

## Comparative evaluation of the effect of antioxidants on the chilled-stored ram semen

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### Summary

The aim of this investigation was to assess the effect of different antioxidants on the motility and membrane integrity of the ram semen during storage at 5°C for 0, 12, 24, 48 and 72 hrs after collection and dilution. A total of 57 ejaculates from 12 Bakhtiary rams was collected by artificial vagina and were extended 1 : 3 with egg yolk-citrate buffer, which was containing one of the following antioxidants: BSA 1% (w/v), BSA 3%, BSA 1% + sucrose 10%, BSA 3% + sucrose 10%, vitamin E (1 mg), vitamin E (2 mg), ascorbic acid (0.9 mg/ml) and control group without any antioxidant. Percent of motile sperm (PMS), rate of forward movement (RFM) and membrane integrity in extenders contained vitamin E groups were significantly higher up to 48 hrs than other groups (P<0.05). Although, addition of 2 mg vitamin E was more effective than 1 mg vitamin E in sperm protection, however, the difference was not statistically significant. Likewise, addition of BSA alone or in combination with sucrose was superior to ascorbic acid in maintenance of sperm motility and membrane integrity. It seems that sucrose has not positive effect on these 3 parameters in combination with BSA. In conclusion, adding vitamin E to the extender of ram semen during dilution is superior in maintenance of sperm kinematic parameters up to 48 hrs during storage at 5°C.

**Key words:** Ram, Semen, Antioxidant, Motility, Membrane integrity

### Introduction

Artificial insemination with chilled-stored semen has become a technique in sheep breeding (Arthur *et al.*, 1996; Ax *et al.*, 2000). Because of low dilution rate of ram semen for intracervical insemination, semen of many individual rams does not respond well to cryopreservation. Therefore, maintaining the motility and fertility of spermatozoa during short-term (typically less than 72 hrs) storage at 5°C is an important consideration in the use of liquid semen (Arthur *et al.*, 1996).

The use of chilled-stored semen is limited by its relatively short time fertilizing capacity (Aurich *et al.*, 1997). Oxidative damage of spermatozoa during storage is a potential cause of the decline in motility and fertility during hypothermic storage of liquid semen (Ball *et al.*, 2001). The survival of ejaculated sperm in seminal plasma alone is

limited to a few hours. To maintain sperm for longer periods and to cool or cryopreserve semen, dilution with a protective solution is necessary (Ax *et al.*, 2000). However ram semen fertility in egg yolk-buffer or milk-based diluents which presently used as extenders, maintains only for 12-24 hrs (Arthur *et al.*, 1996).

Efforts to improve the preservation of cooled ram semen have focused on alteration of extenders (Marti *et al.*, 2003) as well as the addition of specific components to maintain membrane integrity, prevent oxidative stress or preserve motility of spermatozoa in the ram (Watson and Anderson, 1983; Maxwell and Stojanov, 1996; Sanchez-Partida *et al.*, 1997; Uperti *et al.*, 1998; Sarlos *et al.*, 2002). The outcome of such studies has had varied success in terms of improvement of either maintenance of motility of cooled spermatozoa or fertility of cooled semen.

In some studies on stallion semen, bovine serum albumin (BSA) has had protective effects on the spermatozoa from the harmful effects of liquid peroxidation and maintenance of sperm motility was improved by including BSA in semen extenders (Dixon and Kreider, 1981; Kreider *et al.*, 1985; Klem *et al.*, 1986). But, in the recent study, BSA did not improve the maintenance of equine sperm motility during liquid semen storage at 5°C (Ball *et al.*, 2001). However, there is no literature pertaining to the effects of BSA on the ram semen during storage at 5°C.

The aim of this study was to compare the effects of some antioxidants including BSA 1 and 3% alone and in combination with sucrose 10%, vitamin E and ascorbic acid on the total motion, progressive forward movement, as an important indicator of sperm fertilizability (Hafez, 2000), and membrane integrity of ram spermatozoa.

## Materials and Methods

### Animals and experimental procedures

This study was conducted in the farm of Lorestan University, Lorestan Province, Iran. A total of 57 ejaculates were collected from 12 Bakhtiary rams (the native sheep breeds in Iran) aged between 11 and 14 months, during November and December (2004). Semen collection was achieved by artificial vagina and using two sexually receptive and restrained ewes treated with estrogen 1 to 2 days previously (1 mg estradiol benzoate, Aburailhan Pharmaceutical Co., Iran). Antioxidants were purchased from Merck Company (KgaA, Darmstadt, Germany) unless otherwise indicated.

