IJAS Iranian Journal of Applied Animal Science

Effect of Antioxidant D-Aspartic Acid and Thawing Rate on the Freeze-Thawing Process of Ram Semen

Research Article

H. Daghigh Kia[1*] and S. Vatankhah[1]

[1] Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

Received on: 21 May 2018
Revised on: 7 Jul 2018
Accepted on: 31 Jul 2018
Online Published on: Jun 2019

*Correspondence E-mail: daghighkia@tabrizu.ac.ir

Online version is available on: www.ijas.ir

ABSTRACT

The study was conducted to determine the influence of D-aspartic acid (D-Asp) as antioxidant supplement and thawing rates on ram sperm motility, membrane integrity, abnormality, viability, mitochondria activity, malondialdehyde and antioxidant activities, and total antioxidant capacity after freezing-thawing process. Semen samples from five mature rams (3-4 years old) were diluted with extenders (1.5% soybean lecithin, 7% glycerol) containing no supplements (control) and D-Asp (5, 10, and 15 mg/L) and cryopreserved. Frozen straws were thawed at water bath temperatures at 37 ˚C for 30s and at 60 ˚C for 6s. Addition of 10 mg/L of D-Asp improved significantly progressive motility, average path velocity, and straight-line velocity percentages after freeze-thaw (P<0.05). Plasma membrane integrity, mitochondria activity, viability, total antioxidant capacity, and glutathione peroxidase were higher in group receiving 10 mg/L in comparison to other treatments (P<0.05). Meanwhile, total abnormality significantly decreased at this concentration (10 mg/L) in comparison to the level of 15 mg/L and control group (P<0.05). Our results revealed that malondialdehyde level was lower in group receiving 10 mg/L D-Asp compared to other treatments (P<0.05). There were no significant interactions between concentrations of D-Asp and thawing protocols for any semen samples. Also, significant improvement in sperm viability and mitochondria activity were observed at 37 ˚C to 30 s thawing method (P<0.05). In overall, results of the present study demonstrate that addition of D-Asp at level of 10 mg/L have beneficial effect on quality of post-thawed ram semen cryopreserved in an extender. Therefore, this antioxidant in the suitable dose can be recommended as an additional component of ram freezing extender. The 60 ˚C to 6s thawing procedure is not an appropriate replacement for 37 ˚C to 30 s.

KEY WORDS antioxidants, cryopreservation, D-aspartic acid, ram, thawing rate.

INTRODUCTION

It is necessary to use the proper diluent, sperm dilution rate, cooling rate and thawing rate in order to obtain the satisfactory results in cryopreservation of mammalian sperm (Purdy, 2006). However, the storage of semen, especially the effect of reducing temperature, has a great impact on sperm physiology (Maxwell and Salamon, 1993; Bucak *et al*. 2008). During sperm cryopreservation, it is exposed to cold shock and osmotic shock, resulting in increased oxidation due to raised oxidative reactions. It reduces sperm motility, viability, and ultimately reduces fertility (Yue *et al*. 2005; Bucak *et al*. 2008). Oxidative stress occurs when oxidants are more potent than antioxidants (Bansal and Bilaspuri, 2011; Mahat *et al*. 2015). Hence, increased production of reactive oxygen species (ROS) caused by oxidative stress leads to lipid peroxidation (LPO), apoptosis, and DNA damage (Bucak *et al*. 2008; Bansal and Bilaspuri, 2011; Budai, 2014). Generally, the most important effect of LPO on cells is membrane disruption, structure and func-

tion (Mahat *et al*. 2015). In addition, ROS is needed at the physiological level for sperm function, including membrane fluidity, capacity, hyperactivation, acrosome reaction, and fertility ability (Lenzi *et al*. 1996; Aitken *et al*. 1997; Sies, 1997; Agarwal and Anandh Prabakaran, 2005). However, excessive production of ROS destroys the sperm motility, fertility capacity, and the initiation of sperm apoptosis (Bansal and Bilaspuri, 2011; Çoyan *et al*. 2011).

Ram sperm has a high rate of polyunsaturated fatty acid (PUFA) and lower molar cholesterol to phospholipid levels than other species (Alvarez and Storey, 1992; Agarwal and Said, 2003; Çoyan *et al*. 2011). Due to the high levels of PUFA, sperm cells are highly sensitive to lipid peroxidation (Zarghami and Khosrowbeygi, 2004; Khosrowbeygi *et al*. 2008). Endogenous antioxidant capacity may not be sufficient to prevent LPO during prolonged storage (Bucak *et al*. 2008). The addition of antioxidants to the freezing extender can improve the sperm motility and acrosomal integrity and decrease cell damage rate (Maia Mda *et al*. 2010). In order to confront the effects of LPO, the use of antioxidants is necessary (Bansal and Bilaspuri, 2011). Indeed, by reducing the formation of free oxygen radicals, antioxidants alter cellular conditions to maintain sperm motility. They maintain their homeostasis and physiological function eliminating free radicals (Ogbuewu *et al*. 2010).

