The effect of vitamin E on post-thawed buffalo bull sperm parameters

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Abstract: The sperm cells protection against oxidative reactions during cryopreservation process done by antioxidant and amino acids agents. The purpose of this study was evaluation of the effects of vitamin E on Azerbaijan Buffalo bull´s sperm cells after thawing. Therefore for definition the percentage of motility, acrosomal membrane integrity, and live ratio of sperm cells, ejaculations from five mature buffalo bulls after preparation in tris-yolk base medium was added with five levels of vitamin E (0.1, 0.5, 1 or 1.5 mM) separately and frozen process was performed. One month latter, five piote selected randomly and after thawing in 37 °C water bath in twenty seconds, sperm cells motility evaluated with 37 °C warm plate microscope. On the other hand, the one step eosin-nigrosin staining for evaluation of live ratio percentage and formal citrate for acrosomal membrane integrity was performed, then slides evaluated with 1000x light microscope and 200 sperm per slide was counted. The result showed significant difference between blank and vitamin E groups and sperm motility was higher in vitamin E ($P<0.05$). On the other hand sperm motility in vitamin E 1.5 mM was higher than other vitamin E groups ($P<0.05$). Between vitamin E groups, the percentage of live-ratio was higher in vitamin E 1.5 mM and lower in vitamin E 0.1 mM ($P<0.05$) and the lowest was in control group ($P<0.05$).
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1. Introduction:

Buffalo population of the world was estimated to be more than 140 million (Sansone *et al.* 2000). Artificial insemination (AI) is one of the important reproductive biotechnologies, which causes the widespread propagation of semen, limiting the spread of sexually transmitted diseases, and chiefly facilitating genetic improvement programmes in livestock (Andrabi et al. 2008; Numan Bucak et al. 2009). Nevertheless, cryopreservation generates sub-lethal injury to the sperm due to chemical, osmotic, thermal, and mechanical stresses, which may result in loss of viability, motility, damage of deoxyribonucleic acid (DNA), destruction of acrosomal and plasma membrane (Numan Bucak et al. 2007; Rasul et al. 2001) . furthermore change of biochemical factors have been recognized during cryopreservation, including depletion of amino acids and lipoproteins, release of glutamic-oxaloacetic transaminase (GOT), decrease in phosphatase activity, decrease in loosely bound cholesterol protein, inactivation of acrosin enzyme and hyaluronidase, prostaglandins diminution, increase in sodium, decrease in potassium content, reduction of ATP and ADP synthesis and decrease in acrosomal proteolytic activity (Barbas et al. 2009). The extenders is an important factor in cryopreservation process. These mediums must have adequate pH and buffering capacity, appropriate osmolality and should protect spermatozoa from cryogenic lesion. The Tris extenders are an important mediums that often used for semen freezing of bulls, rams, bucks and buffaloes (Barbas et al. 2009; Rasul et al. 2001).

In fact sperm plasma membrane is a primary target for freezing or cold shock injury (Numan Bucak et al. 2009).

Reactive oxygen are responsible to sperm dysfunction due the lipid peroxidation of membranes (Arabi et al. 2001). Agrawal et al (2005) demonstrated that antioxidants are the major defensive mechanism against oxidative stress. It has been now documented that vitamin E is the major antioxidant agent of sperm cells which is a potent scavenger of free radicals and is able to protect plasma membrane from damages mediated by ROS and LPO (Yousef et al. 2003; Gurel et al. 2005; Sinclair, 2000). It has been established that presence of vitamin E is necessary for normal function of male reproductive system and traditionally, vitamin E is called as anti-sterility (Momeni et al. 2009).

Bansal et al (2010) and Ball et al (2001) reported that E is a main chain-breaking antioxidant in membranes because it may directly quench the free radicals such as superoxide anion (•O2-), hydrogen peroxide (H2O2) and hydroxyl radical (OH•).

The present study aimed at finding out the efficacy of E, a biological antioxidant, in reversing

the free radical-mediated oxidative damage on sperm motility, acrosome integrity and viability.

2.Material and methods

2.1. Chemicals

The anti-oxidant used in this study such as E, and other chemicals were obtained from Sigma–Aldrich.

