CHOLESTEROL SUPPLEMENTATION IN EXTENDER IMPROVES QUALITY OF FROZEN-THAWED NILI-RAVI BUFFALO BULL SEMEN

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ABSTRACT

The study was designed to investigate the effect of cholesterol supplementation in extender on post-thaw quality of cryopreserved Nili-Ravi buffalo bull spermatozoa. Semen was collected from three Nili-Ravi buffalo bulls with artificial vagina (42°C) for five weeks ie., two ejaculates/ week and bull (replicate; n=30). Two consecutive ejaculates from each bull were mixed and processed for initial evaluation, semen from each bull was split into four aliquots and diluted (37°C) in *tris*-citric acid extender having cholesterol 0.0 (control), 5.0, 10.0 and 20.0 ng/mL Diluted semen was cooled to 4°C in 2 h and equilibrated for 4 hours at 4°C. Cooled semen was filled in 0.5 ml French straws at 4°C, kept on liquid nitrogen vapours for 10 min. and plunged in liquid nitrogen for storage. Frozen semen was thawed after 24 h at 37°C for 30 seconds. Sperm progressive motility, plasma membrane integrity and viability were higher ($P \leq 0.05$) in extender containing 5.0 ng/mL of cholesterol. However, cholesterol did not provide any significant benefit for chromatin integrity of buffalo spermatozoa. In conclusion, cholesterol supplementation in extender at a concentration of 5.0 ng/mL improved the post-thaw quality of cryopreserved Nili-Ravi buffalo bull spermatozoa.

Keywords: cryopreserved, buffalo, bull spermatozoa, cholesterol

INTRODUCTION

Several cryopreservation protocols and freezing diluents have been developed for cryopreservation of buffalo bull semen (Ansari *et al.*, 2010; 2012; Akhter *et al.*, 2012; Ejaz *et al.*, 2014) but conception rate using frozen thawed semen is low (Akhter *et al.*, 2007; Akhter *et al.*, 2010). The low conception rate with cryopreserved semen is attributed to many stresses on spermatozoa during cooling and freezing steps, including destabilization of the plasma membrane (Steponkus *et al.*, 1983).

Cholesterol controls membrane structure by interacting with the phospholipid hydrocarbon chains (Darin-Bennett and White, 1977) and at temperatures below the phase transition, forces the chains apart, making the membrane more stable (Quinn, 1989). Cryo-damage during freeze-thawing can be minimized by providing cholestrol in freezing diluents (Wessel and Ball, 2004). It was reported that membrane cholesterol to phospholipid ratio influences the sperm sensitivity to cold shock

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damage (Holt, 2000), sperm possessing high cholesterol/phospholipid ratios (rabbit and human sperm; 0.88 and 0.99 respectively; Davis, 1981) are more resistant to the "cold shock" damage than sperm having low cholesterol/phospholipid ratios like boar, ram and bull sperm (White, 1993). i.e; 0.35, 0.37 and 0.45 respectively (Davis, 1981). Buffalo bull semen contain 91.84±3.91–141.88±3.12 mg⁄ 100 ml of cholesterol (Mohan *et al.*, 1979) and is more susceptible to cold shock than cattle bull (Andrabi *et al.*, 2008) having 104–412 mg⁄ 100 ml (Roy Choudhury, 1970) of cholesterol in whole semen. Cholesterol content in bull spermatozoa is 300 µg $l0^{-9}$ sperm (Darin-Bennett and White, 1977). The cholesterol to phospholipid ratio in bull spermatozoa is 0.529±0.097and in seminal plasma is 0.548±0.188 (Jain and Anand, 1976). Chloestrol has been extensively studied for improving semen quality directly and/or indirectly through cholesterol loaded methyl-β-cyclodextrins and found beneficial in bull (Purdy and Graham, 2004; Combes *et al.*, 1998) stallion (Combes *et al.*, 2000; Moore *et al.*, 2005) donkey (Alvarez *et al.*, 2006) boar (Galantino-Homer *et al.*, 2006) rabbit (Serin *et al.*, 2011) ram (Morrier *et al.*, 2004; Awad, 2011) goat (Konyali *et al.*, 2013) and bison (Hussain *et al.*, 2013).