Immediately after collection, semen samples were extended 1 : 3 (0.5 ml semen + 1.5 ml extender) with egg yolk (BioMark laboratory, Belgium) citrate buffer (pH = 7.2) containing 100,000 IU/100ml and 100mg/100ml penicillin and dihydrostreptomycin, respectively. After 30 min, extended semen was stored at 5°C. Extenders were designed to eight groups contained one of the following antioxidants: BSA 1 and 3% (w/v) alone, BSA 1 and 3% in combination with sucrose 10%, 1 and 2

mg vitamin E ( $\alpha$ -tocopheryl acetate, Osvah Pharmaceutical Co., Iran), ascorbic acid (0.9 mg/ml). No antioxidant was used in control group. The experiment was replicated fourteen times with each antioxidant. Only one semen sample was omitted due to the low sperm concentration.

### Semen examination

Ejaculates were evaluated based on the method of Kreider *et al.*, (1985) for percent of motile spermatozoa (PMS), rate of forward movement (RFM) and sperm membrane integrity (SMI). PMS was determined by estimating the percentage of motile spermatozoa (cells showing any kind of movement) in field under a microscope at  $\times 400$ . RFM (scale 0 to 4, with 4 being the highest rating) was based on progressive forward movement of spermatozoa. SMI was assessed by sperm stained with dual stain technique using eosin 1% and aniline blue 4%. Spermatozoa that stained with eosin red dye throughout their length, were considered to be damaged, while all others were classified as intact membrane (Ax *et al.*, 2000). All of the above examinations were performed by a same person with at least 200 sperms.

### Statistical analysis

Results were analysed using the SPSS version 10. Before analysis, data were assessed for equality of variances by Levene's test. Upon this evaluation, PMS, RFM and SMI were compared among antioxidants using one-way ANOVA and Bonferroni test as post hoc, to determination of difference among groups (Petrie and Watson, 1999). Data were presented as mean  $\pm$  SEM. Values were considered to be statistically significant at  $p < 0.05$ .

## Results

### Percent of motile spermatozoa

Means for PMS in semen exposed to the extender with different antioxidant groups are presented in Table 1. PMS was not different among groups at time 0 after dilution ( $P > 0.05$ ). However, PMS was gradually decreased with time in all of antioxidants except for vitamin E group. The

PMS with 1 and 2 mg of vitamin E was 85.1, 40.6, 16.3 and 87.6, 44.1, 18.8 at 12, 24 and 48 hrs after dilution, respectively, which was significantly higher than other antioxidants ( $P < 0.05$ ). There was a tendency for higher PMS with 2 mg of vitamin E than 1mg ( $P = 0.06$ ). Likewise, the motility with BSA was significantly higher than ascorbic acid, so that were 25% and 11.5% at 24 hrs, respectively. However, there was no difference in PMS among different concentrations of BSA or in combination with sucrose 10% in all of evaluations except for BSA 3%+sucrose 10% at hour 24.

It should be considered that the motility of spermatozoa in most groups at 48 hrs was mainly vibratory and represented less or no forward motion. At 72 hrs after collection, all of spermatozoa were immotile in all of treatments.

#### **Rate of forward movement**

Means for RFM of spermatozoa in different treatment diluents are presented in Table 2. Examining the data revealed a precipitous decline in RFM with time especially after 12 hrs except for vitamin E groups, which was observed higher RFM until 48 hrs than other groups ( $P < 0.05$ ). BSA alone or with sucrose as well as ascorbic acid had no high positive effect on RFM than the control group. The RFM of semen in vitamin E and other groups declined rapidly by 48 and 24 hrs, respectively.

#### **Sperm membrane integrity**

Means for SMI in different antioxidants are shown in Table 3. Sperm membrane integrity declined with time in all of antioxidants groups. However, SMI was significantly higher in vitamin E groups than other groups up to 48 hrs ( $P < 0.05$ ). There was no significant difference in SMI between 1 and 2 mg of vitamin E ( $P > 0.05$ ), except at hour 24, which was higher in 2 mg vitamin E group.

#### **Discussion**

Oxidative stress is one of the factors associated with decline in fertility during semen storage. The sperm plasma membrane

contains a high amount of unsaturated fatty acids and therefore is particularly susceptible to peroxidative damages with subsequent loss of membrane integrity, impaired cell function and decreased motility of spermatozoa (Aurich *et al.*, 1997; Ball *et al.*, 2001). Efforts for adding of antioxidants in the extenders are in order to prevent or decrease this peroxidation process.

