IJBPAS, September, 2015, 4(9): 5848-5859

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

INFLUENCE OF ADDED VITAMIN C ON CHILLED AND FROZEN-THAWED RAM SPERM CRYOPRESERVED IN TRIS EXTENDER

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ABSTRACT

The present study aimed to evaluate quality of pre and post frozen-thawed ram semen processed with extender containing different levels of vitamin C as antioxidant. Pooled semen's were collected from 6 rams and diluted with Tris – egg yolk (TEY) extender. The diluted semen was divided to 4 aliquots including control (o) and 1, 2 and 3 mg/ml vitamin C and was cooled at 5°C and their qualities were evaluated during pre-freezing and then the cooled semen samples were packaged into 0.25 ml straws. Straws were frozen in the vapor of liquid nitrogen and thawed ten days Routine semen evaluations like sperm motility, progressive motility, viability, hypo-osmotic swelling test (HOST), normal spermatozoa and acrosome defects in pre and post freezing process were conducted. Results showed that the effects of vitamin C in Tris extender on some characteristics of spermatozoa were significant compare with control group, but different levels of vitamin C were not significant. The highest sperm motility, viability, normal spermatozoa and HOST were achieved by addition of 2mg/ml of vitamin C in Tris extender. On the basis of the present results it is concluded that supplementing TEY extender with vitamin C improved quality of Zel ram spermatozoa in chilling and freezing conditions.

Key words: Semen, Antioxidant, Vitamin C, Zel ram

INTRODUCTION

The production of Reactive Oxygen Species (ROS) is a normal physiological event in different organs. However, the over-production of ROS can cause structural damage of spermatozoa membranes. Oxidative stress results in a fall of intracellular ATP levels, which decreases sperm motility and also initiates lipid proxidation (LPO), which has been suggested as the cause of abnormal acrosome reaction and loss of membrane fluidity, culminating in a loss of fertilizing potential of spermatozoa (Ball, 2008). For counteract the dilatory effects of ROS, seminal plasma has an antioxidant system that seems to be very relevant to the protection of sperm. Therefore control the level of ROS and promote motility and viability of sperm, numerous antioxidants have proven beneficial in treating spermatozoa (Batool et al, 2012). The addition of vitamin C in an extender can affect on optimal sperm performance by decreasing cell damage through its continuous radical-scavenging action.

Beconi et al. (1993) had indicated that the presence of 5mM vitamin C in extender exerted an antioxidant effect during freezing and thawing in bovine semen. ROS that cause the 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 dilatory motility, plasmalemma functionality, viability, acrosome and chromatin damages of spermatozoa (Ansari et al, 2010).

The addition of exogenous antioxidants is required to decrease this ROS-mediated damage (Vishwanath and Shannon, 1997).

Batool et al (2012) reported that addition of vitamin C in extender may be improving the freeze ability of zebu bull spermatozoa. Ascorbic acid (vitamin C) is also a naturally occurring free scavenger, and as such, its presence helps various other mechanisms in decreasing numerous disruptive free radical processes, including LPO (Hu et al, 2010).

A positive effect of the addition of vitamin C on preservation of membrane integrity of cooled equine sperm has been observed by Aurich et al (1997) but, Baumber et al (2005) were stated that the addition of ascorbic acid to the cryopreservation extender did not improve the quality of equine spermatozoa after thawing; failing to demonstrate a clear, positive effect on the maintenance of sperm motility or fertility during at 5°C.

Andrabi et al (2008) reported that the addition of natural antioxidants (vitamins C or E) in the semen extender may be a step closer to improve the frozen-thawed quality of buffalo spermatozoa. Therefore, the present study was undertaken to test the efficacy of adding vitamins C to Tris-fructose-egg yolk extender on sperm quality of the Zel ram in pre and post freezing conditions.

METHOD AND MATERIAL

Animals and Semen Collection

These experiments were performed around Golestan province in Iran. In this study six healthy and mature Zel rams with an average body weight of 60.0 ± 5.0 kg were selected and were housed individually in pens on semi-slatted floors. Animals were fed with a diet according to the recommendations of the National Research Council (NRC) based on 80:20 ratio of forage (alfalfa) to concentrate adlibitum and had free access to water. From each ram, eight ejaculates were collected using electro ejaculator as described by Evans and Maxwell (1987).

