The Effects of Different Extenders and Myo-Inositol on Post-thaw Quality of Ram Semen

Article *in* Kafkas Üniversitesi Veteriner Fakültesi Dergisi · January 2011

CITATIONS
6

READS
106

6 authors, including:

Recai Kulaksız
Balıkesir University
61 PUBLICATIONS **305** CITATIONS
SEE PROFILE

Mustafa Numan Bucak
Selcuk University Veterinary Faculty, Konya, Turkey
66 PUBLICATIONS **1,917** CITATIONS
SEE PROFILE

Ergun Akcay
Ankara University
83 PUBLICATIONS **460** CITATIONS
SEE PROFILE

Fatih Sakin
Mustafa Kemal University
36 PUBLICATIONS **624** CITATIONS
SEE PROFILE

Kafkas Univ Vet Fak Derg
17 (2): 217-222, 2011
DOI:10.9775/kvfd.2010.3024

RESEARCH ARTICLE

The Effects of Different Extenders and Myo-Inositol on Post-thaw Quality of Ram Semen [1]

Recai KULAKSIZ * Mustafa Numan BUCAK ** Ergun AKÇAY ***
Fatih SAKİN **** Ali DAŞKIN *** Ahmet ATEŞŞAHİN *****

[1] *This study was supported by grants from TUBİTAK, Turkey (KAMAG-106G005)*
* Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, University of Kafkas, 36100 Kars - TURKEY
** Ministry of Agriculture and Rural Affairs, Lalahan Livestock Central Research Institute, Lalahan, TR-06100 Ankara - TURKEY
*** Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, University of Ankara, TR-06100 Ankara - TURKEY
**** Department of Pharmacology and Toxicology, Department of Physiology, University of Mustafa Kemal, TR-31100 Hatay - TURKEY
***** Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Firat, TR-23100 Elazig - TURKEY

Makale Kodu (Article Code): KVFD-2010-3024

Summary

The study was conducted to evaluate the effects of different extenders and inositol additions on post-thaw semen quality, lipid peroxidation (LPO) and antioxidant activities. Semen was collected from four Karayaka rams from by artificial vagina three times a week. Semen samples showing normospermy quality were pooled. The pooled semen samples were extended in three extenders (Tris, T-, skimmed milk, M- and sodium citrate, NaC) with myo-inositol at two different doses (5 mM, 10 mM) and no antioxidant (control). Nine experimental groups were assigned as follows: T-5I, T-10I, T (control); M-5I, M-10I, M (control); Na-5I, Na-10I, NaC (control). Straws containing extended semen were equilibrated at 4°C for 2 h, frozen in vapor of (15 min at -120°C) liquid nitrogen and stored in liquid nitrogen. Frozen semen was thawed in a water bath at 37°C for 30 seconds. The use of all the extenders supplemented with different doses of myo-inositol did not lead to any significant improvement in microscopic sperm and oxidative stress parameters (P>0.05). Extenders of T and M resulted in higher sperm motility (50.00±2.24% and 55.00±0.42%) and HOST (49.00 3.32% and 48.17±2.97%) rates, compared to NaC (37.00±3.74% and 31.80±2.96%, P<0.01), following the freeze/thawing process. Extenders supplementated with myo-inositol not significantly affect malondialdehyde (MDA) levels and activities of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GSH-PX) in comparison to the control groups (P>0.05), except for MDA level of T extender containing 10 mM inositol. MDA level was found lower (1.22±0.07 nmol/ml) in T than those of the M and NaC (P<0.05). For GSH and GSH-PX activities, T and NaC gave the higher values, compared to M, following the freeze/thawing process (P<0.01).

