



RESEARCH ARTICLE

# Alpha-tocopherol improves frozen-thawed sperm quality by reducing hydrogen peroxide during cryopreservation of bull semen

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## Abstract

This study was conducted to investigate the effects of different levels of  $\alpha$ -tocopherol in cryopreservation media on the bull sperm quality after thawing. Semen samples were collected from six Holstein bulls using artificial vagina twice a week. Semen samples were pooled to eliminate individual differences and then divided into four equal parts for freezing with extenders containing different concentrations of  $\alpha$ -tocopherol according to experimental groups as follows: 0 (control), 1.2 mM (E1), 2.4 mM (E2) and 4.8 mM (E4). Motion characteristics, viability, plasma membrane functionality, lipid peroxidation and  $H_2O_2$  status were determined after thawing. Results showed that malondialdehyde (MDA) concentration was significantly lower in E4 ( $6.1 \pm 0.6$  nmol/mL) than E1 and E2 extenders ( $8.1 \pm 0.6$  nmol/mL and  $8 \pm 0.6$  nmol/mL, respectively). Also, the lowest significant concentration of  $H_2O_2$  was observed in E4 ( $3.2 \pm 0.13$  nmol/mL) compared to E1 ( $4 \pm 0.1$  nmol/mL), E2 ( $5.3 \pm 0.1$  nmol/mL) and control ( $6.7 \pm 0.1$  nmol/mL). Moreover, E2 and E4 produced the highest significant motility ( $74.2 \pm 1.6\%$ ,  $75.9 \pm 1.6\%$ ), viability ( $78.2 \pm 1.8\%$ ,  $76.1 \pm 1.8\%$ ) and membrane functionality ( $73 \pm 1.6\%$ ,  $70.5 \pm 1.6\%$ ) compared to other groups. It can be concluded that  $\alpha$ -tocopherol at the concentration of 4.8 mM can be an efficient antioxidant additive in Bioxcell extender for cryopreservation of bull semen.

**Additional key words:** antioxidant; bioxcell; lipid peroxidation; sperm;  $H_2O_2$ .

**Abbreviations used:** LPO (lipid peroxidation); MDA (malondialdehyde); ROS (reactive oxygen species).

**Authors' contributions:** Designed the experiments: MM and MC. Performed the experiments: MM. Wrote the paper: MS and RM.

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## Introduction

Improvement in livestock production techniques, especially artificial insemination and semen freezing are the leading causes of accelerated rate of genetic selection (Barbas & Mascarenhas, 2009; Forouzanfar *et al.*, 2010). Semen cryopreservation has allowed specific opportunities for conservation of genetic resources through sperm banks, guarantee of a constant commercial supply of semen, and collaboration in breed improvement programs by artificial insemination (Bucak *et al.*, 2007; Masoudi *et al.*, 2017). Semen cryopreservation may lead to oxidative, chemical and physical damages on sperm membrane leading to reduction of sperm viability and fertility (Evans *et al.*, 1987; Watson, 2000; Emamverdi *et al.*, 2014; Najafi *et al.*, 2014a; Sariözkan *et al.*, 2015). Freezing process

mostly leads to loss of motility, acrosomal and plasma membrane functionality of spermatozoa (Tuncer *et al.*, 2011; Najafi *et al.*, 2013; Shahverdi *et al.*, 2015). Moreover, because of high amount of polyunsaturated fatty acids in mammalian spermatozoa (plasma membrane), bull spermatozoa is very susceptible to oxidative stress which can influence on the quality and fertility potential of spermatozoa (Zanganeh *et al.*, 2013). Antioxidant enzymes such as glutathione peroxidase and superoxide dismutase have crucial roles to maintain defense mechanisms against oxidative stress-induced damages in semen (Tuncer *et al.*, 2010). However, antioxidant capacity in spermatozoa may be insufficient to prevent oxidative stress during the freeze-thawing process (Ernster *et al.*, 1992; Najafi *et al.*, 2014b; Masoudi *et al.*,

2016a). Therefore, addition of suitable antioxidants to the extenders is suggested to reduce oxidative damages during freeze–thawing of bull spermatozoa (Büyükleblebici *et al.*, 2014).