Semen Processing

Semen samples were immediately transferred into graduated test tubes after collection by electro ejaculator, placed in a thermo flask at 37°C, and transported to the laboratory for evaluation. The fresh semen was then microscopically examined for volume, wave motility, percent motile sperm, progressive motility, viability, major and minor defects, normality and concentrate spermatozoa. Semen samples that showed more than 80% viability and motility and 90% normality of spermatozoa were selected for this experiment (Table1). The semen samples were pooled to eliminate individual differences and divided into four equal aliquots and kept at 37ºC in water bath. After primary observation, semen samples were diluted at a 1:4 ratio (semen: diluent) in Tris extender. The dilution contained Tris (hydroxyl methyl amino methane) (3.876 g), glucose (0.523 g), citric acid (2.123 g), egg yolk (15%), glycerol (5%), penicillin (100000 IU) and streptomycin (100 mg) double distilled water 85 mL (Evans and Maxwell, 1987).

Semen was split into four parts and different amounts of vitamin C (0 (control), 1, 2 and 3 mg/ml) were added to each groups. Diluted semen was cooled near to +5ºC within 2 hours. After 2h, a part of the samples were investigated. The remaining part of the samples was packaged into 0.25 ml straws. At first, straws were frozen at heights of 4-6 cm above the level of the liquid nitrogen (vapor liquid nitrogen), the frozen straws were then transferred to liquid nitrogen (Evans and Maxwell, 1987) and then characteristics of motility,

progressive motility and viability, plasma membrane integrity (HOST), normality and acrosome damages were examined.

Semen Analysis

Volume

The volume of each ejaculate was recorded by reading the graduated tube (Biswas et al, 2002).

Sperm concentration

Sperm concentration was determined using semen diluted with 3% NaCl. The semen was extender placed on a hemocytometer with the sperm counted in five squares of one chamber (Smith and Mayer, 1955).

Mass motility

To investigate the mass motility, a drop (25µl) of semen was placed on a pre warmed slide and examined under light microscope magnification (100X). The mass motility was scored into 5 scales: 1= no motion, 2= free spermatozoa moving without forming any waves, 3= slow moving waves, 4= vigorous movement with moderately rapid waves and 5= dense, very rapidly moving waves (Biswas et al, 2002).

Visual Motility

For determination of motility of spermatozoa a drop of semen sample was placed on a pre-heated glass slide and cover slipped. Semen scoring was performed at microscopic magnification of (400X) based on the visual estimation of sperm motility (%) and the percentage was - rounded to nearest 5%. Sperm motility was scored from zero to 100% (El-Nattat et al, 2011).

Viability

The viability of the sperm in the sample was evaluated by means of eosin-nigrosin stain according to the method described by Bearden and Fuquay (1992). A total of 100 sperm cells were counted on each slide at (400X) magnification of microscope. Sperm showing partial or complete purple colorization was considered non-viable or dead and only sperm showing strict exclusion of the stain were considered to be alive.

Progressive motility

For evaluated of Progressive motility, a drop of semen diluted in 1:4 ratio with Tris was placed on pre warmed slide (37ºC) and cover slip. The motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400X) of microscope.

Sperm morphology

Assessment of abnormal (head, mid-piece and tail of spermatozoa) and normal spermatozoa was performed using an eosin–nigrosin stain. For morphological assessment, a drop of diluted semen was placed on a slide and covered. A total of 200 sperm cells were counted on each slide. The morphology of the spermatozoa was

assessed under microscopy (magnification 1000X, oil immersion).

Acrosomal damage

For assessment of Acrosomal damages, Gimsa stain was used. The slides examined under the microscope using the oil immersion by counting 200 sperm in different fields of the slide. The Acrosomal cap of the sperm taken the stain was considered to have an intact acrosome and the rest were considered as damaged acrosome (Watson, 1975).

Assessment of spermatozoon membrane integrity

The hypo-osmotic swelling test (HOST) was used to investigate the functional integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 30 ml of semen with 300 ml of a 100 mOsM hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) (Revell and Mrode, 1994) at 37°C for 60 min. After incubation, a drop of the mixture was placed on a clean sterilized dry glass slide with a cover slip. 400 sperm were observed under magnification (400X) with bright-field microscopy. Sperm with swollen or coiled tails were recorded (Buckett et al, 1997).

Sperm Motility Recovery Rate

The sperm motility recovery rate was evaluated by comparing the motility of pre freeze (Mpr) and post thaw (Mps) spermatozoa using the formula: Recovery rate = Mps/Mpr × 100% (Hui Li et al, 2005).

Sperm progressive Motility Recovery Rate

The sperm progressive motility recovery rate was evaluated by comparing the progressive motility of pre freeze (PMpr) and post freezing (PMps) spermatozoa. Recovery rate = PMps/PMpr × 100%.

Sperm viability Recovery Rate

The sperm viability recovery rate was calculated by comparing the viability of pre freeze (Vpr) and post thaw (Vps) spermatozoa. Recovery rate = Vpr/Vps × 100%.

Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M.). Means were analyzed by one-way analysis of variance, followed by the Duncan post hoc test to determine significant differences in all the parameters among all groups using the SPSS/PC computer program (Version 15.0; SPSS,). Differences with values of $P<0.05$ were considered to be statistically significant.

