The Journal of Animal & Plant Sciences, 23(4): 2013, Page: 964-968
ISSN: 1018-7081

ASSESSMENT OF OPTIMAL OSMOTIC PRESSURE OF CITRATE EGG YOLK EXTENDER FOR CRYOPRESERVATION OF BUFFALO BULL (*BUBALUS BUBALIS*) SEMEN

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ABSTRACT

The study was designed to assess the effects and optimal range of osmotic pressure of citrate egg yolk extender (CEYE) for cryopreservation of buffalo bull semen. Semen samples of four Nili-Ravi buffalo bulls, kept for artificial insemination (AI) purposes, were collected at weekly interval for five weeks. Five solutions of tri-sodium citrate dihydrate and D (-) fructose of varying osmotic pressures (255, 265, 275, 285 and 295 mOsm/kg) were used to prepare citrate egg yolk extender. The semen samples were frozen at -196°C using 20 x 10^6spermatozoa/0.5ml straw. The post-thaw evaluation was carried out in terms of spermatozoa motility, viability, acrosomal integrity, plasma membrane integrity (PMI), spermatozoa DNA integrity and lipid peroxidation. The buffalo bull spermatozoa motility was significantly ($P<0.05$) affected by varying osmotic pressures and the spermatozoa were more motile (50.3 ± 4.2 %) in the semen samples extended with CEYE at an osmotic pressure of 295 mOsm/kg compared to 275, 265 and 255 mOsm/kg. Other spermatozoa characteristics including viability, acrosomal integrity, PMI, DNA integrity and lipid peroxidation were remained same in all the treatments groups. The osmotic pressure of 285-295 mOsm/kg was considered to be most suitable for most of the spermatozoa characteristics for the cryopreservation of buffalo bull semen.

Keywords: Nili Ravi; semen quality; osmotic pressure; citrate extender; cryopreservation

INTRODUCTION

Buffaloes possess a vital position in rural livestock production and their population has increased manifold during the last decades. In Pakistan, rate of natural breeding in buffaloes is higher (83.9%) compared to breeding through artificial insemination (14.1%), whereas in only 2% animals, both breeding methods are being used (Younas *et al*., 2009). It is generally accepted that approximately 50% of buffalo spermatozoa are damaged during semen freezing process (Watson, 2000), which affects spermatozoa motility, acrosomal and chromatin integrity (Rasul *et al*., 2001; Mahmood and Ijaz, 2006; Khan and Ijaz, 2007 and 2008) resulting in low conception rate. A lower conception rate (33.0%) has been reported in buffaloes using cryopreserved semen (Bhosrekar *et al*., 2001).

Different semen extenders are being used to extend spermatozoon survival, increase in volume for multiple breeding. It is documented that buffalo spermatozoa are more susceptible to damage during cryopreservation than cattle spermatozoa (Raizada *et al*., 1990). This damage may be attributed to higher concentration of solute and ice crystal formation (Mazur, 1980). There is no special extender developed for buffalo semen, therefore, bovine semen extenders are commonly used for this purpose. Osmolality of cattle semen is ~ 300 mOsm/kg which is different from that of buffalo bull semen *i.e.* 268 mOsm/kg (Khan and Ijaz, 2008). Due to lower osmolality of buffalo semen, cryopreservation of buffalo bull semen in such extenders exerts an osmotic stress to spermatozoa and causes spermatozoa injury (Meyers, 2005). Semen extension in hypotonic or hypertonic solutions affects spermatozoa either by swelling or shrinkage of spermatozoa due to the movement of the water across the plasma membrane. During freezing and thawing process, spermatozoa again undergo a series of changes that once again affects the spermatozoa survival.

In addition to these factors, oxidants like reactive oxygen substances (ROS) and hydrogen peroxide (H_2O_2) are physiologically produced at low concentration and act as mediators without prominent effect on spermatozoa characteristics. However, ROS production at high concentration disturb enzymatic activity and spermatozoa functions (Baumber *et al*., 2000; Chatterjee and Gangon, 2001; Yousef *et al.*, 2003; Bucak *et al.*, 2007) and lead to low fertility potential of cryopreserved spermatozoa compared to fresh semen (Said *et al.*, 2005).

