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The influence of vitamin E on semen characteristics of ghezel rams in during cooling and frozen process

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ABSTRACT

The aim of this study was investigation of various levels of vitamin E in Tris extender on semen characteristics of Ghezel rams in pre and post freezing conditions. Semen was collected from six healthy and mature rams with average 55±5 Kg body weight by electro ejaculator. High quality samples were mixed and diluted in Tris extender after apply different concentration of vitamin E. Treatments were various levels of vitamin E (0, 1, 2, 3, 4 and 5 m/M). The semen aliquots were chilled and preserved at 5°C and their quality was evaluated in pre freezing, and then cooling semen samples entered into 0.5 ml of straws. At first, straws were frozen upper vapor liquid nitrogen, and then kept in -196ºC. After 10 days straws were thawed and some characteristics of spermatozoa were examined. Analysis of data was carried based on completely randomized design. Results of this study showed that the effect of vitamin E on characteristics of viability, motility, progressive motility and normal of spermatozoa were significant in pre and post freezing conditions (P<0.05), and the highest percentages of this treats were obtained in treatment of 2m/M vitamin E. In conclusion for storage long term of Ghezel rams spermatozoa, using 2mg/ml of vitamin E in semen extender is recommend.

Keywords: Vitamin E, Viability, Progressive Motility, Normal Spermatozo, *Gheze Ramsl.*

INTRODUCTION

Cryopreservation of spermatozoa involves several steps, such as dilution, chilling, freezing and thawing [29]. Each of these steps can damage the plasma membrane structure and normal sperm function [22]. Many studies have been reported the oxidative stress-related mechanisms of sperm damage [12, 21]. Oxidative stress play a key role in the etiology of detective sperm function, induction of per oxidative damage to plasma membrane, [34 and 43] decline of sperm motility and cell quality that results in insufficient numbers of viable spermatozoa and fertility [34].

By sperm processing (excessive centrifugation, cryopreservation/thawing) and low scavenging and antioxidant levels in seminal plasma or sperm-processing media, High generation of reactive oxygen species (ROS) accompanied and induced a state of oxidative stress (OS). Lasso et al [27], reported that the freezing-thawing process declined the antioxidant defense capacity of sperm cell, the loss of superoxid dismutase activity and the decrease in glutathione levels with 78% given fresh semen. The non-enzymatic antioxidants provide protection mechanisms by three ways: 1) prevention, 2) interception, and 3) repair [42 and 44]. It is well known that vitamin E act as a lipid-soluble antioxidant in cell membranes and it effectively control lipid oxidation. It is required for growth, development and prevention of chronic diseases [26 and 32]. Because its lipo solubility, vitamin E is the first line of defense against the peroxidation of the polyunsaturated fatty acid of membranous phospholipids structure [9]. Traditionally, vitamin E is called as anti-sterility because it is necessary for normal function of male reproductive system. Bansal et al [8], and Balla et al [7], reported that vitamin E is a main chain-breaking antioxidant in membranes because it may directly scavenging the free radicals such as superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\bullet$).

Askasi et al [6] and Al-Khanak et al [3], found that vitamin E (α-tocopherol) increased viability and motility of spermatozoa by preventing oxidative stress to the plasma membranes. Therefore, this study is designed to find out the optimal concentrations of vitamin E and its effect on chilled and frozen ram semen.

MATERIALS AND METHODS

Animals and Semen Collection

These experiments were performed around Azerbaijan province in Iran. In this study Six *Ghezel* rams with average 3- 4 years old and 55 ± 5 kg body weight were selected and were housed individually in pens on semi-slatted floors. Animal were fed a diet according to NRC recommended based on 60:40 forage to concentrate ad libitum and had free access to water. From each ram 10 ejaculates were collected by using electro ejaculator as described by Evans and Maxwell [20].

Semen Processing

After semen collection by electro ejaculator, they transferred into graduated test tubes, placed in a thermo flask at 37°C, and transported to the laboratory for evaluation. Fresh undiluted semen was then microscopically evaluated for semen volume, wave motion, percent motile sperm, progressive motility, viability and normal spermatozoa. Sperm motility was evaluated after collection. Semen samples that showed more than 80% motility and viability and wave motion more than 4 were selected for this experiment (Table1). The semen samples were pooled to eliminate individual differences and divided into 6 equal aliquots and kept at 37ºC in water bath. After primary observation, semen samples were diluted at a 1:4 ratio (semen: diluent) at 37ºC with Tris extender. The dilution contained Tris (hydroxyl methyl amino methane) (3.876g), glucose (0.523 g), citric acid (2.123 g), egg yolk (15%), glycerol (5%) penicillin (100000 IU) and streptomycin (100 mg) [20].

