

# Changes in motility, morphology, plasma membrane and acrosome integrity during stages of cryopreservation of buck sperm

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Changes in sperm structure and function occur during the processing of semen. The present study was designed to investigate the effect on buck sperm during different stages of semen preparation including dilution, cooling, equilibration and freeze-thawing. Semen ejaculates from three mature bucks (replicates = 5) were diluted with tris-citric acid egg yolk glycerol extender at 37 °C, cooled to 4 °C over 90 min, equilibrated at 4 °C for 2 h, transferred to 0.5 mL straws, placed in nitrogen vapour, frozen and thawed and then analysed. Sperm samples were assessed for percentage motility, acrosomal and plasma membrane integrity, live sperm, and morphology after dilution, cooling, equilibration and thawing. Mean percentage motility after dilution ( $86.0 \pm 1.4\%$ ) was reduced significantly ( $p < 0.05$ ) due to cooling and equilibration ( $77.6 \pm 1.3\%$  and  $74.6 \pm 1.4\%$  respectively); furthermore, it decreased significantly ( $p < 0.05$ ) after freezing and thawing ( $42.3 \pm 2.5\%$ ). Mean percentage of live sperm was higher ( $p < 0.05$ ) after dilution ( $89.3 \pm 1.4\%$ ) compared with cooling ( $84.8 \pm 1.8\%$ ) and equilibration ( $80.2 \pm 2.5\%$ ) and further reduced ( $p < 0.05$ ) after freezing and thawing ( $56.0 \pm 3.4\%$ ). Sperm morphology dropped significantly ( $p < 0.05$ ) from  $96.4 \pm 0.3\%$  after dilution to  $88.8 \pm 1.3\%$  at cooling and further decreased ( $p < 0.05$ ) after freezing and thawing ( $81 \pm 1.9\%$ ). Mean percentage of sperm with normal plasma membrane after dilution ( $82.2 \pm 1.1\%$ ) was significantly reduced ( $p < 0.05$ ) at cooling or equilibration ( $73.8 \pm 1.8$ ) and further decreased ( $p < 0.05$ ) after freezing and thawing ( $50.1 \pm 2.9\%$ ). The percentage of sperm with normal acrosomes did not differ significantly due to dilution, cooling or equilibration ( $85.8 \pm 1.7\%$ ,  $83.2 \pm 1.6\%$ ,  $81.7 \pm 1.8\%$ ) but was significantly reduced after freezing and thawing ( $45.2 \pm 2.8\%$ ). In conclusion, frozen thawed sperm showed maximum damage to motility, morphology, plasma membrane and acrosome integrity following cooling.

## Introduction

Globally, about 90% of goats are found in Asian countries, including China, India, Pakistan and Bangladesh (Iqbal *et al.* 2008). In tropical regions, goats are kept to provide milk and meat. Goats are embedded in the culture and are socially accepted to alleviate poverty, particularly in developing countries. Against this background, research has been undertaken on reproductive biotechnology including artificial insemination using fresh or frozen semen.

Cryopreservation facilitates the supply of genetic material for artificial insemination (AI) in goats (Leboeuf, Restall & Salamon 2000). The greatest obstacle to the exploitation of frozen semen is that the freeze-thawing process of mammalian sperm generally leads to a decrease in motility and viability of sperm cells as a result of damage to membrane integrity and ultrastructure (Watson 2000). Frozen thawed sperm are subjected to chemical, osmotic, thermal, and mechanical trauma that occurs during dilution, cooling, equilibration and thawing. It has been demonstrated that cryopreservation leads to a decrease in sperm motility in the goat (Dorado *et al.* 2009). However, the extent of damage at consecutive stages of cryopreservation of buck sperm has not been determined. Some of the key semen assays related to functional significance and fertility include hypo-osmotic swelling as a measure of plasma membrane integrity (Jeyendran *et al.* 1984), the presence of a normal acrosomal ridge (Gillan, Evans & Maxwell 2005), and sperm morphology, which has been considered as a robust clinical test (Blom 1973).