Alpha-tocopherol is a well-known lipid peroxidation inhibitor in biological membranes, acting as a scavenger of reactive oxygen species (ROS), preventing oxidative damage during cryopreservation of bull semen (O’Flaherty *et al.*, 1997). A water-soluble vitamin E analogue (Trolox) improved sperm motility and mitochondrial membrane functionality during liquid storage of boar semen (Cerolini *et al.*, 2000). In a study by Dalvit *et al.* (1998), vitamin E increased the rate of fertilization when added to the bull semen extender. Alpha-tocopherol also improved total motility and viability of bull spermatozoa after freeze-thawing (Nasiri *et al.*, 2012). This observation may be related to the protective effects of  $\alpha$ -tocopherol against ROS (Towhidi & Parks, 2012). Moreover, dietary supplementation of vitamin E has been reported to increase reproductive capacity in chicken (Khan *et al.*, 2012), boar (Brzezińska-Ślebodzińska *et al.*, 1995), rabbit (Yousef, 2010), ram (Masoudi *et al.*, 2016b) and buck (Majid *et al.*, 2015).

Although there are several reports for beneficial effects of  $\alpha$ -tocopherol on cryopreservation of bull spermatozoa, there is no report for consideration of cellular parameters such as  $H_2O_2$  and sperm parameters after thawing. Therefore, the present study was conducted to determine the potential effects of different concentrations of  $\alpha$ -tocopherol in Bioxcell extender for cryopreservation of bull semen.  $H_2O_2$  concentration and lipid peroxidation were assessed as an ROS scavenging in sperm cells. Moreover, sperm parameters, including motion characteristics, viability, membrane functionality and morphology were also evaluated after freeze-thawing-thawing.

## Material and methods

The chemicals used in this study were purchased from Sigma (St. Louis, MO, USA), and Merck (Darmstadt, Germany), unless otherwise indicated.

### Animal and semen collection

Six fertile Holstein bulls (aged 3–6 years) under uniform conditions with feeding based on National Research Council (NRC, 2001) were used in this study. Semen samples were collected using artificial vagina from six bulls twice a week during three weeks (in six replicates). Immediately after collection, semen volume and sperm concentration, sperm motility and morphology were evaluated. Semen samples were accepted for

experiment if the following criteria were met: volume of 5–10 mL, concentration of  $>1 \times 10^9$  spermatozoa/mL, total motility  $> 70\%$ , and abnormal morphology  $< 10\%$ . Then, ejaculates from bulls in each day were pooled and divided into four groups according to experimental treatments.

### Antioxidant treatments, semen dilution and cryopreservation

Alpha-tocopherol was added to the Bioxcell (IMV Technologies, L’Agile, France) to yield four different final concentrations: 0 (control), 1.2 mM (E1), 2.4 mM (E2) and 4.8 mM (E4). Because the  $\alpha$ -tocopherol was not soluble in water, ethanol 0.05% was used to solve it before addition to extender. Pooled semen was divided into four equal aliquots and diluted with four extenders containing different concentrations of  $\alpha$ -tocopherol to a final concentration of approximately  $60 \times 10^6$  spermatozoa/mL ( $15 \times 10^6$  total spermatozoa in each 0.25 mL straw). Diluted semen samples were cooled to 4°C for 4 h. Subsequently semen was frozen at a programmed rate of  $-3^\circ\text{C}/\text{min}$  from +4 to  $-10^\circ\text{C}$ ;  $-40^\circ\text{C}/\text{min}$  from  $-10$  to  $-100^\circ\text{C}$ ; and  $-20^\circ\text{C}/\text{min}$  from  $-100$  to  $-140^\circ\text{C}$  in a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen for storage. For sperm evaluation, straws were thawed individually at  $37^\circ\text{C}$  for 30 s in a water bath. Sperm evaluation was performed on all semen samples immediately after thawing.

### Motility and velocity parameters

Motion characteristics of rewarmed sperm were measured using a computer-assisted sperm analysis (CASA, CEROS vers. 12.3; Hamilton-Thorne Biosciences, Beverly, MA, USA). Sperm sample was placed in a chamber ( $38^\circ\text{C}$ , Leja 4; 20 mm height; Leja Products, Luzernestraat B.V., Holland) and then loaded chamber placed on the warm stage of the microscope ( $37^\circ\text{C}$ ). Afterwards, three randomly selected microscopic fields were examined. Motility data was characterized as follows: total motility (MOT,%); progressive motility (PROG,%); average path velocity (VAP,  $\mu\text{m}/\text{s}$ ); straight line velocity (VSL,  $\mu\text{m}/\text{s}$ ); curvilinear velocity (VCL,  $\mu\text{m}/\text{s}$ ); amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ); straightness (STR,%); linearity (LIN,%). At least 200 spermatozoa were assessed in each CASA analysis.

### Membrane functionality (HOST)

Hypo-osmotic swelling test (HOST) was designed to determine membrane functionality as described by Revell & Mrode (1994), with a slight modification.