All chemicals used were purchased from Merck Chem. Semen was split into 6 parts and different amounts of vitamin E (0 (control), 1, 2, 3, 4 and 5 m/M) were added to each group. Diluted semen was cooled gradually to +4ºC within 2 hours. After 1.5-2 h a part of samples were investigated. Reminded samples entered into 0.5 ml of straws. At first, straws were frozen at heights of 4 and 6cm above the level of the liquid nitrogen, the frozen straws were transferred to liquid nitrogen. After 10 days straws were thawed in a water bath at 37ºC [31 and 35], and then parameters of motility, progressive motility, viability and abnormality of spermatozoa were examined.

Semen evaluation

Sperm Volume

The volume of semen ejaculate was measured by reading the graduated tube [13].

Sperm Wave motion

To evaluate the wave motion, a drop (25µl) of semen was placed on pre warmed slide without any cover slip and examined under microscope equipped with phase- contrast optics (100X). The wave motion was scored into 5 scales: 1= no motion, 2= free spermatozoa moving without forming any wave, 3= small, slow moving waves, 4= vigorous movement whit moderately rapid waves and eddies 5= dense, very rapidly moving waves and eddies [13].

Sperm Motility

Individual sperm motility was assessed subjectively under a phase-contrast microscope (magnification 400X) equipped with a heated stage adjusted to 37ºC. Motility estimations were performed from 5 different fields in each sample by the same person throughout the study, the mean value averaged from 5 successive estimations and calculation of 100 spermatozoa was used as the final motility score [11].

Sperm Progressive motility

A drop of semen diluted was placed on a clean pre warmed slide (37ºC) and cover slip. The progressive motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400X).

Sperm viability

A 50 µL drop of diluted semen was placed on a pre-warmed slide and mixed with 50 µl drop of the supra vital stain [1% (w/v) eosin B, 5%)w/v) nigrosin in 3% tri-sodium citrate dehydrate solution] to prepare a thin smear [38].

After drying, the smears were evaluated by microscope with magnification (400X). Two hundred spermatozoa were counted for unstained heads of spermatozoa (live) and partial stained heads of spermatozoa (dead).

Abnormal Spermatozoa Rate

To investigate the abnormal spermatozoa rate, eosin-nigrosin preparations were made according to the method described by Blom [15 and 48]. For morphological assessment, a drop of semen diluted was put on a slide and covered. A total of 100 sperm cells were counted on each slide. The morphology of the spermatozoa was evaluated under phase contrast microscopy (magnification 1000X, oil immersion). Spermatozoa were classified as normal or as having one of the following abnormalities: abnormal heads, detached heads, coiled tails and distal cytoplasmic droplets.

Sperm Motility Recovery Rate

The sperm motility recovery rate was evaluated by comparing the motility of pre freeze (Mpr) and post thaw (Mps) sperm. If Mpr and Mps are the sperm motility percentages before and after freezing, then the recovery rate would be Mps/Mpr × 100% [25].

Sperm progressive Motility Recovery Rate

The sperm progressive motility recovery rate was evaluated by comparing the progressive motility of pre freeze (PMpr) and post thaw (PMps) sperm. If PMpr and PMps are the sperm progressive motility percentages before and after freezing, then the recovery rate would be 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. If Vpr and Vps are the sperm viability percentages before and after freezing, then the recovery rate would be Vps/Vpr × 100%.

Statistical analysis

The mean and standard error of mean (S.E.M.) were calculated for all values. The analysis of variance (ANOVA) was performed to test for significant differences between treatments. The data for motility, progressive motility, viability, and total normal spermatozoa rate were examined by Fishers post hoc test following one-way analyses of variance (ANOVA) using the Mini tab Statistical Package. Comparisons between the treatments were done using tukey-test. Analyses were considered as statistically significant at $(P<0.05)$.

