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The Effects of Different Concentrations of Glycine and Cysteine on the Freezability of Moghani Ram Spermatozoa

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ABSTRACT : Two experiments were designed to evaluate the effects of the amino acids glycine and cysteine on cryopreservation of ram spermatozoa. After primary evaluation of collected ejaculates, the semen samples were pooled and diluted 1:4 before cooling (experiment 1) and freezing (experiment 2) with Tris-Citrate-Fructose-Yolk (TCFY) extender supplemented with different concentrations of glycine and cysteine (5, 10, 15 and 20 mM). As the control, semen was diluted and frozen in the extender without amino acids. Motility, viability and membrane integrity were assessed as the parameters for semen quality in the first experiment. In the second experiment, motility, progressive motility, viability, membranes and acrosome integrity were evaluated after the freezing-thawing process. The results of the first experiment indicated that the addition of 10 and 15 mM cysteine compared to the control (basic) extender significantly increased ($p<0.01$) the motility, viability and membrane integrity of spermatozoa after cooling. However, further increasing these amino acids up to 20 mM had a significant negative effect ($p<0.05$). Our results showed no significant differences ($p>0.05$) between 5 mM glycine compared to 5 mM cysteine and between 20 mM glycine and 20 mM cysteine. The results of experiment 2 showed that the amino acids significantly improved post-thaw motility, progressive motility, viability, membranes and acrosome integrity of ram spermatozoa. These positive effects were observed at concentrations between 5 to 15 mM of glycine and cysteine, with the best results at 15 mM. Further increasing of amino acid concentrations significantly decreased the post-thaw characteristics of spermatozoa, but the results showed that cysteine was better than glycine and control extenders. The data indicated that addition of glycine or cysteine to the freezing extender can be recommended for cryopreservation of ram spermatozoa. However, further studies are still needed to determine the effect of such addition on fertility in farm animals. (**Key Words** : Cryopreservation, Glycine, Cysteine, Moghani Ram Spermatozoa)

INTRODUCTION

Cryopreservation induces partially irreversible damage to sperm membranes (Watson, 2000; Moore et al., 2005), which may decrease sperm motility, viability and the fertilization rate after artificial insemination (Matsuoka et al., 2006). Damage during cryopreservation has been attributed to cold shock, ice crystal formation, oxidative stress, cryoprotectant toxicity, osmotic changes and lipid-protein reorganizations within the cell membranes (Watson and Martin, 1975; Drobnis et al., 1993; Bailey et al., 2000; Purdy and Graham, 2005). It is suggested that membrane is thought to be a primary target of chilling or freezing damage in cells (Morris et al., 1981) and occurs when cell membrane undergo the phase transition from liqiud crystalline to a gel phase (Darin-Bennett and White, 1977), which results in a loss of selective permeability and integrity of the plasma membrane (Morris and Clarke, 1987).

On the other hand, differences among species in the sensitivity of their sperm to cooling are largely attributable to compositional variations of the sperm plasma membrane (Bailey et al., 2000). Mammalian sperm characteristically contain a high concentration of polyunsaturated fatty acids in their membranes and they lack a significant cytoplasmic component containing antioxidants. Therefore, sperm cells are highly susceptible to Lipid peroxidation (LPO) by free radicals such as O_2^- and H_2O_2 (Sinha et al., 1996; Gadella et al., 2001), which lead to the structural damage of sperm membranes during the freezing-thawing process (Sinha et al., 1996). It is well known that the ram sperm have a higher

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polyunsaturated/saturated fatty acids ratio and lower cholesterol/phospholipid molar ratio than other species (Evans and Maxwell, 1987). This can make the ram sperm more sensitive to cold shock and peroxidative damage than those of other species such as bull, rabbit, or even the human with subsequent loss of membrane integrity of the acrosomal region and impaired cell function (Watson, 1981; Fiser and Fairfull, 1989; Aitken and Fisher, 1994). Based on this information, the composition of extender and suitable cryoprotectants seem to play an important role for successful semen cryopreservation (Hammerstedt et al., 1990; Curry et al., 1994). Therefore, in recent years, different antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation and free radicals are eliminated by antioxidant systems (Baumber et al., 2000).

