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Original Article

Influence of Added Vitamin C and Vitamin E on Frozen-Thawed Bovine Sperm Cryopreserved in Citrate and Tris-Based Extenders

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Abstract

The objective of this study was to evaluate quality of frozen-thawed bull semen processed with extenders containing vitamin C and E as antioxidants. Pooled semen's were collected from 5 bulls and diluted to a concentration of 30×10^6 sperm/mL with citrate –egg yolk (CEY) or Tris – egg yolk (TEY) extenders. The diluted semen was divided to 5 aliquots including control and 4 experimental groups. Each aliquot was further diluted with an equal volume of CEY or a Tris (hydroxymethyl) aminomethane (TRIS) - based extender without (control) or containing vitamin C 1mM or 2mM and vitamin E 0.1mM or 0.2mM, and routine semen evaluations like sperm motility, viability and measurement of lipid peroxidation (LPO) were conducted. Significant reductions of LPO were achieved by addition of 1mM vitamin C and 0.1 mM vitamin E to CEY extender ($P < 0.05$). The highest sperm viability was achieved by addition of 0.1mM vitamin E to CEY extender. Supplementing CEY extender with 2mM vitamin C and 0.1mM vitamin E improved the sperm motility compared with the control group. On the basis of the present results it is concluded that vitamin C and E are very efficient antioxidants in CEY extender.

Key words: Antioxidant, Sperm, Lipid peroxcidation, Cryopreservation

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Introduction

Mammalian sperm cells contain a high proportion of polyunsaturated fatty acids, and are therefore particularly susceptible to peroxidative damage. Their vulnerability especially increases following cryopreservation with a subsequent loss in membrane integrity, impaired cell function and decreased motility and the fertilizing capability of the sperm for artificial insemination (AI).[1-2] The production of Reactive Oxygen Species (ROS) is a normal physiological event in various organs. However, the over-production of ROS can cause structural damage of sperm membranes.[3] To counteract the destructive effects of ROS, seminal plasma has an antioxidant system that seems to be very relevant to the protection of sperm.[4]

To control the level of ROS and promote motility and survival of sperm, numerous antioxidants have proven beneficial in treating male infertility.[5] Ascorbic acid and vitamin E are naturally occurring free radical scavenger and their presence also assist various other mechanisms in decreasing numerous disruptive free radical processes, including LPO.[6, 7]

Vitamin C (ascorbic acid, ascorbate) represents the major water-soluble antioxidant in plasma. Ascorbic acid is required *in vivo* as a cofactor for at least eight enzymes, [8] and can also act as an antioxidant by reacting with free radicals.[9] The addition of vitamin C in an extender can impact on optimal sperm performance by reducing cell damage through its continuous radical-scavenging action. Beconi *et al*. (1993) had indicated that the presence of 5mM vitamin C in the freezing diluent exerted an antioxidant effect during freezing and thawing of bovine sperm.[10]

Vitamin E includes a group of lipid soluble compounds, tocopherols and tocotrienols that act as antioxidants defending the organism against oxidative stress. Vitamin E is believed to be the primary components of the antioxidant system of the spermatozoa,[11] and is one of the major membrane protectants against ROS and LPO.[12] It is not synthesized by mammalian cells and once membrane tocophorol is consumed during period of oxidative stress, cellular lipids are subject to peroxidation which can result in toxic damage.[13] Vitamin E can inhibit LPO reaction in the membrane by eliminating peroxyl ($ROO^{\bullet}$),alkoxyl ($RO^{\bullet}$), and other lipid-derived radicals.[14] Supplemental food by vitamin E has been shown to increase total sperm output and sperm concentration in boars,[15-17] cattle,[6] rabbits,[18] and rams.[19-21] However, there are limited data regarding the effects of the vitamin C and E in different extenders on semen quality. Therefore, the objective of this study was, to test the hypothesis that different levels of vitamin E and vitamin C might effectively protect bull semen from oxidative damage during cryopreservation in different extenders resulting in higher sperm viability and motility values after thawing.

Materials and Methods

Animals and semen collection. Semen from 10 fertile Holstein bulls aged 3 to 6 yr and weighing 300 to 900 kg at the start of the experiment, was used in this study. Forty ejaculates (four per each cow) were collected twice weekly using the artificial vagina (45°C), from ten Holstein bulls. The bulls utilized in this study had their fertility previously established based on both in vivo and in vitro fertility trials. The ejaculates were transferred to the laboratory and immersed in a water bath (34˚C) until semen evaluation.