Keywords: *Ram semen, Inositol, Cryopreservation, Semen parameters, Antioxidant activities*

Koç Spermasının Çözüm Sonu Kalitesi Üzerine Farklı Sulandırıcıların ve Myo-İnositolün Etkileri

Özet

Bu çalışma koç spermasının çözüm sonu kalitesi, lipit peroksidasyonu ve antioksidan aktiviteleri üzerine farklı sulandırıcıların ve inositolün etkilerini değerlendirmek amacıyla yapıldı. Sperma 4 baş Karayaka koçundan suni vajen yardımıyla haftada üç kez alındı. Alınan sperma örneklerinden normospermi özellik gösterenler birleştirildi. Birleştirilen sperma örnekleri iki farklı dozda myo-inositol (5, 10 mM) içeren ve içermeyen (kontrol) üç farklı sulandırıcı (tris, yağsız süt tozu, sodyum sitrat) ile sulandırıldı. Örnekler dokuz ayrı çalışma grubuna ayrıldı: T-5I, T-10I, T (kontrol); M-5I, M-10I, M (kontrol); Na-5I, Na-10I, NaC (kontrol) sulandırılmış sperma içeren payetler 4°C'de 2 saat ekilibre edildi, sıvı azot buharında (-120°C'da 15 dakika) dondurulduu ve sıvı azot (-196°C) içinde saklandı. Dondurulmuş spermalar su banyosunda 37°C'de 30 saniyede çözdürüldü. Sulandırıcılara eklenenen myo-inositol mikroskopik sperm ve oksidatif stres parametelerine önemli bir etkiye neden olmadı (P>0.05). T ve M sulandırıcıları, NaC sulandırıcısına göre donma-çözünme sonrası spermatozoon motilitesinde (%50.00±2.24% ve 55.00±3.42) ve HOS testte (%49.00±3.32% and 48.17±2.9) daha yüksek oranlar verdi (P<0.01). Myo-inositol ilave edilen sulandırıcılar MDA seviyeleri ile CAT, SOD, GSH ve GSH-PX aktivitelerini, 10 mM inositol içeren T sulandırıcısının MDA seviyesi hariç, kontrol gruplarına göre önemli oranda etkilemedi (P>0.05). MDA seviyesi T sulandırıcısında (1.22±0.07 nmol/ml), M ve NaC sulandırıcılarına göre daha düşük bulundu (P<0.05). GSH ve GSH-PX aktiviteleri için, T ve NaC sulandırıcıları M sulandırıcısına göre daha yüksek değerler verdi (P<0.01).

Anahtar sözcükler: *Koç sperması, İnositol, Kryopreservasyon, Sperma parametreleri, Antioksidan aktivite*

İletişim (Correspondence)
+90 474 2426807/1236 GSM: +90 505 3220756
recaikulaksiz@gmail.com

INTRODUCTION

Karayaka sheep are one of the most economically important skin and meat-breed of sheep reared in Turkey [1]. Little is known regarding the reproductive performance of Karayaka rams and ewes surviving under normal environmental conditions in Turkey. The Karayaka population has been decreasing in recent years, and effective procedures should be applied for the preservation of the valuable genetic material. Preservation of genetic material such as sperm, embryo and tissue samples, could be archieved by freezing process.

The survival of frozen-thawed ram sperm is affected by many factors, extensively reviewed by Salamon and Maxwell [2]. These factors include the kinds and concentrations of ingredients and cryoprotectants in the semen extenders and the quality of semen used for freezing. However, even the presence of cryoprotectans such as glycerol and egg yolk, the freeze-thaw process causes damage to sperm plasma membranes and the reduces the motility and fertilizing ability of spermatozoa [3,4].

A defective antioxidant capacity associated with high unsaturated: saturated fatty acids ratio and low cholesterol content of sperm plasma membrane have a major influence in the extensive damages inflicted to ram sperm during cryopreservation [5].

Myo-inositol (MI), belonging to vitamin B complex, is isoforms of inositol, and widely distributed in nature [6]. Inositol is present in high concentrations in the epididymal fluid and seminal plasma of several species including the ram [7] and is considered to be important for the survival of spermatozoa in the epididymis [8]. In addition, inositol protects enzymes from damage caused by freezing or lipid peroxidation [9] and maintains the acrosome integrity of frozen-thawed ram spermatozoa [10].