Since the discovery of the biological effects of amino acids in prevention of cell damage during freezing stress (Chu et al., 1974), numerous studies investigated their protective effects on different type of animal cells against freezing damage including sperm (Kruuv and Glofcheski, 1992; Khlifaouia et al., 2005; Ali Al Ahmad et al., 2008; Atessahin et al., 2008; Sheshtawy et al., 2008). Furthermore, after the detecting of several amino acids in seminal plasma (e.g cysteine, glycine, proline and histidine), they used successfully as cryoprotectants to cryopreserve spermatozoa of many mammalian species (Khlifaouia et al., 2005; Ali Al Ahmad et al., 2008; Atessahin et al., 2008; Sheshtawy et al., 2008). The published reports suggested that addition of amino acids to extender improved post-thawing sperm motility, viability, acrosome integrity and membrane integrity in goat (Bilodeau et al., 2001; Ali Al Ahmad et al., 2008; Atessahin et al., 2008; Uysal and Bucak, 2008), Zebu, buffalo and bull (Iqbal and Hunter, 1989; Dhami and Shani, 1994; Dhami et al., 1995; Bilodeu et al., 2001; Sheshtawy et al., 2008), ram (Scanchez- Partidata et al., 1992; Uysal and Bucak, 2007), stallion and donkey (Trimèche et al., 1999; Li et al., 2003; Khalifaoui et al., 2005) and boar (Szczesniak-Fabianczyk et al., 2006). However, by which mechanism these amino acid components protect spermatozoa during freezing-thawing process, have not clearly understood and are still unclear. Therefore, based on the presented results of these investigations and few studies about the role of amino acids on ram spermatozoa quality after freezing and thawing, the main goal of this study was to study the effect and optimal concentrations of glycine and cysteine on freezability of Moghani ram sperm during freezing and thawing.

MATHERIAL AND METHODS

Animals and location

This experiment was performed at the testing station, located in Jafar- Abad, 39°, 26′, 20.6″ N latitude and 48°, 5′, 26.4″ E longitude and lasted from April to May. Four mature Moghani rams, 3 to 4 years of age and 60-65 kg were used in the study. Moghani sheep is one of the most important fat-tailed meat breed in Iran and is well known for its large size, high tolerance to harsh environment and capability to produce heavy lambs. The animals were kept under natural photoperiod and nutritional levels were adjusted to meet maintenance requirements. They had free access to salt lick and fresh water.

Semen extenders

The base extender consisted of 3.07 g Tris (hydroxymethyl-aminoethane, Merck 64271, Germany), 1.64 g citric acid (BHD 1081, England), and 1.26 g fructose (BDH 28433, England) in 100 ml distilled water, containing 5.0% (v/v) glycerol (Merck, 2400 Germany) and 15% (v/v) egg yolk (Kumar et al., 2003).

Semen collection and evaluation

Semen was collected, twice a week for 8 weeks, from four fertile rams that had been trained to serve an artificial vagina (42-43°C). The prepuce was wiped clean prior to collection to prevent contamination of the semen. Semen was collected in the morning and transported to the laboratory at 37°C within 10 to 15 minutes and placed in a water bath at 37°C. Ejaculates were evaluated for volume (ml), sperm concentration ($\times 10^9$ sperm/ml), mass activity (%, undiluted semen), motility (%, diluted with normal saline), progressive motility using an arbitrary scale of 1 to 5 (1, 2, 3, 4 or 5 = 10 to 25%, 25 to 50%, 50 to 70%, 70 to 90% or 90 to 100% of the motile spermatozoa), viability (%, using eosin-nigrosin staining) and morphological normal acrosome (%). Only semen samples with adequate motility more than 70% and sperm concentration more than 3×10^7sperm/ml were used for cryopreservation. Semen volume was estimated in a calibrated semen collection tube, and sperm concentration was determined by Neubaur hemocytometer, diluting (1:200) a small sample of the semen with 2% eosin solution. To evaluate motility and progressive motility, a sample of the diluted spermatozoa was placed under a cover slip in the centre of a pre-warmed (37°C) slide and it was transferred to a heated microscope stage set at 37°C and subjectively assessed by phase contrast microscopy (×400 magnification). The rate of motility and progressive motility was determined in percentages. Viability was performed using a modification of the eosin-nigrosin stain procedure described by Evans and Maxwell (1987). A mixture of 10 μl of diluted spermatozoa and 10 μl eosin-nigrosin stains was smeared on a slide and allowed to air dry in a dust-free environment. Two hundred spermatozoa from different microscopic fields were examined under a bright-field microscope using a

400× objective, and the number of nonstained (viable) spermatozoa was counted.