Semen processing. Fresh semen was assessed for ejaculate volume, concentration, motility and viability of the sperm. The volume of ejaculates was measured in a conical tube at 0.1 ml intervals, the sperm concentration

determined using hemocytometer and sperm motility was evaluated at 400× magnification based on the visual estimation. Sperm viability was determined by Eosin-Nigrosin stain [22]. Only ejaculates of 5 bulls with motility > 70 % and sperm concentration of 1×10^9 sperm mL^{-1} were included in this study.

Experimental extenders. Pooled semen was collected from 5 bulls and diluted to a concentration of 30×10^6 sperm mL^{-1} with CEY extender (sodium citrate2.9 g dL^{-1}, pencillin 1000 IU mL^{-1}, streptomycine 1000 µg mL^{-1}) and double distilled water to make the volume 100 mL) or a Tris–egg yolk (TEY) extender (Tris (hydroxymethyl) aminomethane (TRIS) 3.028 g; citric acid monohydrate1.675 g; fructose 1.25 g; penicillin G sodium 1,000 IU mL^{-1}; streptomycin sulphate 1,000 µg mL^{-1}; and double distilled water to make a volume of 100 ml) containing 20 % egg yolk and 7 % glycerol (Merck, Germany). The diluted semen was divided 5 aliquots including control and 4 experimental groups.

Each aliquot was further diluted with an equal volume of CEY or a Tris (hydroxymethyl) aminomethane (TRIS) - based extender without (control) or containing 1 mM, 2 mM vitamin ; 0.1 mM, 0.2mM vitamin E. In both experiments, semen extended was equilibrated in an equilibration chamber at 5°C for 4 h before filling in 0.5ml French straws. The straws were placed on steel racks and held in liquid nitrogen at -140°C for 10 min. Frozen straws were then immediately immersed in liquid nitrogen (-196°C) and stored for 4 weeks until further assessment. At the time of analysis, three straws of semen from each treatment were thawed at 37°C for 30s to perform the following semen quality parameters. Each experiment was conducted in triplicate.

Post-thawed sperm motility. Progressive sperm motility was assessed using a phase contrast microscope (200× magnification), with a warm stage maintained at 37°C. A wet mount was made using a 5 µl drop of semen placed directly on a heated (37°C) microscope slide and overlaid with a cover slip. Sperm motility estimations were performed in three microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score.

Live and dead sperm. This was estimated as per standard staining procedure as described by Sidhu and Guraya(1985). The staining solution contains Eosin (0.67 g /100 mL) and Nigrosin (5 g /100 mL) and water to make the volume 100 ml. A drop of diluted semen mixed with eight drops of stain was incubated at 30°C for 5 min. Then smears made on pre-warmed slides were allowed to dry at 30°C. The excess stain was washed off in running tap water. The slide was then immersed briefly in ethanol to remove water. Then mounted smear was observed under 400× objective lens of the phase contrast microscope. Approximately 400 sperm were counted.

Lipid peroxidation (LPO). An aliquot (500µl) of semen from each sample was centrifuged at 800× *g* for 10 min; sperm pellets were separated and washed by resuspending in PBS buffer and were re-centrifuged (three times). After the last centrifugation, 1mL of deionized water was added to sperm [23] and they were snap-frozen and stored at -70°C until further analysis. The samples were thawed before the lipid peroxidation assay. The concentrations of malondialdhyde (MDA), as indices of the LPO in the sperm samples, were measured using the thiobarbituric acid reaction according to the method of Placer *et al.* (1966). The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption at 532 nm with the standard curve of MDA equivalents generated by the acid catalyzed hydrolysis of 1, 1, 3, 3-tetramethoxypropane. The MDA concentrations were expressed in nmol 10^{-9}.

Statistical analysis. The experiment was conducted in triplicate. Results are quoted as Mean ± SEM. Statistical analyses were carried out using the General Linear Model procedures (GLM) of SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), followed by the Tukey's post-hoc test. The *P*-values less than 0.05 were considered to be significant.

