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The effect of glycosaminoglycan enzymes and proteases on the viscosity of alpaca seminal plasma and sperm function

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ABSTRACT

In order to advance the development of cryopreservation and other assisted reproductive technologies in camelids it is necessary to eliminate the viscous component of the seminal plasma without impairing sperm function. It has been postulated that glycosaminoglycans (GAGs) or proteoglycans are responsible for this viscosity. This study investigated the effect of the GAG enzymes hyaluronidase, chondroitinase ABC and keratanase and the proteases papain and proteinase K on seminal plasma viscosity and sperm function in order to aid identification of the cause of seminal plasma viscosity and propose methods for the reduction of viscosity.

Sperm motility, DNA integrity, acrosome integrity and viability were assessed during 2 h incubation. All enzymes reduced seminal plasma viscosity compared to control ($P < 0.001$) although papain was most effective, completely eliminating viscosity within 30 min of treatment. Sperm motility and DNA integrity was not affected by enzyme treatment. The proportion of viable, acrosome intact sperm was reduced in all enzyme treated samples except those treated with papain ($P < 0.001$).

These findings suggest that proteins, not GAGs are the main cause of alpaca seminal plasma viscosity. Papain treatment of alpaca semen may be a suitable technique for reduction of seminal plasma viscosity prior to sperm cryopreservation.

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1. 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 interaction 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. 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., 2000, 1999). 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 present in human seminal plasma (Binette et al., 1996)

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and have recently been identified in the seminal plasma of ram and alpaca (Kershaw-Young et al., 2012). All five classes of GAGs 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., 2012). 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., 2012) 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.

2. Materials and methods

2.1. Animals

This study was performed using five male alpacas under authorization from the University of Sydney animal ethics committee. As Australian alpacas are not considered seasonal breeders (Smith et al., 1994) the study was conducted from February to November 2009. All males were >3 y, had a body condition score >3 and had testes more than 3 cm long.

2.2. 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, $n = 15$) during summer (February and March) using an artificial vagina fitted inside a mannequin as described previously (Morton et al., 2010). Within 5 min of collection, semen was assessed for volume, viscosity, sperm motility and concentration as described below.

Only samples with a volume >1 mL, viscosity ≥15 mm, motility ≥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 μL diluted semen plus 20 μL 0.01% BSA, (2) Hyaluronidase: 380 μL diluted semen plus 20 μL of 100 U/mL hyaluronidase (final concentration 5 U/mL), (3) Chondroitinase ABC: 380 μL diluted semen plus 20 μL 4 IU/mL chondroitinase ABC (final concentration 0.2 IU/mL), (4) Hyaluronidase and chondroitinase ABC: 380 μL diluted semen plus 10 μL 200 U/mL hyaluronidase (final concentration 5 U/mL) and 10 μL 8 IU/mL Chondroitinase ABC (final concentration 0.2 IU/mL), (5) Papain: 380 μL diluted semen plus 20 μL 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, $n = 15$) during winter (June–August) 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 μL diluted semen plus 20 μL 0.01% BSA (2) Keratanase: 380 μL diluted semen plus 20 μL 10 U/mL keratanase (final concentration 0.5 U/mL), (3) Proteinase K: 380 μL diluted semen plus 20 μL 20 mg/mL proteinase K (final concentration 1 mg/mL), (4) Papain: 380 μL diluted semen plus 20 μL 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.

2.3. 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, diluted in 0.02 M PBS) to a stock concentration of 100 U/mL or 200 U/mL. Chondroitinase ABC from Proteus

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