The morphological acrosome abnormality was assessed by viewing wet mount of diluted spermatozoa fixed in buffered Formalin-Citrate solution described by Weitze (1977). A drop of the fixed spermatozoa was placed on slide and covered with a cover glass. The slides were examined by phase-contrast microscopy using a 100× oil immersion objective and white light. Spermatozoa (n = 200/slide) were examined and the percentage with normal acrosomes determined. The hypo-osmotic swelling test (HOS-test) was used to evaluate the functional integrity of the sperm membrane, and was performed by incubating 20 µl of semen with 200 µl of a 100 mOsm hypo-osmotic solution (9 g fructose+4.9 g sodium citrate/1 distilled water) at 37°C for 60 min. After incubation, 100 µl of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm were counted in at least 5 different microscopic fields. The percentage of sperm with swollen and curled tails was then recorded (Revell and Mrode, 1994).

Experimental procedure

Two experiments were concluded in this study. First experiment was designed to investigate the influence of different concentrations of glycine and cysteine (5, 10, 15 or 20 mM) on cooled ram spermatozoa (without freezing process). The semen extenders (basic extender containing different concentrations of glyciene and cysteine) were prepared and kept in a water bath at 37°C, on the day of semen collection. Semen samples were pooled and diluted 1:4 with prepared extender. As control, semen was diluted in the same rate with basic extender without amino acids. The extended semen samples were packaged in 0.25-ml French straws and the open end of the filled straws was sealed with polyvinyl chloride powder. Straws were kept at 5°C for 3.5 h for the equilibration. After equilibration time, the cooled samples were placed again in the water bath at 37°C min and rewarmed semen samples were assessed to on motility, viability and membrane integrity.

The second experiment was designed to study the effects of glycine and cysteine during freezing step. The preparation and procedures of second experiment till before freezing were according to experiment 1. The cooled semen samples were then held 4-5 cm above the surface of the liquid nitrogen for 10 min before being plunged into liquid nitrogen for storage (-196°C). After 24 h, the straws were thawed in water bath at 37°C for 2 min. The froze-thawed semen assessed to motility, progressive motility, viability, morphological acrosome abnormality and hypo-osmotic swelling test.

Statistical analysis

The experiments were conducted as a completely randomize design, and statistical analysis of data was performed by the General Linear Model (GLM) procedure of the SAS (1996). All percentage data were arcsine transformed before statistical analysis. Back-transformed data are reported as mean±SEM. A probability level of $p \leq 0.05$ was considered as significant.

RESULTS

Macroscopic and microscopic seminal characteristics in primary evaluation are presented in Table 1. The average of volume (ml), sperm concentration (10^9 ml^{-1}), mass activity (1-5), motility (%), progressive motility (%), live sperm (%) and Normal acrosome (%) in the ejaculates of Moghani ram spermatozoa were 1.2, 4.2, 4.55, 88.85, 82.54, 87.63 and

Table 1. Macroscopic and microscopic characters of seminal plasma of Moghani ram spermatozoa

Seminal characters	n	Mean	SEM	Min- Max
Volume (ml)	64	1.24	0.06	0.8-2.6
Sperm concentration (10^9 ml^{-1})	64	4.4	0.09	3.54-5.25
Mass activity (1-5)	64	4.55	0.08	3-5
Motility (%)	64	88.85	0.69	70-95
Progressive motility (%)	64	82.54	0.68	68-90
Live sperm (%)	63	87.63	0.84	80-89
Normal acrosome (%)	58	88.54	0.94	85-95

Table 2. Effect of different concentrations of amino acids on characteristics of Moghani ram sperm after cooling (mean±SEM)

	Control	Cysteine (mM)				Glycine (mM)				SEM
		5	10	15	20	5	10	15	20	
Motility (%)	69.37^{g}	74.68^{d}	80.18^{b}	82.37^{a}	72.25^{ef}	72.93^{de}	77.10^{c}	78.06^{c}	70.81^{fg}	0.64
Viability (%)	72.43^{d}	75.29^{c}	82.57^{a}	84.20^{a}	74.70^{cd}	74.86^{cd}	78.42^{b}	80.29^{b}	72.85^{d}	0.80
Membrane integrity (%)	59.46^{e}	64.26^{e}	72.93^{a}	74.81^{a}	62.78^{ed}	62.06^{ed}	69.12^{b}	72.22^{a}	60.81^{de}	0.95

Values with different letter(s) in the same row are significantly different ($p<0.05$, Duncan-test).

