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# Cryopreservation of *in situ* cool stored buffalo (*Bubalus bubalis*) epididymal sperm

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## Summary

The objectives of this study were to investigate the effects of testis-epididymis cool storage on viability and progressive motility of buffalo epididymal sperm (EP) and to compare the influences of two basic semen extenders on post thaw EP viability and progressive motility. Abattoir collected buffalo testicles were allotted to three storage times (0 h: n = 10; 24 h: n = 10 and 48 h: n = 12). Following storage, isolated sperm were subjected to cryopreservation with two different cryoprotective media: whole cow milk-7%glycerol (MG) or egg yolk-tris-citrate-7%glycerol (EYG). Pre freeze and post thaw sperm progressive motility and viability were evaluated. Results indicated that viability and progressive motility of sperm decreased after 24 h cool storage of the epididymis ($P<0.05$). There was no difference between 24 and 48 h of storage on sperm viability ($P>0.05$), but progressive motility decreased across storage times (24 h *versus* 48 h: $P<0.05$). Cryopreservation severely influenced viability and progressive motility of EP ($P<0.05$). Milk-7%glycerol protected viability and progressive motility of EP against cold shock more efficiently than EYG ($P<0.05$). The results of this study demonstrate that it is possible to preserve buffalo EP within the epididymis at 4°C short term, but that it has poor freezability upon recovery by basic semen freezing protocols.

**Key words:** *Bubalus bubalis*, River Buffalo, Epididymal sperm, Cryopreservation, Cool storage

## Introduction

There is a considerable population (>500,000 head) of river buffalo (*Bubalus bubalis*) in Iran which play a significant role in rural life by producing milk and meat while tolerating the impact of harsh environmental conditions (Naderfard and Qanemy, 1997). This species is not in danger of extinction in Iran, but the rate of fertility, which is directly correlated with production, is low (Barile, 2005). Although buffalo semen has been stored successfully in liquid and frozen states (Sansone *et al*., 2000), artificial insemination (AI) has not yet been developed mainly due to oestrus detection issues (Barile, 2005). With the new approaches developed for the management of ovulation (e.g., Ovsynch program), assisted reproductive technologies (ART) could help to overcome reproductive insufficiency in the buffalo species (Barile, 2005).

Epididymal sperm (EP) have been successfully used for AI and *in vitro* production of embryos (IVP) in several species (Kikuchi *et al*., 1998; Hori *et al*., 2004; Hori *et al*., 2005). Motile sperm have been isolated from cool stored epididymis of boar (Kikuchi *et al*., 1998), cattle (Nichi *et al*., 2007), dog (Stilley *et al*., 2000; Yu and Leibo, 2002), stallion (Braun *et al*., 1994; Bruemmer *et al*., 2002), African buffalo (*Syncerus caffer*) (Bartels *et al*., 1999), goat (Blash *et al*., 2000), ram (Kaabi *et al*., 2003), camel (Wani *et al*., 2005), Iberian red deer (*Cervus elaphus hispanicus*) (Soler *et al*., 2005; Fernández-Santos *et al*., 2006) and Sika deer (*Cervus nippon*) (Hishinuma *et al*., 2003). Tomcat EP (Hermansson and Axnér, 2007) has been diluted in a chemical medium and was motile up to 72 h under refrigerator condition.

Influences of different types (Herold *et al*., 2004; Wani *et al*., 2005) and concentrations (Herold *et al*., 2004;

Fernández-Santos *et al*., 2006; Herold *et al*., 2006; Morton *et al*., 2007) of cryoprotectants, additives to freezing medium (Herold *et al*., 2004) and determination of equilibration time (Herold *et al*., 2004; Herold *et al*., 2006) have been investigated for EP cryopreservation. Morton *et al*. (2007) compared citrate based, tris based and lactose based diluents for alpaca EP freezing. Herold *et al*. (2004, 2006) used AndroMed® (a chemical defined medium) and Triladyl™ (an egg yolk based medium) for cryopreservation of African buffalo (*Syncerus caffer*) EP. They reported the optimal equilibration time of 4 h for African buffalo (*Syncerus caffer*) EP freezing (Herold *et al*., 2006). Cool stored epididymal Iberian red deer (*Cervus elaphus hispanicus*) (Fernández-Santos *et al*., 2006) and stallion (Braun *et al*., 1994; Bruemmer *et al*., 2002) sperm were successfully cryopreserved.

