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Effect of α-Tocopherol Acetate and Ascorbic Acid in Extender on Quality of Zebu Bull Spermatozoa

Arfa Batool,[1] Khadija Mehboob,[1] Saima Qadeer,[1] Muhammad Sajjad Ansari,[2] Bushra Allah Rakha,[1] Nemat Ullah,[3] Syed Murtaza Hassan Andrabi[4] and Shamim Akhter[1]*

[1]Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, 46300, Pakistan
[2]Department of Zoology, University of Gujrat, Gujrat, Pakistan
[3]Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi
[4]Animal Reproduction Laboratory, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, Pakistan

Abstract.- The experiments were designed to determine the effect of α-tocopherol acetate and ascorbic acid in extender on post-thaw motility, plasmalemma functionality, viability and chromatin damage of Zebu bull spermatozoa. Semen collected from three bulls of Sahiwal breed (*Bos indicus*) was diluted with tris-citric acid extender either containing α-tocopherol acetate or ascorbic acid at 0.5, 1.5, 2.5 and 3.5 mM or without any supplement (control) and cryopreserved. For each extender, thawing of three semen straws was performed at 37°C for 30 seconds for the assessment of sperm quality parameters. Sperm motility (%) remained similar in extenders containing α-tocopherol at 0.5. 1.5, 2.5 mM and control. Sperm plasmalemma functionality (%) was recorded higher ($P < 0.05$) in extender containing 0.5, 1.5 and 2.5 mM α-tocopherol acetate than control. Sperm viability (%) was observed higher ($P<0.05$) in extender containing 0.5 mM α-tocopherol acetate. Sperm chromatin damage was similar ($P > 0.05$) in all experimental extenders containing α-tocopherol acetate and control. Extenders containing ascorbic acid 0.5, 1.5, 2.5, 3.5 mM did not improve sperm motility, plasmalemma functionality, viability and chromatin integrity. In conclusion, α-tocopherol acetate (0.5 mM) in extender improved the plasmalemma functionality and viability of frozen-thawed Zebu bull spermatozoa.

Keywords: Zebu bull spermatozoa, α-tocopherol, ascorbic acid.

INTRODUCTION

Cryopreservation is an oxidative process that reduces the viability of bovine semen upto 50% (Bilodeau *et al*., 2000). Sperm cells are more prone to reactive oxygen species (ROS) molecules (Baumber *et al*., 2005) which cause lipid peroxidation due to higher contents of polyunsaturated phospholipids present in sperm membrane (Lenzi *et al*., 2002).

Freeze-thawing of spermatozoa accelerate the production of ROS molecules in semen that can damage motility, plasmalemma functionality, viability, acrosome and induce sperm chromatin damage (Aitken *et al*., 1998; Ansari *et al*., 2010). There are neither protections nor repair systems for sperm integrity and therefore the addition of exogenous antioxidants are required to reduce this ROS-mediated damage (Vishwanath and Shannon, 1997). Alpha tocopherol and ascorbic acid are naturally occurring antioxidants to protect the spermatozoa against ROS-mediated damages in bovine semen (Andrabi, 2009). It is believed that freeze-thawing process reduces the level of naturally occurring antioxidative agents and increases the lipid peroxidation of sperm membrane system by increasing ROS during oxidative stress (Bilodeau *et al*., 2001).

It was hypothesized that addition of α-tocopherol and ascorbic acid in extender may improve the freezability of zebu bull spermatozoa. Therefore, experiments were designed to study the effect of α-tocopherol acetate and ascorbic acid in extender on motility, plasmalemma functionality, viability and chromatin damage of frozen-thawed Zebu bull spermatozoa.

* Corresponding author: sashraf1993@gmail.com
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MATERIALS AND METHODS

Extender preparation

The stock extender was prepared using tris-citric acid as a buffer (pH 7.0; Osmotic pressure 320 mOsmol/Kg) that consisted of 1.56% citric acid, 3.0% tris–(hydroxymethyl)-amino methane 0.2% fructose, 7% glycerol and 20% egg yolk in distilled water. Alpha tocopherol acetate was added to stock extenders at the rate of 0.5, 1.5, 2.5, 3.5 mM to make experimental extenders, while extender without any supplement was kept as control.