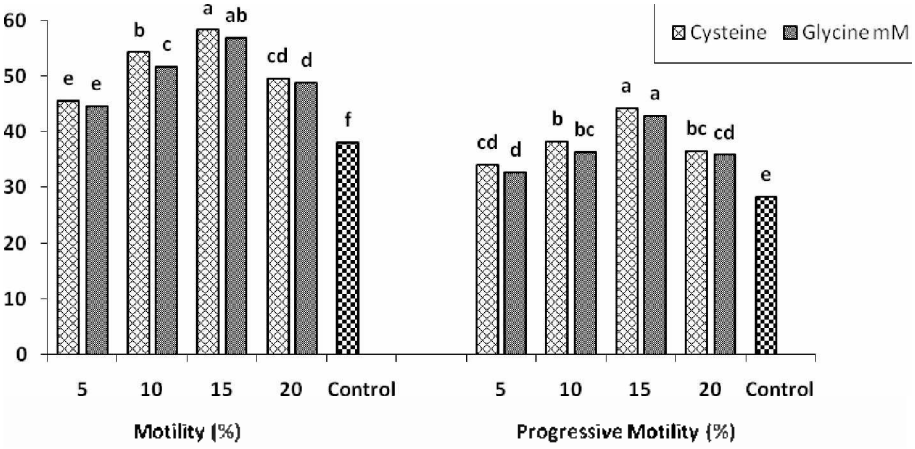

Figure 1. The effect of different concentrations of cytein and glycine on motility and progressive motility of frozen-thawed Moghani ram spermatozoa, Duncan'smultiple range test, $p<0.05$.

88.5 respectively.

Table 2 shows the effect of supplementation of basic tris extender with different concentrations of glycine and cysteine on cooled ram spermatozoa. The results indicate that the addition of 10 and 15 mM cysteine to the control (basic) extender increased significantly ($p<0.01$) the sperm motility, viability and membrane integrity after cooling. However, further increasing of these amino acids up to 20 mM affected significantly negative ($p<0.05$). Furthermore, there were no significant differences ($p>0.05$) between 5 mM glycine compared to 5 mM cysteine and 20 mM glycine compared to 20 mM cysteine. Also, the results showed no significant difference in the motility, viability and membranes integrity of semen samples cooled in extender containing 20 mM glycine and control extender.

Figures 1-3 present the results of different concentrations of used amino acids on post-thaw motility, progressive motility, viability, membranes and acrosome integrity of ram spermatozoa. There was a significant differences ($p<0.0001$) in assessed parameters by adding of amino acids to basic extender (control extender). However, the cryoprotection profiles of glycine and cysteine were dosage dependent. Initially, with increasing concentrations of glycine or cysteine, the post-thaw motility, progressive motility, viability, membranes and acrosome integrity increased and reached a best level (15 mM), but with further increasing of amino acids concentrations the post-thaw characteristics decreased.

Furthermore, the observed results in this study demonstrated that both cysteine and glycine treatments showed positive effects on post-thaw motility, progressive motility, membranes and acrosome integrity when added to basic extender at 15 mM concentration. All levels of cysteine and glycine significantly ($p<0.0001$) decreased the percentage of abnormal sperm. Extender containing 15 mM cysteine decreased significantly sperm abnormalities compared to other treatments ($p<0.05$) and control ($p<0.0001$) in froze-thawed samples. However, there were

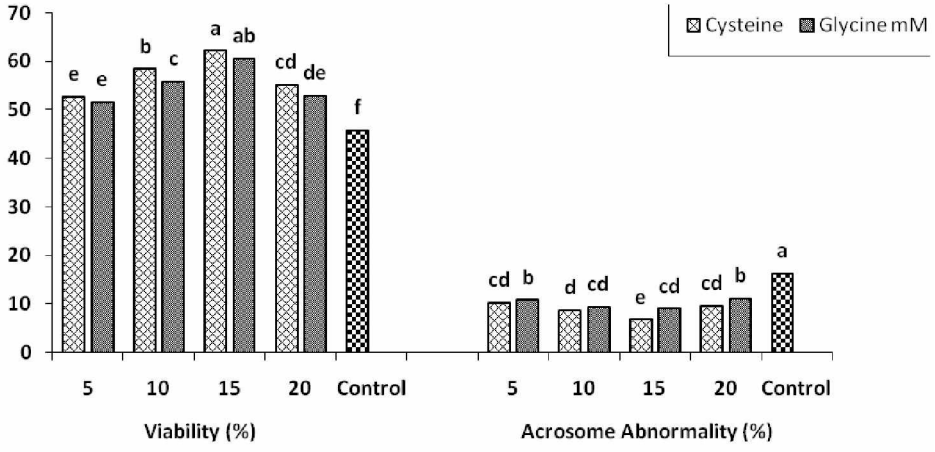

Figure 2. The effect of different concentrations of cyteine and glycine on viability and acrosome abnormality of frozen-thawed Moghani ram spermatozoa, Duncan'smultiple range test, $p<0.05$.

