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EFFECT OF NON-ENZYMATIC ANTIOXIDANTS IN EXTENDER ON POST-THAW QUALITY OF BUFFALO *(BUBALUS BUBALIS)* BULL SPERMATOZOA

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ABSTRACT

The objective of the present study was to determine the effect of non-enzymatic antioxidants (vitamin C or E) in tris-citric acid buffer (TCA) on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. Split pooled buffalo bull ejaculates were diluted in TCA egg yolk glycerol extender containing either vitamin C (TCAC), vitamin E (TCAE) or without antioxidant (TCAN) at 37°C. Extended semen was cooled to 4°C in 2 h and equilibrated for 4 h at 4°C. Cooled semen was then filled in 0.5 ml straws at 4°C and frozen in programmable cell freezer. Thawing of semen was performed at 37°C for 30 seconds. Sperm motility, plasma membrane integrity and sperm morphology (acrosome integrity, head, mid-piece and tail abnormalities) of each semen sample were evaluated. Percentage of post-thaw spermatozoal motility assessed visually at 0 and 6 h and the post-thaw percentage of spermatozoa with intact plasma membranes at 0 h were higher ($P<0.05$) with TCAC and TCAE compared to control. However, the differences in both these parameters between the former two groups was non significant. The post-thaw percentage of spermatozoa with normal acrosomes was higher ($P<0.05$) in TCAE extender than control. Mean sperm abnormalities in samples cryopreserved with extender having TCAC, TCAE or TCAN were similar ($P>0.05$). In conclusion, non-enzymatic antioxidants, particularly vitamin E, in the tris citric acid extender may improve the quality of frozen-thawed buffalo bull spermatozoa.

Key words: Buffalo-spermatozoa, antioxidants, cryopreservation.

INTRODUCTION

One of the major changes in the spermatozoa during cryopreservation is the increased sensitivity to reactive oxygen species (ROS), which are involved in lipid peroxidation. The most common ROS are superoxide anion, hydrogen peroxide, peroxyl radicals, hydroxyl radicals, nitric oxide and peroxynitrite anion (Sikka, 1996). ROS can cause alterations in sperm plasma membrane and reduction in motility and fertilizing ability of spermatozoa (Maxwell and Stojanov, 1996; Dalvit *et al.,* 1998; Chatterjee and Gagnon, 2001; Raina *et al.,* 2002).

The addition of natural antioxidant (vitamin E; 1 mg/ml) in the freezing diluent exerted a protective effect against lipid peroxidation, thereby preserving the metabolic activity and cellular viability of bovine spermatozoa (Beconi *et al.,* 1993). A greater protective effect against lipid peroxidation has been observed in bovine semen samples frozen with vitamin E and then incubated with vitamin E after thawing versus samples incubated without the antioxidant (Beconi *et al.,* 1991). Another non-enzymatic antioxidant (vitamin C) has been proposed as electron donor for some trans plasma membrane redox systems. Vitamin C (Ascorbic acid) may act as an oxidant at low concentrations and as an antioxidant at high concentrations (Affranchino *et al.,* 1991; Breininger *et al.,* 2005). Ascorbic acid, at a concentration of 5 mM in the freezing diluent acts as an antioxidant during freezing and thawing of bovine spermatozoa (Beconi *et al.,* 1993). *In vitro* studies strongly suggest that the antioxidant effect of ascorbate is related to direct vitamin E regeneration by reducing the tocopheroxyl radical in the one-electron redox cycle (Packer *et al.,* 1979; Dalvit *et al.,* 1998).

