



Effect of Antioxidant combinations on sperm quality of cross breed rams during liquid storage

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ABSTRACT

The aim of this study was to evaluate the effects of the addition of antioxidants (vitamin E, β -mercaptoethanol and their combination) to semen extender. Progressive motility, viability and pH of fresh ram semen were examined. Ejaculate samples were collected with artificial vagina from three Ghezel-Merino rams (3 years old) during non-breeding reproduction season. Semen was diluted with a Citrate-base extender containing group1, without antioxidants, group2, vitamin E 5mM, group3, β -mercaptoethanol (β -ME) 50mM and group4, vitamin E(5mM) & β -mercaptoethanol 50m. Diluted semen was cooled to 5° C and storage at same temperature for 72h. Semen was investigated after 24, 48 and 72h. Result showed that progressive motility and viability in Control group were higher than other treatment groups. The progressive motility in vitamin E supplemented group was significantly higher than other groups supplemented with antioxidant. The addition of vitamin E to the extender increased ($P < 0.05$) sperm viability compared to other groups supplemented with antioxidants. Progressive motility and viability were significantly higher than other ($P < 0.05$) in the vitamin E group. Viability and progressive motility were significantly higher in vitamin E & β -ME groups than in β -ME. In conclusion vitamin E supplementation of citrate –egg yolk extender improved progressive motility and viability fresh sperm during storage at 5° C for 72h. Sperm storage time had a significant effect on sperm viability and progressive motility. Based on this study, there were no combinations more efficient at combating semen quality than control group.

Key words: Semen, Extender, Fresh semen

INTRODUCTION

Ram sperm deteriorates rapidly at room temperature thus making fresh semen transportation to remote areas is impossible (Shamsuddin et al., 2000). When semen is stored at 4-5°C, there is a gradual decline in motility and fertility (Maxwell and Salamon, 1993). One cause of this decline may be attributed to the action of reactive oxygen species generated by the cellular components of semen (Alvarez and Storey, 2005). Mammalian sperm membranes are rich in unsaturated fatty acids (Hong et al., 2010). Lipid peroxidation by reactive oxygen species during preservation could be a detrimental factor due to high

contents of unsaturated fatty acids and a low level of antioxidants in the spermatozoa (Funahashi and Sano, 2005). The addition of various antioxidants to sheep semen diluents extend the period of storage of semen, improve the motility, reduce the degree of cellular damage, improve the acrosomal integrity, and increase the viability and fertilization capacity of sperm in vitro (Maxwell and Stojanov, 1996; Maxwell and Watson, 1996). These compounds may be present naturally or through artificial synthetic to be added (Upreti, 1997). Vitamin E is one of the most important lipid-soluble primary defense antioxidants, and its principal role as an antioxidant is believed to be in chain-breaking by scavenging lipid peroxy radicals which propagate lipid peroxidation (Burton et al., 1985). A variety of tocopherol isoforms exist as natural and synthetic molecules with vitamin E activity. The distinct chemical structure of VitE, a hydrophilic polar phosphate group attached to the chroman head and a hydrophobic hydrocarbon tail, interacts in a quite unique and efficient mechanism of action (Rezk et al., 2004) making it more potent than any other tocopherol esters against lipid peroxidation (Negis et al., 2007; Salamon and Maxwell, 1995). A recent report by (Bilodeau et al., 2000) thiol such as mercaptoethanol prevented from loss of sperm motility and can reduce many type of reactive oxygen species. Some work has been published on the influence of ROS and beneficial use of antioxidants on ram sperm functions (Bucak et al., 2007; Stefanov et al., 2004; Upreti et al., 1998) many of which show some beneficial effects. However, there are indications from other tissues that a mixture of two or more antioxidants administered in combination may be more protective than single antioxidants working alone to reduce oxidative stress. The objective of this study was to evaluate the effect of vitamins E and β -ME alone or mixture of two on the motility and viability of spermatozoa when ram semen is stored at refrigerator temperature and determine whether antioxidant supplementation could improve sperm function during storage at 5°C.

MATERIAL AND METHODS

2.1. *Animals*

Three Ghezel-Merino rams (approximately 3 years old) were used in this study. These animals were raised on the farm as semen donor for AI purpose since 2012. They were maintained under uniform feeding, housing and lighting conditions. The experimental animals were subjected to the same feeding program of the farm. Animals were fed twice daily in order to achieve a predetermined feed intake of 2.5% body weight (on dry matter basis) per ram per day. Water was ad libitum.