D-Asp is an endogenous amino acid and the most extensive amino acid in mammals and human tissues (D'Aniello *et al*. 2007). The presence of D-Asp was first discovered by D'Aniello and Giuditta in nervous system of marine mollusks (D'Aniello and Giuditta, 1977). D-Asp has a direct effect on the production of sperm. There is a direct relationship between the concentration of D-Asp and the semen quality in humans (Gualtieri *et al*. 2005; Topo *et al*. 2009). Combination of D-ASP, Zn, and coenzyme Q10 could prevent motility reduction, DNA fracture, and lipid oxidation of sperm during storage (Talevi, 2013). Oral intake of D-Asp improved rooster sperm quality, except for sperm abnormality percentage (Ansari *et al*. 2016). So far, there is no study about the antioxidant effect of D-Asp on post-thawed sperm quality.

During freezing, all physiological activity of the sperm is stopped until thawing, and with thawing it returns to life and the physiological state (Nicolae *et al*. 2014; Athurupana *et al*. 2015; Borah *et al*. 2015). Moreover, fast freezing of cells requires rapid thawing (Mazur, 1984). Various studies have shown that, rapid thawing is one of the most effective factors in sperm survival, and leads to an increase in the sperm motility and the intact acrosomes (Dhami and Sahni, 1993; Fiser *et al*. 1993; Eriksson and Rodriguez-Martinez, 2000). Hence, finding an optimal thawing procedure by using the suitable temperature for any species is necessary (Borah *et al*. 2015).

Regardless of all studies on the cryopreservation of ram semen, it seems that there are many vague points about the effect of antioxidants on the maintenance of sperm parameters as well as various thawing rates. The aim of this study was to investigate the effect of different levels of D-Asp as a synthetic antioxidant for improving semen quality and comparing the temperature of thawing during the freeze-thawing process.

MATERIALS AND METHODS

Reagents

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

Semen collection

Semen samples were obtained twice a week from five mature Ghezel rams (3 years old) with superior genetic merit and fertility capacity under the same environmental and nutritional conditions using artificial vagina. Immediately after collection, the ejaculates were transferred to the laboratory, and immersed in a water bath (37 ˚C), until semen evaluation. Semen was evaluated and samples with volume > 0.75 mL, > 80% progressive motility, < 10% abnormal morphology and > 3×10^9 sperm mL^{-1} sperm concentration were selected for further examination. To eliminate individual effects, samples were pooled together.

Semen extending, freezing and thawing

In the present study, Tris-based extender (223.71 m*M* Tris, 55.50 m*M* fructose, 72.87 m*M* citric acid (320 mOsm/kg, pH 7.2)) and glycerol at 7% v/v were used. Soybean lecithin (P3644 Sigma L-α-phosphatidylcholine from soybean) was added to the basic extender at 1.5% (wt/vol). Experimental treatments included four extenders supplemented with different levels (0, 5, 10, and 15 mg/L) of D-Asp. The experiments were replicated six times.

The diluted semen was gently equilibrated at 4 ˚C for 2 h, then, aspirated into 0.25 mL French straws (IMV, L'Aigle, France) (4×10^8 mL^{-1}, spermatozoa per straw). Cooled semen samples, finally, frozen in liquid nitrogen vapor (LN), (4 cm above the LN), for 7 min and plunged into LN for storage. For the purpose of different assessments, the straws were thawed individually in a water bath using two different conditions: at (i) 37 ˚C for 30 Seconds, (ii) at 60 ˚C for 6 seconds.

Evaluation of spermatozoa after thawing

Sperm motility

Immediately after thawing, sperm motility parameters were analyzed using computer assisted sperm analysis (CASA)

(Animal Version 12.3 CEROS, Hamilton-Thorne Biosciences, Beverl, MA, USA). Total motility (TM), progressive motility (PM), average path velocity (VAP), curvilinear velocity (VCL), straight linear velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) parameters were recorded.

Sperm viability

Sperm viability was assessed using eosin-nigrosin (eosin-Y 1.67 g, nigrosin 10 g, and sodium citrate 2.9 g, dissolved in 100 mL distilled water) staining method (Najafi *et al*. 2014). Sperm suspension smears were prepared by mixing 10 µL of sperm sample with 20 µL of stain on a warm slide. Viable and nonviable sperm were assessed by counting sperm (200/slide) using phase-contrast microscopy (CKX41; Olympus, Tokyo, Japan) × 1000.

Sperm abnormalities

Sperm abnormality was assessed by Hancock solution (Mehdipour *et al*. 2016) using phase-contrast microscopy (CKX41; Olympus, Tokyo, Japan) × 400. Sperm (200/slide) were evaluated and the percentage of total sperm abnormalities (head abnormalities, detached heads, abnormal mid-pieces and tail defects) was determined.

Functional membrane integrity

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails (Dodaran *et al*. 2015). Ten µL of semen with 100 µL of a 100 mOsm hypo-osmotic solution (9 g/L fructose+4.9 g/L sodium citrate in 1 L distilled water) was incubating at 37 ˚C for 30 min. Then, 200 spermatozoa were evaluated and sperms with coiled and swollen tails were determined in each sample under light microscopy (CKX41; Olympus, Tokyo, Japan) at 400 × magnification.

