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Research Article

EFFECT OF VITAMIN C ON THE SEMINAL AND BIOCHEMICAL PARAMETERS OF MURRAH BUFFALO BULL SEMEN DURING DIFFERENT STAGES OF FREEZING

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ABSTRACT

The objective of the present study was to test the efficacy of adding vitamin C to the Tris citric acid yolk glycerol extender on seminal and biochemical parameters of semen at the end of equilibration time (pre freezing stage) and after 24 h freezing and thawing (post freezing stage) of buffalo semen. Semen was collected twice a week from four Murrah buffalo bulls (6 replicates/bull). Each ejaculate was divided into two parts. First part was diluted with Tris citric acid yolk glycerol extender that served as a control and the second part was diluted with the same extender containing vitamin C (2.5mM). The extended semen was cryopreserved in liquid nitrogen. The semen samples were examined for various seminal and biochemical parameters at pre-freezing stage, i.e., before cryopreservation and post-freezing stage, i.e., after cryopreservation. Significant improvement in progressive sperm motility, livability percent, acrosomal integrity and malondialdehyde (MDA) was observed in semen supplemented with vitamin C during pre-freezing and post-freezing stages. However, the release of glutamic oxaloacetic transaminase (GOT) did not differ significantly in both the groups. Thus inclusion of vitamin C in the extender for diluting the semen has shown improvement in post thaw quality of buffalo bull semen.

Key words: Antioxidants, cryopreservation, Murrah bull semen, seminal parameters

The buffalo is the skeletal base of Indian dairy industry. Genetic improvement of buffaloes by maximum utilization of elite sires for artiûcial insemination (AI), can enhance the production potential as well as help in upliftment of the progressive farming community. This requires high quality cryopreserved buffalo semen and greater AI coverage. Although AI with frozen semen has made tremendous improvement in farm animals, but the conception rate to frozen thawed semen in buffalo is reported to be only about 33% (Anzar *et al*., 2003). There are numerous factors that affect male fertility, one of these being the oxidative stress (OS) during cryopreservation of semen. The sperm cells under aerobic conditions are always attacked by reactive oxygen species (ROS). The oxidative damage leads to malfunction of sperm due to induction of lipid peroxidation (LPO) and high polyunsaturated fatty acids (PUFAs) content in sperm plasma membrane. This leads to alteration in sperm plasma membrane reducing the motility, viability and acrosome integrity of spermatozoa (Bilodeau *et al.*, 2000; Ansari *et al.*, 2010). In order to control the level of ROS and promote motility and livability of sperm, numerous antioxidants viz. vitamin C, vitamin E, glutathione and coenzyme Q-10 have proven beneficial in treating male infertility (Sinclair, 2000).

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The role of vitamin C in ameliorating the adverse effects of reactive oxygen and nitrogen radicals has been well established (Padayatty *et al*., 2003). The addition of vitamin C in an extender improves the optimal sperm performance by reducing cell damage through its continuous radical scavenging action. The antioxidant effect of vitamin C is related to direct vitamin E regeneration by reducing the tocopheroxyl radical in the one electron redox cycle (Dalvit *et al.*, 1998). Therefore, the present study was designed to observe the effect of supplementation of vitamin C in the extender on the seminal and biochemical parameters of Murrah buffalo bull semen during the different stages of freezing.

MATERIALS AND METHODS

Extender: Tris-citric acid containing 2.115 g citric acid and 3.786 g tris (hydroxymethyl)-aminomethane in 74 ml distilled water was used as a buffer (pH 6.8) for the experimental extender.. Egg yolk (20% v/v), fructose (1%; w/v), glycerol (6.8%; v/v), penicillin G sodium (1000 I.U/ml) and streptomycin sulphate (1000 µg/ml) were added to the extender. The extender was divided into two parts: first part of extender did not contain any antioxidant that served as control (TY-G) and the second part of extender contained vitamin C as sodium ascorbate, 2.5 mM (TY-G+vitamin C). Aliquots of each extender were stored at -20^{0}C and thawed immediately before use.