Results

Effect of vitamin C on frozen-thawed bull semen in CEY extender. Results in Table 1 showe that lowest production of MDA, as an indicator of LPO, was obtained by addition of 1 mM vitamin C to CEY extender. The percentage of sperm viability did not change significantly with the addition of different concentration of vitamin C to CEY extender compared with the control group. Sperm motility was significantly higher ($P < 0.05$) in samples extended with 1 mM and 2 mM vitamin C compared with the control group.

Effect of different concentration of vitamin C on frozen- thawed bull semen in TEY extender. Data for influence of vitamin C on frozen-thawed bull semen in TEY extender are shown in Table 2. The concentration of LPO did not change significantly with the addition of the vitamin C to TEY extender compared with the control group. There were no significant difference in post-thaw sperm viability and motility following the supplementation of the TEY extender with vitamin C.

Effect of vitamin E on frozen-thawed bull semen in CEY extender. Data for influence of vitamin E on frozen-thawed bull semen in CEY extender are presented in Table 3. The lowest production of MDA, as an indicator of LPO, was obtained by addition of 0.1 mM vitamin E to semen extender compared with the control group ($P < 0.05$). Supplementing extender with 0.1 mM vitamin E led to the highest ($P < 0.05$) post-thaw sperm viability and motility compared with the control group (Table 3).

Effect of different concentration of vitamin E on frozen-thawed bull semen in TEY extender. Data for influence of vitamin E on frozen-thawed bull semen in TEY extender are presented in Table 4. The concentration of LPO did not change significantly with the addition of the vitamin E to TEY extender compared with the control group. There were no significant differences in post-thaw sperm viability and motility following the supplementation of the TEY extender with vitamin E.

Table 1. Effect of vitamin C on post-thawed bull semen quality cryopreserved in a citrate - egg yolk (CEY) extender and lipid peroxidation (MDA) (n = 5, mean ± SEM)

Parameters	Concentration of vitamin C		
	Control	1mM	2mM
MDA(nmol 10^{-9})	1.90 ± 0.27^{a}	1.41 ± 0.32^{b}	2.14 ± 0.98^{c}
Sperm viability (%)	60 ± 3^{a}	69 ± 3^{a}	73 ± 4^{a}
Sperm motility (%)	46 ± 1^{a}	59 ± 3^{b}	62 ± 4^{b}

The heterogeneous letters (a, b & c) in rows indicate significant differences :($P < 0.05$)

Table 2. Effect of vitamin C on post-thawed bull semen quality cryopreserved in a tris - egg yolk extender (TEY) and lipid peroxidation (MDA) (n = 5, mean ± SEM)

Parameters	Concentration of vitamin C		
	Control	1mM	2mM
MDA(nmol 10^{-9})	6.45 ± 0.96^{a}	5.74 ± 0.33^{a}	5.90 ± 0.42^{a}
Sperm viability (%)	53 ± 4^{a}	65 ± 3^{a}	60 ± 1^{a}
Sperm motility (%)	46 ± 1^{a}	55 ± 5^{a}	55 ± 0.00^{a}

The homogenous letters in rows indicate non-significant differences

Table 3. Effect of vitamin E on post-thawed bull semen quality cryopreserved in a citrate - egg yolk (CEY) extender and lipid peroxidation (MDA) (n = 5, mean ± SEM)

Parameters	Concentration of vitamin E		
	Control	0.1mM	0.2mM
MDA(nmol 10^{-9})	3.88 ± 0.04^{a}	0.98 ± 0.06^{b}	1.25 ± 0.18^{b}
Sperm viability (%)	51 ± 1^{a}	66 ± 2^{b}	61 ± 1^{b}
Sperm motility (%)	43 ± 3^{a}	56 ± 4^{b}	51 ± 5^{b}

The heterogeneous letters (a, & b) in rows indicate significant differences :($P < 0.05$)

Table 4. Effect of vitamin E on post-thawed bull semen quality cryopreserved in a tris - egg yolk (TEY) extender and lipid peroxidation (MDA) (n = 5, mean ± SEM)

Parameters	Concentration of vitamin E		
	Control	0.1mM	0.2mM
MDA(nmol 10^{-9})	7.69 ± 0.16^{a}	5.45 ± 1.42^{a}	6.07 ± 0.54^{a}
Sperm viability (%)	46 ± 3^{a}	55 ± 4^{a}	50 ± 5^{a}
Sperm motility (%)	40 ± 1^{a}	50 ± 5^{a}	43 ± 6^{a}