Malondialdehyde concentration

Malondialdehyde (MDA) concentrations, as an index of LPO in the semen samples, were measured using the thiobarbituric-acid reaction (Esterbauer and Cheeseman, 1990). In order to evaluate MDA in sperm samples, one ml of the diluted semen (25010^6 spermatozoa/mL; four straws per each replicate) was mixed with one ml of cold 20% (w/v) trichloroacetic acid to precipitate protein. To inhibit lipid oxidation, one mL of butylated hydroxytoluene (2% BHT solution in ethanol) and one mL of ethylene diamine tetra acetic acid (EDTA) (1 m*M* final concentration) were added to the sample before trichloracetic acid (TCA) precipitation. The precipitate was pelleted by centrifuging (1200 g for 15 min), and one mL of the supernatant was incubated with one mL of 0.67% (w/v) thiobarbituric acid in a boiling water bath at 100 ˚C for 10 min. After cooling, the absorbance was evaluated by a spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK) at 532 nm, and the results were reported as nmol/mL.

Glutathione peroxidase activity

Seminal plasma glutathione peroxidase activity (GPx) was measured using a commercial kit (Randox, Crumlin, UK). GPx catalyzes glutathione oxidation using cumene hydro peroxide. In the presence of the glutathione reductase and NADPH, the oxidized glutathione was immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured by a spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK). GPx activity was reported as IU/g protein.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was analysed using a commercial kit (Randox, Crumlin, UK), with xanthine and xanthine oxidase to generate superoxide radicals which react with 2- (4-iodophenyl) -3- (4-nitrophenol)-5-phenyltetrazoliumchloride (I.N.T) to form a red formazan dye. The amount of enzyme activity is measurable by inhibiting this reaction and preventing color formation. One unit of SOD prevents reduction of INT by 50% under the conditions of the assay. The absorbance was recorded at 505 nm by a spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK). SOD activity was reported as U/mg protein.

Total antioxidant capacity

Total antioxidant capacity (TAC) was measured using a commercial kit (Randox, Crumlin, UK). Accordingly, frozen semen was thawed in a water bath using two different conditions: at (i) 37 ˚C for 30 s, (ii) at 60 ˚C for 6 s. Immediately the antioxidant capacity of the samples was evaluated. The absorbance of the samples was measured at 600 nm wave length by a spectrophotometer (T80 UV/VIS PG Instruments Ltd, UK). TAC values were reported as mmol/l protein in seminal plasma samples.

Mitochondrial activity

Mitochondrial activity of sperm was assessed by a Rhodamine 123 (R123; Invitrogen TM, Eugene, OR, USA) and PI (Graham *et al*. 1990). In summary, 10 mL of R123 solution (0.01 mg/mL) was added to 500 mL of diluted semen (50×10^6 spermatozoa/mL) and incubated at 37 ˚C for 20 min in the dark. Then, samples were centrifuged at 1200 g for 3 min and the sperm pellets were re-suspended in 500 mL tris buffer. Also, 10 mL of PI solution (1 mg/mL) was added to samples and evaluated by flow cytometry.

The percentage of sperm mitochondria activity was assessed by R123 high fluorescence and no PI fluorescence.

Statistical analysis

The experiment was conducted as a completely randomized design. Experimental treatments included four extenders supplemented with different levels (0, 5, 10, and 15 mg/L) of D-Asp. The experiments were replicated six times. The data were analyzed by general linear method (GLM) procedure of SAS version 9.1 (SAS, 2002). Mean comparisons were performed using least square means and the significant level was considered P<0.05. The Tukey-Kramer's test was used for mean comparisons. Differences with values of P < 0.05 were considered to be statistically significant. The results were expressed in least squares means ± standard error of the mean. The model used in the present study was as follows:

$Y_{ij} = \mu + T_i + e_{ij}$

Which:

Y_{ij}: observed depended variables including sperm variables. μ: mean of the population.

T_i: effect of treatment.

e_{ij}: random residual error.

RESULTS AND DISCUSSION

The results showed that samples receiving 10 mg/L D-Asp had the highest TM and PM percentages compared to other treatment (P<0.05) (Table 1).

Group receiving 10 mg/L D-Asp had higher percentage of VAP compared to 15 mg/L and control groups (P<0.05). The addition of D-Asp at level 10 mg/L provided a higher VSL, in comparison with the 15 mg/L and control groups (P<0.05). No significant differences were observed in other motility parameters (Table 1).

Membrane integrity was significantly higher in the group receiving 10 mg/L D-Asp compared to other groups (P<0.05) (Table 2).

Groups supplemented with 5 and 10 mg/L of D-Asp had the lowest sperm abnormality compared to other groups. Viability was significantly higher in group with 10 mg/L D-Asp, compared to other groups. Moreover, mitochondrial activity significantly was improved in 10 mg/L compared to 15 mg/L and control groups (P<0.05) (Table 2). Supplementation of semen extender with 10 mg/L D-Asp significantly reduced MDA level in comparison with the control group (P<0.05) (Table 3).