2.2. Animals, Semen collecting and processing

The four mature buffalo-bulls (3–4 years of age, and 600–650 kg of body weight) maintained at the northwest buffalo Research Institute, Ministry of Agriculture, Urmia, Iran, under uniform feeding, housing and lighting conditions were used as a semen donor. A total number of 20 ejaculates were collected from buffalo-bulls with the aid of an artificial vagina at weekly intervals for 5 weeks. Immediately after collection, the ejaculates were transferred to the laboratory, and evaluated for sperm motility and sperm concentration.

The sperm concentration of each ejaculate was determined by means of a spectrophotometer. Only ejaculates containing sperm with >80% progressive motility and a concentration higher than 1.5×10^9 sperm/ml were pooled, in order to eliminate the bull effect. The base extender was Tris-based extender (Tris 2.66 g/100 ml, citric acid 1.47 g/100 ml, fructose 0.63 g/100 ml, egg yolk 20% (v/v), glycerol 7% (v/v), pH 6.8) was used as the basis extender. Each mixed ejaculate was split into 11 equal aliquots and diluted at 37 ◦C to concentration of 6×10^6 sperm/mL with the base extender containing E (0.1, 0.5, 1 or 1.5 mM), and no anti-oxidants (control), respectively.

Diluted semen samples were aspirated into 0.5 ml French straws, sealed with polyvinyl alcohol powder and cooled horizontally from 37 to 4 ◦C, and maintained at 4 ◦C for 4 hrs. The straws were frozen in vapor 4cm above liquid nitrogen for 10 minutes and plunged into liquid nitrogen. After being stored for one month, 5 frozen straws from each group were thawed individually at 37 ◦C for 20 s in a water bath for microscopic evaluation.

2.3. Semen evaluation

- *Sperm viability*

A 50 µL drop of frozen–thawed semen was placed on a pre-warmed slide and mixed with 50 µl drop of the supravital stain [1% (w/v) eosin B, 5% (w/v) nigrosin in 3% tri-sodium citrate dehydrate solution] to prepare a thin smear [20]. After air-drying, the smear was evaluated by microscope with 400× magnification. Two hundred spermatozoa were counted for unstained heads of spermatozoa (live) and/or stained/partial stained heads of spermatozoa (dead) (Ijaz *et al.* 2009).

- *Post-thawed sperm motility*

Percentage motility was assessed using a phase-contrast microscope (×40). A 5 µL drop of thawed semen placed directly on a pre-warmed slide and covered with a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample by the same researcher. The mean of three observations was considered as a single data point.

- *Normal Acrosomes*:

A 500-µL portion of each semen sample was fixed in 50 µL of a 1% solution of formal citrate containing 2.9% (w/v) trisodium citrate dihydrate (Merck) and 1% (v/v) commercial formaldehyde. Two hundred spermatozoa were counted with a phase contrast microscope (1000×) for their normal apical ridge.

- *Statistical analysis*

The study was replicated three times. Results are expressed as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to assess differences among treatments on motion characteristics, plasma membrane integrity and normal acrosome morphology. When the F ratio was significant (P<.05), Tukey's post hoc test was used to compare treatment means (Version 12.0; SPSS, Chicago, IL).

3. Results

- *Sperm parameters (percentages of motility, viability, acrosome Normality):*

The effects of antioxidants on the sperm characteristics of frozen buffalo semen were evaluated in the five independent experiments.

The post-thaw sperm motility improved significantly ($P<0.05$) in the groups as compared to their respective values.

As set out in Table 1, the inclusion of vitamin E led to a higher motility, compared to the control group ($P < 0.05$). The motility of sperm cells post thaw increased significantly when adding of 0.1, 0.5, 1 or 1.5 Mm, E (56.98 ± 0.05, 61.08 ± 0.07, 63.16 ± 0.17 and 65.03 ± 0.09 respectively) to the extender as compared to the control group (45.86 ± 0.18).

No significant differences were observed in acrosome damage, following the supplementation of the freezing extender with antioxidants, following the freeze–thawing process.

