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THE INFLUENCE OF TAURINE SUPPLEMENTATION IN LACTOSE EGG YOLK GLYCEROL EXTENDER FOR CRYOPRESERVATION OF BUFFALO BULL (*BUBALUS BUBALIS*) SEMEN

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ABSTRACT

The objective of this study was to assess the influence of supplementing various concentrations of taurine in lactose egg yolk glycerol extender (LEGE) on spermatozoa characteristics. Ejaculates of four routinely used Nili- Ravi buffalo bulls were collected once in a week for five weeks using an artificial vagina at Semen Production Unit, Qadirabad, Sahiwal, Pakistan. Pooled semen samples were diluted at 37°C in LEGE extender containing taurine (TA) (0.0, 20.0, 40.0, 60.0 mM) and aspirated into French straws (0.5 ml) having 20×10^6 spermatozoa. Filled and sealed semen containing straws were cooled from 37 to 4°C in a cold cabinet for 4 h before placing it 4 cm above liquid nitrogen (LN) vapors for 20 minutes and stored in LN. These stored straws were thawed individually at 37°C for 30 s in a water bath for evaluation of spermatozoa characteristics such as motility, viability, acrosomal/plasma membrane/DNA integrity and lipid peroxidation. The results indicate that spermatozoa motility (%) significantly decreased ($P<0.05$) in TA 60.0 mM supplemented group compared to TA 0.0 and 20.0 mM. However, the spermatozoa motility difference was non significant ($P>0.05$) among control (TA 0.0), TA 20.0 and TA 40.0 mM supplemented groups. Similarly, spermatozoa DNA integrity rate (%) was significantly decreased ($P<0.05$) in TA 40.0 and TA 60.0mM supplemented groups as compared to control group. In conclusion, TA supplementation in LEGE at 20.0, 40.0 and 60.0 mM was unable to produce significant effect on spermatozoa characteristics such as viability, acrosomal integrity, plasma membrane integrity and lipid peroxidation compared to control group.

Key words: Nili Ravi; semen quality; taurine; lactose extender; cryopreservation

INTRODUCTION

Rapid genetic improvements in mammals demand augmentation of artificial insemination (AI) Johnson *et al.,* (2000). Application of AI with frozen thawed semen is limited in buffalo due to poor freezability of buffalo bull spermatozoa compared to cattle bull spermatozoa (Kumaresan *et al*., 2005). The main hindrance to utilize cryopreserved buffalo bull semen is damage of spermatozoa plasma membrane during cryopreservation and thawing, which reduces post-thaw spermatozoa motility and viability (Holt, 2000). Spermatozoa must retain intact and functional plasma membrane during freezing and thawing process (Marti *et al.,* 2003). For this reason, semen extender is supplemented with cryoprotectants.

The efficacy of semen extender can be further enhanced by adding antioxidants such as taurine, trehalose and vitamin E (Aboagla and Terada, 2003). Presence of high phospholipid and unsaturated fatty acid contents in mammalian spermatozoa makes them more responsive to lipid peroxidation (LPO) (Aitken *et al.,* 1993). Protection to spermatozoa against LPO is naturally provided in seminal plasma via its constituent antioxidants (Strzezek *et al.,* 1999). For long term semen storage, these antioxidants levels are insufficient and may increase oxidative stress during the cryopreservation. It has also been reported that freeze-thaw process may also elevate reactive oxygen species (ROS) in semen and leads to further damage (Chatterjee and Gagnon, 2001). Antioxidants provide resistance to spermatozoa against oxidative stress (Cabrita *et al*., 2010) and prevent the excessive free radicals production (Aurich *et al*., 1997).

Taurine (2-aminoethane sulfonic acid) is the most abundant low-molecular-weight, free amino acid (Ekremoğlu *et al.,* 2007) found in the retina, heart, brain, skeletal muscle, leukocytes and semen of bull, pig and human (Zini *et al*., 2009; Ibrahim and Boldizsár, 1981). Its presence in the mammalian oviducts assists in spermatozoa capacitation, fertilization and protects spermatozoa against ROS (Sariözkan *et al*., 2009). Taurine has also been used to study the effects on spermatozoa motility, viability and plasma membrane integrity of rabbit, ram, bull, cat and buffalo (Uysal *et al*., 2007; Foote *et al.,* 2002) using different extenders. In the recent years, many studies have focused on modifying extenders to improve the quality of stored buffalo spermatozoa. To the best of our knowledge, the effect of taurine on the cryopreserved buffalo bull semen extended in LEGE has not been reported. Therefore, main

objective of the present study was to determine the effect of taurine supplementation in LEGE on post-thaw buffalo bull spermatozoa quality parameters such as motility, viability, acrosomal, plasma membrane, DNA integrity and lipid peroxidation.