2.2. *Evaluation of microscopic sperm parameters*

Semen was collected by artificial vagina once a week during the non-breeding season. Immediately after collection, each ejaculate was immersed into a water bath maintained at 37 °C prior to evaluation. The semen samples were evaluated for volume, wave motion, sperm concentration, pH, progressive motility and viability. The volume of ejaculate was determined by collecting semen into a graduated tube (Shamsuddin et al., 2000). To evaluate the wave motion a drop 1-2 of undiluted semen was placed on a pre-warmed slide 37 °C without a coverslip and examined under phase contrast microscope (100×) (Nikon, Eclipse, E200, Japan). The wave motion was scored 0 = no motility, 1 = few sperm with weak movement (<20%), 2 = some motile spermatozoa (20–40%) without wave movement, 3 = slow wave movement (40–60%) with motile spermatozoa, 4 = rapid wave movement without whirlpool (60–80%) with motile spermatozoa and 5 = vary rapid wave movement with clear whirlpools (>80%) motile spermatozoa (Avdi et al., 2004). The sperm progressive motility was estimated subjectively by preparing a wet mount of diluted semen by placing a 1-5 drop of fresh semen under coverslip at magnification of

about 200× under phase contrast microscope. At least 200 spermatozoa, selected randomly from a minimum of four microscopic fields, were examined. The mean of four successive estimations was recorded as the final motility. The sperm concentration was determined by means of a haemocytometer. Sperm viability of the samples was assessed by means of the nigrosin–eosin staining (Evans and Maxwell, 1987). The stain was prepared as: eosin-Y 1.67 g, nigrosin 10 g, sodium citrate 2.9 g, 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 assessed by counting 200 cells under the phase-contrast microscope at magnification 400×. Sperm showing partial or complete purple coloring was considered non-viable and only sperm showing strict exclusion of the stain were considered to be alive.

2.3 Semen processing

One ejaculate of each ram was divided in the 4 treatments. Only ejaculates with a sperm progressive motility greater than 70% were used in the study. Each ejaculate was divided into 4 equal aliquots and was diluted to a concentration of 4×10^8 sperm/ml with an Egg- yolk- citrate (EYC) extender containing sodium citrated hydrate (2.9g/dl), egg yolk(20ml/dl), penicillin(1000 unit/ ml) streptomycin(1000µg/ml) and distilled water to make the volume 100 ml. Samples of diluted semen were supplemented with either 5mM vitamin E (group2), other aliquot sample was supplemented with the 50mM β mercaptoetanol (group3) and another aliquot sample was supplemented with mixture of vitamin E & β-ME (group4) as an antioxidant with a final concentration of 4×10^8 spermatozoa/ml. Other aliquot sample was without antioxidant (group 1). Diluted semen samples were aspirated into 0.25ml French straws. The aliquot after cooling for 1.5-2h was stored at 5°C in a refrigerator to measure the parameters such as pH, sperm viability and progressive motility at 72h.

2.4 statistical analyses

The progressive motility and viability and pH for semen samples were analyzed using Proc MIXED of SAS (SAS, Version 9, Carry, NC) in an initial model with fixed effects for antioxidants, storage time, counting storage time and antioxidants. Animal considered as random effect.

3. RESULTS

Characteristics of three ram have been shown in Table1.

Effect of time storage, adding of antioxidant and Effect of time storage and antioxidants on semen pH was not significant.

Table1. Descriptive statistics of qualitative and quantitative traits.

Variable	number	mean	SD	min	max
pH	12	6.7	0.2	6.5	7
volume(ml)	12	0.8	0.2	0.6	1.1
Wave motion(1-5)	12	4.94	0.17	4.5	5
Viability%	12	91.62	2.72	86	95
Progressive%	12	87.12	2.64	82	90
concentration $\times 10^9$	12	4.56	0.09	4.4	4.6
PH after	191	6.55	0.2	6.4	6.7
Viability% after	191	65.13	6.11	38	93
Progressive% after	191	61.92	6.33	34	89

Effect of antioxidant on sperm viability

Effect of antioxidant on sperm viability has been shown in fig 1.