RESULTS

The characteristics of fresh spermatozoa are shown in Table 1. The influence of vitamin C on the cooled semen parameters of *Zel rams* were shown in Table 2. The effects of vitamin C on percentages of

motility, progressive motility, viability, plasma membrane integrity, and normal spermatozoa were significant ($p \leq 0.05$) compare with control group. The effects of vitamin C in the post freezing extender on sperm parameters were shown in Table 3. The percentages of motility, progressive motility, viability, plasma membrane integrity, acrosome integrity and normal spermatozoa were significant ($p \leq 0.05$) compare with control group but different levels of vitamin C were not significant although this treats were higher in 2mg/ml of vitamin C. Recovery rate of motility and viability of spermatozoa are shown in Table 4. The highest motility (36.10 ± 1.64), progressive motility (32.7 ± 2.44) and viability recovery rate (39.47 ± 0.77) were observed in Tris extender containing 2mg/ml of vitamin C.

Table 1: Characteristics of Zel ram spermatozoa, under fresh condition

Volume	Mass motility	Motility	Progressive motility	Viability	Major defects	Minor defects	Acrosome defects	Normal sperm	Concentration
1.30	4.5	80	75	85	2.2	3.5	1.2	93.1	3.54×10^9

Table 2: Characteristics of Zel ram spermatozoa, stored at 5°C in diluents supplemented with different levels of Vitamin C (mean ±S.E.M)

Vitamin C (mg/ml)	Motility (%)	Progressive Motility (%)	Viability (%)	Major Defects (%)	Minor Defects (%)	HOST (%)	Acrasome defects (%)	Normal sperm (%)
Control	72 ± 1.22^b	67.2 ± 1.35^b	75.8 ± 1.01^b	2.6 ± 0.5	13 ± 1.30^a	72.4 ± 1.12^b	4 ± 0.44	80.04±0.9^b
1	76.4 ± 0.97^a	72 ± 1.22^a	81 ± 0.83^a	2.2 ± 0.37	9.8 ± 2.41^a	77.8 ± 0.96^a	3.8 ± 0.37	84.2±2.85^{ab}
2	77.6 ± 1.12^a	73.6 ± 1.56^a	81.6 ± 0.74^a	2.4 ± 0.24	6.2 ± 0.80^b	78.2 ± 0.91^a	3.6 ± 0.20	87.6±1.16^a
3	76.4 ± 0.98^a	72.2 ± 1.15^a	79.8 ± 0.96^a	2 ± 0.31	8 ± 0.94^b	76.8 ± 0.96^a	3.8 ± 0.37	86.6± 1.40^a

Different superscript letters (a to b) within the same column showed significant differences among the groups (P<0.05)

Table 3: Characteristics of Zel ram spermatozoa, after frozen-thawed in diluents supplemented with different levels of Vitamin C (mean ±S.E.M)

Vitamin C (mg/ml)	Motility (%)	Progressive Motility (%)	Viability (%)	Major Defects (%)	Minor Defects (%)	HOST (%)	Acrasome defects (%)	Normal sperm (%)
Control	16.4 ± 0.97^b	13 ± 1.54^b	19.4 ± 1.28^b	2.4 ± 0.24	19.2 ± 0.48^a	16.2 ± 1.06^b	7.8 ± 0.66^a	70.6± 1.28^b
1	26.6 ± 1.29^a	22.4 ± 1.12^a	3 ± 0.94^{ab}	2.2 ± 0.37	14.4 ± 0.50^b	27.4 ± 1.12^{ab}	5.8 ± 0.2^b	77.6± 0.74^a
2	28 ± 1.22^a	23.6 ± 1.56^a	32.2 ± 0.48^a	2 ± 0.44	14.8 ± 0.66^b	28.8 ± 0.73^a	4.8 ± 0.58^b	78.4± 0.5^a
3	25.2 ± 1.59^a	22.4 ± 1.91^a	28.2 ± 1.35^{ab}	2.4 ± 0.24	15.6 ± 0.40^b	25 ± 1^b	5.8 ± 0.2^b	76.2 ±0.37^a

Different superscript letters (a to c) within the same column showed significant differences among the groups (P<0.05)

Table 4: Recovery rate of Characteristics of Zel ram spermatozoa, in diluents supplemented with different levels of Vitamin C (mean ±S.E.M)

Vitamin C (m/M)	Recovery of Motility (%)	Recovery of Progressive motility (%)	Recovery of Viability (%)
Control	22.85 ± 1.62[b]	19.46 ± 2.55[b]	25.69 ± 2.07[b]
1	34.7 ± 0.90[a]	31.04 ± 1.06[a]	37.02 ± 1.05[a]
2	36.10 ± 1.64[a]	32.7 ± 2.44[a]	39.47 ± 0.77[a]
3	32.95 ± 1.95[a]	31.84 ± 1.64[a]	35.2 ± 1.42[a]

Different superscript letters (a to b) within the same column showed significant differences among the groups (P<0.05).