This study was undertaken to determine 1) the effect of duration of cool storage of excised epididymes on viability and progressive motility of river buffalo EP, and 2) the influence of two basic diluents (milk-7%glycerol (MG) and egg yolk-tris-citrate-7%glycerol (EYG)) on post thaw viability and progressive motility of buffalo EP.

## Materials and Methods

Tyrodes lactate (TL) solution components, fructose, citric acid, and glycerol were purchased from Merck, Germany. *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulfonic acid (HEPES) buffer, bovine serum albumin (BSA; Fraction-V), pyruvic acid, Tris (TRIZMA®BASE), phenol red and gentamycin were provided from Sigma-Aldrcih, St. Louis, USA. Other materials are specified through the text.

### Media preparation

Sperm TL medium was tyrodes lactate medium (100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.29 mM $NaH_2PO_4$ $H_2O$, 21.6 mM Na Lactate, 2.1 mM $CaCl_2$ $2H_2O$, 0.4 mM $MgCl_2$ $6H_2O$ and 10 mM HEPES buffer; Parrish *et al*., 1988) plus 0.006 g/ml BSA, 1 mM sodium pyruvate, 25 µg/ml gentamycin and 10 mg/L phenol red (pH = 7.4 and osmolarity = 290-305 mOsm/L). The cryoprotective media (pH = 6.8-7.4) were egg yolk-citrate-glycerol (199.8 mM TRIZMA®BASE, 45 mM citric acid, 55.5 mM D-fructose, 20% (v/v) egg yolk 7% (v/v) glycerol and 0.5 mg/ml gentamycin; Salisbury and Fuller, 1941) and cow milk (Choopan dairy industries, Tehran, Iran) - glycerol (whole cow milk with 7% (v/v) glycerol and 0.25 mg/ml gentamycin; Zaugg and Almquist, 1972). Each of the media was prepared separately in two parts (part A: media without glycerol and part B: media with 14% glycerol). One batch of each part was prepared and frozen for subsequent use in 10 ml aliquots. On the day of use, the parts A were thawed and warmed to room temperature (22-25°C) and the parts B were thawed at 4ºC until liquefied.

### Testes preparation and EP isolation

Mature buffalo bull (>2.5-year-old) testes were collected from the local abattoir, placed on ice and transported to the laboratory. The testes were separated from each other and allotted to three different times of cool storage (0 h: n = 10; 24 h: n = 10; 48 h: n = 12). After storage, each testicle was brought to room temperature in less than 30 min and cauda epididymis was dissected from testis-epididymis, minced and placed into a 60 mm tissue culture dish containing sperm TL medium for 15 min. Then the tissue was removed and the remaining medium was centrifuged (Behdad®, Tehran, Iran) at 700 g for 6 min to create a sperm pellet. Supernatant was removed and the isolated sperm analyzed for sperm concentration, viability and progressive motility. Finally, a sperm suspension ($300 \times 10^6$ sperm/ml) was created by adding sperm TL medium. The diluted EP (provided from each testis-epididymis) was subjected to cryopreservation.

### Cryopreservation and thawing procedures

The two step dilution procedure was used for sperm cryopreservation (Salisbury and Fuller, 1941). The sperm suspension was divided equally and added to part A of the diluents (EYG and MG) at room