To our knowledge, information on the effect of cholesterol addition in semen extender for the cryopreservation of buffalo spermatozoa is lacking in published literature. It was hypothesized that cholesterol addition in extender may improve post-thaw quality of Nili-Ravi buffalo bull spermatozoa. Therefore, this study was conducted to determine the effect of different concentrations of cholesterol in extender on post-thaw motility, plasma membrane integrity, viability and chromatin integrity of buffalo bull spermatozoa.

MATERIALS AND METHODS

Preparation of extenders

Tris-citric acid (pH 7.0; osmotic pressure 320 mOsmol Kg^{-1}) was used as a buffer, consisted of 1.56% citric acid (Fisher Scientific, Loughborough, Leicestershire, UK), 3.0% tris–(hydroxymethyl)-aminomethane (Research Organics, Cleveland, OH, USA), 0.2% w/v fructose (Scharlau, Barcelona, Spain), 7.0% glycerol (Merck, Darmstadt, Germany), egg yolk 20% v/v, antibiotics; benzyl penicillin (1000 IU mL^{-1}) and streptomycin sulphate (1000 µg mL^{-1}) in 74 mL distilled water. Four different experimental extenders were prepared having 0.0 ng/ml (extender I), 5.0 ng/ml (extender II) 10.0 ng/ml (extender III) and 20.0 ng/ml (extender IV) of cholesterol (Sigma Chemical Co., St. Louis, MO, USA). Because of the insolubility of cholesterol in water, ethanol 0.05% was used. Cholesterol was mixed in 0.05% of ethanol and then added in extender.

Semen collection and initial evaluation

Two consecutive ejaculates were collected from each bull/week in a graduated tube with the aid of artificial vagina (42°C) at weekly intervals for a period of five weeks (replicate). Two consecutive ejaculates from each bull were mixed in a graduated tube and immediately transferred to the laboratory for initial evaluation (volume, motility and concentration). Visual motility of the spermatozoa was assessed under phase contrast microscope (400x; 37°C; ANSARI *et al.*, 2012). Sperm concentration was assessed by Neubauer haemocytometer. The qualified semen ejaculates (>0.5 ml volume, >60% motility, >0.5 × 10^9 sperm ⁄ ml concentration) from three bulls were split into four aliquots and held for 15 minutes at 37°C in the water bath before dilution in four different

experimental extenders.

Semen processing

Each semen aliquot was extended in Tris citric acid extender (37°C; 50 × 10^6 motile spermatozoa / ml) containing different levels of cholesterol 5.0, 10.0 and 20.0 ng/ml (Sigma Chemical Co., St. Louis, MO, USA). while extender without cholesterol was kept as control. Diluted semen was cooled to 4°C in 2 h at the rate of 0.275°C $minute^{-1}$ and equilibrated for 4 h at 4°C. Semen was then filled in 0.5 ml French straws (IMV, France) with the aid of a suction pump at 4°C in the cold cabinet unit and kept on liquid nitrogen vapours (5 cm above the level of liquid nitrogen) for 10 minutes. Straws were then plunged and stored into liquid nitrogen (-196°C). After 24 h, semen straws were thawed in a water bath at 37°C for 30 seconds. For each treatment, semen from three straws (from same replicates) was pooled and incubated at 37°C for assessment of post-thaw semen quality after thawing.

Sperm progressive motility

A drop (5 µl) of thawed semen sample was placed on a prewarmed glass slide and covered with cover slip, progressive motility was assessed under phase contrast microscope (400X; 37°C) with closed circuit television (Akhter *et al.*, 2011).

Structural and functional integrity of sperm plasma membrane

Structural and functional integrity of sperm plasma membrane was assessed using supravital hypo-osmotic swelling test (HOST) as described by Tartaglione and Ritta (2004). Solution for HOS assay was consisted of 0.73 g sodium citrate (Merck) and 1.35 g fructose (Scharlau, Barcelona, Spain) in 100 ml distilled water (osmotic pressure 190 mOsmol/kg). For the assessment of structural and functional integrity of sperm plasma membrane, 50 µL of the semen sample was mixed with 500 µL of the pre-warmed HOS solution, and incubated at 37°C for 30-40 minutes. After incubation a drop of mixture was placed on a slide, cover slipped and visualized microscopically (400X magnification), one hundred spermatozoa per experimental extender were evaluated in five different fields. Swollen tails of sperm were indicated as intact, biochemically active sperm membranes, while unswollen tails were indicated as disrupted, inactive, non functional sperm membranes (Chan *et al.*, 1991).