GPx activity was significantly increased in 10 mg/L compared to control group (P<0.05). Total antioxidant capacity (TAC) was significantly elevated in the group with D-Asp at 10 mg/L dose compared to the other groups. No significant differences were observed in SOD activities among treatment (Table 3).

The effects of thawing rates on post-thaw motility parameters and oxidative status of ram spermatozoa are presented in Tables 4 and 5. There were no significant differences between different thawing method (37 ˚C to 30 s and 60 ˚C to 6 s). Viability and mitochondria activity significantly were improved at thawing rate of 37 ˚C to 30 s compared to 60 ˚C to 6s (P<0.05) (Table 6).

Moreover, no significant change was observed in the plasma membrane integrity and total abnormality between different thawing method (37 ˚C to 30 s and 60 ˚C to 6 s) (Table 6).

ROS production during cryopreservation may have resulted in reduction of sperm motility, DNA integrity, viability, and fertilizing potential of sperms (Malo *et al*. 2010). A strategy of preventive antioxidation could be formulated as prevention by diversion, i.e. by channelling an attacking species into a less harmful product, thereby lowering the risk of major damage (Sies, 1997). Moreover, rapid thawing, ensures rapid passage from a danger temperature zone (-50 to -30 ˚C and -30 to 0 ˚C), reduces the possibility of recrystallization, hydration, and damage to the cytoplasm and membrane of the sperm (Fiser *et al*. 1993; Eriksson and Rodriguez-Martinez, 2000). Therefore, the sperm is transmitted directly from the glassy state to the liquid state (Perry, 1955).

There is a direct relationship between semen quality and the concentration of D-Asp (D'Aniello *et al*. 2007). In general, D-Asp could significantly improve some of ram sperm parameters and a mild dose of D-Asp effectively protected ram spermatozoa against cryo-injuries that occur during freeze-thawing process. In this context, the highest protective effect of D-Asp acid was identified at a concentration of 10 mg/L. At this dose, some sperm motility parameters (TM, PM, VAP, VSL) were significantly improved. Results of other studies showed positive effect of D-Asp oral administration on semen concentration, motility, and progressive motility in humans, rabbits, and rooster (Macchia *et al*. 2010; D'Aniello *et al*. 2012; Ansari *et al*. 2017). Under external conditions, using a concentration of 500 μL/mL, D-Asp could maintain the total motility and progressive motility over a six-hour storage period (Talevi *et al*. 2013).

Also, roosters sperm motility increased with exposure to glutamate, N-methyl-d-aspartate receptors (NMDA) (Froman, 2003), and d-homocysteinesulfinic acid, an NMDA receptor agonist (Froman *et al*. 2006).

Previous studies, have reported that D-Asp directly affects the viability of sperm by inhibiting DNA fragmentation, lipid peroxidation (Talevi *et al*. 2013), and activating the cellular waterfalls (Di Fiore *et al*. 2014).

Table 1 Effect of D-aspartic acid on motile parameters of frozen–thawed sperm

Variable	Treatment				SEM
	Control	5	10	15	
Total motility (TM, %)	47.5^{c}	54^{b}	62.2^{a}	46^{c}	1.6
Progressive motility (PM, %)	19.6^{b}	22.6^{b}	27.4^{a}	18.6^{b}	1.2
Average path velocity (VAP, µm/s)	62.1^{b}	63.2^{ab}	67.4^{a}	60.8^{b}	1.3
Straight-line velocity (VSL, µm/s)	46.2^{ab}	48^{ab}	51.7^{a}	45.1^{b}	1.6
Curvilinear velocity (VCL, µm/s)	106.3	108.2	113.5	103.5	3.6
Linearity (LIN, %)	43	44.7	46	43.6	1.8
Straightness (STR, %)	72.9	76.3	77.3	74.3	2.5
Amplitude of lateral head displacement (ALH, µm)	8.1	7.9	7.4	7.9	0.38
Beat/cross frequency (BCF, Hz)	16.6	17	18	16	1

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 2 Effect of D-aspartic acid on viability membrane integrity, morphological appearance and mitochondrial activity of frozen–thawed sperm

Variable	Treatment				SEM
	Control	5	10	15	
Membrane integrity (%)	43.4^{b}	47.8^{b}	57^{a}	43.2^{b}	1.3
Total abnormality (%)	22.6^{a}	19.7^{ab}	17.2^{b}	23.6^{a}	1.2
Mitochondria activity (%)	41.8^{c}	46.9^{b}	54.2^{a}	40^{c}	0.2
Viability (%)	48.7^{c}	57.4^{b}	69.9^{a}	48.6^{c}	1.8

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Effect of D-aspartic acid on malondialdehyde (MDA), glutathione peroxidase (GPx), superoxide dismutase (SOD) and total antioxidant capacity (TAC) ofram sperm

Variable	Treatment				SEM
	Control	5	10	15	
MDA (nmol/mL)	3.8^{a}	2.5^{b}	1.7^{c}	3.3^{ab}	0.2
GPx (IU/g protein)	36.7^{b}	46^{ab}	50.9^{a}	38.9^{ab}	3.3
SOD (U/mg)	73.1	74.6	81.5	70.8	2.9
TAC (mmol/L)	1.3^{b}	1.5^{ab}	2^{a}	1.4^{b}	0.1

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Sperm viability improvement in group receiving 10 mg/L D-Asp is in agreement with previous studies.