Table 1. Effect of different concentrations of vitamin E on buffalo-bull semen Motility after freezing-thawing

Treatments	Motility (%Mean ± SE)
E 0.1 mM	56.98 ± 0.05 [d]
E 0.5 mM	61.08 ± 0.07 [c]
E 1 mM	63.16 ± 0.17 [b]
E 1.5 mM	65.03 ± 0.09 [a]
Control	45.86 ± 0.18 [e]

Values are (mean ± standard error of mean). Different letters within a column indicates significant differences (P<0.05).

Table 2. Effect of different concentrations of vitamin E on buffalo-bull semen acrosomal normality after freezing-thawing

Treatments	acrosomal normality (%Mean ± SE)
E 0.1 mM	68.38 ± 0.14
E 0.5 mM	68.03 ± 0.12
E 1 mM	67.91 ± 0.11
E 1.5 mM	68.26 ± 0.14
Control	68.38 ± 0.11

Table 3. Effect of different concentrations of vitamin E on buffalo-bull semen viability after freezing-thawing

Treatments	Live sperm (%Mean ± SE)
E 0.1 mM	66.86 ±0.06 [d]
E 0.5 mM	71.03 ±0.28 [c]
E 1 mM	73.03 ±0.10 [b]
E 1.5 mM	74.7 ±0.14 [a]
Control	55.51 ±0.16 [e]

Values are (mean ± standard error of mean). Different letters within a column indicates significant differences (P<0.05).

The viability of sperm cells post thaw increased significantly when adding of 0.1, 0.5, 1 or 1.5 mM, vitamin E (66.86 ±0.06, 71.03 ±0.28, 73.03 ±0.10 and 74.7 ±0.14 respectively) to the extender as compared to the control group (P<0.05) and the highest viability of sperms were seen at 1.5 mM vitamin E treatment. Also, there are significant differences between the E treatments (P<0.05).

4. Discussions

Buffalo sperm is susceptible to cold stress than other species such as bull, rabbit and human (Andrabi et al., 2008; Fise et al., 1989). Of course, different reactions to temperature tensions is result from differences in membrane lipid composition as buffalo sperm has high levels of saturated and unsaturated fatty acids and has lowest ratio of cholesterol/phospholipids compared with other species (Evans et al, 1987).

Antioxidant capability in sperm cells is limited because of deficiency cytoplasmic components having antioxidant effects to expunction of reactive oxygens. Thus, mammal's sperm haven't enough ability to encountering with peroxidation during the freezing and thawing processes (Alvarez et al, 2005; Bilodeau et al, 2000 and Lapointe et al, 2003). Reactive oxygen is known to play a major role in sperm membrane damage and directly damage sperm DNA that cause reduces of the sperm's motility, acrosomal membrane integrity and sperm metabolic alterations. In recent years, adding antioxidants to semen extenders for improvement of sperm quality were studied.

Addition of antioxidants such as vitamin E and vitamin C to the semen freezing diluent, may prevents or diminishes cryodamage to spermatozoa metabolism and antioxidant capacities (Anghel et al, 2009; Andrabi et al, 2008; Beheshti et al, 2011).

Beconi et al (1991) demonstrated that vitamin E prevents lipid peroxidation of frozen bovine semen. It has been demonstrated that this improved sperm quality and fertility in human sperm.

In some studies have been documented low levels of vitamin E would allow for production of physiological level of ROS that are necessary for capacitation, acrosome reaction and *in vitro* fertilization (DalEt al, 1998). Addition of vitamin E had a beneficial effect on sperm motility in liquid ram semen (Upreti et al, 1997), fresh human semen (Donnelly et al, 1999) and to little effect in equine chilled semen (Ball et al, 2001). In the present study, the inclusion of different levels of vitamin E to extender before freezing causes significant improvement in sperm quality parameters such as motility and viability of sperms than control group (P<0.05).

This study shows that all doses of vitamin E improve the buffalo sperm quality parameters such as motility and viability of sperms than control group (P<0.05). These findings were similar to results obtained with Ijaz et al (2009), Kheradmand et al (2006), Bansal and Bilaspuri (2009).

Supplementation the incubation medium with all doses of vitamin E improved the percent sperm motility compared to control group (P<0.05). It is suggested that addition of E may be useful in preventing the rapid loss of motility that occurs during semen freezing.

However, we haven't seen significant differences in normal acrosome percent between treatment and control groups. This finding was in contrast with results of Bansal (2010) that may be due to the differences in semen extender and animal species.

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