Semen Collection: A total of 24 ejaculates were collected by artificial vagina at 42^0C from four adult Murrah buffalo bulls *(Bubalus bubalis*) of known fertility. The bulls were of 4-6 years of age with 600-650 kg body weight and were maintained under uniform feeding and handling conditions during the entire period of study. Ejaculates were collected twice a week for a period of 3 weeks (six replicates). Visual motility of each ejaculate was assessed at 37^0C using a phase contrast microscope. Progressive motility of spermatozoa, sperm concentration and percent livability were assessed using standard procedures. Ejaculates containing more than 60% progressively motile spermatozoa and 0.5×10^9 spermatozoa/ml were used for the present study.

Semen Cryopreservation: Buffalo bull semen samples were diluted with two experimental extenders to a concentration of 120×10^6 motile spermatozoa/ml using slow rate of extension in 3-4 steps within 15-20 min at room temperature (22-25^{0}C). French mini straws (0.25 ml capacity) were then filled with the semen and the straws were cooled to 5^0C in 2 h and equilibrated for 4 h at 5^0C. Freezing of semen straws was done in liquid nitrogen (-120 to -130^{0}C) for 8-9 min. Straws were then directly plunged into liquid nitrogen (-196^{0}C) for storage.

The semen samples were assessed for progressive sperm motility, live spermatozoa, intact acrosomes, release of GOT and lipid peroxidation at the end of equilibration time, i.e., at pre-freezing stage, just before cryopreservation of semen and at post-freezing stage, i.e., 24 h after storage. Progressive sperm motility, live spermatozoa and intact acrosomes were evaluated using standard procedures. GOT is primarily associated with the spermatozoa and considered to be an indicator as to what may be occurring to the spermatozoa during cryopreservation, although it should not be mistaken for the enzyme necessary for fertility (Pace and Graham, 1970). The release of GOT from spermatozoa was measured by the method used by Yatzidis (1960) with slight modifications. The semen samples were centrifuged at 3000 rpm for 10 min at 16^0C. Initially, 0.1 ml supernatant was mixed with 0.9 ml of normal saline. GOT substrate (0.5 ml) was added and the test tubes were kept in a water bath at 37^0C for 60 min. Afterwards, 0.5 ml of aniline citrate solution was added to stop the reaction and the tubes were then kept at room temperature. Subsequently, 0.5 ml of 2, 4-dinitrophenyl hydrazine was added and mixed vigorously. Finally 3 ml of sodium hydroxide was added and mixed by inverting the tubes. The color produced was measured after 30 min (the color remained stable for 1 h) at 500 nm wavelength against the blank set at zero optical density in the spectrophotometer. Level of lipid peroxidation was measured by determining the amount of malondialdehyde (MDA) as per the method of Ottolenghi (1959). 0.5 ml of extended semen was mixed with 1.5 ml of 80% trichloroacetic acid (TCA). Then 2 ml of 0.67 % solution of 2-thiobarbituric acid (TBA) in 10% TCA solution was added and kept in boiling water bath. After 15 min the tubes were immediately cooled in ice cold water for 5 min and centrifuged at 2000 rpm for 10 min. The red chromogen of MDA and TBA formed in the supernatant was measured at 535 nm wavelength in the spectrophotometer.

The means and standard errors, multivariate analysis of variance, Duncan's Multiple Range Test for means and linear product moment of biological parameters were calculated using SPSS/PC student ware computer software (Norusis, 1988).

RESULTS AND DISCUSSION

The results revealed that pre-freezing and post-freezing progressive sperm motility (%) was significantly ($P<0.05$) higher in vitamin C supplemented semen samples as compared to control group (Table 1), that could be due to inhibition in lipid peroxidation damage. This finding is in agreement with those of other workers who also reported that supplementation of vitamin C at the time of extension had resulted in increased progressive sperm motility in canine semen (Wittayarat *et al*., 2012), Awssi ram semen (Azawi and Hussein, 2013) and bull semen (Stolbov and Rimanova, 1983; Raina *et al.*, 2002). The greater number of motile spermatozoa present in the samples frozen with natural antioxidants would increase the fertilizing potential of post thaw spermatozoa (Breininger *et al*., 2005). However, the differences in pre-freezing and post-freezing progressive sperm motility can be attributed to many factors like initial quality of semen, type of extender used, concentration and nature of cryoprotective agents and freezing rates of semen.