As AI industry in the country is making progress, and there is a dire need to optimize the buffalo bull semen extenders. Therefore, the present study aimed at evaluating the effects of different osmotic pressures of citrate egg yolk extender on post-thaw semen characteristics and to deduce an optimal osmotic pressure

of the extender for cryopreservation of buffalo bull semen.

MATERIALS AND METHODS

Animal housing and management: Four (Nili-Ravi) buffalo bulls, maintained at the Semen Production Unit, Qadirabad, Sahiwal, Pakistan were used for the collection of semen samples. The breeding bulls were fed seasonal fodder at 10 % of the body weight along with 2-3 kg concentrate on the daily basis. These bulls were reared under standard management conditions and had free access to drinking water during the entire study.

Preparation of extender: The extender was prepared as described earlier (of Nair *et al*., 2006) with little modification. Briefly, 30.0 g of tri-sodium citrate dihydrate (TSCD), (Merck 64271 Darmstadt, Germany) and 10 g D (-) Fructose (BDH Laboratory supplies, England) was dissolved in bi-distilled water to attain a final volume of 1000 ml. The pH of the solution was adjusted at 7.0. Later on, the solution was divided in five equal parts to prepare required osmotic pressures *i.e*. 295, 285, 275, 265 and 255 mOsm/Kg with bi-distilled water using an osmometer (Osmomat 030, Gonotec Berlin, Germany). These solutions were kept at 65°C for half an hour in a hot water bath, and then each osmotic pressure maintained solution was further divided in five parts and stored at -40 °C (MDF-U5411, Sanyo, Japan). These stored solutions were maintained at 37°C prior to extender preparation and to prepare 100 ml citrate egg yolk extender of each osmotic pressure, benzyl penicillin (1000 I.U/ml; Sinochem, China), streptomycin sulphate (1000 µg/ml; China National Medicine and Health Products Chongqing, China), egg yolk (20%; v:v) and glycerol (7%; vol/vol; Scharlab S.L. Spain) were added. These extenders were mixed and stored overnight at 4 ^{o}C and maintained at 37°C before semen extension.

Semen collection, evaluation and processing: Artificial vagina maintained at 42°C, was used to collect semen (Andrabi *et al*., 2008). Two consecutive ejaculates were collected from each bull at weekly interval for 5 weeks during the months of December and January. After collection, semen was maintained at 37°C for 15 minutes in a hot-water bath (holding time) before dilution. Semen samples having more than 70% motility were selected for further processing. Equal volume of each ejaculate was mixed to get enough semen volume and replicates were prepared to remove bull to bull variation**.** The pooled semen was extended with each osmotic pressure maintained extender to attain a final concentration of 20 x 10^6 spermatozoa per 0.5 ml straw (Foote and Kaproth, 1997). Semen straws were processed and stored in liquid nitrogen at -196°C for evaluation.

The following characteristics were evaluated to assess the effect of CEYE for the cryopreservation.

Spermatozoa motility: Thawing of frozen semen straws was carried out at 37°C for 30 seconds in a hot-water bath and spermatozoa motility rate was assessed following the procedure of Ijaz *et al*. (2009) at X400 under a phase-contrast microscope (Labomed Lx 400, U.S.A)

Spermatozoa viability: A thin smear of thawed semen and Eosin and Nigrosin as described by Zemjanis (1970) was prepared on a microscopic slide and evaluated it under a phase contrast microscope (X1000). Heads of live spermatozoa remained unstained, while stained or partially stained heads of the spermatozoa were considered as dead spermatozoa.

Spermatozoa acrosomal integrity: For the evaluation of acrosomal integrity, 500 µl of thawed semen was mixed in a test tube with 50 µl of 1% formaldehyde citrate. The spermatozoa (200 in number) were evaluated for their normal apical ridge using a phase-contrast microscope at X1000 (Khan and Ijaz, 2007).