MATERIALS AND METHODS

Animals and semen collection: Four Nili-Ravi buffalo bulls were housed at the Semen Production Unit (SPU), Qadirabad, Sahiwal, Pakistan, under uniform management and feeding conditions during the entire study. Semen was collected using an artificial vagina during the months of March and April. Two ejaculates were collected from each bull at weekly interval for 5 weeks. Immediately after semen collection, the ejaculates were placed in a water bath at 37°C for their assessment. Before the assessment fifteen minutes holding time was also observed, ejaculates with more than 70% motile spermatozoa were chosen for processing. At each collection, equal volumes of first ejaculate from four bulls were pooled to eliminate individual difference and divided in four equal aliquots. Three aliquots were diluted by either LEGE supplemented with TA 20.0, 40.0 and 60.0 mM, while the fourth aliquot was diluted in LEGE without TA and served as a control group.

Extender preparation: The composition reported by Chaudhari and Mshelia (2002) was used with slight modification. Briefly, 100.0 g of D (-) Lactose monohydrate (Scharlau Chemie, S.A., Spain) was dissolved in bi-distilled water to attain final volume of 1000 ml and thoroughly mixed using a vortex mixer. This solution was confirmed to have an osmotic pressure >300 mOsm/kg using a cryoscopic osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany).

The pH of this solution was maintained at 7.0 by adding N/10 NaOH solution drop by drop. By adding bi-distilled water, the osmotic pressure of this solution was maintained at 295 mOsm/kg. This solution was divided in to five equal parts and stored at -40°C in a biomedical freezer (MDF, U5411, Sanyo, Japan) until use. One night before the day of semen collection, one part of this solution was warmed in a water bath at 37°C and then pasteurized at 65°C for 30 min. Later on, this solution was cooled down to 37°C and divided in to four equal parts for the preparation of the extender.

To prepare LEGE, benzyl penicillin and streptomycin sulphate (1000 I.U/ml and 1000 µg/ml respectively), egg yolk (20%, vol/vol) and glycerol (5%, vol/vol) were added in each part, well mixed with a mixer and stored overnight at 4°C in a refrigerator. At each collection, these four prepared extenders were maintained at 37°C and TA was added in three LEGE at 20.0, 40.0 and 60.0 mM respectively, while the fourth extender was kept as the control group (TA 0.0).

Semen processing: Semen straws of 0.5 ml capacity having spermatozoa concentration of 20 x 10^6 (Foote and Kaproth, 1997) were filled, sealed and cooled from 37°C to 4°C in a cold cabinet and equilibrated for four hours (Andrabi *et al.,* 2006). These straws were placed horizontally on freezing grills in a freezing tank at 4 cm above liquid nitrogen (LN) for 20 minutes before merging them in to LN and stored in LN container for a week before evaluation. The following post-thaw spermatozoa characteristics were evaluated to study the effect of various concentrations of taurine supplemented in LEGE.

Spermatozoa motility: Frozen semen straws were thawed at 37°C for 30 seconds in a hot water bath. Percentage of motility was judged under a phase-contrast microscope at X400 as previously described by Asr *et al.* (2011).

Spermatozoa viability: A 50 µl drop of the thawed semen was placed on a pre-warmed (37°C) glass slide and mixed with 50 µl of Eosin and Nigrosin (Zemjanis, 1970) using an applicator stick. A uniform smear was prepared with the help of another microscopic slide. This smear was air dried before evaluating it under a phase-contrast microscope (X1000). The heads of two hundred spermatozoa were evaluated to find out viable spermatozoa (%). The spermatozoa with stained or partially stained heads were considered as dead spermatozoa. Whereas, the spermatozoa having unstained heads were counted as viable spermatozoa.

Spermatozoa acrosomal integrity: A 500 µl of thawed semen was mixed in a test tube with 50 µl of 1% formaldehyde citrate. Two hundred spermatozoa were evaluated for their normal apical ridge using a phase-contrast microscope at X1000 as described by Asr *et al*. (2011). Abnormalities in spermatozoa such as ruffled, swollen or absent acrosome were counted as damaged/abnormal apical ridge.

Spermatozoa plasma membrane integrity (SPMI): To assess SPMI, 500 µl of hypo-osmotic swelling (HOS) solution (0.735 g of tri-sodium citrate dihydrate and 1.351 g D (-) fructose) of 75 mOsm/kg was incubated with 50 µl of thawed semen in a hot water bath for 45 min at 37°C. Observation of small drop of incubated mixture was carried out under a phase-contrast microscope (X400) and two hundred spermatozoa were counted for swelling/coiling of tail (Adeel *et al*., 2009). The mean of three readings was counted as single data point.