The aim of the present study was to improve the procedure for freezing of Karayaka ram semen. We focused on three factors affecting cryopreservation success: (i) type of extender, (ii) the effect of inositol additions to the extenders and (iii) interactions between extenders and inositol.

MATERIAL and METHODS

Animals and Semen Collection

The animals were housed at The Education Research and Practise Farm, Faculty of Veterinary Medicine, University of Ankara, and maintained under uniform feeding and housing conditions. Semen samples were obtained from 4 mature Karayaka rams (2 and 3 years of age). A total number of 20 ejaculates were collected the rams with the aid of artificial vagina three times a week during the non-breeding season.

Extenders

In the present experiment, three semen extenders (Tris [T], Skimmed milk [M] and Na citrate [NaC]) and two different doses of myo- inositol (5 mM and 10 mM) were tested. The contents of extenders formed as nine different groups are given in *Table 1*. For freezing, nine experimental groups were assigned as follows: T with 5 mM inositol (T-5I), T with 10 mM inositol (T-10I), T with no additive (T); M with 5 mM inositol (M-5I), M with 10 mM inositol (M-10I), M with no additive (M); NaC with 5 mM inositol (Na-5I), NaC with 10 mM inositol (Na-10I), NaC with no additive (NaC). The pH of extenders was adjusted at 7.0.

Table 1. *Extender compositions **
Tablo 1. *Sulandırıcı içerikleri*

Contents	**Unit**	**T**	**T-5I**	**T-10I**
Tris base	mM	375	375	375
Citric acid	mM	125	125	125
Glucose	mM	41	41	41
Inositol	mM	-	5	10
Inositol	mM	-	-	-
		M	**M-5I**	**M-10I**
Skimmed milk	g/l	100	100	100
Glucose	g/l	9	9	9
Inositol	mM	-	5	10
Inositol	mM	-	-	-
		NaC	**Na-5I**	**Na-10I**
Na-citrate	g/l	29	29	29
Inositol	mM	-	5	10
Inositol	mM	-	-	-

** All the extenders contained 50 mg streptomycin and 50.000 UI penicillin, 100 ml egg yolk and 50 ml glycerol per litre (1.000 ml)*
T: *Tris with no additive (control),* ***T-5I:*** *Tris with 5 mM inositol,* ***T-10I:*** *Tris with 10 mM inositol,* ***M:*** *Skimmed milk with no additive (control),* ***M-5I:*** *Skimmed milk with 5 mM inositol,* ***M-10I:*** *Skimmed milk 10 mM inositol,* ***NaC:*** *Na citrate with no additive,* ***Na-5I:*** *Na citrate with 5 mM inositol,* ***Na-10I:*** *Na citrate with 10 mM inositol*

Semen Extending, Freezing and Thawing

Only ejeculates, between 1 and 2 ml in volume, spermatozoa with >80 % motility and concentration higher than $3x10^9$ spermatozoa/ml, were pooled, balancing the sperm contribution of each male to eliminate individual differences. Each of the pooled ejaculate was split into nine equal aliquots and diluted (at a concentration of $400x10^6$ sperm/ml) in the extenders given above. Diluted samples were aspirated into small-sized (0.25 ml) French straws, sealed with polyvinyl alcohol powder and equilibrated at 4°C for 2 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen, for 15 min and plunged into liquid nitrogen for storage. After stored for a week, the frozen straws were thawed

individually at 37°C for 30 s in a water bath for semen evaluation.

Semen Evaluation

Subjective motility was assessed using a phase-contrast microscope (x100), with a warm stage maintained at 37°C. A wet mount was made using a 5-µl drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample.