Current study shows that addition of 10 mg/L D-Asp significantly increased mitochondria activity after thawing. Similarly, Ansari *et al*. (2017) reported that administration of D-Asp to rooster sperm improved mitochondrial activity. During sperm cryopreservation, mitochondrial activity dramatically decreases (Partyka *et al*. 2012), which is necessary for the spermatozoa to reach oocyte and penetrate its zona pellucida. Mitochondria are the major site of production of ATP which is an essential substrate for maintaining flagella movement of the sperm. In the present study, the addition of D-Asp clearly improved mitochondrial activity, and subsequently increased sperm motility. Therefore, supplementation of extender with D-Asp may lead to higher production of ATP. However, other study by Chandrashekar and Muralidhara (2010) in testis of prepubertal rats, showed that high concentrations of D-Asp treatment caused significant oxidative dysfunctions in testis mitochondria as revealed by elevated ROS generation, hydroperoxide levels and MDA levels.

In the current study, addition of 15 mg/L of D-Asp strongly reduced the sperm mitochondrial activity.

Reducing intracellular antioxidant and seminal plasma substances such as superoxide dismutase and glutathione peroxidase is one of the major causes of frozen sperm's vulnerability to fresh sperm's (Bilodeau *et al*. 2002). Furthermore, TAC in seminal plasma has a key role in infertility (Giulini *et al*. 2009).

In this study, GPx and TAC in the group treated with 10 mg/L D-Asp improved significantly relative to the control group. In a study on bull sperm, it has been shown that less activity of antioxidant enzymes results in lipid peroxidation (Nair *et al*. 2006).

Hence, supplementation of the freezing extender with additive D-Asp may prevent cryodamage to spermatozoa, antioxidant capacities and DNA integrity (Bucak *et al*. 2008). A positive relationship was observed between seminal plasma TAC and sperm motility (Shi *et al*. 2006). Therefore, as the total antioxidant capacity increases, motility will also improve. However, the exact mechanism behind this effect is not clear.

Table 4 Effect of different thawing method on motile parameters of ram sperm

Thawing rates	Motility parameters								
	TM (%)	PM (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	LIN (%)	STR (%)	ALH (µm)	BCF (Hz)
37 ˚C to 30 s	53.8	22.9	63.9	48.6	109.4	44.3	75.6	7.6	17
60 ˚C to 6 s	50.7	21	62.8	46.9	106.3	44.3	78.8	8	16.8
SEM	1.1	0.9	0.9	1.1	2.5	1.3	1.8	0.3	0.7

TM: total motility; PM: progressive motility; VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; LIN: linearity; STR: straightness; ALH: amplitude of lateral head displacement and BCF: beat/cross frequency.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 5 Effect of different thawing method on malondialdehyde (MDA), glutathione peroxidase (GPx), superoxide dismutase (SOD) and total antioxidant capacity (TAC) of ram sperm

Thawing rates	Oxidative status			
	MDA (nmol/mL)	GPx (IU/g protein)	SOD (U/mg)	TAC (mmol/L)
37 ºC to 30 s	2.8	42.8	75.7	1.5
60 ºC to 6 s	2.9	43.4	74.3	1.5
SEM	0.1	2.3	2	0.1

SEM: standard error of the means.

Table 6 Effect of different thawing method on plasma membrane integrity, mitochondria activity, total abnormalities, and viability of ram sperm

Thawing rates	Parameter			
	Plasma Membrane integrity (%)	Total abnormality (%)	Mitochondria activity (%)	Viability (%)
37 ºC to 30 s	49	20.5	47.2[a]	58[a]
60 ºC to 6 s	46.6	21	44.2[b]	54.4[b]
SEM	0.9	0.9	0.7	1.3

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In the present study, group receiving 10 mg/L D-Asp had the lowest MDA and probably had a major influence on sperm survival following thawing. Decrease of MDA level might be attributed somehow to the antioxidant effect of D-Asp on lipid peroxidation of spermatozoa. Similarly, our results are in agreement with Mehdipour *et al*. (2016) showing that addition of substance with antioxidant properties at 10 mg/L significantly decreased MDA levels. Also, Talevi *et al*. (2013) reported that *in vitro* treatment of human spermatozoa with zinc, D-Asp and CoQ_{10} prevented lipid oxidation during storage.

Improvement of sperm membrane integrity in the presence of 10 mg/L D-Asp following cryopreservation are in agreement with Ansari *et al*. (2016). This indicates that at this dose, D-Asp inhibit peroxidation of membrane lipids during storage and thus has protective effects on sperm membranes. Cryopreservation disrupts the transbilayer phospholipid asymmetry in the plasma membranes of the sperm. Higher amounts of ROS induce lipid peroxidation resulting in oxidative stress which leads to the destruction of sperm motility and membrane integrity. Due to the inability of sperm to synthesize membrane components and low antioxidant capacity, spermatozoa become more susceptible to damage by ROS (Jones and Mann, 1977).

Addition of 10 mg/L D-Asp reduced post-thawed abnormalities which are in agreement with the results of Ansari *et al*. (2016).