Spermatozoa plasma membrane integrity: To assess PMI for each osmotic pressure of the extender, hypo-osmotic swelling (HOS) test as described by Adeel *et al*. (2009) was used. Observation was made under a phase-contrast microscope (X400) and two hundred spermatozoa were counted for swelling/coiling of tail.

Spermatozoa DNA integrity: Acridine Orange (AO) staining technique was employed to test spermatozoa DNA integrity following the procedure of Tejada *et al*. (1984). The spermatozoa were assessed at X1000 under a fluorescent microscope (Labomed, Lx 400, U.S.A.). Spermatozoa with intact (double stranded) DNA fluoresced green, while spermatozoa with red or yellow-orange to red fluorescence were counted as spermatozoa with single stranded DNA.

Lipid peroxidation: Malondialdehyde, a stable lipid per-oxidation product was determined by thio-barbituric acid assay as previously described (Ohkawa *et al*., 1979) and absorbance was measured at 532 nm using a spectrophotometer (UV 2800, BMS, Canada). Results were expressed as nm of MDA.

Statistical analysis: Data were analyzed using Statistical Package for Social Science (Version 13, SPSS Inc., USA). The results were presented as Mean ± S.E. The data were analyzed by using one-way analysis of variance (ANOVA). Results were considered significant at $P<0.05$ and the differences in groups were compared by the Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

The effect of different osmotic pressures on various spermatozoa characteristics of buffalo bull semen

is presented in Table 1. The spermatozoa motility was significantly (P<0.05) affected by varying osmotic pressures, however, all other parameters including spermatozoa viability, acrosomal integrity, plasma membrane integrity, DNA integrity and lipid peroxidation were not influenced (P > 0.05) by the changes in the osmolality. The spermatozoa were more motile (50.3%) at osmotic pressure of 295 mOsm/kg compared to that for 255, 265, 275 mOsm/kg. During semen cryopreservation, spermatozoa have to face an-isosmotic environment before, during and after cooling (Woods *et al*., 2004) and this environment must be maintained within 92-103% in order to maintain 90% motility rate (Guthrie *et al*., 2002). The higher post-thaw motility along with complete functional integrity of various components of spermatozoa is considered a desirable trait to assess the fertility potential. In the present study, the maximum spermatozoa motility (50.3%) was noted in the CEYE at an osmotic pressure of 295 mOsm/kg. During the current study, the osmotic pressure of the buffalo bull semen recorded was 289.4 mOsm/kg. The spermatozoa motility rate at osmotic pressure of 285 and 295 mOsm/kg is higher compared to 275, 265 and 255 mOsm/kg in this study. This confirms the findings of Guthrie *et al*. (2002) that maximum spermatozoa motility rate can be achieved when the osmotic pressure of the extender is towards iso-osmotic. The results of the spermatozoa motility of the present study are similar to the results of Anzar and Graham (1995) who reported 51% spermatozoa motility in Holstein bulls using osmotic pressure of 300 mOsm/kg.

The spermatozoa viability is another characteristic to evaluate the semen quality. The spermatozoa viability results of the current study demonstrated that different osmotic pressures had non-significant (P>0.05) effect. However, it was interesting to know that spermatozoa viability rate increased as the osmotic pressure was lowered to 255 mOsm/kg. This indicates that semen samples may have lower spermatozoa motility rate with higher viability rate. Our findings at different osmotic pressures of CEYE are better as compared to those of Shoae and Zamiri (2008), who used egg yolk sodium citrate extender in Holstein bulls and recorded 51.7% viable spermatozoa.