Artificial insemination (AI) is one of the major reproductive biotechnologies of the modern era through which rapid genetic improvement in livestock has been achieved in the developed countries. To raise genetic potential of livestock, AI with frozen-thawed spermatozoa was introduced in most of the developing countries more than three decades ago, yet it has not been applied in buffaloes on a large scale (Anzar *et al.,* 2003, Andrabi, 2008). It is reported that viability or fertility of frozen thawed semen is lower in buffalo compared to cattle (Rasul *et al.,* 2000; Andrabi *et al.,* 2001; Andrabi *et al.,* 2008; Andrabi, 2008). On the basis of previous information on cattle spermatozoa, (Affranchino *et al.,* 1991; Beconi *et al.,* 1991; Beconi *et al.,* 1993) we hypothesized that addition of natural antioxidants (vitamins C or E) in the semen extender may also be a step closer to improve the frozen-thawed quality of buffalo spermatozoa. Therefore, the present study examined the effect of non-enzymatic antioxidants (vitamins C or E) in tris-citric acid (TCA)

extenders on post-thaw motility, membrane integrity, and morphology of buffalo bull spermatozoa.

MATERIALS AND METHODS

Experimental extenders

Tris-citric acid (TCA) containing 1.56 g citric acid (Merck, Germany) and 3.0 g tris(hydroxymethyl)-aminomethane (Sigma, USA) in 74 ml distilled water was used as a buffer for the experimental extenders. The pH of buffer was 7.00 and the osmotic pressure was 320 mOsmol/Kg. Egg yolk (20% vol/vol), fructose (0.2%; wt/vol; Riedel-DeHaen, Switzerland), glycerol (7%; vol/vol; Merck, Germany), benzyl penicillin (1000 I.U/ml; Hebei, China) and streptomycin sulphate (1000 µg/ml; Sigma, USA) were added to each of the three experimental extenders (Andrabi *et al.,* 2008).

The first extender contained vitamin C (TCAC) as sodium ascorbate (Sigma, USA), which was added at the rate of 5 mM (Beconi *et al.,* 1993; Raina *et al.,* 2002). The second extender contained vitamin E (TCAE) available as α-tocopherol acetate (Sigma, USA), added at the rate of 1 mg/ml (Beconi *et al.,* 1993; Raina *et al.,* 2002). The third extender did not contain any antioxidant and served as control (TCAN). Aliquots of each extender were stored frozen at -20°C and thawed before use.

Semen collection

Ejaculates were collected by artificial vagina at 42°C from three adult Nili-Ravi buffalo bulls (*Bubalus bubalis*) of known fertility. The bulls were kept under uniform feeding and handling conditions during the entire study. Ejaculates were collected at weekly intervals for a period of 5 weeks (replicates; n = 5). The frequency of collection from each bull was two ejaculates on one day each week. Visual motility of each ejaculate was assessed at 37°C using a phase contrast microscope (X 400; Leica, Leitz Wetzlar, Germany) observed on closed circuit television by two operators. Progressive motility of spermatozoa was assessed to the nearest 5%. Sperm concentration was assessed by digital photometry (Dr. Lange LP 300 SDM, Minitub, Germany) at 546 nm. Ejaculates containing more than 70% progressively motile spermatozoa and 0.5×10^9 spermatozoa/ml were pooled in order to have sufficient semen for a replicate (Rasul *et al.,* 2000, 2001; Andrabi *et al.,* 2008). At least one ejaculate on every collection from each bull did qualify for freezing.

Semen cryopreservation

Buffalo bull semen was cryopreserved according to Rasul *et al.* (2000). After a holding time of 15 min at 37°C, three aliquots of semen were diluted (37°C) in a single step with one of the three experimental extenders to a concentration of 50×10^6 motile spermatozoa/ml. After dilution, the semen was cooled to 4°C in 2 hours and equilibrated for 4 h at 4°C. Pre-cooled 0.5 ml straws were then filled with the cooled semen at 4°C in the cold cabinet unit (Minitub, Germany) and frozen in a programmable cell freezer (KRYO 10 series III, UK) from 4°C to -15°C at the rate of 3°C/minute and from -15°C to -80°C at the rate of 10°C/minute. Straws were then plunged into liquid nitrogen (-196°C) for storage. After 24 h storage, semen straws were thawed at 37°C for 30 seconds.