Spermatozoa DNA integrity: Acridine orange (AO) staining technique following the procedure of Tejada *et al*. (1984) was used. Briefly, one drop of thawed semen was added on a microscopic slide and a thin smear was prepared using another microscopic slide. This smear was

air dried and fixed in Carnoy's solution (methanol and glacial acetic, 3:1) for 2 hours. These slides were removed from the solution, air dried and stained with freshly prepared AO (Scharlau Chemie S.A, Spain) in the dark for 5 minutes and finally washed with distilled water. The spermatozoa were observed at X1000 using fluorescent microscope (Labomed, Lx 400, U.S.A.) to assess intactness of DNA. The normal (double stranded DNA) spermatozoa fluoresced green while single stranded DNA spermatozoa fluoresced red or yellow-orange to red.

Lipid peroxidation: Thio-barbituric acid assay as previously described by Ohkawa *et al*. (1979) was used to determine malondialdehyde (MDA) and its 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 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

Results of the current study reveal that TA supplementation in LEGE at 60.0 mM significantly ($P<0.05$) affected the spermatozoa motility compared to TA 0.0 and 20.0 mM. The highest spermatozoa motility (49.0%) among the TA supplemented groups was observed in the 20.0 mM group. However, the comparison of spermatozoa motility of TA 0.0, 20.0 and 40.0 mM supplemented groups was non-significant ($P>0.05$). Similarly, the spermatozoa DNA integrity (%) was significantly affected in the groups supplemented with TA at 40.0 and 60.0 mM compared to the TA 0.0 mM group. The difference between TA supplemented groups (TA 20.0, 40.0 and 60.0 mM) was non significant ($P>0.05$). Supplementation of TA in LEGE did not produce any significant effects on the viability, acrosomal integrity, plasma membrane integrity and lipid peroxidation of the spermatozoa (Table 1).

Taurine (TA) is an intracellular amino acid found in majority of the mammalian tissues and plays its role in cell proliferation, viability, osmoregulation and prevents injuries induced by oxidants in many tissues (Chesney, 1985). It also maintains the stability of bio-membranes, scavenges ROS, minimizes the end products of lipid peroxidation (Huxtable, 1992), modulates Ca^{2+} uptake (Singh *et al*., 2012) and inhibits protein phosphorylation (Kumar and Atreja, 2012). The spermatozoa motility in LEGE supplemented with TA exhibited significant ($P<0.05$) effects and it decreased the motility by increasing the concentrations of TA. The findings of spermatozoa motility in the control group (49.7%) are similar to the reports of Sariözkan *et al.* (2009) and Bucak *et al*. (2007) who reported 49.6 and 47.5% motile spermatozoa with Bioxcell extender for bull semen and Tris based extender for ram semen, respectively. The results of the present study are comparatively higher than those reported by Ahmad, (1984) i.e., 40.0% in buffalo bulls, and relatively lower at 40.0 and 60.0 mM of TA as compared to the reports of Reddy *et al*. (2010) and Bucak *et al*. (2007) using 50 mM of taurine in Murrah buffalo bulls and rams, which were 48.3 and 56.0%, respectively. As per early report of Uysal *et al*. (2007), it is not necessary that all spermatozoa characteristics are affected at the same time under same supplemented concentration in the extender. Similarly, previous reports indicate that TA addition improved post-thaw motility of ram semen but it did not enhance spermatozoa motility using whole milk in bulls (Chen *et al*., 1993) and fish (Ekici *et al*., 2012). Different factors including: specie difference, technique used for semen collection, composition of the extender, concentration of the cryoprotectant used (Reddy *et al*., 2010), thawing duration, time taken by the observer, and disordered functioning of axonemal proteins may contribute towards the low motility rate. In the current study LEGE supplemented with various concentrations of TA could not produce significant effect on various spermatozoa characteristics compared to the control group. It might be due to the reasons: we used lactose media with maintained osmotic pressure (295 mOsm/kg) in preparing LEGE and secondly we used different extender (LEGE) for supplementation of TA. Currently, there is no data available regarding the use of LEGE supplemented with TA in buffalo bull semen.