The spermatozoa have a cap like structure over the anterior end, surrounded internally and externally by an acrosomal membrane. Healthy spermatozoa must retain this membrane throughout freeze-thaw process and start acrosomal reaction in female tract (Thérien and Manjunath, 2003). The spermatozoa with damaged acrosome may be motile and viable, but may not be able to fertilize an ovum (Graham, 2001). The findings of this study indicated that different osmotic pressures had non-significant (P>0.05) effect on the spermatozoa acrosomal integrity. It was an interesting observation that acrosomal integrity was dropped (67.6 to 58.8%) as the osmotic pressure was dropped to 285 mOsm/kg. This indicates that maximum acrosomal integrity of spermatozoa can be maintained when the osmotic pressure of the extender is close to osmtic pressure of the semen. Current findings of the study at different osmotic pressures are relatively higher compared to the results of Shoae and Zamiri (2008), who reported spermatozoa acrosomal integrity of 50.5% for Holstein bulls. On the other hand, Rasul *et al*. (2000) reported 69.2 % acrosomal integrity in tri-sodium citrate dihydrate extender having an osmotic pressure of 301 mOsm/kg. It is speculated that this difference in spermatozoa acrosomal integrity might be due to change in pH of the buffer, composition of the extender, transition and storage period of spermatozoa in male reproductive system. Likewise, the spermatozoa PMI response was also non-significantly (P>0.05) affected at various osmotic pressures. However, plasma membrane swelling rate increased as the osmotic pressure was dropped to 285 mOsm/kg. This result confirms the finding of Nie and Wenzel, (2001) that spermatozoa PMI increased as the osmolarity was lowered. Our results are comparatively better than those reported by Rasul *et al*. (2000), with *tri*-sodium citrate di-hydrate with an osmotic pressure of 301 mOsm/kg (45.2%). This variation in results might be due to the change in pH and composition of the extender used with different osmotic pressure of the media.

Reproductive potential of bovines also depends on the quality of DNA present in the spermatozoa head. Any defect in chromatin structure and integrity negatively affect the fertility of animals. The osmotic pressures in the present study failed to exert any significant (P>0.05) effects on spermatozoa DNA integrity. However, our findings at 295mOsm/kg are closely in agreement to the findings of Martin *et al*. (2007), who reported 0.5% spermatozoa with damaged DNA. In buffalo bull semen comparatively higher DNA fragmentation rate was recorded by Kadirvel *et al*. (2012). Oxidative stress exerts pronounced affect on buffalo bull spermatozoa compared to cattle (Nair *et al*., 2006). The varying levels of osmotic pressure on the lipid peroxidation response in the current study was found to be non-significant (P>0.05).

In conclusion, the spermatozoa were more motile in the semen samples extended with CEYE with an osmotic pressure of 295 mOsm/kg. However, the other parameters were remained same in all the treatments groups. The osmotic pressure of 285-295 mOsm/kg was considered to be most suitable for most of the spermatozoa characteristics for the cryopreservation of buffalo bull semen. This study reports a preliminary data and envisages for further studies in a larger time span with a larger population and sample size.

Table 1. Post-thaw spermatozoa characteristics under various osmotic pressures of buffalo bull semen.

Osmotic pressure (mOsm/kg)	Spermatozoa characteristics (%)					Lipid peroxidation (nm)
	Motility	Viability	Acrosomal integrity	PMI	DNA integrity	
255	37.0 ± 3.6[b]	65.1 ± 2.5	58.8 ± 2.9	58.19 ± 2.9	97.6 ± 0.4	42.8 ± 8.2
265	36.0 ± 3.4[b]	66.5 ± 3.8	59.7 ± 2.3	54.85 ± 5.0	97.8 ± 0.2	27.1 ± 5.0
275	38.7 ± 3.7[b]	63.0 ± 4.4	59.8 ± 3.7	55.12 ± 3.6	98.1 ± 0.2	46.3 ± 9.1
285	45.7 ± 3.8[ab]	61.2 ± 4.3	67.6 ± 2.9	49.71 ± 3.9	98.0 ± 0.1	53.1 ± 12.7
295	50.3 ± 4.2[a]	60.3 ± 3.7	64.0 ± 3.1	54.18 ± 3.3	98.3 ± 0.2	41.3 ± 10.6

Values are represented as Mean ± S.E. [a,b]Means in column with letter are significant ($P < 0.05$).

Acknowledgement: The authors thank the Higher Education Commission, Pakistan, for providing financial support to carry out this trial.

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