On the other hand, Anghel *et al*. (2010) showed that addition of antioxidants to extender reduces abnormalities in bucks.

Results of Mazur (1985) showed that freezing and thawing rates are effective on sperm plasma membrane integrity and viability of sperm. It seems that, to improve the sperm function, fast thawing is essential. Probably ice crystalline formation during freezing may grow during a slow thawing process (Deka and Rao, 1987; Dodaran *et al*. 2015). Significantly higher values for post-thaw viability was found for straws thawed at 37 ˚C to 30 s compared with those thawed at 60 ˚C to 6 s. This result is in agreement with Correa *et al*. (1996) who reported a positive effect of thawing at 37 ˚C on post-thaw viability of bull sperm. Hashemi *et al*. (2007) recomended 38 ˚C for 12-30 s for thawing ram semen. Researchers recommended 38-42 ˚C and 75 ˚C temperatures for thawing ram semen (Aamdal and Andersen, 1968; Andersen *et al*. 1973; Salamon and Maxwell, 1995).

Our results agree with Pontbriand *et al*. (1989) who reported no positive effect of the thawing temperature (37 ˚C for 20s and 60 ˚C for 8 s) on the motility and acrosome integrity post-thawing process. Abnormality was improved with thawing at 37 ˚C for 30 s, but this increase was not different with results of other researchers (Marshall, 1984; Vazquez *et al*. 1997).

Significant correlation was found between thawing procedure and increased mitochondrial activity. Freeze-

thawing causes damage to the morphology, mitochondria, acrosome and the tail of sperm (Woolley and Richardson, 1978). Frozen-thawed ram semen at 39 ˚C and 50 ˚C significantly improved mitochondrial activity, viability, motility and plasma membrane integrity (Nicolae *et al*. 2014).

CONCLUSION

Freezing extender with 10 mg/L D-Asp had higher protection effect on membrane integrity, mitochondria activity, glutathione peroxidase, total antioxidant capacity**,** malondialdehyde, and total abnormality of sperm. It had higher motility parameters compared to other groups. More research is needed to clarify physiological effects of D-Asp on sperm quality parameters. The results of this study indicate that thawing rate of 60 ˚C for 6s did not provide a suitable replacement for the thawing rate of 37 ˚C for 30 s, and there is a significant improvement in viability and mitochondrial activity at a lower temperature.

ACKNOWLEDGEMENT

The authors would like to acknowledge the financial support of University of Tabriz for this research.

REFERENCES

Aamdal J. and Andersen K. (1968). Freezing of ram semen in straws. Pp. 977-980 in Int. Congr. Anim. Reprod. Artif. Insem., Paris, France.

Agarwal A. and Anandh Prabakaran S. (2005). Oxidative stress and antioxidants in male infertility: a difficult balance. *Iranian J. Reprod. Med*. **3,** 1-8.

Agarwal A. and Said T.M. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum. Reprod. Update*. **9,** 331-345.

Aitken R.J., Fisher H.M., Fulton N., Gomez E., Knox W., Lewis B. and Irvine S. (1997). Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol. Reprod. Dev*. **47,** 468-482.

Alvarez J.G. and Storey B.T. (1992). Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J. Androl*. **13,** 232-241.

Andersen V.K., Aamdal J. and Fougner J.A. (1973). Intrauterine and deep cervical insemination with frozen semen in sheep. *Reprod. Domest. Anim*. **8,** 113-118.

Anghel A., Zamfirescu S., Dragomir C., Nadolu D., Elena S. and Florica B. (2010). The effects of antioxidants on the cytological parameters of cryopreserved buck semen. *Romanian Biotechnol. Lett*. **15,** 26-32.

Ansari M., Zhandi M., Kohram H., Zaghari M. and Sadeghi M. (2016). The effect of D-aspartic acid on sperm quality of broiler breeder roosters. *Iranian J. Agric. Sci*. **47,** 165-174.

Ansari M., Zhandi M., Kohram H., Zaghari M., Sadeghi M. and Sharafi M. (2017). Improvement of post-thawed sperm quality and fertility of Arian rooster by oral administration of d-aspartic acid. *Theriogenology*. **92,** 69-74.

Athurupana R., Ioki S. and Funahashi H. (2015). Rapid thawing and stabilizing procedure improve postthaw survival and *in vitro* penetrability of boar spermatozoa cryopreserved with a glycerol-free trehalose-based extender. *Theriogenology*. **84,** 940-947.

Bansal A.K. and Bilaspuri G. (2011). Impacts of oxidative stress and antioxidants on semen functions. *Vet. Med. Int*. **32,** 1-7.

Bilodeau J.F., Blanchette S., Cormier N. and Sirard M.A. (2002). Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: Protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. *Theriogenology*. **57,** 1105-1122.

Borah B.K., Deka B.C., Biswas R.K., Chakravarty P., Deori S., Sinha S. and Ahmed K. (2015). Effect of thawing methods on frozen semen quality of yak (*Poephagus grunniens*) bulls. *Vet. World*. **8,** 831-834.