In the present study, the effects of BSA, vitamin E and ascorbic acid on motility and membrane integrity of diluted ram semen during storage at 5°C were investigated. Independent of their concentration, positive as well as no effects were found. According to study of Ball *et al.*, (2001), adding the ascorbic acid did not improve the maintenance of motility of cooled equine spermatozoa during the 96 hrs storage period. This result is consistent with the observation of Aurich *et al.*, (1997) who also did not detect a positive effect of adding vitamin C to cooled, extended equine spermatozoa. The recent authors did, however, observe a positive effect of adding vitamin C on the maintenance of sperm membrane integrity during cooled storage.

In another study (Sanchez-Partida *et al.*, 1997) in the ram, adding the ascorbic acid in diluent and its effect on the post-thaw motility characteristics and fertility of spermatozoa was examined. They found no positive effect from ascorbic acid, even the presence of more than 50  $\mu$  M ascorbic acid significantly reduced all motility characteristics compared with the control diluent. In the present study, adding ascorbic acid (0.9 mg/ml) did not improve the motility or membrane integrity in compare with control group during 72 hrs of storage. However, there is no study about the protective effect of ascorbic acid on the ram semen at 5°C for comparison with our study.

Vitamin E functions as an intracellular antioxidant scavenging for free reactive oxygen and lipid hydroperoxidas and converting them to non-reactive forms. Thus, maintains the integrity of membrane phospholipids against oxidative damage and peroxidation (Smith and Akinbamijo, 2000). However, adding vitamin E to stored semen has resulted a variable success in improving

**Table 1: Motility of spermatozoa (%) in extender containing different antioxidants**

Time (hr)	BSA 1%	BSA 3%	BSA 1% + Sucrose 10%	BSA 3% + Sucrose 10%	V. E 1 mg	V. E 2 mg	Ascorbic acid	Control	Overall Mean ± SEM
0	90.3 <sup>a</sup>	89.1 <sup>a</sup>	89.6 <sup>a</sup>	89.0 <sup>a</sup>	89.9 <sup>a</sup>	89.5 <sup>a</sup>	90.1 <sup>a</sup>	90.0 <sup>a</sup>	89.7 ± 0.18
12	79.9 <sup>a</sup>	79.3 <sup>a</sup>	81.0 <sup>a</sup>	79.5 <sup>a</sup>	85.1 <sup>b</sup>	87.6 <sup>b</sup>	78.6 <sup>a</sup>	80.5 <sup>a</sup>	81.6 ± 0.37
24	25.0 <sup>a</sup>	22.0 <sup>a</sup>	24.5 <sup>a</sup>	21.0 <sup>b</sup>	40.6 <sup>c</sup>	44.1 <sup>c</sup>	11.5 <sup>d</sup>	11.3 <sup>d</sup>	25.4 ± 1.20
48	4.8 <sup>a</sup>	5.0 <sup>a</sup>	4.9 <sup>a</sup>	4.0 <sup>a</sup>	16.3 <sup>b</sup>	18.8 <sup>b</sup>	2.7 <sup>a</sup>	2.1 <sup>a</sup>	7.5 ± 0.66
72	0	0	0	0	0	0	0	0	0

Means with different superscripts (a, b, c, d) within each row are significantly different (P<0.05)

**Table 2: Rate of forward movement (RFM) of spermatozoa in extender containing different antioxidants**

Time (hr)	BSA 1%	BSA 3%	BSA 1% + Sucrose 10%	BSA 3% + Sucrose 10%	V. E 1 mg	V. E 2 mg	Ascorbic acid	Control	Overall Mean ± SEM
0	3.7 <sup>a</sup>	3.6 <sup>a</sup>	3.6 <sup>a</sup>	3.5 <sup>a</sup>	3.6 <sup>a</sup>	3.5 <sup>a</sup>	3.5 <sup>a</sup>	3.6 <sup>a</sup>	3.6 ± 0.05
12	3.1 <sup>a</sup>	3.0 <sup>a</sup>	3.0 <sup>a</sup>	3.1 <sup>a</sup>	3.6 <sup>b</sup>	3.5 <sup>a</sup>	3.0 <sup>a</sup>	2.8 <sup>c</sup>	3.2 ± 0.06
24	2.0 <sup>a</sup>	1.9 <sup>b</sup>	2.0 <sup>b</sup>	2.0 <sup>b</sup>	2.6 <sup>c</sup>	2.7 <sup>c</sup>	1.7 <sup>b</sup>	1.6 <sup>b</sup>	2.1 ± 0.06
48	0.5 <sup>a</sup>	0.4 <sup>a</sup>	0.3 <sup>b</sup>	0.4 <sup>a</sup>	1.0 <sup>c</sup>	1.0 <sup>c</sup>	0.2 <sup>b</sup>	0.3 <sup>b</sup>	0.5 ± 0.05
72	0	0	0	0	0	0	0	0	0