Post-thaw spermatozoal evaluation

Visual motility

The motility of spermatozoa was assessed at 0 and 6 h post-thaw. Thawed semen sample was placed on a pre-warmed glass slide and cover-slipped. Visual motility of spermatozoa was assessed at 37°C using phase contrast microscope observed on closed circuit television by two operators.

Plasma membrane integrity

Plasma membrane integrity (PMI) of spermatozoa was assessed by hypoosmotic swelling (HOS) assay, as described earlier (Jeyendran *et al.,* 1984) at 0 h post-thaw. The HOS solution contained 0.73g tri-sodium citrate dihydrate (Merck, Germany) and 1.35 g fructose (Riedel-DeHaen, Switzerland), dissolved in 100 ml distilled water (osmotic pressure ~190 mOsmol/Kg). The assay was performed by mixing 50 µl of frozen-thawed semen sample to 500 µl of HOS solution and incubating at 37°C for 40 min. After incubation, a drop of semen sample was examined under phase contrast microscope (X 400; Olympus BX40, Japan). Two hundred spermatozoa were counted for their swelling characterized by coiled tail, indicating intact plasma membrane (Ahmad *et al.,* 2003).

Sperm morphology

Acrosomal integrity and morphological abnormalities (head, mid-piece and tail) of spermatozoa were determined at 0 h post-thaw. For this purpose, frozen-thawed semen (100 µl) was fixed in 500 µl of 1% formal citrate (2.9g tri-sodium citrate dihydrate; Merck, Germany and 1 ml of 37% formaldehyde solution of Merck, Germany dissolved in 100 ml of distilled water). Intactness of acrosome characterized by normal apical ridge (NAR) of 200 spermatozoa was assessed using phase contrast microscopy (X 1000; Ahmad *et al.,* 2003). Also morphological abnormalities of 200 spermatozoa were assessed using phase contrast microscope under oil immersion, as described by Akhter *et al.* (2007).

Statistical analysis

Results are presented as means ± SD. Effect of non-enzymatic antioxidants for different variables was analyzed by the analysis of variance (ANOVA). When the F–ratio was significant (P<0.05), Tukey's Honestly significant difference was used to compare the treatment means (SYSTAT, 1996).

RESULTS

Data on the effect of non-enzymatic antioxidants in the cryodiluent on motility of spermatozoa at 0 and 6 h post-thaw are presented in Table 1. Post-thaw motility of spermatozoa assessed visually at 0 and 6 h was higher (P<0.05) with TCAC and TCAE compared to the control (TCAN), difference between the former two groups was non significant.

Table 1: Effect of non-enzymatic antioxidants in extender on motility of buffalo bull spermatozoa at 0 and 6 h post-thaw

Time post-thaw (h)	Treatment	Visual motility (%)
0	TCAN	54.0 ± 2.2 [a]
	TCAC	59.0 ± 2.2 [b]
	TCAE	60.0 ± 0.0 [b]
6	TCAN	35.0 ± 5.0 [a]
	TCAC	45.0 ± 6.1 [b]
	TCAE	44.0 ± 6.5 [b]

Means (± SD) with different superscripts show significant difference (P<0.05) between treatments at a given time (TCAC: vitamin C; TCAE: vitamin E; TCAN: without antioxidant/control).

Data pertaining to the effect of natural antioxidants in media on PMI of spermatozoa at 0 h post-thaw are presented in Table 2. The post-thaw percentage of spermatozoa with intact plasma membranes was higher (P<0.05) with TCAC and TCAE compared to the control (TCAN), difference between the former two groups was non significant.

Table 2: Effect of non-enzymatic antioxidants in extender on intact plasma membrane and acrosomal integrity of buffalo bull spermatozoa at 0 h post-thaw

Treatment	Intact plasma membrane (%)	Normal acrosome (%)
TCAN	76.9 ± 4.5 [a]	80.2 ± 0.8 [a]
TCAC	84.0 ± 2.1 [b]	85.7 ± 3.9 [ab]
TCAE	82.9 ± 2.5 [b]	86.5 ± 5.1 [b]

Means (± SD) with different superscripts within column show significant difference (P<0.05) between treatments.