The viability of sperm in the sample was assessed by means of a eosin-nigrosin stain [11]. The sperm smears were prepared by mixing a drop of semen with two drops of stain on a warm slide and spreading the stain immediately with a second slide. The viability was assessed by counting 200 cells with bright-field microscopy (magnification 400x). Sperm showing partial or complete purple colorization was considered non-viable or dead. Only sperm showing strict exclusion of the stain were considered to be alive.

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 10 µl semen with 100 µl semen of a 100 mOsm hypo-osmotic solution (fructose and sodium citrate) at 37°C for 30 min. After incubation 0.1 ml of the mixture was spreaed with a cover slip on a warm slide two undres sperms were evaluated (magnification 400x) with bright-field microscopy. Sperm with swollen or coiled tails were recorded [12].

Biochemical Assays

Biochemical assays were performed on the sperm samples immediately after thawing and without washing.

Malondialdehyde (MDA) Concentrations

The concentrations of MDA, as indices of the LPO in the sperm samples, were measured using the thiobarbituric acid reaction and according to the method of Placer et al.[13] The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption with the Standard curve of MDA equivalents generated by the acid catalyzed hydrolysis of 1.1,3.3-tetramethoxypropane. The MDA concentrations were expressed in nmol/ml.

Glutathione (GSH) Levels and Gluthione Peroxidase (GSH-PX), and Catalase (CAT) Activities

The GSH content of sperm was measured using the method of Sedlak and Lindsay [14]. The samples were precipitated using 50% trichloracetic acid and then centrifuged at 1.000×*g* for 5 min. The reaction mixture contained 0.5 ml supernatant, 2.0 ml Tris-EDTAbuffer (0.2 mol/l and pH 8.9) and 0.1 ml 0.01 mol/l 5.5 dithiobis-2-nitrobenzoic acid. The solution was maintained at room temperature for 5 min, and then recorded at 412 nm using a spectrophotometer. The levels of GSH were expressed as nmol/ml.

The GSH-PX activity was determined according to the method of Lawrence and Burk [15]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 EU/ml oxidized glutathione (GSSG)-reductase, 1 mM GSH and 0.25 mM H_2O_2. An enzyme source (0.1 ml) was added to 0.8 ml of the above mixture, and this mixture incubated at 25°C for 5 min before the initiation of the reaction-induced by the addition of 0.1 ml of the peroxide solution. The absorbance at 412 nm was recorded for 5 min using a spectrophotometer. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The blank value (enzyme replaced with distilled water) was subtracted from each value and the GSH-PX activity was expressed as international units (IU)/g protein for the sperm samples.

The method described by Goth [16] was used for the determination of the CAT activity in the sperm. A 0.2 ml sperm volume was incubated in 1 ml substrate (65 mol of hydrogen peroxide in 60 mmol/l sodium potassium phosphate buffer, pH 7.4) at 37°C for 60 s. One unit of CAT decomposes 1 mol of hydrogen peroxide per min, under these conditions. The enzymatic reaction was terminated using 1 ml 32.4 mmol/l ammonium molybdate, and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm using a spectrophotometer. Values of CAT activity were expressed as *k*U/l, where *k* was the first-order rate constant.

Statistical Analysis

The study was repeated 5 times and the results were expressed as the mean±SEM. Means were analyzed using a one-way analysis of variance, followed by Tukey's post hoc test to determine significant differences in all the parameters between all groups using the SPSS/PC computer program (version 12.0; SPSS, Chicago, IL). Differences with values of $P<0.05$ were considered to be statistically significant [17].

RESULTS

Sperm Characteristics (Motility, Viability and Membrane İntegrity)

The effect of different extenders and myo-inoistol on standard semen quality parameters in frozen Karayaka ram semen were evaluated in 5 independent experiments. As set out in *Tables 2-5*, the use of all the extenders supplemented with different doses of myo-inositol did not lead to any significant improvement in microscopic sperm and oxidative stress parameters ($P>0.05$, *Table 2,*

3 and 4). Extenders of T and M resulted in higher motility (50.00±2.24% and 55.00±3.42%) and HOST (49.00±3.32% and 48.17±2.97%) rates, compared to NaC (37.00±3.74% and 31.80±2.96%, P<0.01, respectively), following the freeze/thawing process.