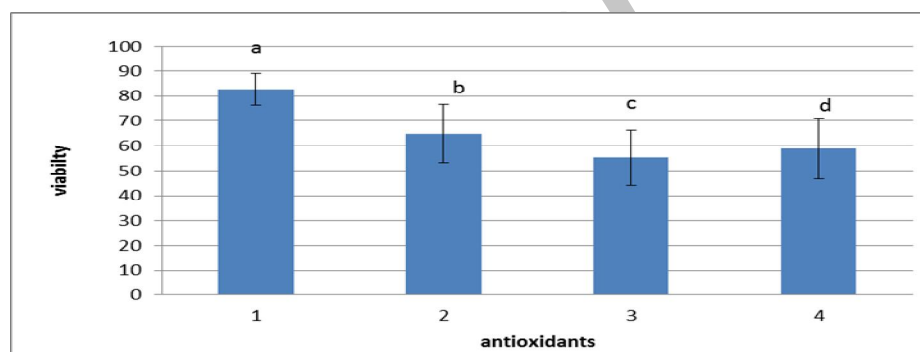


Fig 1. Effect of antioxidant on sperm viability: 1, control samples; 2, vitamin E; 3, β mercaptoethanol; 4, β mercaptoethanol & vitamin E. Least squares means with different letters are significantly different ($P < 0.05$).

Antioxidant had a significant effect on sperm viability and this effect was not dependent to time of storage. Viability of the control group was the highest. Viability of Vitamin E treated group had better than vitamin E + β -ME. Vitamin E & β -ME had better than β -ME.

Effect of antioxidant on sperm progressive motility

Effect of antioxidant on sperm progressive motility has been shown in fig2. Antioxidant had a significant effect on sperm progressive motility. This effect was not dependent diluents type. Sperm Progressive motility of the control group was the highest. Sperm Progressive motility of Vitamin E treated group had better than vitamin E & β -ME. Vitamin E & β -ME had better than β -ME.

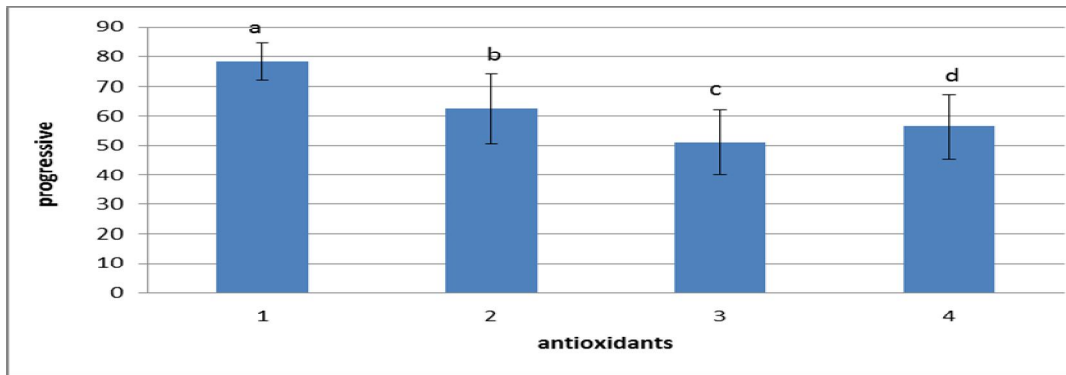


Fig 2. Effect of antioxidant on progressive motility: 1, control samples; 2, vitamin E; 3, β mercaptoetanol; 4, β mercaptoetanol & vitamin E. Least squares means with different letters are significantly different ($P < 0.05$).

Effect of storage time on sperm viability

Effect of storage time on sperm viability has been shown in fig 3. Sperm storage time had a significant effect on sperm viability. Reversely with the increasing storage time sperm viability decreased.

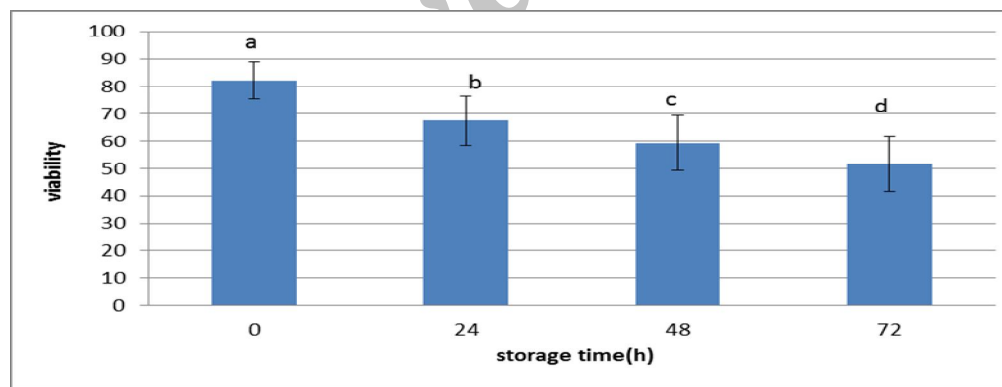


Fig 3. Effect of storage time (h) on sperm viability Least squares means with different letters are significantly different ($P < 0.05$).