RESULTS

The effect of vitamin E on the semen parameters of *Ghezel* rams were evaluated in pre freezing as shown in Table 2. Effect of vitamin E in Tris extender on motility percentage was significant ($P<0.05$). All of doses of vitamin E in Extender led to improve motility percentages, in comparison to control group. Also, the addition of 2 m/M vitamin E to semen extender led to higher motility sperm with average (80.4±1.56), in comparison to control group with average (60.2±1.56). Effect of vitamin E on progressive motility and viability were significant and addition of 2m/M of vitamin E to semen extender led to higher progressive motility and viability in comparison to control group. The percentage of abnormal sperm was significantly lower in 2m/M vitamin E and the highest were obtained in control group. Higher percentage of normal spermatozoa with average (96.4±1.12) was observes in extender with 2m/M vitamin E. Also, the effects of the presence of vitamin E in the post freezing extender on cryopreservation sperm characteristics were shown in Table 3. The highest percentage of motility spermatozoa was (44.8 ±1.73) in treatment of 2m/M Vitamin E, after thawing. The increase in the vitamin E level, the motility spermatozoa rate was decreased. The progressive motility, viability and normal spermatozoa were improved with the presence of vitamin E in the extender compared with the control. The higher percentage of this treats were obtained in Tris extender with 2m/M of E vitamin. The recovery of sperm parameters were shown in table 4. The addition of vitamin E on semen dilution could significant effect on motility, progressive motile and viability recovery rate. The higher motility, progressive motile and viability recovery rate with average (55.7±1.6), (52.6±1.6) and (60.9±1.70) were observed in Tris extender with 2m/M of vitamin E respectively.

Table1. Volume, motility wave motility, progressive motility, viability, abnormality and normal spermatozoa in *Ghezel rams*, in fresh condition

Fresh semen	Volume (ml)	Wav motion	Motility (%)	Progressive Motility (%)	Viability (%)	Abnormality (%)	Normal Spermatozoa (%)
	1.4	4.6	87	80	90	4.1	95.9

Table2. Motility, progressive motility, viability, abnormality and normal spermatozoa of *Ghezel rams*, stored at 5°C in diluent supplemented with vitamin E

Vitamin E	Motility (%)	Progressive motility (%)	Viability (%)	Abnormality (%)	Normal spermatozoa (%)
Control	60.2^{c}	55.03^{c}	65.4^{c}	10.2^{a}	89.8^{a}
E (1m/M)	68.1^{b}	65.20^{b}	72.0^{b}	6.8^{b}	93.2^{b}
E (2m/M)	80.4^{a}	74.10^{a}	84.4^{a}	3.6^{c}	96.4^{c}
E (3m/M)	73.55^{b}	70.70^{b}	76.2^{b}	6.0^{b}	94.0^{b}
E (4m/M)	70.03^{b}	65.50^{b}	72.1^{b}	5.7^{b}	94.3^{b}
E (5m/M)	69.5^{b}	64.0^{b}	71.5^{b}	6.0^{b}	94.0^{b}
SEM	± 1.56	± 1.50	± 1.52	± 1.12	± 1.12

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

Table3. Motility, progressive motility, viability, abnormality abnormal spermatozoa *Ghezel rams*, from frozen–thawed semen samples in the presence and the absence of vitamin E

Vitamin E	Motility (%)	Progressive motility (%)	Viability (%)	Abnormality (%)	Normal spermatozoa(%)
Control	25.1^{c}	18.4^{c}	30.4^{c}	18.0^{a}	82.0^{a}
E (1m/M)	32.7^{b}	25.7^{b}	38.2^{b}	13.4^{b}	86.6^{b}
E (2m/M)	44.8^{a}	39.0^{a}	51.4^{a}	8.2^{c}	91.8^{c}
E (3m/M)	36.8^{b}	31.9^{b}	42.1^{b}	11.7^{b}	88.3^{b}
E (4m/M)	34.2^{b}	29.6^{b}	39.0^{b}	11.0^{b}	89.0^{b}
E (5m/M)	33.3^{b}	28.0^{b}	39.6^{b}	13.0^{b}	87.0^{b}
SEM	±1.73	± 1.65	± 1.89	± 1.94	± 194

Table4. Recovery rate of motility, progressive motility and viability spermatozoa in *Ghezel rams*, in the presence and the absence of vitamin E

Vitamin E	Recovery of Motility (%)	Recovery of Progressive motility (%)	Recovery of Viability (%)
Control	41.6^{c}	32.7^{c}	46.4^{c}
E (1m/M)	48.01^{b}	41.3^{b}	53.05^{b}
E (2m/M)	55.7^{a}	52.6^{a}	60.9^{a}
E (3m/M)	50.03^{b}	45.1^{b}	55.2^{b}
E (4m/M)	48.8^{b}	45.1^{b}	54.09^{b}
E (5m/M)	47.9^{b}	43.75^{b}	55.3^{b}
SEM	±1.60	±1.60	±1.70

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

DISCUSION

Oxidative stress cause impairs sperm motility by alteration of the membrane fluidity [39]. Alteration of the membrane fluidity mainly is related to present of polyunsaturated fatty acid (PUFAs) in high concentration at the sperm membrane, therefore spermatozoa becomes vulnerable to lipid peroxidation [28]. Membrane fluidity plays a major role in regulating ion pump that controls inwards and outwards movement of calcium ion into the spermatozoa. Alteration of the membrane fluidity will cause accumulation of calcium ion which damage sperm motility and consequently endanger the viable sperm [40].

