al. 2010).

The cause of the viscous seminal plasma was previously unknown. It was been postulated that glycosaminoglycans (GAGs) were responsible (Perk 1962; Ali et al. 1976). However, while GAGs are abundant in alpaca seminal plasma (Kershaw-Young et al. 2011), enzymes that degrade GAGs do not completely eliminate the viscosity of semen. Conversely, generic proteases including papain and proteinase K trypsin, fibrinolysin, and collagenase (Bravo et al. 1999; Bravo et al. 2000; Morton et al. 2008; Giuliano et al. 2010) all reduce the viscosity of alpaca seminal plasma, suggesting that proteins, not GAGs, are the predominant cause of the viscosity. We have recently determined that the gel-forming protein mucin 5B is more abundant in highly viscous alpaca seminal plasma compared to seminal plasma with low viscosity, suggesting that mucin 5B is the cause of viscosity (chapter 6). Mucin 5B belongs to a family of large glycoproteins with high molecular weight. Glycoproteins consist of a protein core with numerous carbohydrate chains attached. Whilst it is possible to use enzymes that break the links between the protein core and carbohydrates, such as O-glycosidase F, to degrade mucin 5B, these enzymes do not act rapidly and require a controlled environment to work effectively such as a specific stable pH and stable temperature. Consequently there are no specific enzymes that rapidly degrade mucin 5B and it is necessary to identify a generic protease that is able to degrade mucin 5B, thus reducing seminal plasma viscosity, without impairing sperm function. Cysteine proteases degrade mucin (Moncada et al. 2003) altering its function and papain the cysteine protease enzyme present in papaya (*Carica papaya*) has shown promise as a reducer of viscosity in seminal plasma (Morton et al. 2008). However, when exposed to papain over prolonged periods of time at concentrations of 0.5 – 4mg/ml the acrosomes of alpaca spermatozoa were impaired (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 0.1mg/ml (chapter 5).

Most methods for the cryopreservation of alpaca semen involve chilling of the diluted 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 spermatozoa, it would be advantageous to inhibit the papain following liquefaction. Trans-Epoxy succinyl-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. 1981; Tamai et al. 1981; Barrett et al. 1982). 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.

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 (2) the effect of the papain inhibitor E-64, on alpaca seminal plasma viscosity and sperm motility, acrosome integrity, viability and DNA integrity.

Materials and Methods

Animals

Experiments were performed using male alpacas under authorization from the University of Sydney animal ethics committee. All males were > 3 years, had a body condition score > 3 and had testes more than 3 cm long (Tibary and Vaughan 2006).

Experimental Design

Two experiments were conducted. Experiment 1 determined the effect of concentration and time of exposure to papain on the viscosity of seminal plasma and sperm function, whilst experiment 2 investigated the papain inhibitor E-64 on the viscosity of seminal plasma and sperm function.

Experiment 1

Experiment 1 was performed from February to June 2010. Semen was collected from six male alpacas (≥ 2 ejaculates/male, $n = 15$) using an artificial vagina fitted inside a mannequin as described in chapter 3. Within 5 min of collection, semen was assessed for volume, viscosity, and motility and concentration of spermatozoa as described in chapter 3 and briefly below. Only samples with a volume >1ml, viscosity ≥ 15 mm, motility $\geq 50\%$ and concentration $\geq 10 \times 10^6$ spermatozoa/ml were used. Following collection, 1ml of semen was diluted 1:1 in pre-warmed Tris-citrate-fructose buffer (300mM Tris, 94.7mM citric acid, 27.8mM 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) 390 μ l diluted semen plus 10 μ l 0.02M PBS (control), (2) 390 μ l diluted semen plus 10 μ l 0.04mg/ml papain (final concentration 0.001mg/ml; Sigma), (3) 390 μ l diluted semen plus 10 μ l 0.4mg/ml papain (final concentration 0.01mg/ml;), (4) 390 μ l diluted semen plus 10 μ l 4.0mg/ml papain (final concentration 0.1mg/ml). Samples were incubated for 30min at 37°C in a water bath. Semen viscosity, and motility and acrosome integrity of spermatozoa were assessed immediately after dilution (time 0) and at 5, 10, 20 and 30min after treatment.

Experiment 2

Experiment 2 was performed from June to July 2010. Semen was collected from six male alpacas (≥ 2 ejaculates/male, $n = 15$) using an artificial vagina and assessed for volume, viscosity, and motility and concentration of spermatozoa. As for experiment 1, samples with a volume >1ml, viscosity ≥ 15 mm, motility $\geq 50\%$ and concentration $\geq 10 \times 10^6$ spermatozoa/ml were diluted 1:1 in pre-warmed Tris-citrate-fructose buffer (Evans and Maxwell 1987). Diluted semen samples were allocated to two treatment groups, (1) 792 μ l diluted semen plus 8 μ l 0.02M PBS (control; treatment 1) and (2) 792 μ l diluted semen plus 8 μ l 10mg/ml papain (final concentration 0.1mg/ml; treatment 2), and incubated at 37°C for 20min in a water bath. Each aliquot was then divided further into two treatment groups, (1) 297 μ l semen plus 3 μ l 0.02M PBS (control; treatment A) and (2) 297 μ l semen plus 3 μ l 1mM N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide (E-64; Sigma-Aldrich, St Louis, MO, USA; final concentration 10 μ M; treatment B), and incubated at 37°C for 5min 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 motility, acrosome integrity, viability and DNA integrity of spermatozoa were assessed immediately after dilution (0min), after papain or PBS but prior to E-64 treatment (20min), and after E-64 or PBS treatment (25min).

Analysis of semen viscosity and sperm parameters

Viscosity of semen and concentration and motility of spermatozoa

Sample (10 μ l) was diluted (1:9) in 90 μ l 3% sodium chloride (Sigma) and the concentration of sperm was assessed using a haemocytometer (Evans and Maxwell 1987). Viscosity was assessed using the thread test (Bravo et al. 2000). Briefly, 50 μ l of sample was drawn into a pipette, 25 μ l was pipetted onto a warm glass slide and the pipette lifted vertically forming a thread of sample. The length at which the thread snapped was recorded as the measurement of viscosity. Motility of spermatozoa was assessed subjectively at X 100 magnification under phase contrast microscopy (Olympus, Tokyo, Japan) by placing 10 μ L 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

In experiment 1, acrosome integrity of spermatozoa was assessed via microscopy as described in chapter 3 (Kershaw-Young and Maxwell 2011). Briefly, 20 μ l 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 10⁶/ml. Twenty μ L of resuspended spermatozoa was mixed with 4 μ L fluorescent isothiocyanate-conjugated lectin from *Arachis hypogaea* (working concentration 40 μ g/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

In experiment 2, acrosome integrity was assessed using flow cytometry analysis of FITC-PNA stained sperm as described in chapter 3. Briefly semen was diluted in 1ml 0.02 M PBS to a final concentration of 1 x 10⁶ spermatozoa/ml then incubated with 10 μ l FITC-PNA (working concentration 40 μ g/ml) at 37°C for 15 min,. The samples were fixed with 10 μ l 10% neutral buffered formalin (final concentration 0.1%). Fluorescence was detected using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), equipped with an argon ion laser (488 nm, 15 mW) for excitation and acquisitions were made using CellQuest 3.3 software (Becton Dickinson, San Jose, CA). A minimum of 5,000 gated events were recorded. Acrosomes were considered not intact if the acrosome stained green, and considered intact if there was no staining.

Viability of spermatozoa, experiment 2

Viability, measured as spermatozoa with non-impaired membranes, was assessed following PI and Syto-16 staining on a flow cytometer as described in chapter 3 (Kershaw-Young and Maxwell 2011). Briefly, samples were fixed in 1ml 0.1% neutral buffered formalin at 1 x 10⁶ spermatozoa/ml and stored at 4°C. Next day, samples were incubated with 10 μ l Syto-16 (Molecular Probes, Eugene, OR, USA; working concentration 10 μ M) at room temperature for 20 min, then 10 μ L Propidium iodide (PI, Molecular Probes, Eugene, OR, USA, working concentration 240 μ M) at room temperature for a further 10 min. Viability of spermatozoa was determined using a FACScan flow cytometer as described above. Sperm cells that stained positive for Syto-16 and negative for PI were deemed viable, and those that stained negative for Syto-16 and positive for PI were deemed non-viable.

DNA Integrity of spermatozoa

The integrity of sperm DNA was assessed as described in chapter 3 (Kershaw-Young and Maxwell 2011). Briefly, samples were snap frozen in liquid nitrogen and stored at -20°C until analysis. Samples were resuspended to a concentration of 10 x 10⁶ spermatozoa/ml, smeared onto a glass slide and fixed in 100% ice cold methanol. Next, slides were incubated with Terminal deoxynucleotidyl transferase dUTP nick end

labelling (TUNEL) reaction mixture (Roche Applied Science, Mannheim, Germany) in a humidified chamber at 37°C for 1 h, then counterstained with DAPI (Vector Laboratories, CA, USA). A minimum of 200 spermatozoa was assessed with the BX51 fluorescence microscope. 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 11 (VSN International, Hemel Hempstead, UK).

For experiment 1, viscosity of semen, and 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 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 $P < 0.05$.

Results

Experiment 1

Papain treatment significantly reduced the viscosity of alpaca seminal plasma ($P < 0.001$; fig. 7.1). 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 0mg/ml (control) papain-treated samples. However, after 30min incubation, all papain-treated samples had less viscosity than control samples. Viscosity reduced significantly over time in all treatment groups, although the reduction was most rapid for samples treated with 0.1mg/ml papain ($p < 0.001$; fig. 7.1).

Motility of spermatozoa differed between treatments at each time point ($P = 0.01$; table 7.1). At 10 and 20min after treatment, motility was higher in samples treated with 0.1mg/ml papain than control samples, and at 30min motility was higher in samples treated with 0.1mg/ml papain than in all other treatment groups. A decrease in motility of spermatozoa was observed from 0 to 30min post treatment in all groups ($P = 0.01$; table 7.1).

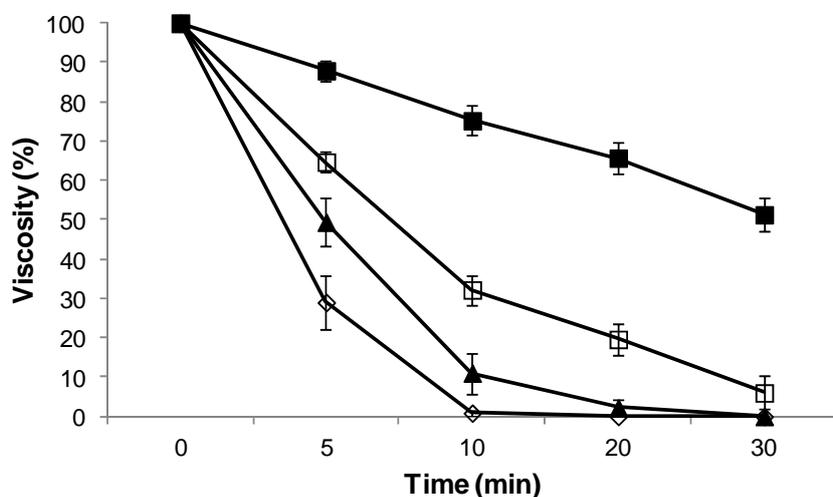


Figure 7.1 Percentage viscosity (mean \pm SEM) of alpaca semen treated with 0 (control, ■), 0.001 (□), 0.01 (▲) and 0.1 (◇) mg/ml papain at 0, 5, 10, 20 and 30 min after treatment.