temperature to create a 30 × $10^6$ sperm/ml sperm dilution. The extended sperm was placed in a 25°C water jacketed tube and cooled to 4°C within 2.5 h in refrigerator. An equal volume of part B of the diluents (EYG and MG) was added in two steps (1:2 and 1:2 of original volume) with a 15 min interval, resulting in a final concentration of 7% glycerol. The glycerolized samples were equilibrated for an additional 1 h at 4°C before being loaded into straws (0.25 ml french straws; IMV, France). The filled straws were sealed, placed 2-4 cm above the liquid nitrogen (LN2) level for 8 min, and then immersed into LN2. At least 48 h after freezing, the straws were removed from LN2, placed 30 sec in air and 30 sec into a 39ºC water bath. The samples were added to 3 ml of sperm TL medium and centrifuged twice at 700 g for 6 min. After the second centrifugation the supernatant was removed and the sperm pellet was resuspended in 2 ml of sperm TL medium for analysis. The efficacies of two cryoprotective media (EYG and MG) on EP freezing were compared across three cool storage times. Two straws per testicle per cryoprotective media (total number of straws = 128) were used for analysis.

### Sperm analyses

An aliquot (5 µl) of the sperm suspension was added to 95 µl of sperm TL medium to make 1:20 sperm dilution. The percentage of motile sperm (on a 10 µl sample of the sperm dilution) was subjectively evaluated (×100) in at least four separated fields on a warmed slide before freezing and immediately after thawing. At each time point an aliquot of the sperm dilution (20 µl) was mixed (1:1 v/v) with an aliquot (20 µl) of stain containing Eosin B/nigrosin, co-incubated for 1 min, smeared across a glass slide and quickly dried with a hair dryer. Stained slides were assessed (200 sperm/slide) using light microscopy (Nikon, Japan). Sperm with a pink or red like acrosome were considered nonviable. Sperm with clear or light acrosome color were considered viable.

### Statistical analysis

The percentage of sperm motility and viability were subjected to arcsin transformation before analysis. The percentages of progressive motile and viable sperm within cool storage times were analyzed by General Linear Model of SAS (SAS, 1996). The effects of cool storage times, types of cryoprotective media and their interaction on sperm viability and progressive motility were examined using the General Linear Model of SAS with Tukey's multiple comparison test (SAS, 1996). Data was presented as least square means ± SE of means.

## Results

Sperm viability (%) decreased (Fig. 1) after 24 and 48 h (76.5 ± 5.3 and 67.9 ± 5.8, respectively; P>0.05) of cool storage in comparison with the control (91.1 ± 5.6). The percentage of progressive motile sperm (0 h: 73.4 ± 3.4, 24 h: 37.5 ± 3.5 and 48 h: 27.1 ± 3.2; P<0.05) decreased across three times of cool storage (Fig. 1).

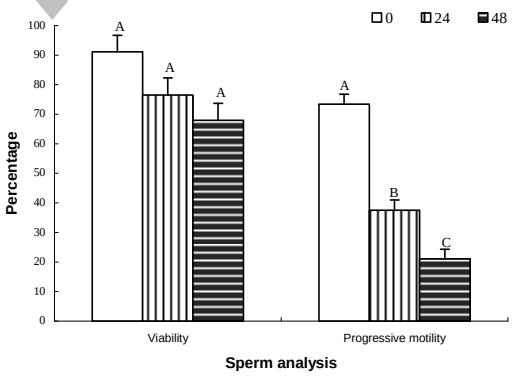


**Fig. 1: Effects of duration of buffalo (*Bubalus bubalis*) testis-epididymal cool storage (0 h: n = 10, 24 h: n = 10 and 48 h: n = 12) on sperm viability and progressive motility. (Different letters (AB) above bars for each variable (viability or progressive motility) indicate significant difference; P<0.05)**

By extruding the effects of types of media and duration of cool storage, the results of the present study show an impact of cryopreservation on viability (>50% decline: Pre freeze: 78.5 ± 2.6 *versus* post thaw: 33.0 ± 2.4) and motility (>80% decline, Pre freeze: 46.0 ± 1.9 *versus* Post thaw: 7.9 ± 2.6) of river buffalo EP. However, MG was more efficient than EYG in protecting the post thaw sperm viability

(P<0.05) across times of cool storage (Table 1).

Cryopreservation (MG or EYG; Table 2) severely affected post thaw sperm progressive motility (Table 2; P<0.05). Post thaw (for MG and EYG) progressive motility was significantly decreased across times of cool storage. Except for 48 h of cool storage, MG protected the sperm motility better (P<0.05) than EYG (Table 2).

