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Evaluation of vitamin E on microscopic parameters of chilled and frozen stored ram semen

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ABSTRACT

The production of reactive oxygen species during the process of cryopreservation, decrease the motility and cellular viability by initiation of lipid per oxidation of bio-membranes. For reduce these damages, antioxidants are used as cryoprotectants against the lipid per oxidation. The aim of this study was to assess the influence of vitamin E adding to the dilution media on standard qualitative parameters (motility, progressive motility, viability, hypo-osmotic swelling test, Acrosomal damages and normal spermatozoa) of pre and post frozen-thawed ram semen. Semen samples were collected by Electro ejaculator from 6 Zel rams, and diluted with a Tris-base extender containing vitamin E (1, 2 and 3 m/M) and without antioxidants (control). Diluted semen was cooled to 5oC and frozen in 0.25 ml straw, prior to being stored in liquid nitrogen. The results show that vitamin E has positive protection effects on the semen characteristics in chilled and frozen thawed. Motility, progressive motility, viability, hypo-osmotic swelling test and total normality of spermatozoa were higher in all groups of vitamin E supplement compare with control group, but highest percentages of treats were observed in 2m/M of vitamin E. Also the effect of vitamin E on Acrosome damages were significant in post frozen conditions and the highest percentages were obtained in control group. Therefore we recommend using 2m/M of vitamin E in Tris extender for short and long storages of Zel ram spermatozoa.

Key Words: Vitamin E, Zel ram, Spermatozoa.

INTRODUCTION

Artificial insemination with cooled stored semen has become a technique in sheep [1]. Because of low dilution rate of ram semen for intra cervical insemination, semen of many individual rams does not react well to cryopreservation. Therefore, protect of motility and survival of spermatozoa during short-term (typically less than 72 hrs) storage is an important consideration in the use of liquid semen [2].

Freezing and thawing process of semen causes functional and ultra structural injury of spermatozoa that lead to decrease of motility, impaired transport and the loss of fertilizing capacity [3]. Although many years of research has established a several methods in order to improve the process, fertility is commonly lower also when liquid stored ram semen is used, however using antioxidants in the extenders could be a promising solution to inhibit damaging membrane changes during the chilling, freezing and thawing process [4]. The advantage effects of antioxidants supply indirect evidence that an oxidative stress happen during cryopreservation [5]. This has been proved by

studies which showed that reactive oxygen species (ROS) are generated during freezing and thawing of bull [6], ram [7] and equine spermatozoa [8].

Lipid peroxidation (LPO) is one of the major biological processes associated with ROS. Mammalian sperm cells are particularly sensitive to lipid peroxidation due to the justly low function of the enzymatic anti-oxidative system and because cellular sperm membranes are rich in polyunsaturated fatty acids, that are easily peroxidisable. In many experiment it had been shown that vitamin E is a major scavenger of lipid peroxyl and alkaoxyl radicals and demonstrated to be having protective effect during semen storage. A significant decline in motility and viability of spermatozoa derived by ROS is associated with semen cryopreservation and thawing processes, and these detrimental effects can be reduced by supplementation of vitamin E to extenders at a dose of 10mmol/liter [9].

These kinds of findings were also noted in other trials that vitamin E supplementation during chilling, lead to good effects on boar sperm motility and mitochondrial membrane potential [10].

Anghel et al [11] showed that addition of 2m/M Vitamin E in extender, protective effects on motility and viability of ram spermatozoa, while oxidative stress was noted to be reduced. Similarly a significant increase in motility and membrane integrity of spermatozoa were observed in cryopreserved buck semen scavenging three types of free radicals i.e. superoxide, peroxyl and hydroxyl radicals [11].