The mean percentage of spermatozoa with normal acrosomes at 0 h was similar for TCAC and TCAN (Table 2). However, the post-thaw percentage of spermatozoa with normal acrosomes was higher (P<0.05) in TCAE extender than the control. Sperm abnormalities (head, mid-piece and tail) at 0 h post-thaw were similar for TCAC, TCAE or TCAN (P>0.05; Table 3).

Table 3: Effect of non-enzymatic antioxidants in extender on sperm abnormalities of buffalo bull at 0 h post-thaw

Treatment	Sperm abnormalities (%)		
	Head	Mid-Piece	Tail
TCAN	0.6 ± 0.6 [ψ]	1.2 ± 0.9	18.5 ± 4.7
TCAC	1.1 ± 0.5	1.2 ± 0.7	16.3 ± 1.2
TCAE	0.8 ± 0.4	1.9 ± 1.6	19.0 ± 4.8

[ψ]Means (± SD) did not differ between treatments.

DISCUSSION

In the present study, the inclusion of non-enzymatic antioxidants (vitamin C or E) in the cryodiluent improved the motility of buffalo spermatozoa at 0 and 6 h after thawing and incubation (37°C). Similarly, Raina *et al.* (2002) found that incorporation of vitamin C or E in TCA based extender improved the motility of liquid buffalo bull semen. The greater number of motile spermatozoa present in samples frozen with natural antioxidants would increase the fertilizing potential of post-thaw spermatozoa (Breininger *et al.,* 2005). However, improvement in fertility was not seen when a combination of gentamycin, tylosin and linco-spectin in extender was compared with srrepto-penicillin (Akhtar *et al*., 2007). Moreover, in the present study, the concentration of natural antioxidants used may have been optimum for preserving buffalo sperm viability, as effect of vitamins C and E may vary with concentration; at higher concentrations vitamin E may act as an oxidation stimulator rather than an antioxidant, while reverse holds true for vitamin C (Affranchino *et al.,* 1991; Breininger *et al.,* 2005).

As in other species, cryopreservation decreases the functional integrity of buffalo bull spermatozoa (Rasul *et al.,* 2001). In the present study, PMI of spermatozoa evaluated post-thawing was significantly higher in TCAC and TCAE than in the control. It is worth mentioning that the present data of PMI is very much supported with that of progressive motility i.e., a similar pattern of effect of antioxidants was observed on visual motility and PMI of cryopreserved spermatozoa.

In this study, percentage of spermatozoa with normal acrosome was significantly higher after freezing and thawing in TCAE than in the control. Thus, supplementing the freezing extender with antioxidants, especially vitamin E, had beneficial effects on acrosome integrity. Furthermore, the percentage of spermatozoa with normal acrosome observed was almost similar to the percentage of

spermatozoa with functionally intact plasmalemma.

Evaluation of sperm morphology is one of the commonest methods to assess viability of frozen-thawed bovine semen (Rocha *et al.,* 2006). In our study, post-thaw sperm head, mid-piece and tail abnormalities were similar in all the three experimental extenders. It is relevant to mention that semen processing does not necessarily increase the proportion of bull spermatozoa with abnormal heads, detached heads, coiled tails and proximal cytoplasmic droplets (Revell, 2003).

In conclusion, non-enzymatic antioxidants particularly vitamin E in the tris-citric acid extender may improve the quality of frozen-thawed buffalo spermatozoa.

REFERENCES

Affranchino, M., G. Trinchero, L. M. Schang and M. Beconi, 1991. Bovine spermatozoa as lipoperoxidation inhibitor. Corn. Biol., 9: 261-274.

Ahmad, Z., M. Anzar, M. Shahab, N. Ahmad and S. M. H. Andrabi, 2003. Sephadex and sephadex ion-exchange filtration improves the quality and freezability of low-grade buffalo semen ejaculates. Theriogenology, 59: 1189-1202.