**Table 1: Effects of duration of testis-epididymis cool storage† and cryoprotective media* on viability (L.S. mean ± SEM) of buffalo epididymal sperm**

| Duration of storage (hours) | No. testis-epididymis | Post-thaw sperm viability (%) | |
|---|---|---|---|
| | | MG | EYG |
| 0 | 10 | $46.2\pm3.4^{Aa}$ | $39.63\pm4.2^{Ba}$ |
| 24 | 10 | $45.5\pm4.3^{Aa}$ | $15.8\pm4.02^{Bb}$ |
| 48 | 12 | $38.45\pm3.3^{Aa}$ | $12.5\pm4.6^{Bb}$ |

† 0, 24 and 48 hours. *MG: Whole Milk-Glycerol; EYG: Whole Egg yolk-Tris-Citrate-Fructose-Glycerol. $^{AB}$Values with different superscript within rows significantly differ (P<0.05). $^{abc}$Values with different superscript within columns significantly differ (P<0.05)

**Table 2: Effects of duration of testis-epididymis cool storage† and cryoprotective media* on progressive motility (L.S. mean ± SEM) of buffalo epididymal sperm**

| Duration of storage (hours) | No. testis-epididymis | Post-thaw progressive motility (%) | |
|---|---|---|---|
| | | MG | EYG |
| 0 | 10 | $16.5\pm2.6^{Aa}$ | $11.6\pm2.5^{Ba}$ |
| 24 | 10 | $8.9\pm2.6^{Aab}$ | $5.4\pm2.6^{Bb}$ |
| 48 | 12 | $4.9\pm2.3^{Ab}$ | $2.4\pm2.5^{Ab}$ |

† 0, 24 and 48 hours. *MG: Milk-Glycerol; EYG: Whole Egg yolk-Tris-Citrate-Fructose-Glycerol. $^{AB}$Values with different superscript within rows significantly differ (P<0.05). $^{abc}$Values with different superscript within columns significantly differ (P<0.05)

## Discussion

Sperm progressive motility decreased significantly across times of storage. The results of this study demonstrated that viable sperm can be harvested from epididymes of river buffalo, at least after 48 h as well as 24 h of cool storage. Buffalo semen was diluted with different extenders and stored successfully at 4°C for at least 72 h (Sansone *et al*., 2000). Motile EP has been collected after cool storage within epididymis or out of epididymis in different species (Braun *et al*., 1994; Kikuchi *et al*., 1998; Stilley *et al*., 2000; Bruemmer *et al*., 2002; Yu and Leibo, 2002; Nichi *et al*., 2007). These findings are in agreement with the studies that reported motility and viability of dog EP did not change up to Days 2 (Stilley *et al*., 2000), 5 (Ponglowhapan *et al*., 2006) or 8 (Bruemmer *et al*., 2002) after cool storage within epididymis. Studies on Sika deer (*Cervus nippon*) (Hishinuma *et al*., 2003) and Iberian red deer (*Cervus elaphus hispanicus*) (Soler *et al*., 2005) EP showed that viable sperm can be isolated up to days 7 and 3 after cool storage, respectively, but sperm motility decreased the storage period continued in both of the studies. A study on cat EP preservation in tris or tris plus 10% egg yolk (Hermansson and Axnér, 2007) at 4°C found that egg yolk-tris promoted the viability of cat EP compared to tris alone. In contrast to the present study, Bissett and Bernard, (2005) reported that only 30% of eland (*Taurotragus oryx*) EP survived at day 3 after epididymis cool storage, and Bartels *et al*. (1999) reported an increase in morphological changes in African buffalo (*Syncerus caffer*) EP with increasing duration of storage at 6°C.

Decreased sperm motility across storage times in the present study may relate to the postmortem changes in epididymal lumen. Prolonged postmortem preservation of epididymis resulted in a severe decrease in the pH of epididymal lumen in wild ruminant (Martinez-Pastor *et al*., 2005). Pyknosis and release of epithelial cells in the lumen of cool stored epididymis were also reported (Hishinuma *et al*., 2003). Therefore, the postmortem changes can occurr even under cool storage and result in reduced pH of epididymal lumen. A strong positive correlation between pH of epididymal lumen and sperm motility was reported (Martinez-Pastor *et al*., 2005).