Beheshti et al [12] reported that supplementation of Vitamin E in extender is effective in preventing the rapid loss of motility and viability of Buffalo bull spermatozoa during cryopreservation. Asadpour et al [13] were observed that addition of 0.1mM vitamin E to CEY extender significantly increase motility and survival of bovine spermatozoa compared with the control group. Effect of vitamin E can be attributed to its antioxidant effects similar to those reported by Cerolini et al [14], who reported that vitamin E supplementation decreased ROS production and improved semen quality in animal. Hence, the semen for its better post thaw quality require to be supplemented with certain natural antioxidants that can counter excess ROS generated during semen freezing process. Vitamin E is the major antioxidant naturally present in mammalian semen that regulates ROS, preserve the sperm from lipid peroxidation and supply higher integrity to plasma membrane and mitochondria as well as better kinematics for sperm post-cryopreservation [15].

Therefore, it was the objective of this study to evaluate effects of vitamin E supplementation in Tris extender on some microscopic parameters of Zel ram spermatozoa.

MATERIALS AND METHODS

Animals and Semen Collection

These experiments were carryout 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 ad libitum and had free access to water. From each ram, eight ejaculates were collected using electro ejaculator as described by Evans and Maxwell [16].

Semen Processing

Immediately after collection, semen samples were pooled to eliminate individual differences and plunge into a water bath maintained at 37^0C prior to evaluation. The semen samples were investigated for volume, mass motility, sperm concentration, pH, motility, progressive motility, viability, major and minor defects and normality of spermatozoa. Semen samples that showed more than 80% viability and motility and 90% normality of spermatozoa were selected for this experiment (Table1). After primary observation, semen samples were diluted at a 1:4 ratio (semen: diluents) 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 [16]. Semen was split into four parts and different amounts of vitamin E (0 (control), 1, 2 and 3 m/M) were added to each groups. Diluted semen was chilled at+5ºC within 2 hours. Then a part of the semen samples were evaluated. The remaining part of the semen samples were filled into sufficient number of 0.25 ml straws and sealed with polyvinyl chloride (PVC) powder and dried. The semen straws were frozen horizontally in liquid nitrogen (LN2) vapor just 4-6 cm above the surface of LN2 for 10 minutes before they were plunged in to LN2. The frozen straws were then transferred to liquid nitrogen. The straws were thawed at 40ºC for 30 sec after of LN2

storage and then characteristics of motility, progressive motility and viability, plasma membrane integrity (HOST), normality and acrosomal damages were examined after 10 days [16].

Semen Analysis

Volume

The volume of ejaculated semen was determined by collecting semen into a graduated tube [17].

Concentration

The sperm concentration was evaluated by means of a hemocytometer [18].

Evaluation of microscopic sperm parameters

Mass motility

To evaluate the mass motility a drop 1-2 of undiluted semen was placed on a pre-warmed slide 37^0C without a cover slip and examined under light microscope (100×). The mass motility was scored 0= no motility, 1= few sperm with weak movement (<20%), 2= some motile spermatozoa (20–40%) without wave motion, 3= slow wave motion (40–60%) with motile spermatozoa, 4= rapid wave motion without whirlpool (60–80%) with motile spermatozoa and 5= dense, vary rapidly moving wave with clear whirlpools (>80%) motile spermatozoa [19].

Sperm motility

Sperm-motility was estimate in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as final motility score. Semen scoring was performed at light microscopic magnification of 400× [20].

Progressive motility

The sperm progressive motility was determinate subjectively by preparing a wet mount of diluted semen by placing a 1-5 drop of semen under cover slip and recorded the proportion of spermatozoa moving progressively straight forward at higher magnification (400×) of microscope. At least 200 spermatozoa, selected randomly from five microscopic fields, were examined. The mean of five successive estimations was recorded as the final motility.

Viability

Sperm viability of the semen samples were assessed by means of the eosin- nigrosin staining [16]. The stain was prepared as: eosin-Y 1.67g, nigrosin10 g, sodium citrate 2.9g, dissolved in 100 ml distilled water. The sperm suspension smears was prepared by mixing a drop of the semen sample with 2 drops of the stain on a warm slide and spreading the stain with a second slide immediately. The viability was determinate by counting 100 cells under the light microscope at magnification 400×. Sperm showing partial or complete purple coloring was considered non-viable or dead and only sperm showing strict exclusion of the stain were considered to believe [21].