Briefly, 10  $\mu\text{L}$  of semen was mixed with 100  $\mu\text{L}$  of a hypo-osmotic solution [100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate] in a 1.5 mL test tube and incubated at 37°C for 30 min. After incubation, the mixture was homogenized and evaluated under a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan). A total of 200 spermatozoa were counted in at least five different microscopic fields at  $\times 400$ . The percentage of spermatozoa with swollen and curved tails was recorded.

### Sperm viability

Viability was assessed using Eosin–Nigrosine staining method (Najafi *et al.*, 2013). Briefly, 20  $\mu\text{L}$  aliquot from sperm suspension was stained by 20  $\mu\text{L}$  Eosin–Nigrosine dye. Then, smears were prepared on a warm slide and spread the stain with a second slide. Twenty hundred sperm were counted under phase-contrast at 1000  $\times$  magnification. Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable. The viability was assessed by counting 200 spermatozoa under phase-contrast at  $\times 1000$  (CKX41, Olympus, Tokyo, Japan).

### Malondialdehyde concentration assay

MDA as an index of lipid peroxidation was measured according to the method described by Esterbauer & Cheeseman (1990). Briefly, 1 mL of sperm suspension ( $250 \times 10^6$  spermatozoa/mL) was mixed with 1 mL of cold trichloroacetic acid (20% w/v). The precipitate was pelleted by centrifuging ( $963 \times g$  for 15 min), and 1 mL of the supernatant was removed and incubated with 1 mL of thiobarbituric acid (0.67% w/v) in a boiling water bath at 100°C for 10 min. After cooling, the absorbance was determined by a spectrophotometer (UV-1200, Shimadzu, Japan) at 532 nm.

### Measurement of hydrogen peroxide

The concentration of  $\text{H}_2\text{O}_2$  in thawed sperm and extenders were measured by the Phenol Red colorimetric method described by da Silva Maia *et al.* (2010). Briefly, a sample of 100  $\mu\text{L}$  of thawed sperm containing approximately  $40 \times 10^6$  spermatozoa was incubated at 37°C for 30 min in 1.0 mL of buffered phenol red solution. After incubation, the samples were centrifuged at  $2000 \times g$  for 10 min, and the supernatant was decanted into a microtube. Then, 10  $\mu\text{L}$  of NaOH solution was added to supernatant. The same procedure was used to determine the concentration of  $\text{H}_2\text{O}_2$  generated in the extender. The assay was performed in duplicate, and the absorbance of the samples was read at 610 nm, at 25°C, in a UV–vis, double beam spectrophotometer (Lambda 25, Perkin Elmer, Beaconsfield, UK). The concentration of  $\text{H}_2\text{O}_2$  in the sample was determined by comparing the absorbance obtained with a standard curve.

### Statistical analysis

All data were checked for normal distribution by Shapiro–Wilk test and analyzed using Proc GLM of SAS 9.1 (SAS Inst, Cary, NC, USA). Six replicates were used for evaluation. Statistical differences among various group means were determined by Tukey’s test and the values of  $p < 0.05$  were considered to be statistically significant. Results are shown as mean  $\pm$  SEM.

### Results

Table 1 shows the percentage of motion parameters in the frozen-thawed bull semen in extenders supplemented with different concentrations of  $\alpha$ -tocopherol. Total motility and progressive motility were significantly higher in E4 ( $75.9 \pm 1.6\%$ ,  $43.3 \pm 1.3\%$ )

**Table 1.** Effect of different extenders on post-thawed bull spermatozoa motility and motion parameters (mean  $\pm$  SEM).

Parameter <sup>[1]</sup>	E0 <sup>[2]</sup>	E1	E2	E4
MOT (%)	61.3 $\pm$ 1.67 <sup>b</sup>	64.1 $\pm$ 1.67 <sup>b</sup>	74.2 $\pm$ 1.67 <sup>a</sup>	75.9 $\pm$ 1.67 <sup>a</sup>
PROG (%)	30.5 $\pm$ 1.34 <sup>b</sup>	31.7 $\pm$ 1.34 <sup>b</sup>	39.1 $\pm$ 1.34 <sup>a</sup>	43.3 $\pm$ 1.34 <sup>a</sup>
VAP ( $\mu\text{m/s}$ )	45.2 $\pm$ 1.70	49.4 $\pm$ 1.70	50.3 $\pm$ 1.70	46.4 $\pm$ 1.70
VSL ( $\mu\text{m/s}$ )	30.1 $\pm$ 0.98	34.4 $\pm$ 0.98	31.3 $\pm$ 0.98	29.5 $\pm$ 0.98
VCL ( $\mu\text{m/s}$ )	70.5 $\pm$ 2.45	75.3 $\pm$ 2.45	81.4 $\pm$ 2.45	79.4 $\pm$ 2.45
ALH ( $\mu\text{m}$ )	2.5 $\pm$ 0.30	2.9 $\pm$ 0.30	2.1 $\pm$ 0.30	2.0 $\pm$ 0.30
LIN (%)	41.8 $\pm$ 1.45	40.7 $\pm$ 1.45	39.3 $\pm$ 1.45	42.0 $\pm$ 1.45
STR (%)	76.7 $\pm$ 2.30	76.8 $\pm$ 2.30	73.0 $\pm$ 2.30	75.0 $\pm$ 2.30