Biochemical Parameters of Post-Thaw Sperm

The effects of different extenders and myo-inoistol on MDA and antioxidant activities following the freeze-thawing of Karakaya ram sperm are shown in *Table*

Table 2. *Mean (±S.E.) of motility, viability, HOST, MDA levels and SOD, CAT, GSH and GSH-PX activities in Karayaka ram semen frozen in tris extender with different myo-inositol doses*

Tablo 2. *Farklı inositol dozları ile tris sulandırıcısında dondurulmuş koç spermasında ortalama motilite, viabilite, HOST ve MDA düzeyleri ile SOD, CAT, GSH ve GSH-PX aktivitesi*

Parameter	T	T-5I	T-10I	P
Motility (%)	50.00±2.24	47.00±2.55	43.00±3.00	-
Viability (%)	52.50±4.93	52.40±3.37	53.00±2.00	-
HOST (%)	49.00±3.32	44.00±2.45	42.40±4.61	-
MDA (nmol/ml)	1.22±0.07 [a]	1.37±0.09 [ab]	1.94±0.33 [b]	*
GSH (nmol/mg protein)	4.28±0.94	2.70±0.11	3.38±0.23	-
GSH-PX (U/g protein)	96.90±2.22	57.15±4.42	76.00±6.71	-
SOD (U/mg protein)	51.43±8.11	50.39±1.62	47.79±2.44	-
CAT (k/g protein)	2.40±2.27	1.48±0.05	2.34±0.33	-

a,b: *Different superscripts within the same line demonstrate significant differences (* P<0.05, **- :** P>0.05)*

Table 3. *Mean (±S.E.) of motility, viability, HOST, MDA levels and SOD, CAT, GSH and GSH-PX activities in Karayaka ram semen frozen in skimmed milk with different myo-inositol doses*

Tablo 3. *Farklı inositol dozları ile yağsız süt tozu sulandırıcısında dondurulmuş koç spermasında ortalama motilite, viabilite, HOST ve MDA düzeyleri ile SOD, CAT, GSH ve GSH-PX aktivitesi*

Parameter	M	M-5I	M-10I	P
Motility (%)	55.00±0.42	54.00±3.67	56.00±4.30	-
Viability (%)	53.00 ± 1.55	53.60±2.29	54.60±4.13	-
HOST (%)	48.17±2.97	47.40±3.57	46.60±4.37	-
MDA (nmol/ml)	1.88±0.27	1.33±0.12	1.36±0.09	-
GSH (nmol/mg protein)	1.53±0.05	1.54±0.11	1.97±0.22	-
GSH-PX (U/g protein)	33.88±0.31	29.82±2.91	42.80±5.10	-
SOD (U/mg protein)	49.0±1.49	59.70±1.34	43.38±1.82	-
CAT (k/g protein)	1.41±0.38	1.18±0.23	1.38±1.41	-

- : *P>0.05*

Table 4. *Mean (±S.E.) percentages of motility, viability, HOST, MDA levels and SOD, CAT, GSH and GSH-PX activities in Karayaka ram semen frozen in sodium citrate with different myo-inositol doses*

Tablo 4. *Farklı inositol dozları ile sodyum sitrat sulandırıcısında dondurulmuş koç spermasında ortalama motilite, viabilite, HOST ve MDA düzeyleri ile SOD, CAT, GSH ve GSH-PX aktivitesi*