Bucak M.N., Ateşşahin A. and Yüce A. (2008). Effect of antioxidants and oxidative stress parameters on ram semen after the freeze–thawing process. *Small Rumin. Res*. **75,** 128-134.

Budai C. (2014). The protective effect of antioxidants on liquid and frozen stored ram semen. *Sci. Pap. Anim. Sci. Biotechnol*. **47,** 46-52.

Chandrashekar K.N. and Muralidhara (2010). D-aspartic acid induced oxidative stress and mitochondrial dysfunctions in testis of prepubertal rats. *Amino Acids*. **38,** 817-827.

Correa J.R., Rodriguez M.C., Patterson D.J. and Zavos P.M. (1996). Thawing and processing of cryopreserved bovine spermatozoa at various temperatures and their effects on sperm viability, osmotic shock and sperm membrane functional integrity. *Theriogenology*. **46,** 413-420.

Çoyan K., Başpınar N., Bucak M.N. and Akalın P.P. (2011). Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters. *Cryobiology*. **63,** 1-6.

D'Aniello A. and Giuditta A. (1977). Identification of D-aspartic acid in the brain of *Octopus vulgaris* lam. *J. Neurochem*. **29,** 1053-1057.

D'Aniello G., Grieco N., Di Filippo M.A., Cappiello F., Topo E., D'Aniello E. and Ronsini S. (2007). Reproductive implication of D-aspartic acid in human pre-ovulatory follicular fluid. *Hum. Reprod*. **22,** 3178-3183.

D'Aniello G., D'Aniello G., Ronsini S., Notari T., Grieco N., Infante V., D'Angelo N., Mascia F., Maddalena Di Fiore M., Fisher G. and D'Aniello A. (2012). D-aspartate, a key element for the improvement of sperm quality. *Adv. Sex. Med*. **2,** 47-53.

Deka B. and Rao A. (1987). Effect of extenders and thawing methods on post thawing preservation of goat semen. *Indian Vet. J*. **64,** 591-594.

Dhami A.J. and Sahni K.L. (1993). Evaluation of different cooling rates, equilibration periods and diluents for effects on deep-freezing, enzyme leakage and fertility of taurine bull

spermatozoa. *Theriogenology*. **40,** 1269-1280.

Di Fiore M.M., Santillo A. and Chieffi Baccari G. (2014). Current knowledge of D-aspartate in glandular tissues. *Amino Acids*. **46,** 1805-1818.

Dodaran H.V., Zhandi M., Sharafi M., Nejati-Amiri E., Nejati-Javaremi A., Mohammadi-Sangcheshmeh A., Shehab-El-Deen M.A. and Shakeri M. (2015). Effect of ethanol induced mild stress on post-thawed bull sperm quality. *Cryobiology*. **71,** 12-17.

Eriksson B.M. and Rodriguez-Martinez H. (2000). Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. *Anim. Reprod. Sci*. **63,** 205-220.

Esterbauer H. and Cheeseman K.H. (1990). Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Methods Enzymol*. **186,** 407-421.

Fiser P.S., Fairfull R.W., Hansen C., Panich P.L., Shrestha J.N. and Underhill L. (1993). The effect of warming velocity on motility and acrosomal integrity of boar sperm as influenced by the rate of freezing and glycerol level. *Mol. Reprod. Dev*. **34,** 190-195.

Froman D. (2003). Deduction of a model for sperm storage in the oviduct of the domestic fowl (*Gallus domesticus*). *Biol. Reprod*. **69,** 248-253.

Froman D.P., Wardell J.C. and Feltmann A.J. (2006). Sperm mobility: deduction of a model explaining phenotypic variation in roosters (*Gallus domesticus*). *Biol. Reprod*. **74,** 487-491.

Giulini S., Sblendorio V., Xella S., La Marca A., Palmieri B. and Volpe A. (2009). Seminal plasma total antioxidant capacity and semen parameters in patients with varicocele. *Reprod. Biomed. Online*. **18,** 617-621.

Graham J.K., Kunze E. and Hammerstedt R.H. (1990). Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biol. Reprod*. **43,** 55-64.

Gualtieri R., Boni R., Tosti E., Zagami M. and Talevi R. (2005). Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium *in vitro*. *Reproduction*. **129,** 51-60.

Hashemi A., Farhoomand P., Pirmohammadi R., Nayebpor M. and Razzaghzadeh S. (2007). Effect of extender and thawing methods on post thawing preservation. *J. Anim. Vet. Adv*. **6,** 1337-1339.

Jones R. and Mann T. (1977). Toxicity of exogenous fatty acid peroxides towards spermatozoa. *J. Reprod. Fertil*. **50,** 255-260.

Khosrowbeygi A., Zargham N. and Farzadi L. (2008). Fatty acid composition in normozoospermic, asthenozoopermic, asthenoteratozoospermic and oligoasthenoteratozoospermic ejaculates. *Int. J. Reprod. Biomed*. **6,** 39-43.

Lenzi A., Picardo M., Gandini L. and Dondero F. (1996). Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. *Hum. Reprod. Update*. **2,** 246-256.

Macchia G., Topo E., Mangano N., D'Aniello E. and Boni R. (2010). DL-aspartic acid administration improves semen quality in rabbit bucks. *Anim. Reprod. Sci*. **118,** 337-343.