Percent live spermatozoa was also significantly ($P<0.05$) higher in vitamin C supplemented semen samples both during pre- and post-freezing stages (Table 1). Kumar *et al.* (1994) reported 48.40% live sperms after freezing of semen in the extender supplemented with sugars like raffinose, sucrose and glucose. These values are similar to 48.21% live sperm obtained in the present investigation after vit. C supplementation. Wittayarat *et al*. (2012) also reported increased sperm viability along with sperm motility in canine semen supplemented with 0.5 mM vitamin C and polyphenol.

Supplementation of vitamin C increased percent intact acrosomes during pre- and post-freezing of semen

Table 1

Seminal and biochemical parameters of Murrah buffalo bull semen during different stages of freezing

Parameters	Stage of freezing	TY-G (Control)	TY-G+vitamin C (2.5mM)
Progressive sperm motility (%)	Pre freezing	$67.70^{b}\pm1.12$	$73.33^{a}\pm1.07$
	Post freezing	$36.25^{b}\pm0.91$	$45.62^{a}\pm0.69$
Live sperm (%)	Pre freezing	$70.77^{b}\pm1.10$	$76.21^{a}\pm1.01$
	Post freezing	$39.36^{b}\pm0.90$	$48.21^{a}\pm0.75$
Intact acrosome (%)	Pre freezing	$73.00^{a}\pm1.10$	$76.50^{a}\pm2.13$
	Post freezing	$41.93^{b}\pm0.94$	$50.68^{a}\pm0.81$
GOT (units/ 120 million sperms)	Pre freezing	$23.29^{a}\pm0.50$	$24.42^{a}\pm0.45$
	Post freezing	$25.61^{a}\pm0.59$	$27.17^{a}\pm0.58$
MDA (concentration ng/120 million sperms)	Pre freezing	$784.50^{a}\pm8.31$	$610.42^{b}\pm12.07$
	Post freezing	$691.25^{a}\pm8.23$	$521.16^{b}\pm8.23$

*Values in a row with different superscripts for a parameter differ significantly ($P<0.05$)
MDA=malondialdehyde; GOT=glutamic oxaloacetic transaminase

of bulls, however, the increase was statistically significant ($P<0.05$) only during post-freezing stage (Table 1). This indicated that vitamin C offered protection to the acrosome and acrosomal membrane during freezing. Bhosrekar *et al*. (1994) reported 89.29% intact acrosomes after freezing and thawing of buffalo semen. Significant improvement in the percent intact acrosomes in good quality (66-79 %) and poor quality (53-68%) spermatozoa in frozen thawed bovine semen (Beconi *et al*., 1993) and 89.29% in buffalo semen (Bhosrekar *et al*., 1994) was observed after addition of vitamin C while extending the semen. However, intact acrosomes in the present study were lower (50.68%) than that of 60.70 to 70.70% reported by Singh (1994). This variation might be due to the factors such as difference in quality of semen, type of extenders, level of egg yolk in extender and method of evaluation of acrosomal intactness.

Non-significant differences were recorded in the GOT release values during pre- and post-freezing of semen in both the control and treated groups (Table 1). However, Tuli *et al.* (1982) reported a significant increase and Singh (1994) observed a decrease in GOT release after freezing and thawing of Murrah buffalo bull semen. The reason for this variation could not be ascertained.

A significant decrease ($P<0.05$) in the production of MDA during pre- and post-freezing of semen was observed in both groups (Table 1). MDA production reduced significantly ($P<0.05$) with the addition of vitamin C in the extender during pre-freezing and post-freezing of buffalo bull semen. These findings are in agreement with Beconi *et al*. (1993) and Singh *et al*. (1989a, 1989b) who also reported that addition of vitamin C and glucose to buffalo spermatozoa inhibited the MDA production and improved percent motility and live spermatozoa, respectively.

On the basis of the present results, it could be concluded that cryopreservation reduces the functional integrity of Murrah buffalo bull spermatozoa. However, addition of vitamin C to a semen extender can reduce the oxidative stress during freezing and thawing of semen thus protecting the buffalo bull semen during cryopreservation as well as improving the longevity and quality of sperms.

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