Another stock extender was prepared in which ascorbic acid was added at the rate of 0.5, 1.5, 2.5, 3.5 mM to make experimental extenders, while extender without any supplement was kept as control.

Semen collection and initial evaluation

Semen was collected from three Sahiwal bulls maintained under standard managemental conditions with artificial vagina (42°C) (three replicate) and initial evaluation was performed. Sperm progressive motility was assessed microscopically (X200) at 37°C and concentration was determined with Neubauer hemocytometer. The semen samples having volume >1ml, concentration >0.5 billion/ml and motility ≥60% were used for further processing.

Semen processing

Semen aliquots were diluted (37°C) with one of the experimental and control extenders at the rate 50×10^6 sperm/ml. The extended semen were cooled to 4°C in 2 hours, equilibrated for 4 hours at 4°C. Semen was then filled in 0.5ml French straws at 4°C and kept on liquid nitrogen (5 cm) vapours for 10 min. For each extender, three semen straws were thawed at 37°C for 30 seconds for the evaluation of semen quality.

Sperm progressive motility

A drop of semen was placed on a pre-warmed glass slide and cover slipped (37°C) for the assessment of progressive sperm motility under light microscope (X200).

Sperm plasmalemma functionality

Sperm plasmalemma functionality was determined with hypo-osmotic swelling (HOS) assay as described by Jeyendran *et al*. (1984). A total of 200 spermatozoa per extender were observed in at least five different fields. Sperm with coiled tails indicated intact, biochemically active sperm membranes, while uncoiled tails indicated disrupted, inactive sperm membranes.

Sperm viability

The viability (live sperm with intact acrosome) of spermatozoa was determined by dual staining procedure as developed by Kovacs and Foote (1992) using supravital trypan-blue and Giemsa stains. Two hundred spermatozoa were evaluated in each smear by light microscopy (X1000). Sperm with no staining in the posterior head region and a purple acrosome were considered live with intact acrosome, while the sperm with blue staining in posterior head region were considered dead.

Sperm chromatin damage

Sperm chromatin damage of Zebu bull spermatozoa was assessed using toluidine blue stain as described by Mello (1982). Briefly, air dried smear of semen sample was fixed in freshly made 96% ethanol-acetone (1:1) at 4°C for 30 min and hydrolyzed in 4 N HCl at 25°C for 30 min. The slides were rinsed and stained with 0.05% toluidine blue prepared in 50% citrate phosphate for 10 minutes. Three hundred spermatozoa were evaluated in at least five different fields in each smear using light microscopy (X1000), sperm heads with intact chromatin stained light blue and sperm with damaged chromatin stained violet or purple.

Statistical analysis

Effect of α-tocopherol acetate and ascorbic acid on different semen quality parameters were analyzed by the ANOVA (analysis of variance) in completely randomized design. When the F-ratio was found significant ($P \leq 0.05$), least significant difference test was used to compare the treatment means (MSTAT-C, Version 1.42).

RESULTS

Effect of α-tocopherol acetate

The data on the effect of α-tocopherol acetate

Table I.- Effect of α-tocopherol acetate and ascorbic acid in extender on post thaw motility, plasmalemma functionality, viability and chromatin damage (%) of Zebu bull spermatozoa.

	Motility	Plasmalemma functionality	Viability	Chromatin damage
α-tocopherol acetate (mM)				
0.0	40.0 ±0.0[a]	57.7 ± 1.5[b]	59.3 ± 2.1[b]	8.33 ± 4.4
0.5	45.0 ±8.7[a]	70.0 ± 3.6[a]	72.3 ± 3.1[a]	7.66 ± 6.5
1.5	43.3 ±2.9[a]	59.3 ± 1.5[a]	59.0 ± 2.6[b]	5.44 ± 4.1
2.5	40.0 ±0.0[a]	60.0 ± 2.6[a]	59.3 ± 1.5[b]	6.55 ± 4.7
3.5	31.7 ± 2.9[b]	51.7 ± 2.1[c]	60.7 ± 1.5[b]	6.11 ± 4.0
Ascorbic acid (mM)				
0.0	33.3 ± 1.7	62.3 ± 4.5	59.3 ± 3.7	3.22 ± 1.1
0.5	40.0 ± 2.9	68.7 ± 3.8	62.7 ± 1.8	3.78 ± 1.6
1.5	38.3 ± 1.7	61.3 ± 8.1	66.3 ± 2.0	4.22 ± 0.8
2.5	36.7 ± 3.3	62.0 ± 6.1	56.7 ± 1.5	4.11 ± 1.3
3.5	38.3 ± 1.7	57.3 ± 2.5	61.7 ± 5.5	4.11 ± 0.2