Mahat R.K., Kumar S., Arora M., Bhale D.V., Mehta R. and Batra J. (2015). Role of oxidative stress and antioxidants in male infertility. *Int. J. Health Sci. Res*. **5,** 324-333.

Maia Mda S., Bicudo S.D., Sicherle C.C., Rodello L. and Gallego I.C. (2010). Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. *Anim. Reprod. Sci*. **122,** 118-123.

Malo C., Gil L., Gonzalez N., Martínez F., Cano R., de Blas I. and Espinosa E. (2010). Anti-oxidant supplementation improves boar sperm characteristics and fertility after cryopreservation: comparison between cysteine and rosemary (*Rosmarinus officinalis*). *Cryobiology*. **61,** 142-147.

Marshall C.E. (1984). Considerations for cryopreservation of semen. *Zoo Biol*. **3,** 343-356.

Maxwell W.M. and Salamon S. (1993). Liquid storage of ram semen: A review. *Reprod. Fertil. Dev*. **5,** 613-638.

Mazur P. (1984). Freezing of living cells: Mechanisms and implications. *American J. Physiol. Cell Physiol*. **247,** 125-142.

Mazur P. (1985). Basic Concepts in Freezing Cells. Oak Ridge National Lab., Oak Ridge, Tennessee, USA.

Mehdipour M., Daghigh Kia H., Najafi A., Vaseghi Dodaran H. and García-Álvarez O. (2016). Effect of green tea (*Camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology*. **73,** 297-303.

Nair S.J., Brar A.S., Ahuja C.S., Sangha S.P. and Chaudhary K.C. (2006). A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim. Reprod. Sci*. **96,** 21-29.

Najafi A., Najafi M.H., Zanganeh Z., Sharafi M., Martinez-Pastor F. and Adeldust H. (2014). Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant. *Reprod. Domest. Anim*. **49,** 934-940.

Nicolae D., Stela Z., Dragomir C. and Hortanse A.A. (2014). Effect of thawing time and temperature variation on the quality of frozen-thawed ram semen. *Romanian Biotechnol. Lett*. **19,** 8935-8940.

Ogbuewu I.P., Aladi N.O., Etuk I.F., Opara M.N., Uchegbu M.C., Okoli I.C. and Iloeje M.U. (2010). Relevance of oxygen free radicals and antioxidants in sperm. *Res. J. Vet. Sci*. **3,** 138-164.

Partyka A., Łukaszewicz E. and Niżański W. (2012). Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. *Theriogenology*. **77,** 1497-1504.

Perry E.J. (1955). Artificial Insemination of Farm Animals. Rutgers University Press, New Brunswick.

Pontbriand D., Howard J.G., Schiewe M.C., Stuart L.D. and Wildt D.E. (1989). Effect of cryoprotective diluent and method of freeze-thawing on survival and acrosomal integrity of ram spermatozoa. *Cryobiology*. **26,** 341-354.

Purdy P.H. (2006). A review on goat sperm cryopreservation. *Small Rumin. Res*. **63,** 215-225.

Salamon S. and Maxwell W.M.C. (1995). Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. *Anim. Reprod. Sci*. **37,** 185-249.

SAS Institute. (2001). SAS®/STAT Software, Release 9.1. SAS Institute, Inc., Cary, NC. USA.

Shi Y.C., Shang X.J., Wang X.L. and Huang Y.F. (2006). Correlation of total antioxidant capacity in seminal plasma with sperm motility of infertile men. *Natl. J. Androl*. **12,** 703-705.

Sies H. (1997). Oxidative stress: Oxidants and antioxidants. *Exp. Physiol*. **82,** 291-295.

Talevi R., Barbato V., Fiorentino I., Braun S., Longobardi S. and Gualtieri R. (2013). Protective effects of *in vitro* treatment with zinc, d-aspartate and coenzyme Q10 on human sperm motility, lipid peroxidation and DNA fragmentation. *Reprod. Biol. Endocrinol*. **11,** 81-91.

Topo E., Soricelli A., D'Aniello A., Ronsini S. and D'Aniello G. (2009). The role and molecular mechanism of D-aspartic acid in the release and synthesis of LH and testosterone in humans and rats. *Reprod. Biol. Endocrinol*. **7,** 120-127.

Vazquez J.M., Martinez E.A., Martinez P., Garcia-Artiga C. and Roca J. (1997). Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. *Theriogenology*. **47,** 913-922.

Woolley D.M. and Richardson D.W. (1978). Ultrastructural injury to human spermatozoa after freezing and thawing. *J. Reprod. Fertil*. **53,** 389-394.

Yue H.X., Li F.P., Jiang M., Lin L. and Xu K.H. (2005). Influence of cryoprotectant with glycerol and freezing-thawing procedure on the motility of human sperm. *Natl. J. Androl*. **11,** 204-206.

Zarghami N.A. and Khosrow beygi A. (2004). Evaluation of lipid peroxidation as an indirect measure of oxidative stress in seminal plasma. *Int. J. Reprod. Biomed*. **2,** 34-39.