The objective of the present study was to assess the damage to sperm motility, plasma membrane integrity (PMI), and the normal apical ridge (NAR) of the acrosome, live-dead and morphology of buck sperm after dilution, cooling to 4 °C, equilibration at 4 °C and thawing after freezing.

## Materials and methods

### Semen collection

Semen was collected from three mature Beetal bucks maintained under optimal conditions of feeding and management in the animal shed of the Department of Theriogenology, University

of Veterinary and Animal Sciences, UVAS, Pakistan during the months of October to December, 2011. Semen collection (replicates = 5) was done twice a week using an artificial vagina maintained at 42 °C. Each ejaculate was transferred to the evaluation room within 5 minutes, kept in a water bath at 37 °C and evaluated for sperm motility and concentration. The ejaculates possessing > 65% percentage motility and > 1.5 billion sperm/mL were used for further processing.

### Semen processing and evaluation

Semen was diluted with tris-citric acid egg yolk glycerol extender (TCEYG, pH 6.8) comprised of tris(hydroxymethyl) aminomethane (3.93% w/v, Fluka, Buchs, Switzerland), citric acid (1.70% w/v, Fluka), egg yolk (20.00% v/v), fructose (0.20% w/v, Merck, Darmstadt, Germany), glycerol (8.00% v/v, Merck), and antibiotics (benzyl penicillin 1000 i.u./mL and streptomycin sulphate (100 µg/mL, Sigma) (Rasul *et al.* 2000) at 37 °C to a final concentration of 100 million sperm/mL. Diluted semen was cooled to 4 °C in 90 minutes (cabinet, Mini tub, France), equilibrated for 2 h and packaged into 0.5 mL French straws. Semen straws were placed in nitrogen vapour for 7 min (4 cm above the liquid level in a closed container), then plunged into and stored in liquid nitrogen until analysed after thawing at 37 °C for 30 s.

Each individual semen sample (3 bucks × 5 replicates = 15 samples) was evaluated for percentage motility, plasma membrane integrity (PMI), normal apical ridge (NAR), live or dead, and morphology at each stage of cryopreservation, that is, after dilution (AD), after cooling (AC), after equilibration (AE) and after freezing and thawing (AT).

### Semen assays

#### Percentage motility

Semen (10 µL) was placed on a pre-warmed (37 °C) glass slide, covered with a cover slip (22 mm × 22 mm) and placed on the pre-warmed stage of a phase contract microscope (Olympus BX51). The percentage of motile sperm was assessed subjectively by viewing 5–6 fields per slide with the aid of closed-circuit television attached to the microscope (200 ×). Sperm possessing linear progressive movement were considered to be motile.

#### Plasma membrane integrity

Plasma membrane integrity (PMI) of sperm was assessed using the hypo-osmotic swelling (HOS) test (Jeyendran *et al.* 1984). Fifty µL of each semen sample was mixed with 500 µL of HOS solution (190 mL Osmol/kg) and incubated at 37 °C for 30 min. After incubation, a 5 µL semen sample drop was examined under a phase-contrast microscope (400 ×). A minimum of 100 sperm were counted for their swelling ability in HOS solution. The sperm characterised by coiling or swelling of the tail of varying degrees were considered to have an intact plasma membrane.

#### Normal apical ridge (NAR)

A 500 µL aliquot of each semen sample was fixed in 50 µL of a 1% formal citrate solution. One hundred sperm were counted

with a phase contrast microscope (1000 ×) for normal apical ridge (NAR). Presence of crescent shaped acrosomes was considered normal.

#### Morphology and live-dead

A 10 µL aliquot of each semen sample was mixed with eosin-nigrosin stain (Ahmad *et al.* 2011). A smear was made and dried and 100 sperm were counted and examined for live-dead and morphology under a phase contrast microscope (400 ×). Sperm without penetration of stain were considered as live.