The homogenous letters in rows indicate non-significant differences

Discussion

Mammalian spermatozoa are highly sensitive to LPO, which occurs as a result of the oxidation of membrane lipids by partially reduced oxygen molecules, e.g. superoxide, hydrogen peroxide and hydroxyl radicals. Spontaneous LPO of the membranes of mammalian spermatozoa destroys the structure of the lipid matrix. These attacks ultimately lead to the impairment of sperm function, such as sperm motility, functional membrane integrity and fertility, leakage of intracellular enzymes and damage to the sperm DNA through the oxidative stress and the production of cytotoxic aldehydes.[2, 24, 25]

Low toxicity and good water solubility of vitamin C has led to its use as an antioxidant additive and has been shown to have protective effects when added to an extender. In the present study positive effect of vitamin C (1 mM) in CEY extender on LPO was noted (Table1), and this is in line with some previous studies (Arabi and Seidaie 2008; Aurich *et al.*, 1997). These results may be explained based on the fact that vitamin C protects the spermatozoa by preventing from endogenous oxidative DNA and membrane damages. It is also believed that vitamin C works by scavenging superoxide anions and singlet oxygen and can protect the lipoproteins from detectable peroxidative damage.[26] This finding suggested that ascorbic acid might be needed to protect sperm against reactive oxygen species. But supplementing TEY extender with different concentration of vitamin C produced no effect on the reduction of LPO compared with the control group (Table 2). In addition, the results of this study show that addition of vitamin C to CEY extender increased sperm motility after thawing. The highest production of MDA was obtained by addition of 2 mM vitamin C to CEY extender. In addition, the results of this study show that addition of vitamin C into CEY extender suppressed deleterious effect of ROS on bovine sperm. Similarly results have been reported by some researchers.[11, 20, 21, 27] They have showed that vitamin C supplementation reduced ROS generation. But supplementing TEY extender with different vitamin C doses not improved the sperm viability or motility compared with the control group. The present finding is consistent with the observation made by Aurich *et al*. (1997) and Ball *et al*.(2001), who also did not detect any positive effect from the addition of

vitamin C to a cooled equine spermatozoa. One major cause of reduced viability after equilibration may be due to the negative effect of glycerol.

The results of the present study suggested that addition of 0.1 mM vitamin E to CEY extender caused a significant reduction in LPO products such as MDA compared with the control group. This agrees with the study carried out by Verma and Kanvar (1999); who observed that the 0.1mM vitamin E reduced the LPO production. The present study confirms a dose-dependent reduction in LPO production when various doses of vitamin E are added to semen samples. Vitamin E, as a lipid soluble antioxidant, also plays a major protective role against oxidative stress and prevents the production of lipid peroxides by scavenging free radicals (particularly strong scavenger of hydroxyl radicals) which are toxic byproducts of many metabolic processes in biological membranes [12,30,31]. But addition of vitamin E to TEY extender concentration of LPO didn't change compared to the control group.

Previous studies showed that vitamin E improved semen quality and quantity. Brzezinska –Slebodzinska *et al*. (1995) found that vitamin E supplementation significantly increased the number of spermatozoa in boars. In addition, effects of vitamin E (tocopherol or Trolox) supplementation on sperm motility varied from being detrimental in fresh human semen [26] and liquid ram semen [32] to little or no effect in equine chilled semen [33, 34] and in human chilled semen[35] In this study addition of 0.1mM vitamin E to CEY extender significantly improved sperm motility and viability compared with the control group. Effect of vitamin E noted in the present study can be attributed to its antioxidant effects similar to those reported by Cerolini *et al*.(2000), Akiyama (1990), Sen *et al* .(2004), Surai *et al*.(1998) who reported that vitamin E supplementation reduced ROS generation and improved semen quality in animal.[41, 12,40,11] However, supplementing TEY extender with different vitamin E doses did not improve the sperm viability or motility compared with the control group.

On the basis of the present results, it is concluded that vitamin C and E are very efficient antioxidant in CEY extender and that their addition to an extender can reduce the oxidative stress provoked by thawing and protects bovine sperm during cryopreservation. However, addition of vitamin C and E to TEY extender cannot improve semen quality. The, results of this study provide a new approach to the cryopreservation of bull sperm and could contribute to the improvement of semen cryopreservation in the cattle industry.

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