Wishart [46] and Cecil and Bakst [18], were stated that the lipid peroxidation begins during semen storage in vitro, but recent studies have reported the lipid peroxidation in sperm begins at the time of ejaculation [14 and 17]. The antioxidants able to neutralize other oxidizable substrate therefore significantly delay or inhibit the oxidation of the substrate at relatively low concentration [45].

In this study, we investigated the effect of vitamin E as antioxidant in extender on some characteristics of spermatozoa in *Ghezel* rams in during pre and post freezing conditions. The results showed that the addition of 2m/M of vitamin E into Tris extender increased, motility, progressive motility, viability and normal spermatozoa. Our observations indicating that the vitamin E was able to protect sperm during storage at 5°C and freezing conditions. Natural antioxidants such as vitamin E, inhibits cell impair by binding to the free radical and neutralizing its unpaired electron mediated by a tocopheryl-quinone' formation [41].

Vitamin E increases intracellular ATP and decline cell permeability and enzyme inactivation peroxidation [4 and 16].

Vitamin E captures and deletes oxygen radicals in the membranes and also the alkoxyl and peroxil radicals which are fuel for the peroxidation chain reaction, that are generated during the conversion of lipid hydro peroxides [1]. Addition of vitamin E in extender had a beneficial effect on sperm motility in liquid ram semen storage [44], fresh human semen [20] and to little effect in equine chilled semen [7]. Vitamin E has been added during short-term storage of liquid semen in different species, the outcome relative to improving motility parameters has varied with

species. May be poor quality semen was treated with additives Vitamin E there is more advantage effect on semen quality and preservability of spermatozoa. In our study supplementation the incubation medium with all doses of vitamin E improvements characteristics of spermatozoa such as motility, progressive motility, viability, normal spermatozoa, Motility and viability Recovery Rate than control group**.**

It is suggested that the addition of vitamin E may be useful in preventing the rapid loss of motility that occurs during semen freezing. In our study the highest percentages of this treats were obtain in extender with 2mM vitamin E and increase level of vitamin E decrease parameters of *Ghezel Ram* spermatozoa that is related to the higher concentration vitamin E may be act as an oxidation stimulator rather than an antioxidant [16].

There were reports on improvement of post-thaw quality from vitamin E addition in bull [2], human [6], and sheep [24] and buffalo semen [33 and 36]. Anghel et al [5] have shown that use, both of 0.1 and 1.0 m/M of vitamin E in extender leads to improved post-thawing cytological parameters for the ram sperm. Hartono [23] in his study showed that the dosage of 0.41 g/100ml vitamin E was the optimum dose in frozen semen of Boer goat.

Also Al Khanak [2], reported that sperm motility and percentage of live-ratio was higher in vitamin E 1.5 m/M and lower in vitamin E 0.1mM and the lowest was in control group.

One of treats that investigated in our study was sperm morphology. Evaluation of sperm morphology is one of the commonest methods to investigated survival of frozen thawed semen [37]. The relationship between sperm morphology and fertility has been evaluated in several studies. Abnormal sperm morphology cause reduced fertility [19].

Vitamin E functions as antioxidants which in turn may provide direct protection of sperm cells from morphological damage by preventing free radical oxygen from damaging sperms. Furthermore, the morphology and the motility of sperm cells would be preserved by the binding of Vitamin E to endoperoxides [30].

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.
Our findings are in conformity with other author's reports, who stated that, Vitamin E supplementation leads to improved motility and normal sperm percentage [47].

Batool et al [10], reported that α-tocopherol acetate (0.5 m/M) in extender improved the plasma lemma functionality.

CONCLUSION

Enhancement of the antioxidant capacity of semen could present a major opportunity for improving ram fertility and in this study vitamin E protects the ram spermatozoa against the damages caused by reactive oxygen species. In conclusion for storage short and long term of *Ghezel* Rams spermatozoa; using 2m/M of vitamin E in semen extender is recommend.

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