#### Preparation of chemicals

A hypo-osmotic solution of 190 mL Osmol/kg was prepared by dissolving Tri-sodium citrate dehydrate (Merck, Germany) and D (-) fructose (Sigma, USA) in 100 mL of de-ionised distilled water (Rasul *et al.* 2000). Formal citrate solution was prepared by dissolving 2.9% (w/v) tri-sodium citrate dehydrate (Merck, Germany) with 1.0% v/v commercial formaldehyde (37.0%, Merck, Germany) (Cabrera *et al.* 2005).

#### Statistical analysis

Data were presented as mean ± SEM and analysed using one-way ANOVA to assess differences amongst stages of cryopreservation, that is, AD, AC, AE and AT for motility, PMI, NAR, live sperm and morphology. Normality of data was determined through the Shapiro-Wilk test. A probability level of < 0.05 was considered to be significant. The least significant difference (LSD) test was used for multiple comparisons. All analysis was performed using statistical software SPSS (Version 13).

## Results

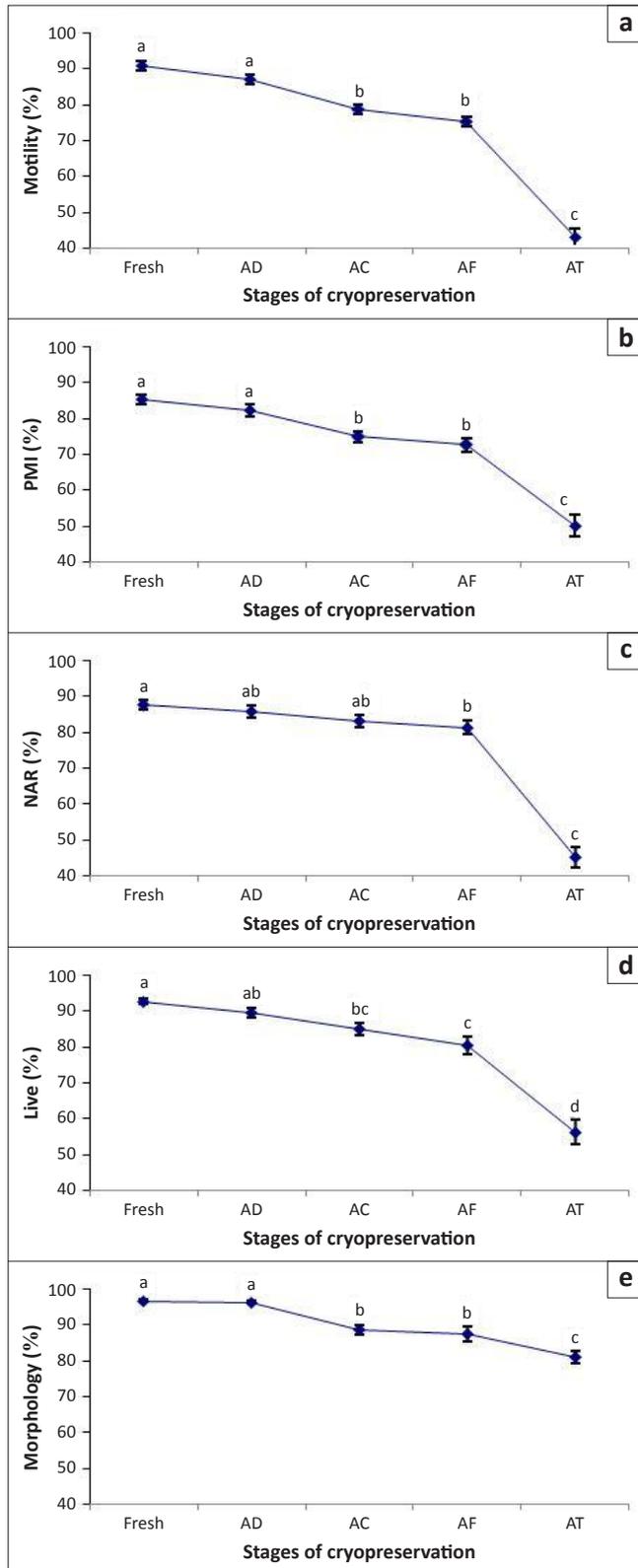
Changes in motility, plasma membrane integrity, normal acrosome, live sperm and morphology at consecutive stages of cryopreservation of buck semen are presented below (Figure 1).