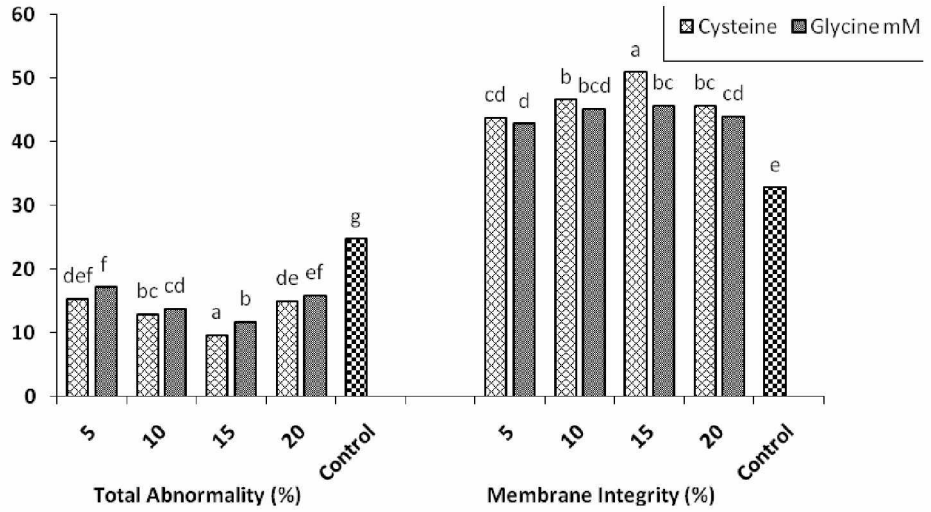

Figure 3. The effect of different concentrations of cyteine and glycine on total abnormality and membrane integrity of frozen-thawed Moghani ram spermatozoa, Duncan'smultiple range test, $p<0.05$.

no significant differences in the post-thaw sperm abnormalities between 5 versus 5, 10 versus 10 and 20 versus 20 mM glycine or cysteine. There were no significant difference in the post-thaw motility, progressive motility, membranes and acrosome integrity of samples frozen in the presence of 5 mM glycine or 5 mM cysteine and 20 mM glycine or 20 mM cysteine.

The results of presented study indicate that cysteine was more effective for preservation of motility, progressive motility, membranes and acrosome integrity sperm parameters (Figure 1, 2 and 3) than glycine and control extenders. In addition, the post-thaw sperm motility, progressive motility, viability and membranes integrity increased significantly in the presence of 15 mM cysteine in extender. However, as compared to control extender, all levels of glycine and cysteine improved the acrosome integrity of the cryopreserved spermatozoa, but 15 mM cysteine level was most effective.

Concerning total abnormality, all with amino acids treatments level decreased significantly. However, the differences in post-thaw abnormal sperm between some treatments was not significant ($p>0.05$).

DISCUSSION

The goal of cryopreservation is to obtain a high number of post-thawing survival normal sperms, but there are many factors during cryopreservation process, which can affect the post-thawing outcome (Eiman and Terada, 2004). Therefore, various methods of cryopreservation including technical aspects of freezing and thawing sperm and of preparing cryopreservation media have been evaluated for their effects on post thawing sperm quality (see Purdy, 2006; Farshad et al., 2009), but currently, the standard methods for freezing-thawing semen which well optimize sperm recovery have not been firmly established yet.

Concerning cryopreservation of ram spermatozoa, various extenders have been described (Salamon and Maxwell, 2000), but there are few reports on the effects of the amino acids on the post-thaw motility and viability of ram spermatozoa. Scanchez-Partidata et al. (1992) demonstrated that the addition of low concentration of proline and betaine glycine (a component related to amino acids) to a medium containing egg yolk and glycerol improved the motility of ram spermatozoa. Uysal and Bucak (2007) and Bucak et al. (2008) presented the positive effects of cystein on motility and membrane integrity.