<sup>[1]</sup> MOT: total motility; PROG: progressive motility; VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; LIN: linearity; STR: straightness. <sup>[2]</sup> E0, E1, E2, E4: 0 mM, 1.2 mM, 2.4 mM and 4.8 mM, respectively. <sup>a,b,c,d</sup> indicate significant differences ( $p < 0.05$ ).

and E2 (74.2±1.6%, 39.1±1.3%) compared to control (61.3±1.6%, 30.5±1.3%), respectively. No significant difference was detected for E0 and E1 for total motility and progressive motility.

Data related to the viability, membrane functionality, MDA concentration and H<sub>2</sub>O<sub>2</sub> are presented in the Table 2.

The higher significant viability in frozen-thawed sperm was observed in E2 and E4 (78.2±1.8%, 76.1±1.8%, respectively) compared to control (61.3±1.8%). Moreover, E2 and E4 produced higher significant membrane functionality (73±1.6%, 70.5±1.6%, respectively) compared to control (60.5±1.6%). No significant difference was detected for control and E1 for viability and membrane functionality.

For MDA concentration, although E4 produced the lowest significant concentration of MDA (6.1±0.6 nmol/mL) compared to E1 and E2 (8.1±0.6 nmol/mL and 8.0±0.6 nmol/mL, respectively), no significant difference was observed between E4 and control (7.2±0.6 nmol/mL). Moreover, the lowest significant concentration of H<sub>2</sub>O<sub>2</sub> was observed in E4 (3.2±0.1 nmol/mL) compared to E2 (4.0±0.1 nmol/mL), E1 (5.3±0.1 nmol/mL) and control (6.7±0.1 nmol/mL). The difference between E2 and E1 was also significant compared to control.

## Discussion

Mammalian spermatozoa contains high amount of polyunsaturated fatty acids in plasma membrane which makes them susceptible to oxidative stress, especially during freeze-thaw process (Purdy, 2006; Tuncer *et al.*, 2011). Damages to membrane matrix causes destruction of structural and biochemical organs of sperm leading to reduction of sperm motility and fertility (Watson, 1976). Frozen-thawed bull spermatozoa is more proxidized than fresh sperm due to lose of intracellular antioxidant capacity in sperm (Tuncer *et al.*, 2010). Therefore, optimization of bull spermatozoa freezing procedure using an antioxidant additive can be an efficient strategy to improve the quality of post-thawed sperm. In this study, 2.4 and 4.8 mM  $\alpha$ -tocopherol showed a

suitable protective effect against freezing damages. However, for H<sub>2</sub>O<sub>2</sub>, extender supplemented with 4.8 mM  $\alpha$ -tocopherol produced better results compared to 2.4 mM. It was clear that ROS accumulated during the cooling, equilibration, freeze-thawing and post-thaw incubation of sperm. Our results showed that  $\alpha$ -tocopherol has suitable cryo-protective effects through its ability to quench ROS accumulation, which is in agreement with several studies that stated analogous of vitamin E in extenders increased the recovery rate of motility and viability of sperm (Donoghue & Donoghue, 1997; Surai *et al.*, 1998; Silva *et al.*, 2013). Moreover, similar to our study, Domínguez-Rebolledo *et al.* (2010) reported that motion characteristics and acrosomal integrity of epididymal red deer spermatozoa were improved when Trolox was added to incubation medium after thawing. However, some studies do not confirm these results because analogues of vitamin E had negative effects when it was added to refrigeration medium of ram (Mata-Campuzano *et al.*, 2014) and red deer (Anel-López *et al.*, 2012) spermatozoa. This discrepancy may be related to selected dose of vitamin E, dilution rate or preservation procedure.