Table 7.1 Percentage motility (mean \pm SEM) of sperm treated with 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-z} Within a column, means without a common subscript differed ($P < 0.05$)

The percentage of spermatozoa with intact acrosomes (mean \pm sem) was higher in samples treated with 0.1mg/ml papain (53.9 \pm 0.50) compared to those containing 0 (51.7 \pm 0.58), 0.001 (51.9 \pm 0.59) and 0.01mg/ml papain (52.3 \pm 0.59; $P < 0.001$). Acrosome integrity decreased significantly over time ($P = 0.007$) and was greater at 0 (52.7 \pm 0.51), 5 (53.48 \pm 0.60) and 10min (52.6 \pm 0.71) compared to 30min (51.4 \pm 0.71 %) after treatment. Acrosome integrity at 20min post-treatment (52.1 \pm 0.63 %) not differ from the other time points.

Experiment 2

As observed in experiment 1, the viscosity of seminal plasma was completely eliminated within 20 min of treatment with 0.1mg/ml papain and viscosity (mean mm \pm sem) was significantly lower in papain-treated samples (0.0 \pm 0.0) compared with the control ($P < 0.001$) at both 20min (pre-E64; 78.7 \pm 5.41) and 25min (post-E64; 66.5 \pm 3.29) of treatment. The papain inhibitor E64 did not affect viscosity ($P = 0.734$).

The motility of spermatozoa (mean % \pm sem) was not different between the control (50.7 \pm 1.16), E64 only (46.7 \pm 2.05), papain only (49.2 \pm 1.40), and papain with E64 (47.3 \pm 2.12) treatment groups. Motility declined significantly ($p < 0.001$) between 0 (54.7 \pm 1.50), 20 (50.3 \pm 1.92) and 25 min (46.8 \pm 1.47 %) of treatment, although the decline in motility was similar for all treatments.

The percentage of spermatozoa with intact acrosomes was higher in papain-treated samples (43.8 \pm 2.71) compared to samples that were not treated with papain (36.1 \pm 2.28 %; $p < 0.01$) but was not affected by E64 treatment or time ($P > 0.05$).

The percentage of viable spermatozoa was not affected by papain or E64 treatment ($p > 0.05$) and did not differ over time ($P > 0.05$). Viability (mean \pm sem) was similar in the control (76.0 \pm 2.36), E64 only (76.4 \pm 3.68), papain only (76.6 \pm 2.72) and papain with E64 (77.7 \pm 3.75) treatment groups.

The percentage of spermatozoa with intact DNA (mean \pm sem) was not different between control (97.5 \pm 0.22), E64 only (97.7 \pm 0.39), papain only (97.6 \pm 0.25) and papain with E64 (97.9 \pm 0.38) treated samples, and did not change over time ($P > 0.05$).

Discussion

The objective of this study was to (1) investigate the effect of time and concentration of papain treatment on semen viscosity and sperm function in order to develop a protocol for the reduction of seminal plasma viscosity in alpacas and (2) to determine the effect of the papain inhibitor E-64 on sperm motility, acrosome integrity, viability and DNA integrity in order to identify a suitable inhibitor of papain for use in the reduction of seminal plasma viscosity in alpacas.

As determined in chapter 4, papain was effective in reducing viscosity. The most rapid reduction in viscosity was observed after 20 min of treatment using concentrations of 0.1mg/ml papain, however viscosity was also

completely eliminated with 30 min when using 0.01mg/ml papain. In order to develop a method for the reduction of seminal plasma viscosity for use within the camelid industry, it is necessary for that method to be rapid, reliable, effective and cost effective. Papain is a relatively cheap protease (\$64 per 50mg) in which one vial can be used to treat 500ml of alpaca semen (250-400 ejaculates) when used at a final concentration of 0.1mg/ml and is thus very cost effective. When used at 0.1mg/ml all seminal plasma samples exhibited 0mm viscosity within 20 min of treatment, indicating that this protocol is reliable and effective in 100% of samples tested. Additionally, using a concentration of 0.1mg/ml initiated more rapid reduction of viscosity than 0.01mg/ml. Consequently we suggest using 0.1mg/ml papain at 37°C for 20 min for the reduction of alpaca seminal plasma viscosity.

For papain to be truly effective it must not only reduce viscosity but also have no detrimental effects on the integrity of sperm. Sperm, being protein-rich are at high risk of degradation by unselective proteases such as papain. Previous studies have suggested that generic proteases including trypsin, fibrinolysin, and collagenase are detrimental to sperm motility, viability and acrosome integrity (Bravo et al. 2000; Morton et al. 2008) in alpacas and llamas. Additionally, papain, when used at concentrations of greater than 1.0mg/ml reduces the percentage of sperm with intact acrosomes (Morton et al. 2008). In the present study, papain concentrations of 0.1 to 0.001mg/ml were not detrimental to sperm motility and acrosome integrity within 30 min of treatment, suggesting that the lower concentrations of papain used were low enough to reduce viscosity without causing sperm damage.

The higher motility observed at 30min post-treatment in samples treated with 0.1mg/ml papain compared to all other treatment groups is mostly likely an effect of the reduction in seminal plasma viscosity observed in these samples. The highly viscous nature of alpaca semen prevents progressive sperm motility and renders sperm slow moving in an oscillatory pattern (Garnica et al. 1993). A reduction in seminal plasma viscosity is associated with an increase in the proportion of progressively motile sperm (Morton et al. 2008; Giuliano et al. 2010). Although we did not observe any progressively motile sperm following enzyme treatment in the present study, it is possible that the reduction of viscosity enabled sperm that were previously non-motile in the viscous seminal plasma medium to move.

The effect of prolonged exposure of sperm to papain is inconclusive. We did not determine any effect of papain treatment over 2 h on sperm motility and acrosome integrity when used at 0.1mg/ml (chapter 5), although acrosome integrity did decrease over time in all treatment groups including control. Conversely at concentrations of 0.5-4.0mg/ml acrosome integrity was significantly lower than control samples within 10min of papain treatment and declined rapidly over time with lower sperm numbers with intact acrosomes in samples treated with higher concentrations of papain (Morton et al. 2008). As the effect of prolonged exposure to papain is undetermined, and the cryopreservation of semen 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. We therefore examined the use of the papain inhibitor E-64 on sperm motility, acrosome integrity, viability and DNA integrity in order to identify an effective reliable inhibitor that does not affect sperm function and integrity.

In a preliminary experiment (not reported) we determined that when 0.1mg/ml papain was incubated with 10 μ M E-64 at 37°C for 5 min then incubated with alpaca semen for 20min at 37°C there was no reduction in viscosity, indicating that 10 μ M E-64 for 5 min at 37°C is effective at inhibiting papain as described previously (Barrett et al. 1982). Consequently, 10 μ M E-64 for 5 min at 37°C was used to inhibit papain following the reduction of alpaca semen viscosity. 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.

In conclusion, this study has demonstrated the potential use of papain as a means of effectively reducing viscosity without compromising sperm integrity. The treatment of alpaca semen with 0.1mg/ml papain for 20 min at 37°C completely eliminated viscosity without compromising sperm integrity. E-64 inhibited the function of papain without impairing sperm function and was therefore successful in preventing any negative effects of long term papain exposure on sperm integrity. The success of this papain and E-64 combination in

reducing semen viscosity without negatively impacting sperm integrity effects may make it a more promising solution to semen viscosity than previously reported and could have significant implications for the development of assisted reproductive technologies in the alpaca. In order to determine whether the effect of reducing semen viscosity is advantageous, it is necessary to examine the cryopreservation potential of papain then E-64-treated alpaca sperm. 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 rates of the sperm, and the optimal storage method (pellet or straws). Additionally, the final seminal plasma concentration during freezing and after thawing is important, as seminal plasma proteins are known to impair or protect sperm during cryopreservation (Maxwell et al. 2007).

Before a reliable, effective, commercially available cryopreservation protocol can be established for alpaca sperm, it is necessary to (1) determine the role of seminal plasma on sperm function in order to optimise the final seminal plasma concentration during or after cryopreservation and (2) determine the effect of semen viscosity reduction on the success of cryopreservation.

8. The effect of Seminal Plasma on Sperm Function

Introduction

The development of semen cryopreservation protocols in camelids, particularly alpacas, is currently limited by the viscous nature of alpaca seminal plasma and a lack of understanding of its role in sperm function. In numerous species, seminal plasma is routinely diluted or removed during processing of semen for cryopreservation; this can have either positive or negative effects on sperm function and fertility (Maxwell et al. 2007). Therefore, development of cryopreservation protocols in alpacas will be hindered until the role of seminal plasma in sperm function is determined.

It is not known whether dilution or removal of seminal plasma prior to freezing will inhibit or stimulate function of alpaca sperm. It is generally considered that excessive dilution of seminal plasma during processing is detrimental to sperm function (Mann 1964), although the effect differs among species (Maxwell et al. 2007). During cryopreservation of stallion semen, seminal plasma was detrimental to sperm function and therefore was either removed by centrifugation or diluted to less than 5% of the freezing medium prior to processing (Moore et al. 2005). Conversely, in the boar (Vadnais et al. 2005) and ram (Ashworth et al. 1994), the presence of 20 or 10% seminal plasma, respectively, during cooling or incubation at 37-39°C was beneficial to the function and integrity of sperm, maintaining both motility and viability.

Understanding the role of seminal plasma in the function of sperm in livestock species has advanced the development of assisted reproductive technologies, e.g. cryopreservation and AI. The addition of seminal plasma to sperm following cryopreservation increased post-thaw motility and fertility in the ram (Maxwell et al. 1999) and enhanced post-thaw sperm function in the boar (Vadnais et al. 2005). Moreover, although detrimental during processing, the addition of seminal plasma to stallion sperm post-thaw increased fertility following AI (Alghamdi et al. 2005). In camelids, viscous seminal plasma prevents successful cryopreservation; therefore, research has focussed on the treatment of semen with non-specific enzymes (Bravo et al. 2000; Giuliano et al. 2010) to reduce viscosity prior to processing. Whilst enzyme treatment of Camelid seminal plasma reduced viscosity, it also adversely affected sperm function and integrity (Bravo et al. 2000), most likely due to non-selective digestion of seminal plasma proteins. Collagenase successfully reduced llama seminal plasma viscosity whilst maintaining sperm function (Giuliano et al. 2010); although these sperm retained their fertilising ability (Conde et al. 2008), the effect of this enzyme on the success of cryopreservation is unknown. Conversely, in the alpaca, collagenase impaired sperm function (Morton et al. 2008) and therefore further research is necessary. In other studies, camelid semen was diluted with cryodiluents at rates ranging from 1:1 to 1:4 in an attempt to moderate the effects of seminal plasma (Deen et al. 2003; Santiani et al. 2005; Niasari-Naslaji et al. 2007; Morton et al. 2009). In these studies, reasonable post-thaw motility was achieved, but fertility was low and not commercially viable after insemination of frozen-thawed sperm.

The objective of the current study was to explore the role of seminal plasma concentration on the motility and functional integrity of alpaca sperm. To determine whether alpaca seminal plasma is beneficial or detrimental to sperm, as well as the optimal dilution rate to maintain sperm function and integrity, the effect of various seminal plasma concentrations on motility, acrosome integrity, DNA integrity, and viability of epididymal and ejaculated alpaca sperm were studied.

Material and Methods

Animals

This study was performed from February to May 2010 using 16 male alpacas under authorization from the University of Sydney animal ethics committee. All males were > 3 years of age, had a body condition score ≥ 3 (mean 3.4 ± 0.1), weighed >70 kg (mean $80. \pm 2.1$), and had testes more than 3 cm long.

Experimental Design

This study was performed as four experiments. Experiments 1 and 2 determined the effect of seminal plasma on epididymal sperm function, and experiments 3 and 4 determined the effect of seminal plasma on ejaculated sperm function.

In experiments 1 and 2, epididymides and testes were collected following castration from 10 male alpacas housed at alpaca studs throughout Australia. Six alpacas were used for experiment 1 (age 43.8 ± 2.5 mo) and 4 alpacas for experiment 2 (age 85.0 ± 24.1 mo). Following castration, epididymides and testes were wrapped in gauze soaked in 0.02M PBS (Sigma-Aldrich, St Louis, MO, USA) then left to cool to room temperature for approximately 2 h prior to transportation to the laboratory overnight at 4°C. Epididymal sperm were then harvested and washed as described below prior to incubation in seminal plasma collected from male alpacas as described below.