Effect of storage time on sperm progressive motility

Effect of storage time on sperm progressive motility has been shown in fig 4. In this fig it is obvious that with enhancement in storage time, progressive motility was reduced regardless to type of the extender supplemented with antioxidants.

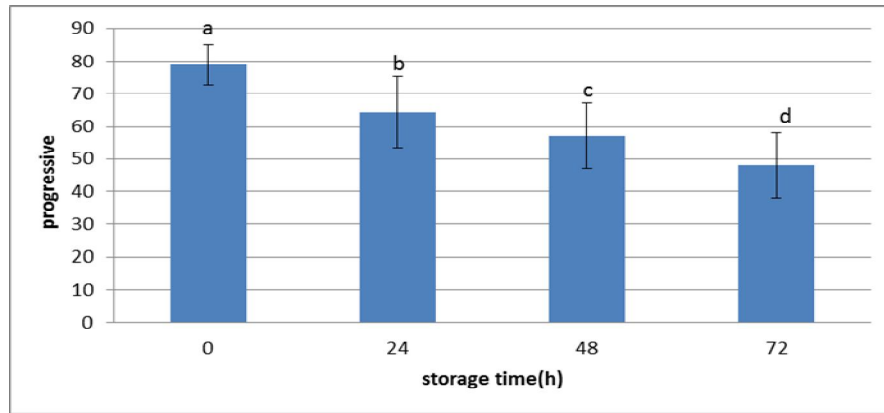


Fig 4. Effect of storage time (h) on progressive sperm motility Least squares means with different letters are significantly different ($P < 0.05$).

Effect of storage time and antioxidants on sperm viability

Effect of different storage times and antioxidants on sperm viability has been shown in fig 5.

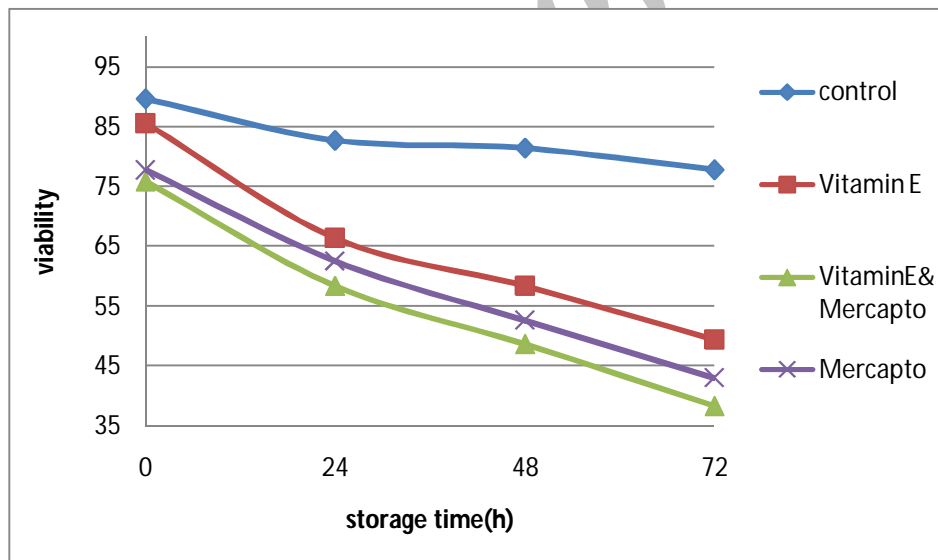


Fig5. Effect of antioxidant & storage time (h) on viability

Effect of storage time and antioxidants was significant ($P < 0.05$). The control group had better performance than other treated groups; vitamin E had better effect than vitamin E + β -ME. Vitamin E & β -ME had better than β -ME.

Effect of storage time and antioxidants on progressive motility

Effect of storage times and antioxidants on sperm progressive motility has been shown in fig 6. This effect was significant ($P<0.05$). The control group had a better performance than treated groups; vitamin E had better effect than vitamin E+ β -ME. Vitamin E & β -ME had better than β -ME.