#### Percentage motility

Fresh semen possessed 89.8 ± 1.26% progressive motility. This declined to 86 ± 4.2% after dilution, which was higher ( $p < 0.05$ ) than at the stages of cooling (77.6 ± 3.9) and equilibration (74.6 ± 4.2). However, mean motility of sperm after freezing and thawing (42.3 ± 7.5) significantly declined ( $p < 0.05$ ) compared with all other stages of cryopreservation.

#### Plasma membrane integrity

Fresh semen contained 85.3 ± 0.92% sperm with an intact plasma membrane. The mean percentage of swollen sperm after dilution (82.2 ± 3.3) differed significantly at cooling (75.0 ± 5.1) and after equilibration (72.6 ± 5.7). However, the mean percentage of swollen sperm after freezing and thawing (50.1 ± 8.7) declined ( $p < 0.05$ ) significantly compared with other stages of cryopreservation.



Note: Superscripts with different letters indicate significant difference ( $p < 0.05$ ). Values are mean  $\pm$  SEM, ( $n = 15$ , semen samples from three Beetal bucks). Motility (a), morphology (e) and plasma membrane integrity (b) decreased ( $p < 0.05$ ) at cooling from fresh and extended semen and further decreased after freezing. Damage to acrosomal ridge (c) increased ( $p < 0.05$ ) due to freezing and thawing. Percentage of live spermatozoa (d) decreased ( $p < 0.05$ ) from dilution to cooling and further to freezing.

**FIGURE 1:** Effect of stages of cryopreservation on motility, after dilution, after cooling, after equilibration, and after freezing and thawing, on the percentile of (a) motility, (b) plasma membrane integrity, (c) normal acrosomeapical ridge, (d) live and (e) morphology of spermatozoa.

## Normal acrosome

Sperm with normal acrosomes were  $87.7 \pm 1.3\%$  in fresh semen. The percentage of sperm with normal acrosomes did not differ significantly as a result of dilution, cooling or equilibration ( $85.8 \pm 5.4\%$ ,  $83.2 \pm 4.8\%$ ,  $81.7 \pm 5.4$  respectively) but decreased significantly ( $p < 0.05$ ) after freezing and thawing ( $45.2 \pm 8.4\%$ ).

## Live dead

Fresh semen had  $92.6 \pm 0.68\%$  live sperm that did not take up the stain. The mean percentage of live sperm after dilution ( $89.3 \pm 4.2$ ) differed significantly ( $p < 0.05$ ) from that after cooling ( $84.8 \pm 5.4$ ) and equilibration ( $80.2 \pm 7.5$ ). However, it declined further ( $p < 0.05$ ) after freezing and thawing ( $56.0 \pm 10.5$ ).

## Morphology

Fresh semen possessed  $96.8 \pm 0.36\%$  morphologically normal sperm. This was similar at dilution ( $96.4 \pm 5.2$ ), but was significantly ( $p < 0.05$ ) lower after cooling ( $88.8 \pm 5.7$ ) and equilibration ( $87.6 \pm 7.5$ ). However, morphology declined significantly ( $p < 0.05$ ) compared with all other stages of cryopreservation after freezing and thawing ( $81.0 \pm 5.7$ ).