In experiment 1 epididymal sperm were harvested and washed and their motility was assessed (time 0 h). Samples were then resuspended to 50×10^6 sperm/ml in 0, 10, 25, 50 or 100% seminal plasma ($r = 4$ replicates/epididymal harvest) containing 0.02M PBS (Sigma), 0.1% BSA (Cohns fraction 5; Sigma), 7.5mg/ml penicillin (Sigma-Aldrich, St Louis, MO, USA), and 5mg/ml streptomycin (Sigma), (herein referred to as PBS-BSA). Samples incubated in 100% seminal plasma contained 0.1% BSA, 7.5mg/ml penicillin and 5mg/ml streptomycin but no PBS. Following dilution, samples were incubated in a water bath at 37 °C and the motility of sperm was assessed after 0.5, 1, 2, 3, 4, 5 and 6 h. To prevent separation of the seminal plasma and PBS-BSA during incubation, the incubation tubes were flicked at 15min intervals and samples were pipetted when motility was assessed.

In experiment 2, epididymal sperm were harvested and washed, and their motility, acrosome integrity and DNA integrity were assessed (time 0 h). Samples were then resuspended to 50×10^6 sperm/ml in 0, 10 or 100% seminal plasma ($r = 4$ replicates/harvest) containing PBS-BSA and incubated in a water bath at 37 °C. Motility and acrosome integrity of sperm were assessed after 1, 2 and 3 h and DNA integrity was assessed after 3 h of incubation. As for experiment 1 the samples were flicked and pipetted during incubation to prevent separation of PBS-BSA from the seminal plasma.

In experiments 3 and 4, semen was collected from 6 male alpacas housed at The University of Sydney, Australia. All six males were used in experiment 3 (age 111.7 ± 7.2 mo) and four of the six males were randomly selected for experiment 4 (age 108.4 ± 10.8 mo).

In experiment 3, ejaculated semen samples were collected from male alpacas ($n = 6$) using an artificial vagina fitted inside a mannequin, as described by Morton et al. (Morton et al. 2008). The motility and concentration of sperm were assessed within 5 min of collection (time 0 h) and only samples containing > 50% motile sperm and $> 50 \times 10^6$ sperm/ml were used. Semen was diluted to 10 (1:9 v/v), 25 (1:3 v/v), 50 (1:1 v/v) or 100 % seminal plasma ($r = 4$ replicates/ejaculate) with PBS-BSA. Homogenisation of seminal plasma and PBS-BSA was performed by manual pipetting for 2 min. All samples were pipetted an equal number of times as pipetting has been shown to reduce seminal plasma viscosity (Morton et al. 2008). Samples incubated in 100% seminal plasma contained 0.1% BSA, 7.5mg/ml penicillin and 5mg/ml streptomycin but no PBS. The samples were incubated in a water bath at 37°C and the motility of sperm was assessed after 0.5, 1, 2, 3, 4, 5 and 6 h. To prevent separation of seminal plasma and PBS-BSA the incubation were tubes flicked at 15 min intervals and samples were pipetted each time motility was assessed.

In experiment 4, ejaculated semen samples were collected from male alpacas ($n = 4$) as described above and the motility and concentration of sperm assessed within 5 min of collection (time 0 h). Only samples containing $> 50\%$ motile sperm and $> 50 \times 10^6$ sperm/ml were used. Samples were then prepared for analysis of acrosome integrity, viability and DNA integrity (time 0 h). Semen was diluted to 10 (1:9 v/v) or 100% seminal plasma ($r = 4$ replicates/ejaculate) with PBS-BSA by manual pipetting for 2 min. Samples incubated in 100% seminal plasma contained 0.1% BSA, 7.5mg/ml penicillin and 5mg/ml streptomycin but no PBS. Next samples were incubated in a water bath at 37°C and motility, acrosome integrity and viability of sperm were assessed after 1, 2 and 3 h and DNA integrity was assessed after 3 h of incubation. The incubation tubes were flicked at 15 min intervals and samples were pipetted each time motility was assessed to ensure the PBS-BSA and seminal plasma remained homogenised.

Harvesting of epididymal sperm

For experiments 1 and 2, epididymal sperm were harvested as described previously (Morton et al. 2007) with some minor modifications. Briefly, epididymides were minced with a sterile scalpel and the sperm allowed to swim out for 30 min in 4ml warm (37 °C) PBS-BSA. Following harvest, sperm were centrifuged at 300 x g for 5 min at room temperature and the supernatant discarded. Sperm were then resuspended in PBS-BSA or in seminal plasma containing 0.1% BSA, 7.5mg/ml penicillin and 5mg/ml streptomycin but no PBS (100% seminal plasma samples) to 1000×10^6 sperm/ml and their motility assessed prior to dilution in seminal plasma. Only samples with at least 100×10^6 sperm retrieved per pair of testes and with sperm displaying greater than 50% motility post-centrifugation were used in the study.

Seminal plasma

To determine the effect of seminal plasma on epididymal sperm function, male alpacas housed at The University of Sydney were trained for semen collection by artificial vagina and used for semen collection and subsequent processing of seminal plasma. Semen was collected from male alpacas ($n = 6$; 2 ejaculates/male: age 114.0 ± 6.5 mo) using an artificial vagina fitted inside a mannequin, as described in chapter 3. Immediately after collection, the semen was centrifuged at 10,000 x g for 30 min, the seminal plasma decanted and sperm pellet discarded, then centrifuged again at 10,000 x g for 30 min and the seminal plasma decanted and stored at -80 °C. To ensure all sperm were removed from the ejaculate, 10 μ L of centrifuged seminal plasma was placed onto a glass slide and observed under a microscope at X 100 magnification for the presence of sperm. The protocol outlined was sufficient to remove all sperm from the ejaculate. Homogenisation of seminal plasma and PBS-BSA was performed by manual pipetting for 2 min.

Analysis of Sperm

Total sperm motility was assessed subjectively at X 100 magnification under phase contrast microscopy as described in chapter 3. All motile sperm whether oscillatory or progressive were considered motile, and progressive and oscillatory motility were not discriminated from each other.

Acrosome integrity was assessed using FITC_PNA fluorescent staining and microscopy as described in chapter 3. A minimum of 200 sperm were observed and 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.

DNA integrity was assessed using TUNEL labelling and microscopic assessment as described in chapter 3. A minimum of 200 sperm was assessed with the BX51 fluorescent microscope and sperm DNA was considered non-fragmented if there was no fluorescence, and fragmented if the sperm head stained green.

Sperm viability, measured as sperm with non-impaired membranes, was assessed using flow cytometry following staining with Propidium iodide (PI, Molecular Probes, Eugene, OR, USA) and Syto® 16 (Molecular Probes, Eugene, OR, USA) as described in chapter 3. Acquisitions were made using CellQuest 3.3 software (Becton Dickinson, San Jose, CA) and were stopped after recording 10,000 gated events. Sperm that stained positive for Syto-16 and negative for PI were deemed viable, and cells that stained negative for Syto-16 and positive for PI were deemed non-viable.

Statistical analysis

Data were analysed using a linear mixed model regression in GENSTAT (Version 11, VSN International, Hemel Hempstead, UK) with post-hoc comparisons using the least significant difference (LSD) test where appropriate. To accommodate the experimental design, the random effects in the model were alpaca ID, replicate nested within alpaca ID, and seminal plasma concentration nested within replicate. The latter term was included to accommodate the splitting of the replicate (ejaculate sample) into sub-samples, each allocated a different seminal plasma concentration. Time, seminal plasma concentration and their interaction were specified as fixed factors. Where data were significantly skewed, they were transformed using arc sine to give a normal distribution prior to statistical analysis.

Results

Experiment 1

The motility of epididymal sperm declined during the 6 h incubation in all samples and the percentage of motile sperm differed between seminal plasma concentrations at each time point ($P < 0.001$) (fig. 8.1). At 0.5 h, motility was higher in samples with 10 or 25% than 0, 50 or 100% seminal plasma. At 1, 2, 3 and 4 h, motility was higher in samples with 10% seminal plasma than all other samples. After 5 and 6 h incubation, there were no differences between seminal plasma concentrations in motility of sperm.

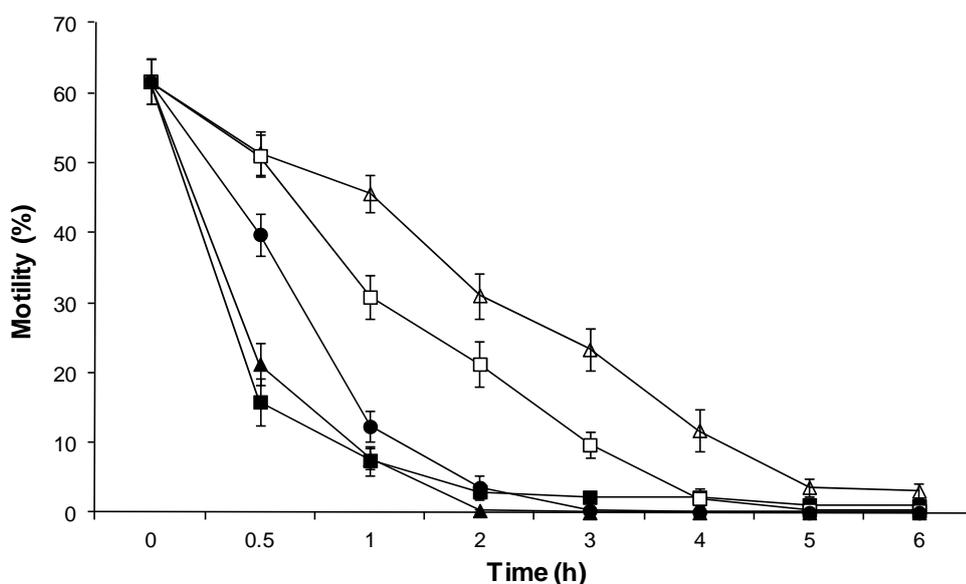


Figure 8.1. Mean \pm SEM motility (%) of epididymal alpaca sperm incubated for 0 to 6 h in 0 (■), 10 (△), 25 (□), 50 (●), or 100% (▲) seminal plasma.

Experiment 2

Motility of sperm differed between seminal plasma concentrations at each time point except at 0 h ($P < 0.001$; table 8.1). At 1 h, motility was higher in samples with 10% seminal plasma than 0 or 100% seminal plasma, and was also better in samples with 100 than 0% seminal plasma. At 2 and 3 h, motility was higher in samples with 10% seminal plasma than 0 or 100% and no differences were observed between 0 and 100% seminal plasma (table 8.1).

Table 8.1. Percent motility (mean \pm SEM) of epididymal alpaca sperm incubated for 0, 1, 2, or 3 h at 37 °C in 0, 10, or 100% seminal plasma.

Time (h)	Seminal plasma (%)		
	0	10	100
0	56.3 ± 2.80 _x	56.3 ± 2.80 _w	56.3 ± 2.80 _x
1	6.0 ± 2.31 ^a _y	43.4 ± 3.67 ^b _x	16.0 ± 3.64 ^c _y
2	3.8 ± 0.94 ^a _y	25.3 ± 3.89 ^b _y	1.9 ± 0.77 ^a _z
3	2.1 ± 0.72 ^a _y	11.6 ± 2.34 ^b _z	0.0 ± 0.00 ^a _z

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

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

The percentage of epididymal sperm with intact acrosomes differed between seminal plasma concentrations at different time points ($P < 0.001$; table 8.2). At 0 h there was no difference between samples, whereas at 1, 2 and 3 h the percentage of sperm with intact acrosomes was significantly higher for 10 and 100 than for 0% seminal plasma. There were no differences observed between samples with 10 or 100% seminal plasma.

Table 8.2. Proportion of intact acrosomes (mean ± SEM) in epididymal sperm incubated for 0, 1, 2, or 3 h at 37 °C in 0, 10, or 100% seminal plasma.