One of the main roles of  $\alpha$ -tocopherol in cryopreservation media is reduction of MDA and consequently improvement in sperm motility (Suleiman *et al.*, 1996). Sperm is highly susceptible to lipid peroxidation (LPO). The spontaneous membrane LPO disrupts the structure of membrane via ROS (Bucak *et al.*, 2008) which ultimately lead to loss of sperm function, such as reduction in membrane functionality, sperm motility and fertility potential (Bansal & Bilaspuri, 2011). Alpha-tocopherol is believed to be the primary component of the antioxidant system of spermatozoa, and is regarded as one of the major membrane protectants against ROS and LPO (Bansal & Bilaspuri, 2009). In the present study, we found that exposure of sperm to the  $\alpha$ -tocopherol resulted in less H<sub>2</sub>O<sub>2</sub> during the cryopreservation process. These results are in agreement with Amini *et al.* (2015) and Martínez-Páramo *et al.* (2012), who obtained lower lipid peroxidation of sperm in response to  $\alpha$ -tocopherol in rooster and sea bass, respectively, after cryopreservation. Moreover, reduction in amount of

**Table 2.** Viability and membrane functionality (HOST) in bull spermatozoa diluted in different extenders (mean±SEM).

Parameter	E0	E1	E2	E4
Viability (%)	61.3±1.8 <sup>b</sup>	64.1±1.8 <sup>b</sup>	76.1±1.8 <sup>a</sup>	78.2±1.8 <sup>a</sup>
Membrane functionality (%)	60.5±1.6 <sup>b</sup>	61.7±1.6 <sup>b</sup>	73.0±1.6 <sup>a</sup>	70.5±1.8 <sup>a</sup>
MDA concentration (nmol/mL)	7.2±0.6 <sup>ab</sup>	8.1±0.6 <sup>b</sup>	8.0±0.6 <sup>b</sup>	6.1±0.6 <sup>a</sup>
H <sub>2</sub> O <sub>2</sub> concentration (nmol/mL)	6.7±0.1 <sup>d</sup>	5.3±0.1 <sup>c</sup>	4.0±0.1 <sup>b</sup>	3.2±0.1 <sup>a</sup>

E0, E1, E2, E4: 0 mM, 1.2 mM, 2.4 mM and 4.8 mM, respectively. MDA: malondialdehyde. <sup>a,b,c,d</sup> indicate significant differences ( $p<0.05$ ).

H<sub>2</sub>O<sub>2</sub> in this experiment in response to  $\alpha$ -tocopherol may be due to potential of  $\alpha$ -tocopherol to the phenoxyl radicals stabilization. Similar results have also been reported by Breininger *et al.* (2005), who stated that  $\alpha$ -tocopherol suppressed the ROS in boar spermatozoa after thawing.

Using  $\alpha$ -tocopherol for reduction of MDA was our interest to evaluate the measurement of MDA in spermatozoa cells and their effects on the sperm performance. Alpha tocopherol can also increase the electron transfer during oxidative stress resulting to stable phenoxyl radical (Davies *et al.*, 1988). However, results of MDA in this study were not as might be expected, because we thought a reduction in MDA after supplementation of our extender with  $\alpha$ -tocopherol would happen. This behavior may be due to the connection of  $\alpha$ -tocopherol because antioxidants may influence on the MDA in the low concentration (Zhandi & Sharafi, 2015).

Taking together, we tested the effects of  $\alpha$ -tocopherol in the wide range (0-4.8 mM) for cryopreservation of bull spermatozoa. The higher results were obtained in high doses of  $\alpha$ -tocopherol (2.4-4.8 mM). It should be noted that the efficiency of antioxidants are affected by various factors such as component of buffer, cryoprotectants and incubation time which resulted to obtain different outcomes in literatures. Cryoprotectants such as egg yolk and soybean or milk have different antioxidant capacity (Alvarez-Rodríguez *et al.*, 2013). It is possible that component of Bioxcell have some effects on the optimum level of  $\alpha$ -tocopherol. Finally, we understand that addition of optimum dose of  $\alpha$ -tocopherol could improve bull sperm quality indices.

In summary, our findings demonstrate that concentrations of  $\alpha$ -tocopherol (2.4-4.8 mM) in Bioxcell can be efficient for preservation of bull spermatozoa in freezing status, although this issue must be tested in fertility trials.