Means with different superscripts (a, b, c, d) within each row are significantly different (P<0.05)

**Table 3: Sperm membrane integrity (%) in extender containing different antioxidants**

Time (hr)	BSA 1%	BSA 3%	BSA 1% + Sucrose 10%	BSA 3% + Sucrose 10%	V. E 1 mg	V. E 2 mg	Ascorbic acid	Control	Overall Mean ± SEM
0	94.0 <sup>a</sup>	93.0 <sup>a</sup>	93.6 <sup>a</sup>	93.1 <sup>a</sup>	93.2 <sup>a</sup>	92.9 <sup>a</sup>	92.1 <sup>a</sup>	92.6 <sup>a</sup>	93.1 ± 0.18
12	81.2 <sup>a</sup>	81.4 <sup>a</sup>	83.3 <sup>a</sup>	80.9 <sup>a</sup>	87.5 <sup>b</sup>	88.7 <sup>b</sup>	80.6 <sup>a</sup>	81.0 <sup>a</sup>	83.2 ± 0.41
24	26.8 <sup>a</sup>	25.9 <sup>a</sup>	25.4 <sup>a</sup>	24.5 <sup>a</sup>	42.1 <sup>b</sup>	47.2 <sup>c</sup>	14.8 <sup>d</sup>	14.7 <sup>d</sup>	28.0 ± 1.1
48	9.6 <sup>a</sup>	9.0 <sup>a</sup>	7.9 <sup>a</sup>	7.5 <sup>a</sup>	18.6 <sup>b</sup>	18.5 <sup>b</sup>	7.2 <sup>a</sup>	7.3 <sup>a</sup>	10.8 ± 0.53
72	0	0	0	0	0	0	0	0	0

Means with different superscripts (a, b, c, d) within each row are significantly different (P<0.05)

the maintenance of motility. According to study of Ball *et al.*, (2001), vitamin E did not significantly alter the maintenance of motility during cooled storage of equine spermatozoa for either 72 or 96 hrs. However, Aguero *et al.*, (1995) reported that adding vitamin E to stallion semen improved the maintenance of motility and viability during 24 hrs cooled storage.

On the other hand, in another study in the rams adding vitamin E impaired the maintenance of motility in stored semen at 15°C (Uperti *et al.*, 1997). Whereas, in our study only vitamin E among other antioxidants had a protective effect on the motility and membrane integrity. So that, these parameters maintained at high value during first 12 hrs and decreased gradually to moderate value up to 48 hrs. Likewise, 2 mg vitamin E was slightly more effective than 1 mg in its protective role. The probable reason for this difference may be due to the semen storage at 15°C carried out by Uperti *et al.*, (1997) and maintaining of sperm metabolism at a high rate in this temperature.

The antioxidants superoxide dismutase (SOD), catalase (CAT), cytochrome and glutathione peroxidase (GP) were added at various concentrations to tris-glucose-yolk diluent (TGY), and their effects on motility, acrosome integrity and fertility of ram spermatozoa were assessed after extension and liquid storage. All the antioxidants improved the motility and acrosome integrity of spermatozoa and a combination of SOD and CAT had an additive effect on the survival of spermatozoa stored at 5°C but not at 25°C. The proportion of oocytes fertilized in vitro declined with time of semen storage and was better for semen diluted with TGY containing SOD or CAT (Maxwell and Stojanov, 1996). However, in the present investigation fertilization rate was not studied. Although pregnancy rate obtained in an insemination trial would be the optimal parameter for determining semen quality, assessment of sperm motility and membrane integrity allows good estimates of fertilizing capacity (Harrison and Vickers, 1990).

BSA as a water-soluble antioxidant has been used in several studies. In one study, BSA 3% (w/v) did not significantly improve

the maintenance of equine sperm motility during cooled storage at 5°C (Ball *et al.*, 2001). BSA has been shown to possess antioxidant properties and has been incorporated into a number of semen extenders. For example, in two studies, adding BSA 1 and 3% alone (Kreider *et al.*, 1985) or in combination with different concentration of sucrose (Klem *et al.*, 1986) did improve maintenance of equine sperm kinematic parameters at 37°C and 38°C, respectively. This difference may be due to the temperature that sperm had been stored. However there is no study concerning the effect of BSA on ram semen. In the present study, BSA alone or in combination with sucrose 10% had not positive effect on sperm kinematic parameters in compare with vitamin E.

It seems that sucrose has not a significant role in protection of oxidative process and maintenance of ram sperm motility. Although, the ability of sucrose solutions to support the viability of stallion spermatozoa has been confirmed by Klem *et al.*, (1986).