Cryopreservation severely affected the viability and progressive motility of buffalo EP. These results are in agreement with the findings of a previous study on cryopreservation of African buffalo (*Syncerus caffer*) EP which reported a more than 50% decline in post thaw sperm viability (Lambrechts *et al*., 1999). In contrast, some studies showed a good freezability of Sika deer (*Cervus nippon*) (Hishinuma *et al*., 2003), dog (Ponglowhapan *et al*., 2006), cat (Hermansson and Axnér, 2007), ram (Kaabi *et al*., 2003) and goat (Blash *et al*., 2000)

EP. Tiplady *et al*. (2002) reported that cryopreservation of cooled equine EP induced a decrease in motility but not in viability from pre freeze to post thaw. On the other hand, comparing ejaculated semen, Raizada *et al*. (1990) reported a higher sensitivity of buffalo semen than cattle against cryopreservation damage. Galli *et al*. (1993) reported poor freezability of buffalo semen and explained it by lower phospholipid contents of buffalo sperm cell membrane.

The results of the present study indicated a superior effect of MG than EYG (20% egg yolk) on freezing of river buffalo EP. There is no report of using whole cow milk for cryopreservation of EP. However, lactose based diluent was more effective than citrate or tris based diluents to protect viability and motility of alpaca EP against freezing damage (Morton *et al*., 2007). Adverse effects of 20% egg yolk in tris-citrate and Triladyl™ compared to 6% egg yolk have been reported for Spanish ibex (*Capra pyrenacia*) EP freezing (Santiago-Moreno *et al*., 2006). The results of the present study are in contrast with the previous report of Herold *et al*. (2004) who compared Andromed® and Triladyl™ for freezing African buffalo (*Syncerus caffer*) EP. They concluded that freezability of African buffalo EP with Triladyl™ was better than Andromed® (Herold *et al*., 2004), and suggested an appropriate equilibration time of equal to or more than 4 h for African buffalo (*Syncerus caffer*) EP freezing (Herold *et al*., 2006). The very low sperm freezability (viability and progressive motility) with egg yolk based diluent in the present study can be related to the susceptibility of buffalo sperm to the egg yolk which was previously reported for buffalo semen cryopreservation (Galli *et al*., 1993; Kumar *et al*., 1993). Various levels of egg yolk in a tris based diluent for cryopreservation of buffalo semen showed that egg yolk beyond 5% did not improve post thaw sperm motility (Sahni and Mohan, 1990). Protective effects of egg yolk on preservation of the lipoprotein sheath of a sperm cell have been established (Kumar *et al*., 1993). However, egg yolk stimulates the enzyme system of sperm to deaminate certain specific amino acids normally present in egg yolk and yield hydrogen peroxide which is toxic to sperm during storage under aerobic conditions (Sahni and Mohan, 1990). These effects may enhance the toxicity of dead sperm, perhaps by providing substrate for lipid peroxidation (Shannon and Curson, 1972) and prolongation of storage by reducing pH (Santiago-Moreno *et al*., 2006). It should be noted that as the yolk concentration increased in the diluent, the pH of the medium decreases (Shannon, 1972). Perhaps this is the reason for the depressing effects of high amounts (20%) of yolk on sperm motility reported in the present and other studies (Sansone *et al*., 2000; Santiago-Moreno *et al*., 2006). Shannon and Curson (1972) reported the depressing effects of enhanced levels of egg yolk (20%) on sperm viability during incubation in 5 and 25°C in combination with dead sperm on the sperm viability that is due to increased proxide formation.

In summary, viable and motile buffalo EP can be isolated after cool storage within epididymis up to 48 h. Cryopreservation has an adverse effect on buffalo EP. Milk-7%glycerol diluent is superior to a 20% level of egg yolk plus glycerol on buffalo EP freezing output. More studies must be done to find the optimized protocol for cryopreservation of river buffalo EP.

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