## References

- Alvarez-Rodríguez M, Alvarez M, Anel-López L, Martínez-Rodríguez C, Martínez-Pastor F, Borrigan S, Anel L, de Paz P, 2013. The antioxidant effects of soybean lecithin-or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (*Ursus arctos*) spermatozoa. *Reprod Fertil Dev* 25: 1185-1193. <https://doi.org/10.1071/RD12181>
- Amini MR, Kohram H, Shahaneh AZ, Zhandi M, Sharideh H, Nabi MM, 2015. The effects of different levels of vitamin E and vitamin C in modified Beltsville extender on rooster post-thawed sperm quality. *Cell Tissue Bank* 16: 587-592. <https://doi.org/10.1007/s10561-015-9506-9>
- Anel-López L, Álvarez-Rodríguez M, García-Álvarez O, Álvarez M, Maroto-Morales A, Anel L, de Paz P, Garde JJ, Martínez-Pastor F, 2012. Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. *Anim Reprod Sci* 135: 37-46. <https://doi.org/10.1016/j.anireprosci.2012.09.001>
- Bansal AK, Bilaspuri GS, 2009. Antioxidant effect of vitamin E on motility, viability and lipid peroxidation of cattle spermatozoa under oxidative stress. *Anim Sci Pap Rep* 27: 5-14.
- Bansal AK, Bilaspuri GS, 2011. Impacts of oxidative stress and antioxidants on semen functions. *Vet Med In* 2011: 1-7. <https://doi.org/10.4061/2011/686137>
- Barbas JP, Mascarenhas RD, 2009. Cryopreservation of domestic animal sperm cells. *Cell Tissue Bank* 10: 49-62. <https://doi.org/10.1007/s10561-008-9081-4>
- Breininger E, Beorlegui NB, O'Flaherty CM, Beconi MT, 2005. Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. *Theriogenology* 63: 2126-2135. <https://doi.org/10.1016/j.theriogenology.2004.08.016>
- Brzezińska-Ślebodzińska E, Ślebodziński A, Pietras B, Wiczorek G, 1995. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biol Trace Elem Res* 47: 69-74. <https://doi.org/10.1007/BF02790102>
- Bucak MN, Atessahin A, Varisli O, Yuce A, Tekin N, Akcay A, 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen-Microscopic and oxidative stress parameters after freeze-thawing process. *Theriogenology* 67: 1060-1067. <https://doi.org/10.1016/j.theriogenology.2006.12.004>
- Bucak MN, Ateşşahin A, Yüce A, 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rumin Res* 75: 128-134. <https://doi.org/10.1016/j.smallrumres.2007.09.002>
- Büyükblebici S, Tuncer PB, Bucak MN, Eken A, Sariözkan S, Taşdemir U, Endirlik BÜ, 2014. Cryopreservation of bull sperm: Effects of extender supplemented with different cryoprotectants and antioxidants on sperm motility, antioxidant capacity and fertility results. *Anim Reprod Sci* 150: 77-83. <https://doi.org/10.1016/j.anireprosci.2014.09.006>
- Cerolini S, Maldjian A, Surai P, Noble R, 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim Reprod Sci* 58: 99-111. [https://doi.org/10.1016/S0378-4320\(99\)00035-4](https://doi.org/10.1016/S0378-4320(99)00035-4)
- Dalvit GC, Cetica PD, Beconi MT, 1998. Effect of  $\alpha$ -tocopherol and ascorbic acid on bovine in vitro fertilization. *Theriogenology* 49: 619-627. [https://doi.org/10.1016/S0093-691X\(98\)00012-0](https://doi.org/10.1016/S0093-691X(98)00012-0)
- da Silva Maia M, Bicudo SD, Sicherle CC, Rodello L, Gallego ICS, 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in