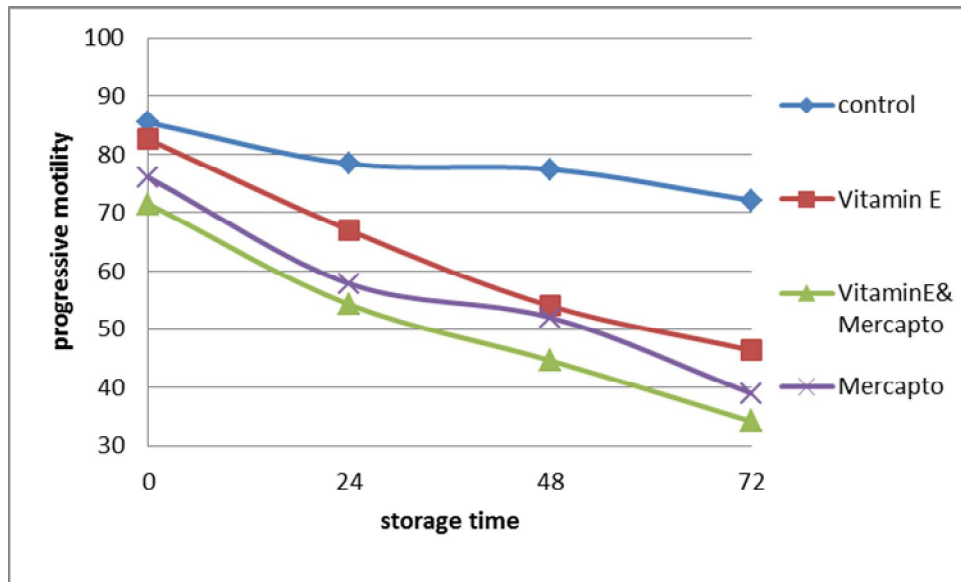


Fig 6. Effect of antioxidant& storage time (h) on progressive motility

DISCUSSION

The mammalian sperm plasma membrane is particularly rich in poly unsaturated Fatty acids (PUFA). This predominance of PUFA renders sperm highly susceptible To Lipid peroxidase (LPO). Spontaneous LPO of the membranes of mammalian sperm destroys the structure of the lipid matrix, as a result of the invasion by reactive oxygen species (ROS). These attacks then ultimately lead to the impairment of sperm function (sperm motility, functional membrane integrity and fertility), through oxidative stress and the production of cytotoxic aldehydes (Alvarez and Storey, 2005). At present study showed that supplementation with vitamin E antioxidants significantly improved semen quality parameters at 24, 48 and 72 h, compared with the control group. Previous studies in equine semen concluded that the addition of vitamin E at concentrations of 1.0, 2.0 and 4.0mM did not significantly alter the maintenance of sperm motility during storage at 5°C and came in contrast with the present results. Furthermore, vitamin E supplementation had also adverse effects on sperm progressive motility of ram spermatozoa stored at 15 °C (Upreti et al., 1997). In agreement with our findings, the α -tocopherol enrichment of boar semen diluents increased cell viability, through its prevention of an oxidative reduction in the levels of the major poly unsaturated fatty acids .The lipid peroxidation reaction was incriminated as a main cause of sperm dysfunction, especially in terms of the loss of membrane fluidity or sperm membrane damage, which can decrease sperm motility and viability (Agarwal et al., 2003). Vitamin E may be the most potent antioxidant to inhibit the propagation stage of lipid peroxidation reaction in the cell membrane (Agarwal et al., 2003; Silva, 2006). Furthermore, vitamin E can be recycled to function again, even when its concentration is low (Agarwal et al., 2003). In this study, the addition of β -ME to semen extender had no significant effect on semen quality. The percentages of sperm progressive motility and viability in the

defined medium supplemented with β -ME were lower than mixture of vitamin E and β -ME treated group. These observations together with obtained results of this study suggest that the diminution sperm quality caused by antioxidants (β -ME) during storage at 4° C. Moreover, in any combination the antioxidants did not appear to be more effective than each of the antioxidants alone, so there was no evidence of synergism or extra effect. This may be due to different mechanism of each antioxidants and diverse materials in composition of the extender.

CONCLUSION

This study showed that use of antioxidant in order to improve semen quality no more positive effect than without antioxidant using.

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