In conclusion, adding vitamin E to the extender of ram semen during dilution is superior in maintenance of motility and membrane integrity of spermatozoa up to 48 hrs during storage at 5°C. However, BSA 1 and 3% alone or in combination with sucrose 10% and ascorbic acid (0.9 mg/ml) had low or no significant effect on sperm kinematic parameters.

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## References

- 1- Aguero, A; Miragaya, MH; Mora, NG; Chaves, MG; Neild, DM and Beconi, MT (1995). Effect of vitamin E addition on equine sperm preservation. *Comun. Biol.*, 13: 343-356.
- 2- Arthur, GH; Noakes, DE; Pearson, H and Parkinson, TJ (1996). *Veterinary reproduction and obstetrics*. 7th. Edn., London, W. B. Saunders Co., PP: 634-647.
- 3- Aurich, JE; Schonherr, U; Hoppe, H and Aurich, C (1997). Effects of antioxidants on

- motility and membrane integrity of chilled stored stallion semen. *Theriogenology*. 48: 185-192.
- 4- Ax, RL; Dally, MR; Didon, BA; Lenz, RW; Love, CC; Varner, DD; Hafez, B and Bellin, ME (2000). Artificial insemination. In: Hafez, B and Hafez, ESE (Eds.), *Reproduction in farm animals*. (7th. Edn.), Philadelphia, Lea and Febiger. PP: 376-389.
  - 5- Ball, BA; Medina, V; Gravance, CG and Baumber, J (2001). Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. *Theriogenology*. 56: 577-589.
  - 6- Dixon, DE and Kreider, JL (1981). Effect of calcium and fatty acids on the isolation of stallion spermatozoa in BSA. *Theriogenology*. 15: 335-344.
  - 7- Hafez, ESE (2000). Preservation and cryopreservation of gametes and embryos. In: Hafez, B and Hafez, ESE (Eds.), *Reproduction in farm animals*. (7th. Edn.), Philadelphia, Lea and Febiger. PP: 431-442.
  - 8- Harrison, RAP and Vickers, SE (1990). Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J. Reprod. Fertil.*, 8: 343-352.
  - 9- Klem, ME; Kreider, JL; Pruitt, JB and Potter, GD (1986). Motility and fertility of equine spermatozoa extended in bovine serum albumin and sucrose. *Theriogenology*. 26: 569-576.
  - 10- Kreider, JL; Tindall, WC and Potter, GD (1985). Inclusion of bovine serum albumin in semen extenders to enhance maintenance of stallion sperm viability. *Theriogenology*. 23: 399-408.
  - 11- Marti, JI; Marti, E; Cebrian-Perez, JA and Muino-Blanco, T (2003). Survival rate of antioxidant enzyme activity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure. *Theriogenology*. 60: 1025-1037.
  - 12- Maxwell, WM and Stojanov, T (1996). Liquid storage of ram semen in the absence or presence of some antioxidants. *Reprod. Fertil. Dev.*, 8(6): 1013-1020.
  - 13- Petrie, A and Watson, P (1999). *Statistics for veterinary and animal science*. 1st. Edn., Blackwell Science. PP: 93-100.
  - 14- Sanchez-Partida, LG; Setchell, BP and Maxwell, WM (1997). Epididymal compounds and antioxidants in diluents for the frozen storage of ram spermatozoa. *Reprod. Fertil. Dev.*, 9(7): 689-696.
  - 15- Sarlos, P; Molnar, A; Kokai, M; Gabor, G and Ratky, J (2002). Evaluation of the effect of antioxidants in the conservation of ram semen. *Acta Vet. Hung.*, 50(2): 235-245.
  - 16- Smith, OB and Akinbamijo, OO (2000). Micronutrients and reproduction in farm animals. *Anim. Reprod. Sci.*, 60-61: 549-560.
  - 17- Uperti, GC; Jensen, K; Munday, R; Duganzich, DM; Vishwanath, R and Smith, JF (1998). Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. *Anim. Reprod. Sci.*, 51(4): 275-287.
  - 18- Uperti, GC; Jensen, K; Oliver, JE; Duganzich, DM; Munday, R and Smith, JF (1997). Motility of ram spermatozoa during storage in a chemically defined diluent containing antioxidants. *Anim. Reprod. Sci.*, 48(2-4): 269-278.
  - 19- Watson, PF and Anderson, WJ (1983). Influence of butylated hydroxytoluene (BHT) on the viability of ram spermatozoa undergoing cold shock. *J. Reprod. Fertil.*, 69(1): 229-235.