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

in extender on post-thaw sperm progressive motility, plasmalemma functionality, viability and chromatin damage of Zebu bull spermatozoa are give in Table I. Sperm motility (%) remained similar in extenders containing α-tocopherol at 0.5. 1.5, 2.5 mM and control that was higher than extender containing 3.5 mM α-tocopherol. Sperm plasmalemma functionality (%) was higher ($P<0.05$) in extenders containing 0.5. 1.5 and 2.5 mM α-tocopherol acetate than 3.5 mM and control. Sperm viability (live sperm with intact acrosome) was recorded significantly ($P<0.05$) higher in extender containing 0.5mM α-tocopherol acetate than 1.5, 2.5, 3.5 mM and control.

Effect of ascorbic acid

The data on the effect of ascorbic acid in extender on post-thaw sperm progressive motility, plasmalemma functionality, viability and chromatin damage of Zebu bull spermatozoa is give in Table I. Sperm motility, plasmalemma functionality, viability and chromatin damage did not differ ($P>0.05$) in extenders containing ascorbic acid 0.5, 1.5, 2.5, 3.5 mM and control.

DISCUSSION

The results of the present study indicate that higher level of α-tocopherol acetate (3.5 mM) was detrimental for sperm motility. Sperm plasma lemma functionality was improved with the supplementation of α-tocopherol acetate at 0.5. 1.5 and 2.5 mM compared to control, however, sperm viability (live sperm with intact acrosome) more precisely narrowed down our findings indicating 5 mM as the best concentration. The α-tocopherol works against the superoxide anions and reduces the lipid peroxidation of sperm plasmalemma during freeze-thawing stress (Donnelly *et al.*, 1999). It is suggested that higher sperm plasmalemma functionality and higher percentage of live spermatozoa with intact acrosome in extender containing α-tocopherol acetate is due to lower oxidative stress and lower level of lipid peroxidation (Andrabi *et al.*, 2008). A higher concentration vitamin E may act as an oxidation stimulator rather than an antioxidant (Breininger *et al.*, 2005) and this is evident in present study. Our results are inline with the studies on bovine (Beconi *et al.*, 1993), ram (Ollero *et al.*, 1998) and boar semen (Jeong *et al.*, 2009).

Sperm chromatin integrity is critical for the development of viable embryo after fertilization (Andrabi, 2009). In present study, sperm chromatin damage remained similar in all experimental extenders containing α-tocopherol acetate. Zebu bull semen have better antioxidant potential (Nichi *et al.*, 2006) that might be the reason of lower chromatin damage to Zebu bull semen during cryopreservation and non-significant effect of antioxidant addition in

semen extender.

In previous studies, ascorbic acid supplementation in extender is reported to reduce the ROS mediated damages in bovine semen during cryopreservation (Hu *et al*., 2010) and preserve the fertilization potential of cryopreserved spermatozoa. A recent study reported vitamin C as an efficient antioxidant in citrate egg yolk extender, however, in tris citric egg yolk extender vitamin C failed to improve semen quality in cryopreserved bovine semen (Asadpour *et al*., 2011). Similar results of acrosomal integrity were reported for buffalo semen using tris citric acid extender supplemented with vitamin C (Andrabi *et al*., 2008). In present study, ascorbic acid supplementation of tris citric egg yolk extender did not improve post thaw semen quality parameters of Zebu bull spermatozoa.

It is concluded that α-tocopherol acetate (0.5 mM) in semen extender improved the post-thaw plasmalemma functionality and viability (live sperm with intact acrosome) of Zebu bull spermatozoa.

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