Hypo-osmotic swelling test

The hypo-osmotic swelling test (HOST) was used to estimate the functional integrity of the sperm membrane, based on curled and swollen tails. The test was performed by incubating 30µl semen with 300µl hypo-osmotic solution (100mOsm) at 37°C for 60 min. After incubation 0.2ml of the mixture was spread with a cover slip on a warm slide. At least 400 spermatozoa were evaluated using bright-field microscopy at (400× magnification). Sperm with swollen or coiled tails were recorded [22].

Abnormality

Abnormalities of head, mid-piece and tail were counted simultaneously as per Singh et al [23]. The sperm smears were prepared by mixing a drop of semen with two drops of the eosin–nigrosin stain on a warm slide and spreading the stain immediately with a second slide. A total of 200 sperm cells were counted on each slide.

Acrosomal damages

For evaluated 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 record to have an intact acrosome and the rest were considered as damaged acrosome [18].

Sperm Motility Recovery Rate

The sperm motility recovery rate was estimated by comparing the motility of pre spermatozoa using the formula:

Recovery rate = Mps/Mpr × 100% [24].

Sperm progressive Motility Recovery Rate
The sperm progressive motility recovery rate was determinate 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 evaluated by comparing the viability of pre freeze (Vpr) and post thaw (Vps) spermatozoa. Recovery rate = Vpr/Vps × 100%.

Statistical Analysis
Values were expressed as mean ± SEM (Standard Error of Mean). Statistical evaluation of significant difference between means was performed by one-way analysis of variance (ANOVA) 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,). The significance level considered was $p<0.05$.

RESULTS

The characteristics of fresh spermatozoa are shown in Table 1. The effect of vitamin E on parameters of chilled rams *Zel* spermatozoa were shown in Table2.The highest motility, progressive motility and live spermatozoa after storage at 5˚C were attained in diluents containing 2m/M Vitamin E, but these treats were not significant in groups with 1 and 3 m/M of vitamin E. The percentages of plasma membrane integrity of spermatozoa in four treatments (0, 1, 2 and 3 m/M) were 71.4%, 80.2%, 87% and 80.6%, respectively in pre freezing. The effects of different levels of vitamin E on major defect were not significant, but the highest percentages of minor defects were observed in control group. The influence of various levels of vitamin E on post-thawing sperm is shown in table 3. Results showed that the evaluated parameters (motility, progressive motility, and viability, HOST and total normality) were higher in all groups of vitamin E supplement compare with control group, but highest percentages of treats were observed in 2m/M of vitamin E. Also Effect of vitamin E on Acrosomal damages was significant in post freezing conditions and the highest percentages were obtained in control group. Recovery rate of motility and viability of spermatozoa are shown in Table 4. The highest motility (43.25±0.93), progressive motility (40.2±1.19) and viability recovery rate (44.52±0.67) were observed in Tris extender containing 2m/M of vitamin E.

Table1. Characteristics of Zel ram spermatozoa, under fresh condition

Volume (ml)	Mass motility	Motility (%)	Progressive Motility (%)	Viability (%)	Major defects (%)	Minor Defects (%)	Normal Sperm (%)	Concentration (ml)
1.34	4.5	88	85	93	2.2	3.5	94.3	3.6×10^{9}

Table2. Characteristics of Zel ram spermatozoa with treatment of vitamin E in pre freezing (mean ±S.E.M)