Akhter, S., M. S. Ansari, S. M. H. Andrabi, N. Ullah, and M. Qayyum, 2007. Effect of antibiotics in extender on bacterial and spermatozoal quality of cooled buffalo *(Bubalus bubalis)* bull semen. Reprod. Dom. Anim., 43: 272-278.

Akhtar, S., M. Sajjad, S. M. H. Andrabi, N. Ullah and M. Qayyum, 2007. Effect of antibiotics in extender on fertility of liquid buffalo bull semen. Pakistan Vet. J., 27(1): 13-16.

Andrabi, S. M. H., 2008. Factors affecting the quality of cryopreserved buffalo ***(****Bubalus bubalis****)*** bull spermatozoa. Reprod. Dom. Anim., (in press).

Andrabi, S. M. H., N. Ahmad, A. Abbas and M. Anzar, 2001. Effect of two different antibiotic combinations on fertility of frozen buffalo and Sahiwal bull semen. Pakistan Vet. J., 21: 166-169.

Andrabi, S. M. H., M. S. Ansari, N. Ullah, M. Anwar, A. Mehmood and S. Akhter, 2008. Duck egg yolk in extender improves the freezability of buffalo bull spermatozoa. Anim. Reprod. Sci., 104: 427-433.

Anzar, M., U. Farooq, M. A. Mirza, M. Shahab and N. Ahmad, 2003. Factors affecting the efficiency of artificial insemination in cattle and buffalo in Punjab, Pakistan. Pakistan Vet. J., 23: 106-113.

Beconi, M., M. Affranchino, L. M. Schang and N. M. Beorlegui, 1991. Influence of antioxidants on SOD activity in bovine sperm. Biochem. Int., 3: 545-553.

Beconi, M. T., C. R. Francia, N. G. Mora and M. A. Affranchino, 1993. Effect of natural antioxidants on frozen bovine semen preservation. Theriogenology, 40: 841-851.

Breininger, E., N. M. Beorlegui, C. M. O'flaherty and M. T. Beconi, 2005. Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. Theriogenology, 63: 2126-2135.

Chatterjee, S. and C. Gagnon, 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Develop., 59: 451-458.

Dalvit, G. C., P. D. Cetica and M. T. Beconi, 1998. Effect of α-tocopherol and ascorbic acid on bovine *in vitro* fertilization. Theriogenology, 49: 619-627.

Jeyendran, R. S., H. H. Van Der Ven, M. Perez Pelaez, B. G. Crabo and L. J. D. Zaneveld, 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil., 70: 219-228.

Maxwell, W. M. C. and T. Stojanov, 1996. Liquid storage of ram semen in the absence or presence of some antioxidants. Reprod. Fertil. Dev., 8: 1013-1020.

Packer, J. E., T. F. Slater and R. L. Wilson, 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature, 278: 737-738.

Raina, V. S., A. K. Gupta and K. Singh, 2002. Effect of antioxidant fortification on preservability of buffalo semen. Asian-Austr. J. Anim. Sci., 15: 16-18.

Rasul, Z., N. Ahmad and M. Anzar, 2001. Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. J. Androl., 22: 278-283.

Rasul, Z., M. Anzar, S. Jalali and N. Ahmad, 2000. Effect of buffering system on post-thaw motion characteristics, plasma membrane integrity and acrosome morphology of buffalo spermatozoa. Anim. Reprod. Sci., 59: 31-41.

Revell, S., 2003. Assessment of the morphology of frozen-thawed bull sperm in relation to its cryopreservation for artificial insemination. Vet. Rec., 152: 765-767.

Rocha, A., E. Oliveira, M. J. Vilhena, J. Diaz and M. Sousa, 2006. A novel apical midpiece defect in the spermatozoa of a bull without an apparent decrease in motility and fertility: a case study. Theriogenology, 66: 913-922.

Sikka, S. C., 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. Front. Biosci., 1: e78-86.

SYSTAT, 1996. Statistics (Version 6.0 for Windows), SPSS, Chicago, IL,USA.