## Discussion

To the best of the authors' knowledge, this study reports for the first time in detail the changes in the motility of caprine semen at successive stages of cryopreservation that included dilution, cooling, equilibration and freezing or thawing. In this study, the percentage motility was significantly reduced by up to 42% after freezing and thawing. Similar findings for a decline in motility due to freezing and thawing compared with fresh semen have been reported in Boer (Tuli & Holtz 1994) and Florida goats (Dorado, Munoz-Serrano & Hidalgo 2010). There maybe two reasons for the reduction in sperm motility: firstly, biophysical injuries as a result of formation of ice crystals in the extra- and intracellular environment and increasing solute concentration (Mazur 1984), and secondly, biochemical oxidative stress resulting in irreversible damage to sperm structure, changes in membrane fluidity and enzymatic activity (Aitken, Clarkson & Fishel 1989). More than 50% of mammalian sperm are usually injured by the cryopreservation process (Watson 2000). Similarly, the motility pattern decreased significantly after freezing and thawing in bull (Budworth, Amann & Chapman 1988) and buffalo (*Bubalis bubalis*) sperm (Rasul *et al.* 2000). In order to minimise the cryodamage in goats, altering the freezing rate or addition of membrane stabilisers might be potential areas for future investigation.

Plasma membrane integrity is of prime importance for the freezing and fertility of the sperm cells. The hypo-osmotic swelling assay has been described as a useful test for assessing functional integrity of the plasma membrane in humans (Jeyendran *et al.* 1984). In the existing study, the plasma membrane integrity of the sperm was lowered

by equilibration and declined further after freezing and thawing, as has previously been reported in goat semen (Azerêdo, Esperb & Resendec 2001). A similar decrease in the membrane integrity of sperm after freezing and thawing has been reported in bulls (Correa & Zavos 1994), boars (Vazquez *et al.* 1997) and stallions (Neild *et al.* 1999). Biochemically, these findings are supported by the fact that the lipid components of the plasma membrane of buck semen are significantly reduced after freezing (Holt & North 1984).

The significance of an acrosomal cap is known to be a prerequisite for successful fertilisation. In the current study, the percentage of sperm with a normal acrosome did not change due to the other stages of cryopreservation, but it was reduced by up to 50% after freezing and thawing. Earlier researchers suggested that cryopreservation induces reduction in acrosome integrity of frozen semen samples of goats (Aboagla & Terada 2004). Acrosome membrane-intact sperm ranged between 40% and 70% in Canary (Cabrera *et al.* 2005) and Florida bucks (Dorado, Rodriguez & Hidalgo 2007). It is presumed that loss of the acrosomal cap during freezing and thawing of buck sperm is similar to that demonstrated in bull sperm (Bamba & Cran 1988). Greater release of the acrosomal enzyme hyaluronidase was noticed after freezing and thawing in the sperm of buffalo bulls (Akhtar & Chaudhry 1989). Therefore, it would be meaningful to study the relationship between fertility and acrosome damage due to freezing and thawing in goat semen.

## Conclusion

In conclusion, the cooling of buck sperm from 37 °C to 4 °C has a negative effect on motility. However, equilibration of buck sperm at 4 °C for two hours seems to have very little effect on semen characteristics. Freezing and thawing cause considerable damage to motility, plasma membrane integrity, acrosomal cap, live-dead ratio and morphology of the sperm. Further studies should focus on determining the ultra-biochemical changes at different stages of cryopreservation to enhance the understanding of their more detailed effects on buck sperm.

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## Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

## Authors' contributions

M.A. (University of Veterinary and Animal Sciences) and R.N. (University of Veterinary and Animal Sciences) performed most of the experiments. A.S. (University of Veterinary and Animal Sciences) and N.A. (University of Veterinary and Animal Sciences) provided the conceptual

design for the experiments. H.R. (University of Veterinary and Animal Sciences) and M.A. were involved in writing this manuscript.

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