Vitamin E (m/M)	Motility (%)	Progressive Motility (%)	Viability (%)	Major Defects (%)	Minor Defects (%)	HOST (%)	Acrosomal damages (%)	Normal sperm (%)
Control	72.8 ± 1.15^{c}	68.60 ± 0.69^{c}	76 ± 1.22^{c}	2.4±0.4	11.2 ± 0.37^{a}	71.40 ± 0.97^{c}	4.8 ± 0.2^{a}	81.6 ± 0.67^{b}
E (1 m/M)	80.4 ± 0.4^{b}	76.40 ± 0.97^{b}	84 ± 0.63^{b}	2.2±0.37	6 ± 0.63^{bc}	80.20 ± 0.20^{b}	4.4 ± 1.66^{a}	89.2 ± 1.2^{a}
E (2 m/M)	86.4 ± 0.97^{a}	82.20 ± 1.35^{a}	91.60 ± 0.6^{a}	2.2±0.37	4.4 ± 0.4^{c}	87 ± 1.22^{a}	1.60 ± 0.24^{b}	88.8 ± 1.24^{a}
E- (3m/M)	80 ± 0.44^{b}	75.60 ± 0.60^{b}	83.6 ± 0.4^{b}	2.4±0.4	6.6 ± 0.7^{b}	80.6 ± 0.4^{b}	2.80 ± 0.37^{b}	77.8 ± 0.48^{c}

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

Table3. Characteristics of Zel ram spermatozoa, with treatment of vitamin E in post freezing (mean ±S.E.M)

Vitamin E (m/M)	Motility (%)	Progressive Motility (%)	Viability (%)	Major Defects (%)	Minor Defects (%)	HOST (%)	Acrosomal damages (%)	Normal sperm (%)
Control	16.8±0.91[d]	13.4±1.43[c]	20±0.94[c]	2.4±0.4	17.8±0.86[a]	16.8±0.91[c]	10±0.89[a]	69.8±1.65[c]
E (1 m/M)	26.8±1.65[b]	23±1.22[b]	30±0.94[b]	2.6±0.24	11.4±0.97[b]	26.8±0.92[b]	7.8±0.2[b]	78.2±0.58[b]
E (2 m/M)	37.4±1.12[a]	33±1.24[a]	40.8±0.86[a]	2.2±0.37	6.6±0.44[c]	34.6±2.01[a]	4.4±0.4[c]	87.4±1.12[a]
E- (3m/M)	23.4±1.02[c]	20.2±0.91[b]	28±0.89[b]	2.6±0.24	11.6±1.02[b]	23.80±0.73[b]	8±0.2[b]	78.2±0.91[b]

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

Table4. Recovery rate of characteristics of Zel ram spermatozoa, with treatment of vitamin E (mean ±S.E.M)

Vitamin E (m/M)	Recovery Motility (%)	Recovery Progressive motility (%)	Recovery Viability (%)
Control	23.14±1.56[c]	19.63±2.33[c]	26.39±1.57[c]
E (1 m/M)	33.34±1.22[b]	30.15±1.79[b]	35.71±1.14[b]
E (2 m/M)	43.25±0.93[a]	40.12±1.19[a]	44.52±0.67[a]
E- (3 m/M)	29.25±2[b]	25.65±0.77[b]	33.49±1.12[b]

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

DISCUSSION

The evaluation of motility alone is inadequate for the assessment of sperm viability after thawing [25]. The integrity and functional activity of sperm membrane are of major importance in the fertilization process and useful indicators of the fertilizing ability of the spermatozoa [26].

Highly motile spermatozoa can be damaged in structure or functions of membranes and conversely, highly immotile sperm cells can have intact plasma membrane and thus viability. Therefore, composed tests (HOST test and motility) are necessary for sperm cells post thawing evaluation. In this study, we investigated the effect of vitamin E as antioxidant in extender on some characteristics of spermatozoa in Zel rams in during pre and post freezing conditions. The results showed that the addition of 2m/M of vitamin E in Tris extender increased, motility, progressive motility, viability, HOST and normal spermatozoa. The lower percentage of progressive motile sperms in control group may be related to excessive production of ROS by dead, immature and abnormal spermatozoa during sperm processing (e.g., extending, freezing, thawing process), accompanied by low scavenging and antioxidant concentrations in seminal plasma and semen extender inducing oxidative stress [27], while the higher values obtained in vitamin E groups may be attributed to the effective of vitamin E through its protective action on the sperm cell membrane against oxidative stress and lipid peroxidation during cryopreservation of semen [28].