Acrosome intact live sperm

Acrosome intact live sperm was assessed using trypan blue Giemsa stain as described by Kovacs and Foote (1992). Briefly, equal drops of trypan blue and semen were placed on a slide, mixed quickly and air-dried. The samples were fixed with formaldehyde-neutral red for 5 minutes, rinsed with running distilled water and followed by the application of 7.5% Giemsa stain for 4 h. After rinsing with distilled water samples were air dried at room temperature and mounted with Canada Balsam. One hundred spermatozoa per experimental extender were evaluated in at least five different fields in each smear under phase contrast microscope at 1000X. Trypan blue penetrates nonviable spermatozoa with disrupted membrane, which stain in blue, while live sperms with intact acrosome appeared unstained. Giemsa accumulates in spermatozoa with an intact acrosome, staining the acrosome region in purple.

Sperm chromatin integrity

Sperm chromatin integrity was assessed as practiced by Ejaz *et al.* (2014). Air dried smears

of semen samples were fixed in 96% ethanol-acetone (1:1) at 4°C for 30 minutes, hydrolyzed with 4N HCl at 25°C for 10-30 minutes. Smears were suspended in distilled water, three times for two minutes each. The slides were stained with toluidine blue in McIlvaine buffer (sodium citrate-phosphate) for 10min. Samples were air dried and mounted with Canada Balsam. One hundred spermatozoa per experimental extender were evaluated in at least five different fields in each smear under light microscope at 1000X. Toluidine blue stain penetrates in sperms having damaged chromatin staining from dark blue to purple while spermatozoa having intact chromatin were stained light blue.

Statistical analysis

The data on sperm post-thaw quality parameters were analyzed using analysis of variance (ANOVA) in randomized complete block design using fixed effect model and were presented as mean (±SE). When F-ratio was found significant ($P<0.05$), LSD test was used to compare the treatment means.

RESULTS AND DISCUSSION

Mammalian sperm have varying degrees of cold shock susceptibility depending on the membrane phospholipid composition as well as the membrane cholesterol to phospholipid ratio (Holt, 2000). The molar ratio of cholesterol: phospholipid in bull spermatozoa is 0.45 (Darin-Bennett and White, 1977). Cryopreservation causes loss of cholesterol from the sperm plasma membrane, leading to a premature acrosome reaction and shorter life (Bailey *et al.*, 2000). In present study, effects of cholesterol supplementation in extender were studied for motility, plasma membrane integrity, viability and DNA integrity of buffalo bull spermatozoa. The results on the effect of cholesterol in extender on progressive motility, structural and functional integrity of sperm plasma membrane, acrosome intact live sperm and chromatin integrity (%) of buffalo bull spermatozoa are presented in Table 1.

Sperm progressive motility was higher (41.33±3.481; in extender containing 5.0 ng/ml of cholesterol while it decreased in extender containing 10.0 (32.00±0.577) and 20.0 ng/ml (31.67±1.668) of cholesterol compared to control ($P < 0.05$). Membrane phase separation during

Table 1. Effect of cholesterol addition in extender on post-thawed semen quality of buffalo bull spermatozoa.

Cholesterol (ng/ml)	Sperm progressive motility (%)	Structural and functional integrity of sperm plasma membrane (%)	Acrosome intact live sperm (%)	Sperm chromatin integrity (%)
0.00	38.333±2.60 ^{b}	67.33±2.628 ^{b}	34.63±1.71 ^{b}	96.6±0.43
5.00	41.33±3.481 ^{a}	71.16±2.5 ^{a}	37.56±1.87 ^{a}	96.56±0.65
10.00	32.00±0.577 ^{c}	67.93±1.338 ^{b}	34.16±0.97 ^{b}	96.66±1.05
20.00	31.67±1.668 ^{c}	67.66±0.185 ^{b}	34.4± 1.40 ^{b}	96.83±0.71

The values with different superscript within the column differ significantly ($P<0.05$).

cryopreservation can result in loss of intracellular molecules, which for sperm can result in irreversible motility loss (Drobnis *et al.*, 1993). Cholesterol addition in extender might stabilize the membrane phase transition resulting in increased sperm progressive motility, while higher concentrations of cholesterol in extender might stiffen sperm membrane and decrease its motility. Similar studies reported improvement in percentage of motile sperm when cholesterol-loaded methyl-β-cyclodextrin were used in cryopreserved bull (Awad and Graham, 2002) stallion (Combes *et al.*, 1998; Graham, 1998) ram (Bailey *et al.*, 2002) and boar (Blackburn, 2003) semen.