Time (h)	Intact acrosomes (%)		
	0	10	100
0	76.6 ± 1.08 ^a _x	76.6 ± 1.08 ^a _x	76.6 ± 1.08 ^a _x
1	39.4 ± 3.73 ^a _y	75.3 ± 1.20 ^b _x	77.4 ± 0.90 ^b _x
2	35.6 ± 4.04 ^a _y	71.6 ± 0.84 ^b _x	74.5 ± 2.47 ^b _x
3	36.0 ± 3.53 ^a _y	71.5 ± 1.33 ^b _x	70.7 ± 2.89 ^b _x

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

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

The percentage of epididymal sperm with non-fragmented DNA did not differ between samples with 0, 10 or 100% seminal plasma ($P > 0.05$) but did decrease from 0 to 3 h (mean ± SEM; 97.7% ± 0.15 vs. 95.6% ± 0.40 respectively; $P < 0.001$).

Experiment 3

The motility of ejaculated sperm declined during the 6 h incubation in all samples, and differed between seminal plasma concentrations at each time point ($P < 0.001$; fig. 8.2). At 0 h there were no differences between samples, however from 0.5 to 5 h, motility was higher in samples with 10% seminal plasma than those with 25, 50 or 100% ($P < 0.001$). From 0.5 to 4 h, motility was also higher in samples with 25% seminal plasma compared to those with 50 or 100% ($P < 0.001$; fig. 8.2).

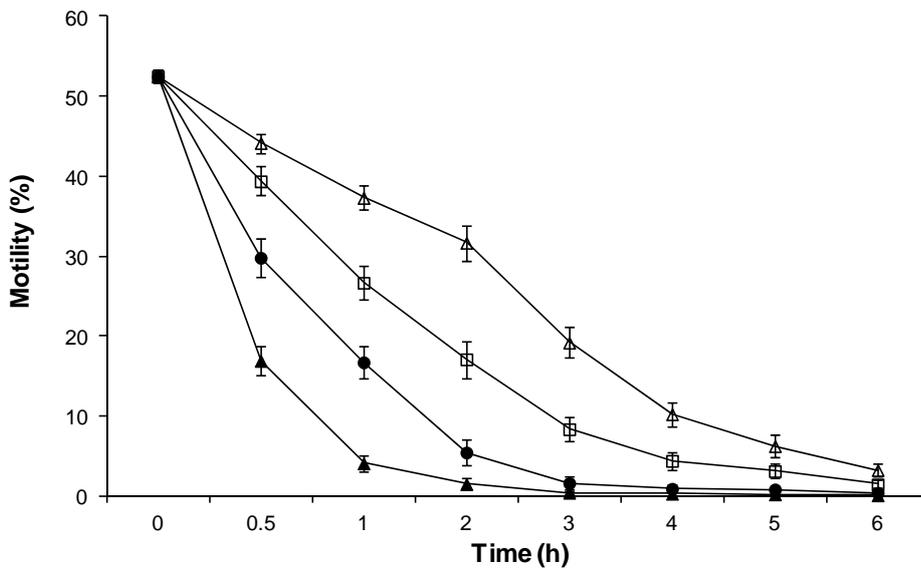


Figure 8.2 Mean \pm SEM motility (%) of ejaculated alpaca sperm incubated for 0 to 6 h in 10 (Δ), 25 (\square), 50 (\bullet), or 100% (\blacktriangle) seminal plasma.

Experiment 4

Motility of sperm declined from 0 to 3 h of incubation and differed between seminal plasma concentrations ($P < 0.001$; table 8.3). At 0 h, there were no differences between samples with 10 or 100% seminal plasma. However, after 1, 2 and 3 h motility was better in samples with 10% seminal plasma than those with 100%.

Table 8.3 Percent motility (mean \pm SEM) of ejaculated alpaca sperm incubated for 0, 1, 2, or 3 h at 37 °C in 10 or 100% seminal plasma.

Time (h)	Seminal plasma (%)	
	10	100
0	62.5 \pm 2.14 ^{a_w}	62.5 \pm 2.14 ^{a_w}
1	51.6 \pm 1.18 ^{a_x}	36.9 \pm 3.82 ^{b_x}
2	35.3 \pm 1.80 ^{a_y}	9.2 \pm 1.86 ^{b_y}
3	18.4 \pm 2.08 ^{a_y}	2.8 \pm 1.2 ^{b_z}

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

^{w-z} Within a column, means without a common subscript differed ($P < 0.05$).

The percentage of sperm with intact acrosomes did not differ between samples with 10 or 100% seminal plasma ($p > 0.05$) but did decline from 0 to 3 h ($P < 0.001$). The percentage of intact acrosomes (mean \pm sem) was highest at 0 h (60.1 \pm 1.05) then declined after 1 h (53.7 \pm 1.31) and 2 h (49.5 \pm 1.37), and was lowest after 3 h incubation (47.3 \pm 1.14).

The percentage of ejaculated sperm with non-fragmented DNA did not differ between samples containing 10 and 100% seminal plasma (mean \pm sem; 95.0 \pm 0.55 vs. 95.5 \pm 0.45 respectively; $P > 0.05$), nor between samples assessed after 0 and 3 h incubation (mean \pm sem; 95.8 \pm 0.27 vs. 94.7 \pm 0.64 respectively; $P > 0.05$).

The percentage of viable sperm differed between seminal plasma concentrations and between time points ($P < 0.001$; Table 8.4). At 0 h there was no difference in viability between samples with 10 or 100% seminal plasma. However after 1, 2 and 3 h there were more viable sperm in samples with 10 than 100% seminal plasma ($P < 0.001$; Table 8.4).

Table 8.4 Percent viability (mean \pm SEM) of ejaculated alpaca sperm incubated for 0, 1, 2, or 3 h at 37 °C in 10 or 100% seminal plasma.

Time (h)	Seminal plasma (%)	
	10	100
0	46.9 \pm 1.86 _x	46.9 \pm 1.86 _x
1	36.2 \pm 4.68 ^a _y	12.7 \pm 2.33 ^b _y
2	30.1 \pm 3.06 ^a _y	9.2 \pm 1.50 ^b _{yz}
3	23.1 \pm 4.07 ^a _z	7.0 \pm 0.70 ^b _z

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

x-z Within a column, means without a common subscript differed ($P < 0.05$).

Discussion

This study is the first to describe the effects of seminal plasma dilution rate on the motility, acrosome integrity, DNA integrity, and viability of alpaca sperm.

When epididymal and ejaculated sperm were incubated for up to 6 h, 10% seminal plasma maintained motility longer than 0, 25, 50, or 100% seminal plasma; therefore, we concluded that alpaca semen should be diluted at least 1:9 to prolong sperm function. These findings were in agreement with studies in the boar (Tso and Lee 1980), stallion (Love et al. 2005), and ram (Ashworth et al. 1994), in which 10% seminal plasma was optimal to maintain sperm motility. In alpacas, a 1:4 dilution rate was superior to 1:2 or 1:1 for maintenance of sperm motility (Morton et al. 2009). It is likely that further dilution of the semen prior to storage would enhance sperm function, because in the present study 10% seminal plasma maintained motility longer than 25% seminal plasma.

The effect of the absence of seminal plasma from ejaculated sperm on their function could not be determined in the present study, due to the high viscosity of the seminal plasma which prevented its removal by centrifugation or other means, without impairing sperm function. However, in the absence of seminal plasma, the motility of epididymal sperm declined rapidly within 30 min. This was in contrast to studies in the ram and bull in which the motility of epididymal sperm was maintained in the absence of seminal plasma (Graham 1994). This difference may be explained by the Tyrode's medium used by Graham et al. (Graham 1994) which contained protective factors to help maintain sperm function, as opposed to PBS with 0.1% BSA used in the present study. Perhaps using a diluent containing protective components would sustain alpaca sperm motility in the absence of seminal plasma. However, based on the findings from the present study, we inferred that some seminal plasma components were beneficial for alpaca sperm function. As a commercial cryodiluent for the preservation of alpaca sperm is not yet available, and the optimal dilution ratio to use for cryopreservation has not been determined, we infer that diluting alpaca semen 1:9 may provide a starting point for development of cryopreservation protocols.

The proportion of ejaculated sperm with non-intact acrosomes did not differ in the presence of 10 or 100% seminal plasma. These findings were in agreement with Morton et al. (Morton et al. 2009), who reported no effect of dilution rate on the acrosome integrity of ejaculated alpaca sperm. However, in the absence of seminal plasma, the acrosome integrity of epididymal alpaca sperm declined and there was a higher proportion of sperm with non-intact acrosomes after incubation without seminal plasma than with 10 or 100% seminal plasma. In contrast to our findings, epididymal alpaca sperm can be liquid stored without seminal plasma for up to 24 h without any detrimental effect on acrosome integrity (Morton et al. 2009). This difference may be explained by the beneficial components in the diluent used by Morton et al. (Morton et al. 2009) compared to the present study. In this study, epididymal sperm were all re-suspended to a concentration of 50×10^6 /ml; therefore, it was the concentration of seminal plasma, and not the concentration of sperm, that inhibited or stimulated acrosome integrity. Seminal plasma proteins prevent or reverse capacitation-like changes and reduce the proportion of sperm with non-intact acrosomes in numerous species (Maxwell et al. 2007), including sheep (Maxwell and Johnson 1999), pigs (Vadnais et al. 2005; Vadnais et

al. 2005), and horses (Schembri et al. 2002). Seminal plasma contains decapacitation factors (Chang 1957) which stabilise sperm cell membranes, preventing capacitation-like changes. As the proportion of sperm with non-intact acrosomes did not change in epididymal or ejaculated sperm in the presence of 10 or 100% seminal plasma, but increased in the absence of seminal plasma, we speculated that alpaca seminal plasma may contain decapacitation factors which prevent capacitation-like changes. Consequently in order to enhance their function, alpaca sperm should not be removed from their seminal plasma or excessively diluted prior to processing and storage for cryopreservation, as sperm with non-intact acrosomes are unlikely to be fertile.

The incubation of epididymal or ejaculated sperm with 0, 10, or 100% seminal plasma had no significant effect on DNA fragmentation. This was in agreement with studies in the bull (Khalifa et al. 2008) in which DNA fragmentation did not differ among samples diluted 1:1, 1:9, or 1:19. Conversely, in the stallion, the absence of seminal plasma was beneficial to DNA integrity compared to 10 or 20% seminal plasma (Love et al. 2005). This may be explained by the long incubation period in the latter study (48 h compared to 3 h in the present study). Based on the outcome of the present study, we concluded that the absence or presence of seminal plasma for 3 h did not cause or prevent DNA fragmentation in alpaca sperm.

The viability of ejaculated sperm was better when incubated in 10 than 100% seminal plasma. The detrimental effect of 100% seminal plasma on the viability of alpaca sperm has not been previously documented. In the donkey, ejaculated sperm had higher viability when incubated in 10 than 20 or 50% seminal plasma (Miro et al. 2009) and bull sperm had higher viability when diluted to a concentration of 10 than 60×10^6 /ml (Prathalingam et al. 2006). Thus, high concentrations of seminal plasma were detrimental to the viability of sperm in other species. Conversely in the llama, sperm viability did not differ between diluted and undiluted semen samples (Giuliano et al. 2010), although viability was only assessed 8 min post-dilution compared to 1 to 3 h in the present study. The cause of this reduced viability is not known, but may be associated with increased osmotic stress and lower pH (Prathalingam et al. 2006). Additionally low dilution rates and thus high concentrations of sperm may increase the concentration of reactive oxygen species, reducing sperm viability. Furthermore, alpaca seminal plasma, like that of the stallion, may contain a protein that is toxic to sperm viability, although this requires further investigation.