Reduction of live sperms in control group can be attributed to peroxidation of polyunsaturated fatty acids to generate lipid peroxides that have an adverse effect on spermatozoa, interfere with the mitochondrial electron transport chain and elevate cellular production of superoxide anion, resulted in membrane damage, leakage of ATP, disrupting DNA integrity and a rapid loss of sperm motility and viability [29].

Vitamin E, if not the only, is the major chain-breaking antioxidant in membranes. It scavenges all the three types of free radicals, such as superoxide, peroxyl and hydroxyl radicals. These radicals will induce the peroxidation of phospholipids in the mitochondria of the sperm cell and thus to their ultimate immobility [30].

Vitamin E in fact producing stable ROOH groups. It has been suggested that vitamin E provides biological stability to the spermatozoa plasmalema [31]. Azawi and Hussein [32] were observed that addition of antioxidants such as vitamin C and E to semen preservation extender improved longevity and quality of chilled ram spermatozoa. Also, Guzman et al [33] reported that vitamin E improved, sperm motility, concentration of spermatozoa, decline of abnormalities, maturation of spermatozoa and higher ATP concentration in the semen and spermatogenesis with participation in the synthesis of antioxidant enzymes [34]. AminiPour et al [35] studied the various levels of vitamin E in Tris extender on semen characteristics of Ghezel rams pre and post freezing conditions and stated that the vitamin E had significant effect on viability, motility and progressive motility of spermatozoa in cryopreservation. In during freezing process, cryopreservation influence the osmotic tolerance and ion homeostasis of sperm enhances production rate of O_2 – and H_2O_2 or in the intracellular concentration of free calcium ions (Ca^{2+}) [36] that lead to

premature acrosomal reaction, altered mitochondrial function, decrease of motility and oxidative DNA injury or fragment all of which damage the acrosome and affect the fertility of the sperm cells [37].

In this study, percentage of spermatozoa with normal acrosome was significantly higher after freezing and thawing in all group of vitamin E than in the control. Thus, supplementation of vitamin E in freezing extender had beneficial effects on acrosomal integrity.
Also, evaluation of sperm morphology is one of the commonest methods to assay viability of frozen thawed sperm [38].

AminiPour et al [35] were observed that the highest percentage of abnormal spermatozoa in control group compare with vitamin E groups. In our study, in during cooling and freezing shocks, the highest defects such as head, mid-piece, cytoplasmic drop and tail abnormalities of spermatozoa were observed in control group. It is relevant to mention that chilling and freezing semen processing without vitamin E in extender, increase the proportion of abnormal of ram spermatozoa. Also, addition of vitamin E above 2m/M, reduced motility, progressive motility, survival rate, HOST, Acrosome integrity and normal of spermatozoa. These events may be is related to pyruvate contained in the ram semen extender reduce the Hydrogen peroxide (an oxidant) to water so that addition of more antioxidant will not enhance motility or viability [39]. In fact in higher concentration, the Vitamin E is pro-oxidant. Zeitoun and Al-Damegh [40] concluded that vitamin E concentration in ram semen extender must not exceed 5IU/ml to achieve the best post-thaw motility and survival with the highest antioxidant enzyme activity. At a similar level, Anghel et al [41] found that better post-thaw sperm parameters of frozen ram semen. But, Andreea and Stela [42] offer that the concentration of Vitamin E up to 1.0m/M can protection against membrane oxidative stress of frozen ram sperm.

In conclusion, addition of vitamin E to the extender of semen is superior in maintenance of motility, progressive motility, viability, membrane and acrosomal integrity and normal of spermatozoa. Recent studies described that a balance between the benefits and hazard from ROS and antioxidants appears to be importance for the normal functioning of spermatozoa. Seminal plasma also seems to be one of the most powerful antioxidant fluids in the organism, however supplementation of the extenders with 2m/M vitamin E antioxidant is recommended to facilitate the enhancement of Zel ram sperm cryopreservation techniques.

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