The susceptibility of the sperm plasma membrane to undergo lipid phase transitions during cooling is inversely related to the proportion of cholesterol present. Lower cholesterol/ phospholipid ratio is present in bull and ram sperm than rabbit and human which are considered to be sensitive to cooling (Darin-Bennett and White, 1977). Structural and functional integrity of sperm plasma membrane was higher (71.16±2.5; $P < 0.05$) in extender containing 5.0 ng/ml of cholesterol while at higher concentration of cholesterol i.e., 10.0 (67.93±1.338) and 20.0 ng/ml (67.66±0.185) it remained similar ($P \geq 0.05$) to that of control. It has been demonstrated that adding cholesterol to bull sperm membrane prior to cryopreservation improved cell cryosurvival rates by increasing membrane fluidity at low temperature (Purdy *et al.*, 2005). Addition of cholesterol loaded methyl β-cyclodextrin improved membrane integrity in bull sperm (Amorim *et al.*, 2009). In this study, higher concentrations of cholesterol (10.0 and 20.0 ng/ml) have no significant effect on structural and functional integrity of sperm plasma membrane showing that 5.0 ng/ml of cholesterol is adequate quantity to improve structural and functional integrity of cryopreserved buffalo bull sperm plasma membrane.

The trend for the effect of cholesterol on acrosome intact live sperm of buffalo bull spermatozoa remained the same as for structural and functional integrity of sperm plasma membrane. Acrosome intact live sperm was improved in extender containing 5.0 ng/ml of cholesterol (37.56±1.87, $P < 0.05$) while it remained similar in extenders containing 10.0 ng/ml (34.16±0.97) and 20.0 ng/ml (34.4±1.40) of cholesterol and control ($P \geq 0.05$).

Cholesterol plays an important role in stabilizing sperm membrane (Purdy and Graham, 2004). Cholesterol might inhibit cryocapacitation which is initiated by removal of cholesterol from the membrane (Cross 1998; Visconti and Kopf, 1998) that increases membrane fluidity and causes rearrangements in membrane proteins (Flesch *et al.*, 2001; Travis and Kopf, 2002). Cholesterol supplementation in sperm may prevent the cryocapacitation, which in turn increases the longevity of the cells after thawing (Moce *et al.*, 2010) or it may inhibit calcium entry into the sperm, which is a prerequisite for capacitation (Visconti *et al.*, 1999). Previous studies suggested that addition of cholesterol loaded methyle β-cyclodextrin in extender improved viability of stallion (Combes *et al.*, 1998; Graham, 1998) and bull spermatozoa (Amorim *et al.*, 2009) after cryopreservation.

During cryopreservation, increased ROS production due to imbalance of antioxidants in seminal plasma is believed to affect nuclear membrane and spermatozoa DNA (Aitken and Krausz, 2001) and alter sperm chromatin structure (Donnelly *et al.*, 2001; Fraser and Strzezek, 2004; Hammadeh *et al.*, 2001; Peris *et al.*, 2004). In present study, sperm chromatin integrity remained similar in all experimental extenders containing

cholesterol. This indicates that chromatin is not critically damaged in cryopreserved buffalo bull sperm (Andrabi, 2009) due to presence of highly condensed nuclear proteins especially protamine-1 in sperm nucleus (Martins *et al.*, 2007; Van Der Schans *et al.*, 2000).

The exact mechanism by which cholesterol improves sperm cryosurvival is still not known. The additional cholesterol may broaden the phase transition of the sperm membranes, thereby reducing lipid of the same species from aggregating into specific domains within the membrane (Drobnis *et al.*, 1993) as well as increasing membrane stability at lower temperature (Purdy *et al.*, 2005) and by decreasing cold shock sensitivity. In conclusion, cholesterol addition at 5.0 ng/ml improved post thaw semen quality parameters of cryopreserved Nili-Ravi buffalo bull spermatozoa.

ACKNOWLEDGEMENTS

The authors thank to Higher Education Commission, Pakistan for financial support under 5000 PhD indigenous fellowship scheme.

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