Effect of osmotic pressure on spermatozoa characteristics of cryopreserved buffalo bull (Bubalus bubalis) semen

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2. Materials and methods

2.1. Animal housing and management

Nili-Ravi buffalo bulls (n = 4), housed at the Semen Production Unit, Qadirabad, Pakistan, under uniform management and feeding conditions were used in this study. These bulls were...
provided seasonal fodder at 10% of the body weight and 2–3 kg concentrate daily.

2.2. Extender preparation

Extender composition of Ramakrishnan and Ariff (1994) was modified to attain desired osmotic pressures. Briefly, 70.0 g of tris (Hydroxymethyl) aminomethane, (BDH Laboratory Supplies, England), 33.40 g of citric acid monohydrate (Merck, Germany) and 25.0 g of D (−) Fructose (BDH Laboratory Supplies, England) were mixed in bi-distilled water to obtain a final volume of 2000 ml. The osmotic pressure of the solution was measured and its pH was adjusted to 7.0 by adding 1 N HCl. Afterwards, the solution was divided into three equal parts and osmotic pressure of each part was adjusted to 295, 275 and 255 mOsm/kg with bi-distilled water. Each of these solutions was further sub-divided into 10 parts for extender preparation and stored at −40°C. At the time of extender preparation, solutions were thawed at 37°C for 30 min.

Tris egg yolk extenders (TEYE; n = 3) using solution of each osmotic pressure were prepared by adding benzyl penicillin (1000 IU/ml) and streptomycin sulphate (1000 μg/ml), egg yolk (20% vol/vol) and glycerol (7% vol/vol). These extenders were stored overnight in a refrigerator and maintained at 37°C before semen dilution.

2.3. Semen collection and processing

Two ejaculates from each bull were collected at weekly intervals for 10 weeks (November to January), using artificial vagina (Andrabi et al. 2008). Immediately after collection, semen was incubated in a water bath at 37°C for 15 min and thereafter subjected to gross and microscopic evaluation. Ejaculates with ≥70% spermatozoa motility were selected for further processing. Equal volume of ejaculate from each bull was pooled at each collection to get enough semen for experimentation and to remove individual bull variation. The pooled semen was evaluated for sperm concentration, extended with TEYE of different osmotic pressures and filled in 0.5 ml straws, each containing 20 × 10^6 spermatozoa. After sealing the open ends, straws were cooled from 37°C to 4°C in a cold cabinet and equilibrated for 4 hours (Andrabi et al., 2006). During deep freezing, straws were placed horizontally on the freezing grill for 20 min in a freezing tank at 4 cm higher on liquid nitrogen (LN) and then dipped and stored in LN at −196°C for one week before evaluation.

2.4. Post-thaw evaluation of semen

Thawing of frozen semen was done at 37°C for 30 s in a water bath and subjected to post-thaw evaluation in terms of sperm motility, viability, acrosomal integrity, plasma membrane integrity and lipid per-oxidation. Five semen straws of each osmotic pressure of each collection were evaluated. Spermatozoa motility was assessed by using a phase contrast microscope (Ijaz et al. 2009), whereas viability was assessed through vital staining (Khan & Ijaz 2008). For the assessment of spermatozoa acrosomal integrity, each thawed semen sample was mixed with 1% formaldehyde citrate. At least 200 spermatozoa were examined under a phase contrast microscope and a percentage of spermatozoa with normal acrosome was recorded. Plasma membrane integrity of spermatozoa was assessed by hypo-osmotic swelling test. Hypo-osmotic solution was prepared by mixing 0.735 g of tri-sodium citrate dihydrate (Merck, 64271 Darmstadt, Germany) and 1.351 g D (−) fructose in bi-distilled water to make a final volume of 100 ml and its osmotic pressure was reduced to 75 mOsm/kg by adding bi-distilled water. For assessing plasma membrane integrity, thawed semen samples were mixed with hypotonic solution, incubated at 37°C for 45 min and examined under phase contrast microscope (Adeel et al. 2009).

The spermatozoa DNA integrity was estimated by using acridine orange staining technique, as described by Farooq et al. (2015) and examining under fluorescent microscope (Labomed Lx 400, USA). The spermatozoa heads showing green fluorescence were considered as having intact DNA, while heads of spermatozoa with red fluorescence or yellow-orange to red were taken as having damaged DNA. Thio-barbituric acid assay, as described by Wadood et al. (2015), was used to determine lipid per-oxidation. The absorbance of organic layer was taken at 532 nm and results were expressed as nanomole of malondialdehyde.

2.5. Statistical analysis

The data were analysed through Statistical Package for Social Science (Version 13, SPSS Inc., USA), using one-way analysis of variance (ANOVA). In case of significant differences among groups, Duncan’s Multiple Range Test was applied. Results have been presented as mean ± S.E.