Although high concentrations of seminal plasma reduced alpaca sperm viability, excessively low concentrations (<10%) may also be detrimental. In the ram (Ashworth et al. 1994), bull (Baas et al. 1983; Garner et al. 1997) and boar (Garcia et al. 2009), sperm viability was better when seminal plasma was present or when semen was not excessively diluted. Similarly in deer, seminal plasma was beneficial to the viability of epididymal sperm (Martinez-Pastor et al. 2006). Due to the low numbers of sperm retrieved from alpaca testes, the effect of seminal plasma on viability of epididymal sperm was not assessed, and consequently the effect of the absence of seminal plasma is unknown. Alpaca sperm may become non-viable in the absence of seminal plasma, as indicated by our motility data, although further studies are required to confirm this.

In conclusion, 10% seminal plasma was optimal to maintain the motility of ejaculated and epididymal alpaca sperm (incubated for 6h at 37 °C) compared to 0, 25, 50, or 100% seminal plasma. Moreover, 10% seminal plasma maintained the acrosome integrity of ejaculated and epididymal sperm and also reduced the proportion of non-viable ejaculated sperm. Consequently, we recommend that alpaca semen samples are diluted 1:9 (retaining 10% seminal plasma) during processing for liquid or frozen storage, or alternatively that semen samples are diluted to 10% seminal plasma on thawing. As the viscous nature of alpaca semen is currently the major limitation to the development of frozen storage, it is possible that either complete removal of the seminal plasma or enzymatic reduction of viscosity will be necessary to develop cryopreservation protocols in this species. Under these circumstances, care should be taken to include protective factors in the cryodiluent that will maintain the viability and acrosome integrity of the sperm and the beneficial effect of seminal plasma should be considered. As in other species, maintaining or adding 10% seminal plasma to frozen-thawed alpaca sperm may enhance their function, integrity and fertility and consequently warrants further investigation.

The study has highlighted the need to retain 10% seminal plasma during sperm cryopreservation, or alternatively to ensure that upon thawing 10% seminal plasma remains in the sample. This could be achieved

by adding 10% seminal plasma back to the sperm upon thawing (if sperm are removed from the seminal plasma for cryopreservation) or by maintaining at least 10% seminal plasma prior to freezing then diluting to a final seminal plasma concentration of 10% upon thawing. Both approaches have their disadvantages. In adding seminal plasma back to the thawed sperm it is likely that, due to the viscous nature of alpaca seminal plasma, there will not be complete mixing of the sperm with the seminal plasma and thus any protective effects of the seminal plasma on the sperm are likely to be lost. In maintaining at least 10% seminal plasma prior to cryopreservation the viscous nature of the seminal plasma may hinder the success of freezing thereby reducing sperm function and integrity post-thaw due to an inability of the cryoprotectants to mix completely with the semen. In reducing the semen viscosity with enzymes such as papain (chapter 7) it is possible to overcome the detrimental effect of viscosity on cryopreservation whilst retaining at least 10% seminal plasma in the sample prior to freezing. Whether papain treatment of alpaca semen prior to cryopreservation is advantageous to sperm function (due to an ability to homogenise the semen with the cryodiluent) or is detrimental (due to degradation of proteins that protect the sperm during cryopreservation) remains to be elucidated.

9. Cryopreservation of Papain-treated Alpaca Semen

Introduction

Unlike other domesticated farm species such as the sheep, cow, horse and pig, the use of assisted reproductive technologies including sperm cryopreservation and artificial insemination are not utilised for old and new world camelids, including the alpaca. The current major impediment to the development of sperm cryopreservation in camelids is the highly viscous nature of the seminal plasma which makes semen assessment and processing difficult. During sperm cryopreservation, the viscous semen does not evenly homogenise with cryodiluents, preventing adequate contact between the sperm and protective reagents within the cryodiluents during freezing. As a result the sperm become impaired and exhibit limited or no motility upon thawing, rendering them infertile and thus not suitable for use in artificial breeding programmes. In order to improve the success of, and establish a protocol for the cryopreservation of alpaca sperm, it is necessary to reduce the seminal plasma viscosity prior to freezing.

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 for this species, particularly in the bactrian camel (Niasari-Naslaji et al. 2007) with post-thaw sperm motility rates of almost 40%, however, pregnancy rates with frozen-thawed semen are still not commercially acceptable (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). Consequently, research on liquid and frozen storage of alpaca semen has focussed on reducing the viscosity of the seminal plasma by mechanical and enzymatic methods (Bravo et al. 1999; Bravo et al. 2000; Morton et al. 2008; Giuliano et al. 2010). Alpaca and Llama sperm pre-treated with collagenase, then cryopreserved, thawed and used for AI resulted in 5 of 19 (26%) pregnancies in alpacas (Bravo et al. 2000). Although this study shows promise and suggests that a reduction in seminal plasma viscosity prior to cryopreservation may improve the success of AI, the pregnancy rates are still not commercially acceptable and thus further research into an optimal cryopreservation protocol for alpaca sperm is necessary.

Cryopreservation is a complex process that requires the optimisation of numerous factors, including the most suitable cryodiluent reagents (i.e. energy source, concentration of cryoprotectant, egg yolk concentration), the optimal cooling, freezing and thawing rates of the sperm, and the optimal storage method (pellet or straws). Various cryodiluents have been used to preserve camelid sperm including those used made in the laboratory and used routinely for other domestic species such as sheep as well as those that are commercially available for species including horse and pig. The results are conflicting and difficult to interpret as different researchers alter the relative concentration of reagents within the cryodiluent as well as altering the primary energy source and freeze-thaw rates. To date, most success appears to have been achieved using lactose based extenders (Niasari-Naslaji et al. 2006; Morton et al. 2007; Wani et al. 2008), although tris-based extenders with fructose or glucose as an energy source achieve reasonable post-thaw sperm motility rates (Deen et al. 2003) and have been reported to be more successful than lactose based extenders by some researchers (Niasari-Naslaji et al. 2006). In order to develop a successful, reliable, commercially available protocol for the cryopreservation of alpaca semen it is necessary to thoroughly investigate all the components of the extender as well as determine the most suitable method of freezing (freeze and thaw rates and storage type). The ability to conduct this research is however hindered by the viscous seminal plasma which prevents successful cryopreservation and it is therefore necessary to first determine whether a reducing the viscosity of alpaca semen prior to cryopreservation improves sperm motility both during and after cryopreservation.

We have determined that the viscous nature of alpaca seminal plasma is caused by the large gel-forming protein mucin 5B (chapter 6) and the protease papain degrades mucin 5B, thus eliminating viscosity, without

impairing sperm function (chapter 7). In addition, alpaca semen incubated with 0.1mg/ml papain for 20min at 37°C and then with 10µM E-64 (papain inhibitor) for 5min at 37°C becomes non-viscous and the sperm remain motile and intact. Whether reducing alpaca semen viscosity with papain improves sperm function and integrity during freezing and post-thaw remains to be determined.

This study investigated the effect of treatment of spermatozoa with papain and E-64 on the 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. Two extenders were used; lactose and a tris-citrate-fructose extender, both of which have been used previously for cryopreservation of alpaca sperm (Morton et al. 2007).

Materials and Methods

Animals

This experiment was performed using four male alpacas under authorization from the University of Sydney animal ethics committee. All males were > 3 y, had a body condition score >3 and had testes more than 3 cm long.

Experimental Design

This experiment was conducted from October to November 2011. Semen was collected from four male alpacas (≥ 2 ejaculates/male, $n = 10$) using an artificial vagina and assessed for volume, viscosity, and motility and concentration of spermatozoa as described in chapter 3. Only samples with a volume >1ml, viscosity ≥ 15 mm, motility $\geq 50\%$ and concentration $\geq 40 \times 10^6$ 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 (300mM Tris, 94.7mM citric acid, 27.8mM fructose) (Evans and Maxwell 1987) or 11% lactose (w/v) extender 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 (300mM Tris, 94.7mM citric acid, 27.8mM 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 5°C over 2h then frozen as 200µl pellets on dry ice as described previously (Evans and Maxwell 1987), then stored in liquid nitrogen. 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 for alpaca spermatozoa (Kershaw-Young and Maxwell 2011) and sperm motility was assessed at 0, 1 and 3h post-thaw.

Viscosity and Motility Analysis

Semen and sperm parameters were assessed as described in chapter 3. Briefly, sample (10µl) was diluted (1:9) in 90µl 3% sodium chloride (Sigma) and the concentration of sperm was assessed using a haemocytometer (Evans and Maxwell 1987). Viscosity was assessed using the thread test (Bravo et al. 2000) in which 50µl of 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. Motility of spermatozoa was assessed subjectively at X 100 magnification under phase contrast microscopy (Olympus, Tokyo, Japan) (Evans and Maxwell 1987). All motile sperm, whether

oscillatory or progressive, were considered motile and used to generate a value for total motility.

Statistical Analysis

Data were analysed using Genstat version 11 (VSN International, Hemel Hempstead, UK). Viscosity and 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 the random effects.

Results

Viscosity

Papain treatment significantly reduced seminal plasma viscosity ($P < 0.001$) and viscosity was completely eliminated in papain-treated samples within 20 min of treatment. Viscosity (mm; mean \pm sem) did not differ between treatments prior to dilution (56.3 ± 9.11) and following dilution (33.4 ± 3.02) but was less in samples treated with fructose-papain (0 ± 0.0) and lactose-papain (0 ± 0.0) compared to the fructose control post-treatment (24.9 ± 5.81) and post-chill (16.6 ± 3.72) and the lactose control post-treatment (26.5 ± 6.13) and post-chill (15.1 ± 3.63).

Motility

The motility of spermatozoa differed between treatment groups at each time point ($p = 0.03$; fig. 9.1). Prior to, and following dilution, there were no differences between treatments. However, motility was lower in lactose control samples, both post-treatment and post-chill, compared to all other treatment groups. Immediately post-thaw (0 h) motility was less in lactose control samples than fructose-papain and lactose-papain treated samples. Fructose control samples had intermediate motility of spermatozoa. At 1h post-thaw lactose control samples contained significantly less motile spermatozoa than all other treatments, and fructose control samples had lower motility of spermatozoa than fructose-papain samples. At 3h post-thaw there were no significant differences in the motility of spermatozoa between treatment groups, although there tended to be greater motility in samples treated with papain and cryopreserved in fructose extender compared with the other treatments. Motility also differed between time points in each treatment group. Generally, motility of spermatozoa increase after dilution compared to pre-dilution, remained high post-treatment, then declined post-chill to intermediate levels, 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 ($P < 0.001$).

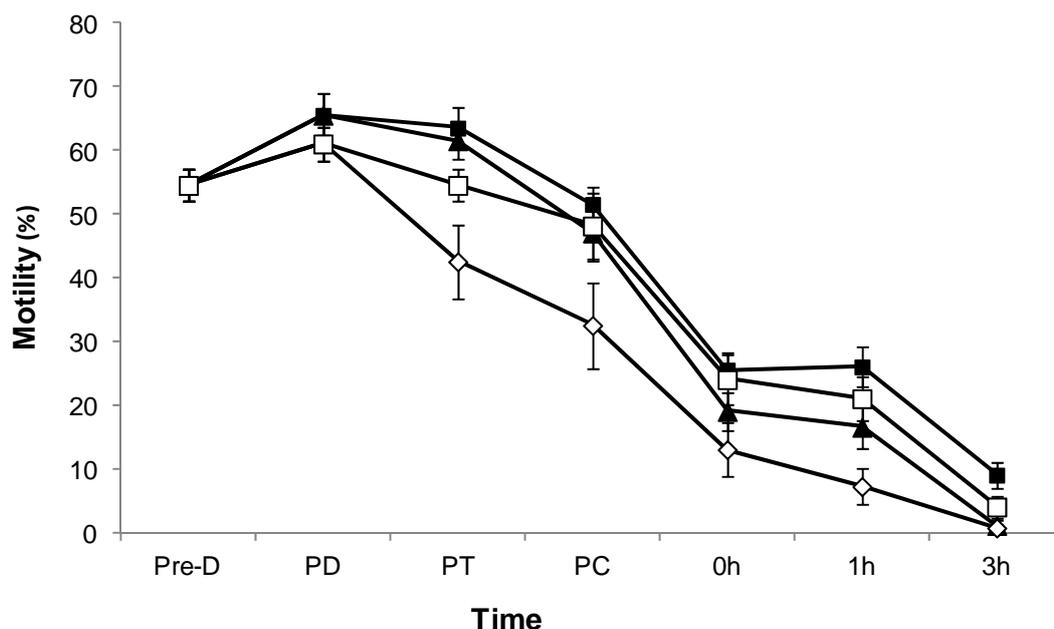


Figure 9.1 Mean \pm SEM motility (%) of ejaculated alpaca sperm pre-dilution (Pre-D), post-dilution (PD),

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.