- extenders with antioxidants. *Anim Reprod Sci* 122: 118-123. <https://doi.org/10.1016/j.anireprosci.2010.08.004>
- Davies MJ, Forni LG, Willson RL, 1988. Vitamin E analogue Trolox CEsr and pulse-radiolysis studies of free-radical reactions. *Biochem J* 255: 513-522.
- Domínguez-Rebolledo ÁE, Fernández-Santos MR, Bisbal A, Ros-Santaella JL, Ramón M, Carmona M, Martínez-Pastor F, Garde JJ, 2010. Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. *Reprod Fertil Dev* 22: 856–870. <https://doi.org/10.1071/RD09197>
- Donoghue A, Donoghue D, 1997. Effects of water-and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. *Poult Sci* 76: 1440-1445. <https://doi.org/10.1093/ps/76.10.1440>
- Emamverdi M, Zhandi M, Shahneh A, Sharafi M, Akhlaghi A, Motlagh M, Dadkhah F, Davachi N, 2014. Flow cytometric and microscopic evaluation of post-thawed ram semen cryopreserved in chemically defined home-made or commercial extenders. *Anim Prod Sci* 55: 551-558. <https://doi.org/10.1071/AN13215>
- Ernster L, Forsmark P, Nordenbrand K, 1992. The mode of action of lipid-soluble antioxidants in biological membranes. Relationship between the effects of ubiquinol and vitamin E as inhibitors of lipid peroxidation in submitochondrial particles. *J Nut Sci Vita* 38: 548-551. [https://doi.org/10.3177/jnsv.38.Special\\_548](https://doi.org/10.3177/jnsv.38.Special_548)
- Esterbauer H, Cheeseman KH, 1990. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Meth Enzym* 186: 407-421. [https://doi.org/10.1016/0076-6879\(90\)86134-H](https://doi.org/10.1016/0076-6879(90)86134-H)
- Evans G, Maxwell WMC, Salamon S, 1987. Salamon's artificial insemination of sheep and goats. vol XI, 194 pp. Butterworths, Sydney; Boston.
- Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, Abedi P, Nili N, Rahmani HR, Nasr-Esfahani MH, 2010. In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology* 73: 480-487. <https://doi.org/10.1016/j.theriogenology.2009.10.005>
- Khan R, Rahman Z-u, Javed I, Muhammad F, 2012. Effect of vitamins, probiotics and protein on semen traits in post-molt male broiler breeders. *Anim Reprod Sci* 135: 85-90. <https://doi.org/10.1016/j.anireprosci.2012.09.005>
- Majid A, Qureshi MS, Khan RU, 2015. In vivo adverse effects of alpha-tocopherol on the semen quality of male bucks. *J Anim Phys Anim Nut* 99: 841-846. <https://doi.org/10.1111/jpn.12284>
- Martínez-Páramo S, Diogo P, Dinis M, Herráez M, Sarasquete C, Cabrita E, 2012. Incorporation of ascorbic acid and  $\alpha$ -tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. *Theriogenology* 77: 1129-1136. <https://doi.org/10.1016/j.theriogenology.2011.10.017>
- Masoudi R, Sharafi M, Shahneh AZ, Towhidi A, Esmaili V, Shahverdi A, Davachi, ND, 2016a. Fertility and flow cytometry study of frozen-thawed sperm in cryopreservation medium supplemented with soybean lecithin. *Cryobiology* 73: 69-72. <https://doi.org/10.1016/j.cryobiol.2016.05.010>
- Masoudi R, Sharafi M, Shahneh AZ, Towhidi A, Zhandi M, Esmaili V, Shahverdi A, 2016b. Effect of dietary fish oil supplementation on ram semen freeze ability and fertility using soybean lecithin and egg yolk-based extenders. *Theriogenology* 86: 1583-1588. <https://doi.org/10.1016/j.theriogenology.2016.05.018>
- Masoudi R, Shahneh AZ, Towhidi A, Kohram H, Sharif AA, Sharafi M, 2017. Fertility response evaluation of artificial insemination methods in sheep with fresh and frozen-thawed semen. *Cryobiology* 73: 77-80. <https://doi.org/10.1016/j.cryobiol.2016.11.012>
- Mata-Campuzano M, Álvarez-Rodríguez M, Tamayo-Canul J, López-Urueña E, de Paz P, Anel L, Martínez-Pastor F, Álvarez A, 2014. Refrigerated storage of ram sperm in presence of Trolox and GSH antioxidants: Effect of temperature, extender and storage time. *Anim Reprod Sci* 151: 137-147. <https://doi.org/10.1016/j.anireprosci.2014.10.006>
- Najafi A, Zhandi M, Towhidi A, Sharafi M, Sharif AA, Motlagh MK, Martinez-Pastor F, 2013. Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology* 66: 275-282. <https://doi.org/10.1016/j.cryobiol.2013.03.002>
- Najafi A, Kia HD, Mohammadi H, Najafi MH, Zanganeh Z, Sharafi M, Martinez-Pastor F, Adeldust H, 2014a. Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender. *Cryobiology* 69: 68-73. <https://doi.org/10.1016/j.cryobiol.2014.05.004>
- Najafi A, Najafi M, Zanganeh Z, Sharafi M, Martínez-Pastor F, Adeldust H, 2014b. Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant. *Reprod Domest Anim* 49: 934-940. <https://doi.org/10.1111/rda.12405>
- Nasiri AH, Towhidi A, Zeinoaldini S, 2012. Combined effect of DHA and  $\alpha$ -tocopherol supplementation during bull semen cryopreservation on sperm characteristics and fatty acid composition. *Andrologia* 44: 550-555. <https://doi.org/10.1111/j.1439-0272.2011.01225.x>
- NRC, 2001. Nutrient requirements of dairy cattle, 7th rev vers. National Research Council, 408 pp. <https://doi.org/10.17226/9825>
- O'Flaherty C, Beconi M, Beorlegui N, 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen/thawed bull sperm. *Andrologia* 29: 269-275. <https://doi.org/10.1111/j.1439-0272.1997.tb00481.x>