Parameter	NaC	Na-5I	Na-10I	P
Motility (%)	37.00±3.74	36.00±2.45	36.25±3.15	-
Viability (%)	47.00±3.57	46.20±2.55	48.60±4.64	-
HOST (%)	31.80±2.96	33.00±2.55	32.25±2.25	-
MDA (nmol/ml)	1.72±0.18	1.38±0.18	1.57±0.15	-
GSH (nmol/mg protein)	4.74±0.34	4.43±0.40	3.05±0.28	-
GSH-PX (U/g protein)	92.36±6.75	85.10±8.87	84.19±4.70	-
SOD (U/mg protein)	67.30±5.78	52.99±3.15	43.20±2.49	-
CAT (k/g protein)	2.85±1.06	1.60±0.98	2.57±0.46	-

-: *P>0.05*

Table 5. *Mean (±S.E.) percentages of motility, viability, HOST, MDA levels and SOD, CAT, GSH and GSH-PX activities in Karayaka ram semen frozen in different extenders without myo-inositol*

Tablo 5. *İnositol içermeyen farklı sulandırıcılarda dondurulmuş koç spermasında ortalama motilite, viabilite, HOST ve MDA düzeyleri ile SOD, CAT, GSH ve GSH-PX aktivitesi*

Parameter	T	M	NaC	P
Motility (%)	50.00±2.24 [a]	55.00±3.42 [a]	37.00±3.74 [b]	**
Viability (%)	52.50±4.93	53.00±1.55	47.00±3.57	**
HOST (%)	49.00±3.32 [a]	48.17±2.97 [a]	31.80±2.96 [b]	**
MDA (nmol/ml)	1.22±0.07 [b]	1.88± 0.27 [a]	1.72±0.18 [a]	**
GSH (nmol/mg protein)	4.28±0.94 [a]	1.53±0.05 [b]	4.74±0.34 [a]	**
GSH-PX (U/g protein)	96.90±2.22 [a]	33.88±0.31 [b]	92.36±6.75 [a]	**
SOD (U/mg protein)	51.43±8.11	49.0±1.49	67.30±5.78	-
CAT (k/g protein)	2.40±2.27	1.41±0.38	2.85±1.06	-

a,b: *Different superscripts within the same line demonstrate significant differences*
*(** P<0.01, **-:** P>0.05)*

2-5. Extenders supplementated with myo-inositol not significantly affect MDA levels and activities of CAT, SOD, GSH and GSH-PX in comparison to the controls (P>0.05), except for MDA level of T-10I. MDA was the lower in T than those of other groups (*Table 2*, P<0.05). MDA was found lower (1.22±0.07 (nmol/ml)) in T than those of the M and NaC. In *Table 5*, for GSH and GSH-PX activities, T and NaC gave the higher values (4.28±0.94 nmol/mg protein and 4.74±0.34 nmol/mg protein for GSH; 96.90±2.22 U/g protein and 92.36±6.75 U/g protein for GSH-PX, respectively), compared to M (1.53±0.05 nmol/mg protein and 33.88±0.31 U/g protein) following the freeze/thawing process (P<0.01).

DISCUSSION

Cryopreservation imposes irreversible damage to sperm membranes, including swelling and distruption of sperm plasma and acrosmal membranes, changes in membrane fluidity, altered calcium influx, and changes in enzymatic activity. The main findings emerging from this study are the decrease in GSH and GSH-PX content observed in ram cryopreserved with M extender, compared to extenders of T and NaC. Bilodeau et al.[18] reported that the cryopreservation reduced sperm glutathione reductase levels by 78%, SOD activity by 50% and lowered the level of GPx activity. Extenders of T and M provided more superiority, compared to NaC for sperm motility and membrane integrity. It can be hypothesized that T and M contained more buffer and antioxidant agents, such as citrit acid for T and casein for M, cryoprotective influence on the integrity of the axosoma and mitochondria, improving post-thawed sperm motility and membrane integrity evaluated the HOST.