Discussion

This study is the first to describe the effect of viscosity reduction on the motility of alpaca sperm following cryopreservation. The use of papain and its inhibitor E-64 significantly increased the motility of alpaca sperm 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 sperm membranes are under stress and it is therefore important that protective agents within the extender are able to interact with or permeate the sperm membrane in order to enhance their protective capacity. It is likely that, in the present study, the reduction in viscosity enabled the protective agents particularly egg yolk and glycerol 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.

After chilling to 4°C for 2h mean motility rates of 32% to 51% were observed. The greatest post-chill motility was observed in papain-treated fructose samples (51%) and lactose papain treated samples (48%). Egg yolk is commonly used in freezing extenders to protect the sperm membrane against cold shock when cooling to 4°C, and it is likely that the 10% egg yolk present in the freezing extenders prevented sperm membrane damage in papain treated samples. The post-chill motility rates observed in this study are similar to those reported previously for epididymal alpaca sperm (Morton et al. 2007; Morton et al. 2010), ejaculated alpaca sperm (Bravo et al. 2000; Santiani et al. 2005), and ejaculated camel sperm (Niasari-Naslaji et al. 2007).

The motility rates (mean) immediately post thaw ranged from 13% in lactose-control samples to 25% in fructose-papain treated samples. These post-thaw motility rates are similar to those observed in other studies in which motility rates of 4-20% (Santiani et al. 2005) and 30-40% (Bravo et al. 2000) were reported for ejaculated alpaca sperm and post-thaw motility rates of 5% to 25% were reported for epididymal alpaca sperm (Morton et al. 2007; Morton et al. 2010). In camels, post-thaw motility rates tend to be slightly higher ranging from 12-35% (Niasari-Naslaji et al. 2007) and more than 50% of ejaculates exhibiting at least 20% motility immediately post-thaw (Deen et al. 2003).

At 1 h post-thaw motility remained above 20% in both lactose and fructose-papain treated samples but was only 16.5% and 7.2% in fructose-control and lactose-control samples respectively, whereas at 3h post thaw motility was 9% and 4% in fructose-papain and lactose-papain treated samples but only 1.1% and 0.7% in fructose-control and lactose-control samples respectively. There are no studies describing the change in post-thaw motility rates over time in ejaculated camelid sperm, however at 3h post-thaw, epididymal alpaca sperm have motility rates of 0-3% (Morton et al. 2007; Morton et al. 2010). Studies on the cryopreservation of camelid epididymal sperm have been conducted in order to overcome the viscous component of the seminal plasma, which is not present when epididymal sperm are extracted from the epididymis of castrated or deceased males. In the present study, post-thaw motility rates in papain-treated samples at 3h post-thaw were superior to those observed in epididymal sperm, suggesting that reducing the viscosity of ejaculated alpaca seminal plasma prior to sperm cryopreservation may be a more suitable method for sperm storage than using epididymal sperm. 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, tris-citrate-fructose extender was superior to lactose in maintaining the motility of ejaculated alpaca sperm when comparing lactose-control and fructose-control, and lactose-papain and fructose-papain samples. This implies that a tris-citrate-fructose extender is more suited to the cryopreservation of alpaca sperm than lactose. Numerous extenders have been used for the cryopreservation of camelid sperm, and the results are conflicting and difficult to interpret. Whilst 11% lactose is 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 (Vyas et al. 1998; Deen et al. 2003; Vaughan et al. 2003; Niasari-Naslaji et al. 2006). In reality, it is almost impossible to compare studies due many factors including;

- differences in the cryoprotectants used i.e. egg yolk or milk, glycerol, DMSO or ethylene glycol for example
- differences in the concentration of cryoprotectants used, for e.g. glycerol concentration of 3 to 8% have been used for camelid sperm
- differences in the type and concentration energy source used i.e. fructose, lactose or glucose
- differences in the type and concentration of buffer used such as tris or citrate
- differences in the methods used for freezing including final dilution factor, cooling and freezing rates and storage type i.e. pellets or straws
- the addition of other additives such as EQUEX paste or antioxidants that have been used successfully to improve post-thaw motility rates in other domesticated species.

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 (Santiani et al. 2005; Morton et al. 2010). The concentration of egg yolk between studies is relatively consistent, however glycerol concentrations show much greater variation. Glycerol initiates its cryoprotective effect by permeating the cell membrane to reduce the formation of ice crystal within the cell which would otherwise cause cell death. Whilst glycerol has protective properties, it is also toxic to sperm cells when used at high concentrations, and there is marked species variation in glycerol tolerance. It is therefore crucial that the optimal glycerol concentration is determined for camelid sperm. Concentrations of 3% or 4% glycerol was better than 2% glycerol in maintaining alpaca epididymal sperm acrosome integrity at 3 h post-thaw and glycerol concentrations of 2-7% have been used for cryopreservation of alpaca sperm (Bravo et al. 2000; Santiani et al. 2005). In the present study we used a final concentration of 6% as 6% glycerol was found to be superior to 4% and 8% for cryopreservation of camel sperm (Niasari-Naslaji et al. 2007).

Based on the findings in the present study in which the majority of variables including egg yolk and glycerol concentration, cooling rates and freeze-thaw rates were equivalent between the extenders, it can be concluded that a tris-citrate-fructose extender is superior to lactose.

In conclusion the reduction of alpaca seminal plasma viscosity with papain and its inhibitor E-64 prior to cooling and cryopreservation improves the motility of alpaca sperm both immediately and 1 h 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. Tris-citrate-fructose appears to be superior to 11% lactose in maintaining sperm motility after chilling and following cryopreservation of viscosity-reduced ejaculated alpaca sperm.

In order to benefit from the optimised method for seminal plasma viscosity reduction 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. Whilst this study indicates that papain and E-64 treatment do improve the success of sperm cryopreservation, post-thaw motility rates are still not commercially acceptable and thus research should now focus on using papain-treated viscosity reduced semen to develop an industry standard semen extender for alpaca sperm cryopreservation.

10. General Discussion of Results

This report presents the findings of RIRDC funded research project PRJ-54 “Advancing Artificial Insemination in Camelids, particularly the Alpaca”

In order to advance the development of AI in camelids, it is essential to first determine the cause of and eliminate the viscous seminal plasma that hinders cryopreservation. The development of a successful reliable protocol for the reduction of viscosity without impairing sperm function will advance establish cryopreservation techniques thus enabling the use of frozen-thawed sperm in AI programmes. The project had 4 main objectives;

- identify the source and constituent of the viscous seminal plasma
- investigate ways to reduce the viscosity of alpaca seminal plasma without impairing sperm function and integrity
- determine the role of camelid seminal plasma on sperm function
- investigate methods for the cryopreservation of alpaca sperm.

This project used a systemic approach to thoroughly investigate each objective and the findings provide new, previously unknown data on the constituents and role of camelid semen as well as describing techniques for the reduction of viscosity that can be implemented in cryopreservation protocols. The findings of the project are summarised as follows:

- Glycosaminoglycans (GAGs), which were postulated to be the cause of viscosity, were abundant in alpaca seminal plasma
- Of the five known GAGs, keratan sulphate was the most abundant and was associated seminal plasma with viscosity
- Glycosaminoglycans were predominantly expressed by the alpaca bulbourethral gland implying that seminal plasma GAGs originate from the bulbourethral gland and not the testis or the prostate
- Enzymes that degrade GAGs reduced seminal plasma viscosity, however the reduction was minimal suggesting that GAGs are not the main cause of viscosity in alpaca seminal plasma
- The proteases papain and proteinase K eliminated alpaca seminal plasma viscosity within 30min of treatment suggesting that proteins are the cause of viscosity in alpaca seminal plasma
- Mucin 5B, a large gel-forming protein was significantly more abundant in highly viscous seminal plasma samples compared to those with low viscosity
- Mucin 5B was identified as the viscosity causing protein in alpaca seminal plasma
- Papain was identified as a suitable enzyme to degrade Mucin 5B protein quickly and effectively
- The treatment of alpaca semen with 0.1mg/ml papain for 20min at 37°C was sufficient to completely eliminate viscosity. Concentrations of 0.01mg/ml papain were also successful after 30min incubation, but concentrations of 0.001mg/ml papain did not completely eliminate the viscous component of seminal plasma within 30min of treatment
- The use of 0.1mg/ml papain for 20min at 37°C did not affect sperm motility, acrosome integrity, viability or DNA integrity

- Whilst the use of 0.1mg/ml papain for 20min at 37°C was not detrimental to sperm function it was anticipated that prolonged exposure of sperm to papain would impair sperm integrity. Therefore the effect of the papain inhibitor E-64 on sperm function was investigated
- The papain inhibitor E-64 when used at 10µM for 5min at 37°C was completely effective in halting digestion of proteins by papain and had no detrimental effect on alpaca sperm motility, acrosome integrity, viability or DNA integrity
- In order to reduce seminal plasma viscosity in alpacas, semen should be diluted 1:1 in a suitable extender immediately following collection then treated with 0.1mg/ml papain for 20min at 37°C prior to the addition of 10µM E-64 at 37°C for 5min
- Seminal plasma plays an important role in the regulation of alpaca sperm function.
- High (50-100%) seminal plasma concentrations appear toxic to sperm, and sperm are not able to maintain motility over prolonged periods in these concentrations. In particular 100% seminal plasma causes sperm death
- Very low (0%) seminal plasma concentrations are detrimental to sperm, and in the absence of seminal plasma sperm become non-motile and their acrosomes become non-intact suggesting that alpaca seminal plasma may contain decapacitation factors
- To prolong the function and integrity of alpaca sperm, seminal plasma should be diluted to a final concentration of 10% during sperm storage. At least 10% seminal plasma should remain in the frozen semen sample, or alternatively if all seminal plasma is removed prior to cryopreservation, 10% seminal plasma should be added back to the sample prior to assessment of thawed sperm and AI
- Papain and E-64-treated viscosity-reduced alpaca sperm are less susceptible to damage during the cryopreservation process. In particular, cryopreserved sperm from viscosity-reduced samples exhibited higher motility rates after chilling and both immediately and 1h post-thaw
- The treatment of alpaca semen with papain and E-64 to reduce viscosity enhances the success of sperm cryopreservation
- Although improved, the post-thaw motility rates of viscosity-reduced cryopreserved sperm are still not sufficient to enable successful pregnancies with AI, most likely due to a lack of understanding of the cryoprotective agents required for successful cryopreservation in camelids
- Further research is required to optimise the components of a semen extender for camelids
- In order to benefit from the findings of this research it is imperative that a semen extender for commercial use within the camelid industry is developed. In addition, further research is required on the use of frozen-thawed sperm for AI in camelids

The findings from this project provide new and important information regarding the components and role of alpaca seminal plasma. Identification of the protein Mucin 5B as the cause of viscosity has led to the development of a protocol for the reduction of seminal plasma viscosity using papain. It is anticipated that this protocol will be adopted by other camelid researchers and the camelid industry in order to reduce viscosity prior to sperm cryopreservation. The viscous nature was previously identified as the major impediment to the development of cryopreservation and AI protocols. The research undertaken in this project has overcome this hurdle and the industry now faces new challenges including optimising the components of a semen extender for sperm cryopreservation as well as developing a protocol for AI with frozen-thawed semen.