The spermatozoa viability of various concentration of TA supplemented extender showed non-significant ($P>0.05$) effect. As compared to this study, higher spermatozoa viability was reported by Bucak *et al*. (2007) in rams using Tris base extender supplemented with TA at 25.0 and 50.0 mM, which were 73.0 and 69.0% respectively. However, the spermatozoa viability at concentration of 40.0 and 60.0 mM of TA was comparatively lower compared to the control group of the present study. These results demonstrated that increasing concentration of taurine did not improve the spermatozoa viability rather it showed negative effects. The spermatozoa acrosomal integrity in the TA supplementation had a non-significant ($P>0.05$) effect. Addition of TA at 20.0 mM depicted acrosomal integrity (72.4%) while, this percentage dropped as the concentration of TA was further increased. Our findings at different concentrations of TA were significantly lower as reported by Sariözkan *et al.* (2009) in bulls and Bucak *et al*. (2007) in rams. This variation in results might be due to the variation in TA concentration, extender, species and freezing protocol used by the authors.

The spermatozoa PMI of TA supplemented groups did not vary significantly. However, the highest PMI was observed by supplementing TA at 40.0 mM (62.9%). These results are comparatively higher than those of Sariözkan *et al.* (2009) in bulls and Bucak *et al*. (2007) in rams, which were 46.6 and 43.6% respectively with TA supplemented at 2.0 and 50.0 mM, respectively. This difference in results of spermatozoa PMI may be due to species variation, concentration of antioxidants and type of extender used. The spermatozoa head DNA integrity was lowered by the supplementation of TA. Exact mechanism responsible for DNA damage is not understood. However, high ROS production due to imbalance of antioxidant in seminal plasma and high contents of unsaturated fatty acids in spermatozoa plasma membrane are believed to affect nuclear membrane and spermatozoa DNA (Aitken and Krausz, 2001). Spermatozoa lipid peroxidation response was non-significant (P>0.05) in TA supplemented groups. However, minimum lipid peroxidation value were recorded at 20.0 mM, which indicate that minimum ROS production was taken place at this concentration of TA and it may stand for good semen quality (Chaudhari *et al*., 2008).

In the current study, non-significant results of spermatozoa viability, acrosomal integrity, SPMI rates and higher DNA damage (%) by supplementation various concentrations of TA might be due to toxic effects of TA, as high antioxidant concentration lowers physiological level of oxidants needed for normal functioning of spermatozoa (Roca *et al*., 2004) and make spermatozoa susceptible to cryo-injury by increasing the plasma membrane fluidity (Shoe and Zamiri, 2008). As a result, it weakens the function of ionic channels/ATPases in membrane and disturbs nutrients entry into the spermatozoa leading to spermatozoa damage. During buffalo semen cryopreservation, higher oxidative stress due to high polyunsaturated fatty acids in spermatozoa plasma membrane affects DNA (Aitken and Krausz, 2001) by breaking their strands (Cordova *et al*., 2002). It transmits defective genome (Sakkas and Alvarez, 2010) and causes fertilization failure or embryo death or poor embryo growth (Aitken *et al*., 1998). It is assumed that spermatozoa damage is a cascade of events that reduce membrane integrity, damages DNA and finally loss of sperm viability. Interestingly, all these spermatozoa characteristics in buffalo semen did not improve by supplementing TA.

Table 1. Post-thaw spermatozoa characteristics under various concentrations of TA extended in lactose egg yolk extender

TA Concentration (mM)	Post-thaw spermatozoa characteristic (%)					Lipid peroxidation (nm)
	Motility	Viability	Acrosomal Integrity	Plasma Membrane Integrity	DNA Integrity	
0.0	49.7 ± 2.5[a]	57.9 ± 3.3	69.8 ± 2.2	59.6 ± 3.4	98.9 ± 0.2[a]	65.9 ± 8.9
20.0	49.0 ± 1.8[a]	58.4 ± 2.4	72.4 ± 2.3	57.2 ± 2.5	98.5 ± 0.2[ab]	57.7 ± 10.6
40.0	45.3 ± 2.0[ab]	57.7 ± 2.6	71.5 ± 1.7	62.9 ± 1.4	98.3 ± 0.2[b]	63.8 ± 8.2
60.0	41.0 ± 2.0[b]	57.4 ± 2.3	70.6 ± 2.0	52.9 ± 3.7	98.2 ± 0.1[b]	61.6 ±8.3

Values are represented as Mean ± S.E.

Different letters (a-b) within the same column indicate significant differences (P<0.05) among the groups

Conclusion: The results of the current study indicate that in lactose egg yolk glycerol extender, the spermatozoa motility significantly decreased in the TA 60.0 mM supplemented group compared to the TA 0.0 group. Similarly, DNA integrity was also lowered in groups supplemented with TA at 40.0 and 60.0 mM. However, supplementation of TA at 20.0 mM and above could not produce any significant effect on the rest of spermatozoa characteristics. This study reports a preliminary data and envisages for further studies of TA supplementation below 20 mM for cryopreservation of Nili-Ravi buffalo bulls using lactose egg yolk glycerol extender.

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