The results of this study demonstrated that supplementation of tirs extender with glycine and cystein, the froze-thawed characteristics of spermatozoa, such as motility, progressive motility, viability, acrosome and membrane integrity, significantly improved. These observations were in agreement with the findings of Uysal and Bucak (2007) and Bucak et al. (2008) for ram; Trimèche et al. (1999) and Khlifaouia et al. (2005), for stallion and Kundu et al. (2001), for goat sperm. Sheshtawy et al. (2008) concluded that addition of 25 mM glutamine, glycine and 5 mM cysteine in conventional freezing medium enhanced post-thaw motility and improved membrane and acrosome integrity of buffalo bull semen. Furthermore, our results indicated that addition of used amino acids was better when added at a concentration of 15 mM. This result was in agreement with the findings of Atessahin et al. (2008). The authors reported that 5 to 15 mM amino acids improved significantly sperm motility of froze-thawed goat spermatozoa.

However, the exact mechanism of sperm protection by amino acids has not been understood and remains unclear. A

variety of hypotheses and speculations have been proposed by various authors to explain the protective mechanism of amino acids during cryopreservation. Kundu et al. (2001) and Anchordoguy et al. (1988) suggested that the protective effects of amino acids may stem from their ability to form a layer on the spermayozoa surface, as these positively charged molecules can combine with the phosphate groups of sperm plasma membrane phospholipids. This may contribute to sperm osmolarity (Billard and Menezo, 1984) and this plays also a positive role in sperm vitality (Roy and Bishop, 1954). Previous studies with carbon 14-labeled glycine indicated that glycine was metabolized by bovine spermatozoa (Filpse and Almquist, 1955). They later reported that glycine was beneficial to sperm by reducing lactic acid accumulation in extenders (Filpse and Almquist, 1956).

Furthermore, it has been speculated that amino acids protect calcium-depende ATPase of sacroplasmic reticulum (Lalonde et al., 1991) and certain enzymes (Noguchi and Matsmuto, 1971) during the changes of state of the freezing medium. Moreover, amino acids have a stabilizing effect on cell membrane, (Rudolph and Crowe, 1985; Lahnsteiner et al., 1992), limited membrane lesion and inhibited plasmolysis through membrane stabilization (Rudolph and Crowe, 1985), prevent lipid peroidaxion of sperm membrane during cryopreservation Alvarez and Storey (1983). A recent report by Bilodeau et al. (2001), demonstrated that thiol-radicals containing amino acids, such as glycine, cysteine, N-acetyl-cysteine and 2 mercaptoethanol, prevented hydrogen peroxide mediated loss of sperm motility in frozen-thawed bull and buffalo semen. However, whether this occurred in our present study remains to be proved.

In addition, the results of this study presented that increasing of used amino acids to 20 mM were detrimental to the freezing of sperm and decreased significantly the assessed characteristics of froze-thawed ram sperm. In agreement with this finding, Trimèche et al. (1999), Li et al. (2003) and Sheshtawy et al. (2008) reported that the high concentrations of amino acids caused a significant reduction in sperm motility, membrane and acrosome integrity, but the authors achieved the negative effects at the concentrations between 80 to 100 mM. However, against our observations and the findings of Trimèche et al. (1999), Li et al. (2003) and Sheshtawy et al. (2008); He et al. (2003) reported that 50 mM glycine significantly improved sperm motility in cooled and frozen-thawed striped bass spermatozoa. The mechanism by which the amino acids decrease the motility and the membrane integrity cannot be clearly understood based on the results of presented study, but the toxicity of higher amino acids concentration during freezing-thawing process has long been recognized in several previous studies (Foote, 1970; Kundu et al., 2001; Khlifaouia et al., 2005; Ali Al Ahmad et al., 2008). Khlifaouia et al. (2005) explained the toxic effect of higher amino acids concentrations is mainly due to their osmotic toxicity and hyper tonicity, which has been speculated by Trimèche et al. (1999). The authors suggested the detrimental effects of amino acids in extender increased when the concentration of used amino acids rides and the reason for these negative effects is the increased osmotic pressure.

CONCLUSION

The results of presented study indicated that additions of selected amino acids (glycine or cysteine) to cryopreservation media significantly enhance motility, progressive motility, membranes and acrosome integrity and viability sperm of ram spermatozoa after freezing and thawing. Concerning the results of used glycine and cystein, all of extenders containing different concentrations of amino acids were significantly better than control extender. However, extender containing 15 mM cystein was the most effective level in this study. Also, based on our results it can suggested, that glycine and cysteine increase the cryoprotecting efficacy of Moghani ram cryopreservation extender and this will help us to define the suitable extender for freezing of ram spermatozoa. However, further studies still needed to recognize the effect of such addition on fertility in farm animals.

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