3. Results

The results regarding post-thaw seminal attributes as affected by varying osmotic pressure of extender are given in Table 1. The spermatozoa motility, acrosomal integrity and DNA integrity were significantly (P < 0.05) affected by different osmotic pressures, whereas other spermatozoa characteristics remained unaffected. Post-thaw sperm motility, acrosomal integrity and DNA integrity were higher (P < 0.05) at 295 and 275 mOsm/kg compared to 255 mOsm/kg. However, differences in these parameters between osmotic pressures of 295 and 275 were non-significant.

4. Discussion

Conventional extenders are being used for buffalo bull semen cryopreservation. The osmolality of the solution used in extender preparation influences post-thaw sperm characteristics. Results of the current study demonstrated that spermatozoa motility reduced significantly (P < 0.05) with decline in osmotic pressure below 275 mOsm/kg, which may signify iso-osmolality of semen extender. The spermatozoa motility at 295 and 275 mOsm/kg was higher (11.33% and 7.66%, respectively) compared to 255 mOsm/kg. The effects of changes in osmotic pressure of the current study are in agreement with the findings
of Mughal et al. (2013), who used citrate egg yolk extender (CEYE). Similar findings of decreasing trend in motility of cattle spermatozoa with variation in osmotic pressure from 300 mOsm/kg using T EYE have also been reported by Liu and Foote (1998). A comparison of the results of sperm motility in the present study using T EYE and the one conducted by Mughal et al. (2013) reveals that T EYE presented a better motility (8.0%, 13.96% and 6.03% at an osmotic pressure of 255, 275 and 295 mOsm/kg, respectively) as compared to CEYE. Since, the reported osmotic pressure for buffalo bull semen by Mughal et al. (2013) is 289.4 mOsm/kg, the motilities at 295 mOsm/kg were significantly higher as compared to other two osmotic pressures. It was interesting to note that similar acrosomal integrity rate was recorded between 295 and 275 mOsm/kg. The acrosomal integrity rates at these osmotic pressures are in agreement with a previous study of Mughal et al. (2013) using CEYE.

The spermatozoa motility and viability are not affected by damaged acrosomes; however their ability to fertilize the ovum is severely disturbed (Graham 2001). The change in osmotic pressure from 295 to 255 mOsm/kg significantly disturbed acrosomal integrity (12.2%) and a pronounced decline was recorded at 255 mOsm/kg compared to other two osmotic pressures. It was interesting to note that similar acrosomal integrity rate was recorded between 295 and 275 mOsm/kg. The acrosomal integrity rates at these osmotic pressures are similar to the findings of Rasul et al. (2000) using tri-sodium citrate dihydrate extender of 301 mOsm/kg.

The spermatozoa are surrounded by the plasma membrane that holds various organelles and intracellular components and maintains chemical gradient of ions and soluble components. Transportation of ions or components also takes place through specific proteins located at the plasma membrane (Schurmann et al. 2002). Any change in its integrity may lead to spermatozoa death or renders them unable to fertilize the ovum. Results of the current study depicted non-significant effect of decreased osmotic pressure (295–255 mOsm/kg) on spermatozoa plasma membrane integrity. These findings are also allied to previous work of Mughal et al. (2013). It was noticed that plasma membrane integrity was non-significantly improved (4.29%) by lowering the osmotic pressure from 295 to 255 mOsm/kg.

The reproductive potential of the animals also relies on the status of the DNA located at the head region of spermatozoa. Any change in its foundation and integrity affects fertility rate. The spermatozoa DNA integrity was significantly affected (P < .05) by change in osmotic pressures. The mechanism responsible for this high DNA damage is not clearly understood. However, it is believed that inappropriate antioxidants in extended semen and high unsaturated fatty acids found in plasma membrane of spermatozoa may contribute towards this DNA damage (Aitken & Krausz 2001). It is assumed that at 255 mOsm/kg, this shield might be compromised. In buffalo bull semen, the presence of lesser amount of natural antioxidants and more unsaturated fatty acids compared with cattle makes spermatozoa highly susceptible to oxidative stress and lipid per-oxidation (Nair et al. 2006). In the current study, however, no difference in lipid per-oxidation was observed when osmotic pressure was lowered from 295 to 255 mOsm/kg.

### 5. Conclusion

The findings of the current study indicate that buffalo bull spermatozoa are sensitive to osmotic pressure of the solution used in extender preparation. Spermatozoa motility, acrosomal and DNA integrity were affected when the osmotic pressure was reduced to 255 mOsm/kg. Consideration of osmotic pressure may be useful in improving the quality of cryopreserved buffalo bull semen and optimum results can be achieved using iso-osmotic extenders. A pronounced change in osmolality may disturb spermatozoa characteristics and fertility rate.

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