DISCUSSION

Mammalian spermatozoa are highly sensitive to lipid peroxidation (LPO), and Spontaneous LPO of the membranes of spermatozoa injury the structure of the lipid matrix. These attacks ultimately lead to the impairment of sperm function, such as sperm motility, membrane integrity, leakage of intracellular enzymes, and damage to the sperm, DNA and production of cytotoxic aldehydes (Aitken et al, 1993). Therefore the antioxidant supplementation in extender has been used to confronting against lipid peroxidation that potentially decreases sperm fertilizing ability. Although the mechanism of Ascorbic Acid (vitamin C) effects on sperm number in animals is not clear, many studies reported positive effect of vitamin C on sperm concentration in human and animals (Metwally and Fouad, 2009).

Vitamin C is required as a cofactor for at least eight enzymes and can also act as an antioxidant by reacting with free radicals (Halliwell and Gutteridge, 1999). Low toxicity and good water solubility of vitamin C has led to its act as an antioxidant and has been shown to have protective effects when added to an extender. In the present study, positive effects of vitamin C in TEY extender on some parameters of spermatozoa compare with control group in pre and post freezing conditions were noted (Table2, 3).

In this study the effect of vitamin C were significant and higher motility, progressive motility, viability, HOST and normal spermatozoa were observed in 2mg/ml vitamin C in TEY extender, but different levels of vitamin C were not significant. These results may be explained that vitamin C protects the spermatozoa by preventing from endogenous oxidative DNA and membrane injuries. It is also believed that vitamin C acts by scavenging super oxide anions and singlet oxygen and can protect the lipoproteins from detectable per oxidative damage (Donnelly et al, 1999).

This finding suggested that ascorbic acid might be needed to protect sperm against reactive oxygen species. The present finding is consistent with the observation made by Asad pour, et al (2011) who also did not detect any positive effect from the

addition of different level of vitamin C on frozen-thawed bull semen.

Metwally and Fouad (2009) reported that the motility of spermatozoa in vitamin C supplementation groups were higher than the control group in Male Grass Carp. Thuwanuta et al (2013) reported that improvement of ram sperm after the addition of antioxidants (vitamins C) to semen extender.

Azwahi and Khudhur Hussein (2013) showed that Awassi ram semen in Tris dilution containing vitamin C could improve sperm motility and viability and also protected acrosome and membrane integrity for 120 hr of preservation at 5 ˚C. These results were in agreement with Hughes et al, (1998) of human semen; Maia et al, (2009, 2010) of ram semen and Singh et al, (1996) in buffalo semen. The beneficial effect of adding vitamin C to ram semen extender was in accordance with the results obtained by Asghari (1999). However, in the presence of transition metal ions (e.g. Fe3+, Cu2+) high levels of ascorbic acid can act as a peroxidation by donating an electron that decrease such ions to forms that, in turn, can react with oxygen molecules to form oxygen radicals. Vitamin C can protect membrane integrity of sperm cells from heat shock during freezing-thawing process of spermatozoa (Lewis et al, 1997).

A probable improvement in semen quality by addition of vitamin C in ram semen extender is more likely related to an inhibition of lipid peroxidation of the sperm plasma membrane as was revealed by Barati et al (2011). Ascorbic acid that prevents chain propagation reactions and regenerates α-tocopherol (Niki, 1987).

Breininger and Beconi (2014) reported that the addition of vitamin C to the semen extender reduced lipid peroxidation and increased sperm motility. Several authors have proved that the addition of molecules with antioxidant capacity to the cryopreservation extender increases oxidative damage (Cabrita et al. 2011). However, contradictory results were obtained in some reports, as the right balance among oxidation and reduction is not easily achieved. The levels of lipid peroxidation increase during incubation (Breininger et al. 2005) and altering sperm motility due to the ROS-induced damage in ATP utilization as repotted by Guthrie and Welch (2012).

In our study vitamin C reduces lipid peroxidation and this is may be indicate the protection of the sperm plasma membrane from per oxidative damages. But ascorbic acid is unstable when exposed to highly oxidative environments, being rapidly oxidized into inactive dehydroascorbate (Linster and Van Schaftingen, 2007).

In our investigate, Vitamin C maybe provided protection of sperm cells from morphological defects by preventing free radical oxygen from damaging sperms and exerted a protective effect compared control group.

CONCLUSION

It could be concluded that the addition of antioxidants such as vitamins C to the preservation media could improve longevity and quality of cooled and frozen sperm in Zel ram semen.

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