A characteristic feature of biological membranes is the asymmetrical arrangement of lipids within the bilayer. The lipid composition of the plasma membrane of mammalian sperm is markedly different from those of mammalian somatic cells. Sperm cells contain a high content of polyunsaturated fatty acids. The ratio of unsaturated to saturated fatty acids in small ruminant sperm membranes is also higher than in other species, making the membranes more susceptible to cold shock and peroxidative damages with a subsequent loss of membrane integrity in the acrosomal region, impaired cell function and decreased motility of the sperm [5,19,20].

Various extenders and cryoprotective agents have been described for cryopreservation of ram semen. Compounds such as taurine, hypotaurine and inositol, which act as antioxidants and present in the epididymis at high concentrations, have been reported to increase the motility, viability and morphological integrity of cryopreserved spermatozoa [10,21,22]. In this study, myo-inositol did not affect post-thaw semen quality when diluted with extenders prior to freezing. The low cryoprotective capacity of inositol has also been reported by Molinia et al.[10] for ram spermatozoa. Additionally, Sanchez- Partida et al.[23] reported that inositol had not significant effect in the post-thaw semen quality when inositol (30- 120 mM) was included in T extender. However, this result contrasts with reports by Abdelhakeam et al.[7] for ram spermatozoa frozen in straws. In Tes-based diluent containing inositol without glycerol, those researhers found a post-thaw motility of 49.6% following freeze-thawing process. The disagreement between current results and data reported by Abdelhakeam et al.[7] could be due to differences in extender composition, dilution rate and freezing protocol.

Inositol has proved to be satisfactory extender component in the freezing of boar sperm [23]. In this study, myo-inositol was not an effective antioxidant for ram spermatozoa. Additionally, inositol did not demonstrate any effectiveness in the prevention of MDA formation. Changes in the preservation protocol and experimental methodology, extender formula, anti-

oxidant concentrations and animal species-breeds may explain different behaviours of antioxidant capacities and why additives do not prevent MDA. This finding related to MDA, is in agreement with the results of studies performed on ram [24] and buck [25] sperm in which no decrease was observed in the level of MDA in the presence of various antioxidants, following the freeze-thawing process. This indicates that MDA is apparently not a major factor influencing sperm quality after thawing. The current results were also contradictory to other studies on rabbit [26] and canine [27] sperm, where inhibition of lipid peroxidation was observed in case of incubation with antioxidants during liquid storage or cryopreservation.

In conclusion, it may be stated that the addition of antioxidant inositol did not improve sperm and biochemical parameters, except for MDA in T-10I. Extenders of T and M resulted in higher motility and HOST rates, compared to NaC following the freeze/thawing process. MDA was found lower in T than those of the M and NaC. For GSH and GSH-PX activities, T and NaC gave the higher values, compared to M, following the freeze/thawing process ($P<0.01$). In order to confirm the contradictory results, further studies are warranted to obtain more concrete results regarding the characterization of enzymatic and non-enzymatic antioxidant systems in cryopreserved ram sperm.

REFERENCES

1. Akçapınar H: Koyun Yetiştiriciliği. s. 111-114, Üsmat Matbaacılık, Ankara, 2000.

2. Salomon S, Maxwell WMC: Storage of ram semen, *Anim Reprod Sci*, 62, 77-11, 2000.

3. Hammerstedt RH, Graham JK, Nolan JP: Cryopreservation of mammalian sperm: What we ask them to survive. *J Androl*, 11, 73-88, 1990.

4. Maxwell WMC, Watson PF: Recent progress in the preservation of ram semen. *Anim Reprod Sci*, 42, 55-65, 1996.

5. White IG: Lipids and calcium uptake of sperm in relation to cold shock and preservation. *Reprod Fertil Dev,* 5, 639-658, 1993.

6. Yoshida K, Yamaguchi M, Morinaga T, Ikeuchi M, Kinehara M, Ashida H: Genetic modification of *Bacillus subtilis* for production of D-chiro-inositol, an investigation drug candidate for treatment of type 2 diabetes and polycystic ovary syndrome. *Appl Environ Microbiol*, 72, 1310-1315, 2006.