- Purdy P, 2006. A review on goat sperm cryopreservation. *Small Rumin Res* 63: 215-225. <https://doi.org/10.1016/j.smallrumres.2005.02.015>
- Revell S, Mrode R, 1994. An osmotic resistance test for bovine semen. *Anim Reprod Sci* 36: 77-86. [https://doi.org/10.1016/0378-4320\(94\)90055-8](https://doi.org/10.1016/0378-4320(94)90055-8)
- Sarıözkan S, Bucak MN, Tuncer PB, Büyükleblebici S, Eken A, Akay C, 2015. Influence of fetuin and hyaluronan on the post-thaw quality and fertilizing ability of Holstein bull semen. *Cryobiology* 71: 119-124. <https://doi.org/10.1016/j.cryobiol.2015.04.011>
- Shahverdi A, Sharafi M, Gourabi H, Amiri Yekta A, Esmaceli V, Sharbatoghli M, Janzamin E, Hajnasrollahi M, Mostafayi F, 2015. Fertility and flow cytometric evaluations of frozen-thawed rooster semen in cryopreservation medium containing low-density lipoprotein. *Theriogenology* 1: 78-85 <https://doi.org/10.1016/j.theriogenology.2014.07.044>
- Silva SV, Soares AT, Batista AM, Almeida FC, Nunes JF, Peixoto CA, Guerra MMP, 2013. Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. *Anim Reprod Sci* 137: 37-44. <https://doi.org/10.1016/j.anireprosci.2012.12.002>
- Suleiman SA, Ali ME, Zaki ZM, El-Malik EM, Nasr MA, 1996. Lipid peroxidation and human sperm motility: protective role of vitamin E. *Andrology* 17: 530-537.
- Surai P, Kostjuk I, Wishart G, Macpherson A, Speake B, Noble R, Ionov I, Kutz E, 1998. Effect of vitamin E and selenium supplementation of cockerel diets on glutathione peroxidase activity and lipid peroxidation susceptibility in sperm, testes, and liver. *Biol Trace Elem Res* 64: 119-132. <https://doi.org/10.1007/BF02783329>
- Towhidi A, Parks JE, 2012. Effect of n-3 fatty acids and  $\alpha$ -tocopherol on post-thaw parameters and fatty acid composition of bovine sperm. *J Assist Reprod Genetic* 29: 1051-1056. <https://doi.org/10.1007/s10815-012-9834-7>
- Tuncer PB, Bucak MN, Sarıözkan S, Sakin F, Yeni D, Çiğerci İH, Ateşşahin A, Avdatek F, Gündoğan M, Büyükleblebici O, 2010. The effect of raffinose and methionine on frozen/thawed Angora buck (*Capra hircus ancyrensis*) semen quality, lipid peroxidation and antioxidant enzyme activities. *Cryobiology* 61: 89-93. <https://doi.org/10.1016/j.cryobiol.2010.05.005>
- Tuncer PB, Sarıozkan S, Bucak MN, Ulutas PA, Akalin PP, Buyukleblebici S, Canturk F, 2011. Effect of glutamine and sugars after bull sperm cryopreservation. *Theriogenology* 75: 1459-1465. <https://doi.org/10.1016/j.theriogenology.2010.12.006>
- Watson P, 1976. The protection of ram and bull sperm by the low density lipoprotein fraction of egg yolk during storage at 5 C and deep-freezing. *J Therm Biol* 1: 137-141. [https://doi.org/10.1016/0306-4565\(76\)90003-6](https://doi.org/10.1016/0306-4565(76)90003-6)
- Watson P, 2000. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 60: 481-492. [https://doi.org/10.1016/S0378-4320\(00\)00099-3](https://doi.org/10.1016/S0378-4320(00)00099-3)
- Yousef MI, 2010. Vitamin E modulates reproductive toxicity of pyrethroid lambda-cyhalothrin in male rabbits. *Food Chem Toxicol* 48: 1152-1159. <https://doi.org/10.1016/j.fct.2010.02.002>
- Zanganeh Z, Zhandi M, Zare-Shahneh A, Najafi A, Nabi MM, Mohammadi-Sangcheshmeh A, 2013. Does rosemary aqueous extract improve buck semen cryopreservation? *Small Rumin Res* 114: 120-125. <https://doi.org/10.1016/j.smallrumres.2013.05.015>
- Zhandi M, Sharafi M, 2015. Negative effect of combined cysteine and glutathione in soy lecithin-based extender on post-thawed ram spermatozoa. *Cell Tissue Bank* 16: 443-448. <https://doi.org/10.1007/s10561-014-9488-z>