Implications

The practical outcomes arising from this research are 1) the development of sophisticated in vitro methods for the assessment of sperm function including acrosome integrity, viability and DNA integrity 2) identification of the cause of viscosity and the development of a protocol for the reduction of seminal plasma viscosity that can be adopted by the camelid industry and research scientists and 3) the recommendation that a final seminal plasma concentration of 10% is maintained prior to cryopreservation or post-thaw in frozen-thawed alpaca sperm.

Whilst these deliverables do not provide answers to the perfect method for sperm cryopreservation and AI in camelids, they enhance the development of such technologies.

The validation of methods for the sophisticated analysis of sperm integrity in camelids will enable other camelid research scientists to fully elucidate the function of camelid semen in order to predict its fertilising ability. This is important, as the ability to perform additional analyses of sperm function and integrity will provide greater data on the effect of cryodiluents, freeze thaw rates and storage methods (pellets or straws), thereby aiding the rapid progression of the further research that is required to establish ARTs in camelids.

The protocol described for the reduction of seminal plasma viscosity will directly benefit the camelid industry as well as benefit camelid researchers. The major impediment to the development of sperm cryopreservation in camelids was the viscous seminal plasma. Whilst there had been attempts to alleviate the viscosity, a lack of understanding of the cause of viscosity prevented identification of a suitable method to reduce the viscous component of the seminal plasma without impairing sperm function. In identifying the protein Mucin 5B as the protein responsible for viscosity, we were able to develop a protocol that degraded this protein, thereby reducing viscosity, without impairing sperm function and integrity. We have thereby overcome the current major problem associated with the development of cryopreservation in alpacas. In doing so, other camelid researchers can now make substantial progress in the development of camelid ARTs by using this protocol prior to cryopreservation in order to improve post-thaw sperm function and fertility. In order to fully benefit from this research outcome, considerable progress must be made in 1) determining the optimal cryodiluent for camelid sperm and 2) optimising the protocol for AI in camelids.

The finding that the presence of 10% seminal plasma is advantageous to sperm motility, viability, and acrosome integrity provides future researchers with a starting point for the development of storage protocols for camelid sperm. Whilst progress in sperm storage for alpacas has been made, the longevity of the sperm during liquid freezing and the post-thaw motility of frozen-thawed sperm are not commercially viable. In using a final seminal plasma concentration of 10% it is likely that greater sperm function will be achieved and hence, this research finding will advance the development of sperm storage protocols in camelids.

In conclusion, the practical outcomes of this research project will advance the development of cryopreservation and AI in camelids facilitating the rapid dissemination of superior genotypes thereby increasing the rate of gain of favourable characteristics such as a high fleece density and low fibre diameter. Producers will benefit from the higher prices obtained for high quality low diameter fleece and higher fleece weights. Cryopreservation will enable the long term storage of sperm and the worldwide transportation of sperm enabling genes from genetically superior males to be preserved indefinitely and breeders to disseminate the genetics of their superior males or inseminate their females both domestically and internationally without the need to transport live animals, thereby reducing shipping costs. Additionally domestic and international demand for cryopreserved semen of genetically superior males will enable breeders to benefit from increased stud fees. The increase in fibre quality gained by the use of these assisted reproductive technologies will increase Australia's reputation as a producer of high quality alpaca fibre, thereby increasing the industry competitiveness in both the domestic and international fibre markets. Additionally the Australian camelid industry will be at the forefront of the development of ARTs in camelids and will benefit from the increased reputation associated with being a world leader in this area.

Recommendations

Based on the findings of the current research the following recommendations are made to the camelid industry, breeding centres and research scientists: 1) Camelid semen should be diluted 1:1 in a suitable semen extender such as tris-citrate-fructose then treated with 0.1mg/ml papain (final concentration) for 20min at 37°C to completely eliminate viscosity of semen. The effect of papain should then be halted by the addition of 10µM E-64 (final concentration) at 37°C for 5min. The viscosity-reduced semen can then be utilised for sperm cryopreservation procedures. 2) A concentration of 10% seminal plasma (dilution rate of 1:9) should be used to preserve the longevity of alpaca sperm function. In particular frozen-thawed sperm should be either diluted to a final seminal plasma concentration of 10% or 10% seminal plasma should be added back to the sperm after thawing. Using a dilution ratio of 1:9 (semen: extender) will help improve the motility of cryopreserved sperm during freezing and post-thaw.

To exploit the key findings of this research project, further research is essential. In determining the cause and source of the viscous seminal plasma in alpaca semen and establishing a protocol that reduces viscosity without impairing sperm function we have overcome the current major impediment to cryopreservation. These findings provide a platform on which to develop a successful cryopreservation and subsequent artificial insemination protocol which can be used commercially within the alpaca industry.

The cryopreservation of camelids semen remains problematic. Despite numerous investigations into the optimal cryodiluent components, post-thaw motility remains low (approximately 20%) and is not commercially viable. In these studies, the viscous component of the seminal plasma was not removed or degraded prior to cryopreservation. Consequently in order to develop a cryopreservation protocol in camelids, it is necessary to optimise the cryopreservation of viscosity-reduced semen. This requires a systemic approach to 1) identify the most effective cryodiluent components for camelid sperm including buffer, energy source and cryoprotectants, 2) investigations on the effect of sperm storage (pellets or straws) on sperm function during cryopreservation and 3) the optimal freeze/thaw rates that maintain sperm function and integrity to be determined.

Of interest is also the role of seminal plasma in sperm function. In numerous species, seminal plasma is routinely diluted or removed during processing of semen for cryopreservation and this can induce either positive or negative effects on the function and fertility of sperm. Camelid semen requires the presence of 10% seminal plasma in order to maintain sperm function and integrity. In the absence of seminal plasma, sperm become acrosome impaired and therefore not fertile. It is therefore important to investigate the addition of seminal plasma proteins either pre-freeze or post-thaw on the fertilising ability of camelid sperm as this may aid the development of artificial insemination through enhanced fertility rates.

In order to fully benefit from the ability to cryopreserve sperm, the AI of females with frozen-thawed sperm must be reliable and effective in achieving pregnancy rates of over 60%. The development of successful AI protocols in camelids is hindered by a lack of understanding of reproductive physiology and knowledge of the optimal methods for fertilisation, in particular the timing of ovulation in female camelids and the optimal time of AI relative to ovulation as well as the sperm numbers required to achieve pregnancy and the optimal site of sperm deposition.

Whilst AI in camelids has been successful using fresh and frozen-thawed semen pregnancy rates were not sufficient for application within the camelid industry. The effect of sperm numbers and site of deposition of semen during AI implies that transcervical insemination is required to achieve reasonable pregnancy rates however the effect of deeper inseminations (i.e. at the uterine horn) is conflicting with some researchers reporting greater fertility and others reporting no difference. Despite attempts to develop AI protocols in camelids, the optimal sperm numbers, site of deposition and timing of insemination have not yet been identified and thus pregnancy rates following AI in camelids do not reach the requirement for commercialisation (at least over 60%). In order to fully commercialise AI in camelids, these questions must be addressed.

In conclusion, in order for the industry to practically apply sperm cryopreservation and artificial insemination, the following must be determined:

- the optimal cryodiluent components including buffer, energy source and cryoprotectants to maintain sperm integrity and function of viscosity-reduced, frozen-thawed alpaca sperm
- the optimal storage (pellets or straws) method for viscosity-reduced, frozen-thawed alpaca sperm
- the optimal free-thaw rates for viscosity-reduced, frozen-thawed alpaca sperm
- the fertilising ability of viscosity-reduced frozen-thawed alpaca sperm in vitro and in vivo.
- the effect of seminal plasma pre-freeze or post-thaw on the fertilising ability of sperm
- the optimal ovulation synchronisation protocol prior to artificial insemination in alpacas
- the optimal sperm dose, site of deposition and timing of artificial insemination for viscosity-reduced frozen-thawed alpaca sperm

In addressing these challenges reliable, effective protocols for sperm cryopreservation and artificial insemination for use within the camelid industry can be established.

Publications Arising from this Research

Peer-reviewed Publications

Kershaw-Young, C. M., Evans, G. and Maxwell, W. M. C. (2011). Glycosaminoglycans in the accessory sex glands, testes and seminal plasma of alpaca and ram. *Reproduction Fertility and Development* **In Press**.

Kershaw-Young, C. M. and Maxwell, W. M. C. (2011). The effect of seminal plasma on alpaca sperm function. *Theriogenology* **76**(7): 1197-1206.

Kershaw-Young, C. M., Stuart, C., Evans, G. and Maxwell, W. M. C. The Effect of Glycosaminoglycan Enzymes and Proteases on the Viscosity of Alpaca Seminal Plasma and Sperm Function. Submitted

Kershaw-Young, C. M., Skidmore, J. A., Billah, M., Evans, G., Rodney, R., Maxwell, W. M. C. Papain and its inhibitor E-64 Reduce Alpaca Semen Viscosity and Allow Freezing and Thawing without Impairing Sperm Function and Fertility. In preparation

Kershaw-Young, C. M., Druart, X., Mactier, S., Evans, G., Maxwell, W. M. C. Identification of the Cause and Source of Viscosity in Alpaca Seminal Plasma. In preparation

Abstracts

Kershaw-Young, C. M., Maxwell, W. M. C. (2009) Glycosaminoglycans in the alpaca reproductive tract. *ISOCARD Abstracts*, Abstract 173 139-140. Oral communication, 2nd conference of the International Society of Camelid Research and Development.

Kershaw-Young, C. M., Heasman, K., Maxwell, W. M. C. (2009) Semen characteristics in alpacas. *ISOCARD Abstracts*, Abstract 187 151-152. Poster presentation, 2nd conference of the International Society of Camelid Research and Development.

Magazine and Newsletter Articles

Kershaw-Young, C. M. (2011) Semen Cryopreservation in Alpacas: A review of 3 years research at The University of Sydney. *Alpacas Online: The Australian Alpaca Association* **December 2011**: 25-26.

Kershaw-Young, C. M. (2011) Semen Cryopreservation in Alpacas. *New Zealand Alpaca* (magazine of the Alpaca Association of New Zealand). **Autumn 2011**: 51-52

Kershaw-Young, C. M. (2009) Advancing Artificial Insemination in Alpacas. *Australian Alpaca Association magazine*. **October 2009 59/09**: 14.

Kershaw-Young, C. M. (2009) Advancing Artificial Insemination in Alpacas. *The Alpaca Breeder* (Australian Alpaca Breeders Association). **August 2009**

Appendices

Appendix 1: Proteins Present in Alpaca Seminal Plasma

60S ribosomal protein L29
Acid sphingomyelinase-like phosphodiesterase 3a precursor
A-kinase anchor protein 8
Alkaline phosphatase, tissue-nonspecific isozyme precursor
Beta-galactosidase precursor
Beta-hexosaminidase subunit beta precursor
Beta-nerve growth factor precursor
Brain cDNA, clone: QccE-11243, similar to human cathepsin D
Cathepsin L2 precursor
Clusterin precursor
Complement factor B
Cysteine-rich secretory protein 1
Dynein heavy chain 6, axonemal
Endonuclease reverse transcriptase
Epididymal secretory protein E1 precursor
Epididymal sperm-binding protein 1 precursor
F-box only protein 41
Gamma-glutamyl hydrolase precursor
Glutathione peroxidase 3 precursor
IGHA1 protein
Immunoglobulin J chain
insulin-like growth factor binding protein 5
Interleukin 18
Lactotransferrin
LAG1 longevity assurance homolog 3
LRRGT00161
MGC152578 protein
Monocyte differentiation antigen CD14 precursor
Muc5b protein
Mucin 5AC, oligomeric mucus/gel-forming
Neuroblastoma suppressor of tumorigenicity 1 precursor
nucleobindin
Peroxioredoxin V (PrxV) protein
Phosphatidylethanolamine-binding protein
Poly-Ig receptor precursor
Pregnancy-associated glycoprotein 6
Protein CYR61 (cysteine rich protein 61) precursor
Putative uncharacterized protein DKFZp686M24262
RNA binding motif, single stranded interacting protein 2
Secreted folate binding protein
Serum albumin precursor
Similar to Nucleobindin 2
Sulfated glycoprotein 1 precursor
Sulphydryl oxidase 1 precursor
Tissue alpha-L-fucosidase
Transmembrane protease, serine 11A
Ubiquitin B
Zinc alpha-2-glycoprotein 1 (Fragment)

Appendix 2: Protocol for the Assessment of DNA Integrity in Alpaca Sperm

Principles of the Assay

TUNEL labelling is used as an indicator of DNA integrity and apoptosis. The TUNEL reaction mixture labels DNA strand breaks which fluoresce green.