7. Abdelhakeam AA, Graham EF, Vazquez IA: Studies on the presence and an absence of glycerol in unfrozen and frozen ram semen: Fertility trials and the effect of dilution methods on freezing ram semen in the absence of glycerol. *Cryobiology*, 28, 36-42, 1991.

8. Voglmayr JK, Aman RP: The distribution of free myo-inositol in fluids, spermatozoa and tissues of the bull genital tarct and observation on its uptake by the rabbit epididymis. *Biol Reprod*, 8, 504-513, 1973.

9. Carpenter JF, Hand S.C, Crowe LM, Crowe JH: Cryoprotection of phosphofructokinase with organic solutes: Characterization of enhanced protection in the presesence of divalent cations. *Arch Biochem Biophys*, 250, 505-512, 1986.

10. Molinia FC, Evans G, Maxwell WMC: Effect of polyols on the post-thaw motility of pellet-frozen ram spermatozoa. *Theriogenology*, 42, 15-23, 1994.

11. Evans G, Maxwell WMC: Salamon's Artificial Insemination of Sheep and Goats. pp. 8-21, 107-141, Butterworths, Sidney, 1987.

12. Revell SG, Mrode RA: An osmotic resistance test for bovine semen. *Anim Reprod Sci*, 36, 77-86, 1994.

13. Placer ZA, Cushman L, Johnson BC: Estimation of products of lipid peroxidation (malonyldialdehyde) in biological fluids. *Anal Biochem,* 16, 359-364, 1966.

14. Sedlak J, Lindsay RHC: Estimation of total, protein bound and non-protein sulfhydryl groups in tissue with Ellmann's reagent. *Anal Biochem*, 25, 192-205, 1968.

15. Lawrence RA, Burk RF: Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun,* 71, 952-958, 1976.

16. Goth L: A simple method for determination of serum catalase activity and revision of reference range. *Clin Chim Acta,* 196, 143-152, 1991.

17. Daniel WW: Analysis of variance. **In,** Daniel WW (Ed): Biostatistic: A Foundation for Analysis in the Health Sciences. pp. 274-320, John Wiley & Sons, Hoboken, 1991.

18. Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C: Levels of antioxidant defenses are 292 decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev*, 55, 282-288, 2000.

19. Aitken RJ, Clarkson JS, Fishel S: Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod*, 41, 183-197, 1989.

20. Alvarez JG, Storey BT: Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev*, 42, 334-346, 1995.

21. Chen Y, Foote RH, Brockett CC: Effect of sucrose, trehalose, hypotaurine, taurine and blood serum on survival of frozen bull sperm. *Cryobiology*, 30, 423-431, 1993.

22. Foote RH, Chen Y, Brockett CC, Kaproth MT: Fertility of bull spermatozoa frozen in whole milk extender with trehalose, taurine or blood serum. *J Dairy Sci*, 76, 1908-1913, 1993.

23. Sanches-Partida LG, Setchell BP, Maxwell WMC: Epididymal compounds and antioxidants in diluents for the frozen storage of ram spermatozoa. *Reprod Fertil Dev,* 9, 689- 696,1997.

24. Bucak MN, Ateşşahin A, Varışlı Ö, Yüce A, Tekin N, Akçay A: The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: Microscopic and oxidative stres parameters after freze-thawing process. *Theriogenology*, 67, 1060-1067, 2007.

25. Ateşsahin A, Bucak MN, Tuncer PB, Kızıl M: Effects of antioxidant additives on microscopic and oxidative parameters of Angora goat semen following the freze-thawing process. *Small Rumin Res*, 77, 38-44, 2008.

26. Alvarez JG, Storey BT: Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol Reprod*, 29, 548-555, 1983.

27. Michael A, Alexopoulos C, Pontiki, Hadjipavlou-Litina D, Saratsis P, Boscos C: Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, 68, 204-212, 2007.