Equipment and Consumables

Glass slides	Coplin jars/slide racks
22 x 22mm coverslips	Humidified Chamber
Pipettes and tips	Tin foil

Reagents

100% Methanol

1 x PBS (0.02M, Sigma-Aldrich, St Louis, MO, USA)

TUNEL enzyme (#11767305001, Roche Applied Science) Dilute enzyme 1:2 in PBS prior to mixing with label.

TUNEL Label Mix (# 11767291910, Roche Applied Science)

VECTASHIELD with DAPI (# VEH1200, Abacus ALS)

Methods

Snap freeze semen (10-20µl) in liquid nitrogen or on dry ice and store at -80°C

Thaw semen and dilute 2:1 in PBS (i.e. 200µl PBS to 100µl semen)

Centrifuge 10 min 1000 x g (may need to be higher for viscous samples)

Decant the supernatant

Resuspend the sperm pellet in PBS at a final concentration of approximately 10 million sperm/ml

Pipette 20ul sample onto slide and smear

Air dry

Fix in ice cold 100% methanol for 3 minutes

Rinse slides 3 x 5min in PBS

Allow slides to dry slightly (not to completion)

For each slide mix 2.5µl diluted TUNEL enzyme with 22.5µl TUNEL label and mix well (make just before use and keep in the dark)

Pipette 25µl TUNEL reaction mixture to each slide

Place 22x22mm coverslip over solution

Place slides in a sealed humidified chamber

Incubate in the dark (cover in tin foil) at 37°C for 60min

Mark placement of coverslip with a permanent marker and remove coverslip

Rinse slides 3 x 5min in PBS

Counterstain with DAPI 1 drop (20-25µl) per slide

Cover with a coverslip and seal with nail polish

Store slides at 4°C in tin foil protected from light

Visualise at an excitation wavelength of 450-500nm and a detection range of 515-565nm

Negative Control

Follow protocol as described above but incubate cells with 25µl TUNEL label solution only (no enzyme).

Positive Control

Follow protocol as described above with the following addition:

After resuspending cells in PBS (point 5), incubate cells with 300U/ml DNase 1 in PBS for 30min at 37°C to induce DNA strand breaks prior to labelling procedures.

Appendix 3: Protocol for the Assessment of Acrosome Integrity of Alpaca Sperm by Fluorescent Microscopy

Equipment and Consumables

Pipettes and tips
Centrifuge
Microscope slides
22 x 22mm coverslips
Fluorescent microscope

Reagents

Neutral buffered formalin
Phosphate buffered saline (0.02M, Sigma-Aldrich, St Louis, MO, USA)
FITC-PNA stain (Sigma)

Method

Fix semen (20 μ l) in 0.1% (final concentration) neutral buffered formalin
Store at 4°C for up to 48h
Bring semen to room temperature and resuspend in 200 μ l PBS
Centrifuge 10 min 1000 x g at room temperature (may need to be higher for viscous samples)
Decant the supernatant
Resuspend the sperm pellet in PBS at a final concentration of approximately 10×10^6 sperm/ml
To 20 μ l resuspended sperm (10×10^6 /ml) add 4 μ l of 40mg/ml FITC-PNA
Incubate 37°C for 15min
Pipette 20 μ l of the stained sperm onto a clean microscope slide
Cover with a 22 x 50mm coverslip and allow to settle for 5min
Visualise under the fluorescent microscope at x 400 magnification using the U-MWIB filter (excitation filter 460-495nm, emission filter 510-550 nm, 505 nm dichromatic mirror).
Count 200 sperm and assess as follows
No fluorescence or equatorial segment stains green: acrosome intact
Acrosome stains green: acrosome not intact

Appendix 4: Protocol for the Assessment of Acrosome Integrity of Alpaca Sperm by Flow Cytometry

Equipment and Consumables

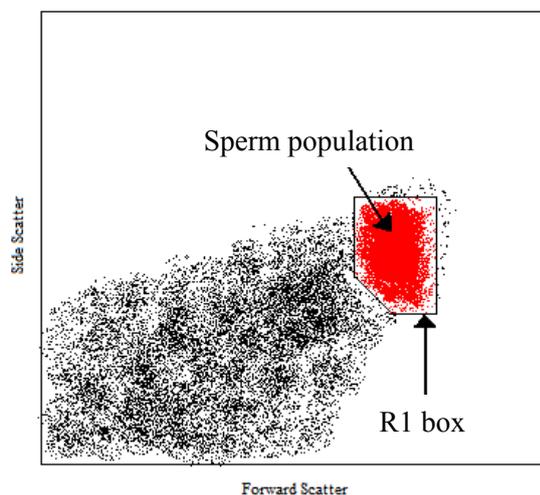
Pipettes and tips
FACScan flow cytometer
5ml falcon polystyrene tubes (12 x 75mm)

Reagents

Neutral buffered formalin
Phosphate buffered saline (0.02M, Sigma-Aldrich, St Louis, MO, USA)
FITC-PNA stain (Sigma)

Method

Immediately following collection, determine sperm concentration in ejaculate
Dilute semen drop-wise in 5ml falcon tubes in 1ml 0.02M PBS pre-warmed to 37°C to a final sperm concentration of 1×10^6 /ml
Add 10 μ l 40mg/ml FITC-PNA
Incubate 37°C for 15min
Fix sperm with 10 μ l of 10% neutral buffered formalin (final concentration 0.1%)
Store samples at 4°C for a maximum of 24h
Bring sample to room temperature
Place sample of FACScan flow cytometer
Select the sperm population and set inside the R1 box
Set the boundaries on the fluorescent plot to ensure the 2 sperm population (fluorescent and non fluorescent) fit within the boundaries
Record a minimum of 5000 gated events
Record the percentage number of fluorescent (acrosome not intact) and non-fluorescent (acrosome intact) sperm.



Appendix 5: Protocol for the Assessment of Acrosome Integrity and Viability of Alpaca Sperm by Fluorescent Microscopy

Equipment and Consumables

Pipettes and tips
Centrifuge
Microscope slides
22 x 22mm coverslips
Fluorescent microscope

Reagents

Neutral buffered formalin
Phosphate buffered saline (0.02M, Sigma-Aldrich, St Louis, MO, USA)
FITC-PNA stain (sigma)
Propidium Iodide (PI) stain (Molecular Probes, Eugene, OR, USA)

Method

Fix semen (30 μ l) in 0.1% (final concentration) neutral buffered formalin
Store at 4°C for up to 48h
Bring semen to room temperature and resuspend in 200 μ l PBS
Centrifuge 10 min 300 x g at room temperature
Decant the supernatant
Resuspend the sperm pellet in PBS at a final concentration of approximately 10×10^6 sperm/ml
To 30 μ l resuspended sperm (10×10^6 /ml) add 6 μ l of 40mg/ml FITC-PNA
Incubate 37°C for 10min
Add 0.5 μ l of 0.6mM PI
Incubate 37°C for 5min
Pipette 20 μ l of the stained sperm onto a clean microscope slide
Cover with a 22 x 50mm coverslip and allow to settle for 5min
Visualise under the fluorescent microscope at x 400 magnification using the U-MWIB filter (excitation filter 460-495nm, emission filter 510-550 nm, 505 nm dichromatic mirror).
Count 200 sperm and assess as follows
No fluorescence: acrosome intact, viable
Acrosome stains green: acrosome not intact
Sperm head stains red: non-viable
Sperm head stains red and acrosome stains green: non-viable acrosome not intact

Appendix 6: Protocol for the Assessment of Viability of Alpaca Sperm by Flow Cytometry

Equipment and Consumables

Pipettes and tips

FACScan flow cytometer

5ml falcon polystyrene tubes (12 x 75mm)

Reagents

Neutral buffered formalin

Phosphate buffered saline (0.02M, Sigma-Aldrich, St Louis, MO, USA)

Propidium iodide stain (Molecular Probes, Eugene, OR, USA)

Syto-16 stain (Molecular Probes, Eugene, OR, USA)

Method

Immediately following collection, determine sperm concentration in ejaculate

Dilute semen to a final sperm concentration of 1×10^6 /ml in 5ml falcon tubes with 1ml 0.1% neutral buffered formalin diluted in 0.02M PBS

Store at 4°C for a maximum of 16h

Add 10µl 10µM Syto-16

Incubate room temperature for 20min

Add 10µl 240µM PI

Incubate room temperature 10min

Place sample of FACScan flow cytometer

Select the sperm population and set inside the R1 box

Set the boundaries on the fluorescent plot to ensure the sperm populations fit within the boundaries

Record a minimum of 10,000 gated events

Record the percentage number of sperm within each boundary and assess as follows:

Syto-16 positive, PI negative: membrane intact, viable

Syto-16 negative, PI positive: membrane not intact, not viable)

No stain: the number of events in this boundary should be minimal. If there are a high percentage of sperm in this boundary either the staining procedure has not worked or the sperm population in the R1 box contains particles that are not sperm such as debris.

Appendix 7: Protocol for the Reduction of Alpaca Seminal Plasma Viscosity

Equipment and Consumables

Pipettes and tips
Water bath set to 37°C
15ml falcon tubes

Reagents

Papain from Carica Papaya (Sigma-Aldrich, St. Louis, MO, USA) at 10mg/ml concentration
N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide (E-64; Sigma-Aldrich, St Louis, MO, USA at 10°M concentration
Tris-Citrate-Fructose semen extender or any alternative extender to be used for semen storage

Methods

Collect semen from male alpaca
Following collection, transfer semen to a pre-warmed 15ml falcon tube using a pasteur pipette and place in water bath at 37°C
Immediately dilute semen drop-wise with an equal volume of pre-warmed 37°C semen extender (i.e. 1ml semen plus 1ml extender)
Pipette up and down slowly using a 1ml pipette to encourage mixing of the semen with the extender
Add papain at a final concentration of 0.1mg/ml (stock 10mg/ml so used at a 1:100 dilution) and pipette up and down to mix papain with diluted semen
Incubate 20min at 37°C in a water bath, at 10min flick 15ml falcon tube to encourage even mixing
Assess viscosity. Viscosity should be 0mm.
Add E64 at a final concentration of 10uM (stock at 1mM so use at 1:100 dilution)
Incubate 5min at 37°C in a water bath
Assess sperm motility and use sample for downstream applications such as cryopreservation or AI

Tris-Citrate-Fructose semen extender

3.634g Trizma base (Sigma)
0.5g D-(-) Fructose (Sigma)
1.99g Citric acid monohydrate (Sigma)
100ml Milli-Q water
Check pH is approximately 7 and osmolarity is 300-320mOsm

Papain from Carica Papaya (Sigma-Aldrich, St. Louis, MO, USA)

Stock concentration is 25mg/ml.
Dilute in 200µl papain in 300µl 0.02M PBS (Sigma) to give a stock concentration of 10mg/ml
Aliquot in 100µl aliquots (enough to treat 10ml diluted semen)
Store at 4°C for up to 2 months
Before use pre-warm to 37°C in a water bath
Discard any warmed papain (do not re-chill and re-use)

N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide (E-64; Sigma-Aldrich, St Louis, MO, USA)

E-64 is provided as 1mg solid containing 2.79µM of E-64
Dilute 1mg E-64 in 2.79ml deionised water to a stock concentration of 1mM
Aliquot to 50µl aliquots
Store in freezer at -20°C for up to 4 months
Do not freeze-thaw aliquots, once thawed discard any unused E-64.

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