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The Effect of Different Concentrations of Glycerol and DMSO on Viability of Markhoz Goat Spermatozoa During Different Freezing Temperatures Steps

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Abstract: The present study was conducted to determine the following; (1) the influence of different concentrations of glycerol (1, 3, 5 or 7%, v/v) in experiment 1 and DMSO (1, 1.25, 1.5 or 1.75% v/v) in experiment 2 added either at 37 or 5°C and (2) the comparing of best concentration of glycerol with the best of DMSO, obtained in this study, on post-thaw motility, progressive motility, viability and normal acrosome of Markhoz goat sperm. In experiment 1, motility, progressive motility and viability of sperm were improved significantly ($p<0.05$) by increasing of glycerol concentrations in the extenders, with the best results obtained with glycerol at 7% added at 37°C. However, the rate of normal acrosome showed an opposite trend, i.e., the extender containing 1% glycerol added at 5°C showed better results ($p<0.05$). In experiment 2, the observed results showed similar tendencies to experiment 1. The data showed that the extender containing 1.75% DMSO concentration (the highest level) added at 37°C was significantly ($p<0.05$) better than others. The percentage of intact acrosomes decreased significantly ($p<0.05$) by increasing of DMSO concentrations, when added at 37°C. Further, the results of 1 % DMSO added at 5°C was ($p<0.05$) better than other groups. In regard to all evaluated parameters, the observed results in experiment 3 showed that extender containing 7% glycerol added at 37°C was significantly ($p<0.05$) better than 7% glycerol added at 5°C and extender containing 1.75% DMSO added at both temperatures. In conclusion, the results of presented study indicated that glycerol is still the cryoprotectant of choice for freezing of Markhoz goat sperm.

Key words: Sperm, cryopreservation, cryoprotectant, goat

INTRODUCTION

The cryopreservation is a complex process (Purdy, 2006) that involves balancing a number of interrelated factors amongst which are freezing technique, the composition of cryoprotective diluents, dilution, cooling rates and thawing methods (Eiman and Terada, 2004). It is generally accepted that a substantial number of spermatozoa are damaged during cryopreservation (Watson, 2000). This damage 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, membrane alteration, cryoprotectant toxicity and osmotic changes (Watson and Martin, 1975). Therefore, the composition of extender, suitable cryoprotectants and optimum freezing and thawing rates are important factors for successful semen cryopreservation (Hammerstedt *et al*., 1990; Curry *et al*., 1994).

Since Polge *et al*. (1949) reported that glycerol is beneficial to sperm preservation, it has been routinely included in most cryopreservation protocols for many types of cells, including mammalian spermatozoa (Fiser and Fairfull, 1989; Hammerstedt *et al*., 1990; Bailey *et al*., 2000; Rasul *et al*., 2007). Glycerol as a penetrating cryoprotectant, causes membrane lipid and protein rearrangement, which results in increased membrane fluidity and permeability for ions and increase ATP consumption (Hammerstedt and Graham, 1992; Holt, 2000), greater dehydration at lower temperature and therefore an increased ability to survive cryopreservation (Holt, 2000). However, there is evidence that the presence of glycerol in the diluted semen can induce osmotic damage to spermatozoa (Purdy, 2006), toxic effects on metabolism of cryopreserved cells (Hammerstedt *et al*., 1990), alters the plasma membrane bilayers of spermatozoa, in particular acrosome integrity and interacts with bound proteins and glycoproteins (Hammerstedt and Graham, 1992; Buhr *et al*., 2001; Farshad and Akhondzadeh, 2008) and depresses fertility (Abdelhakeam *et al*., 1991). With such negative consequences, the use of glycerol is still generally recommended for cryopreservation of semen

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(Morrier *et al*., 2002). On the other hand, since Lovelock and Bishop (1959) first reported that dimethylsulphoxide (DMSO) was superior to glycerol for protecting erythrocytes during freezing, it has been widely used as a cryopreservative agent alone or in combination with other cryoprotectants (Snedeker and Gaunya, 1970; Singh *et al*., 1995; Vicente and Viudes-de-Castro, 1996; Kundu *et al*., 2000, 2002; Han *et al*., 2005). DMSO is, like glycerol, a membrane permeable cryoprotective agent (Purdy, 2006) due to its lower molecular weight (Lovelock and Bishop, 1959) it penetrates in cells rapider (Rasul *et al*., 2007) and therefore, will dehydrate the spermatozoa and minimize the intracellular ice formation (Kundu *et al*., 2002). Although it has a beneficial effect for sperm cryopreservation of bovine (Snedeker and Gaunya, 1970), goat (Kundu *et al*., 2001) and rabbit (Vicente and Viudes-de-Castro, 1996), it cause a damaging effect during freezing and thawing of buffalo spermatozoa (Rasul *et al*., 2007). Based on the results of different investigations, the present study is designed to compare the effect of different concentrations of glycerol and DMSO at two different temperatures on viability of Markhoz goat spermatozoa.

MATERIALS AND METHODS

Animal and location: This experiment was performed at the testing station, located in Sanandaj, 35° 20′ N latitude and 47°E longitude and lasted from September to November. Five mature Markhoz goat bucks, 2 to 4 years of age and 55-60 kg were used in the study. The animals were kept under natural photoperiod and nutritional levels were adjusted to meet maintenance requirements. Goats were fed twice a day with a diet of 530 g alfalfa hey, 190 g barley straw and 300 g concentrates. They had free access to salt lick and fresh water. The basic extenders were Tris (3.786%), citric acid (2.172%), fructose (1%) (Evans and Maxwell, 1989). Solutions were adjusted to pH 7.0 and isotonic extender.

Collection and evaluation of semen: Semen was collected twice a week (8 weeks) by artificial vagina (42-43°C) using an estrous female as a mount. Within 2-3 min after collection, the semen was taken to the laboratory and kept in a water bath at 37°C. Ejaculates were evaluated for volume, sperm concentration (sperm cells were counted in four squares of a hemocytometer after 1:200 dilution of semen with 0.5% eosin solution), mass activity (undiluted semen), motility (diluted with normal saline), progressive motility, viability (eosin-nigrosin stain) and morphological normal acrosome. Ejaculates with a motility more than 70% and sperm concentration more than 3×10^7 (sperm mL^{-1}) were selected, pooled and processed further.

The assessment of froze-thawed spermatozoa included motility (%), progressive motility (%), viability (%) and normal acrosome (%). 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 (x400 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 (1989). 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 400x objective and the number of nonstained (viable) spermatozoa was counted. The morphological normal acrosomes were assessed by viewing wet mounts of diluted spermatozoa fixed in buffered Formalin-Citrate solution described by Weitze (1977). A drop of the fixed spermatozoa was placed on a slide and covered with a cover glass. The slides were examined by phase-contrast microscopy using a 100x oil immersion objective and white light. Spermatozoa (n = 200/slide) were examined and the percentage with normal acrosomes determined.

Experimental procedure: Three experiments were concluded in this study. First experiment was designed to investigate the influence of different concentrations of glycerol (1, 3, 5 and 7%, v/v) at two different temperatures on motility, progressive motility, viability and morphological normal acrosome. In the first part of this experiment, semen diluted (1:4) by one-step-method at 37°C. The Tris-citric acid-fructose (basic extenders) contained egg yolk (2.5 %, v/v) and glycerol (1, 3, 5 or 7%, v/v). The extended semen was then packaged in French straws (0.25 mL). The open end of the filled straws was sealed with polyvinyl chloride powder. Straws were kept at 5°C for 3 h before being exposed to the vapour of liquid nitrogen (4-5 cm from the LN2 surface level) for 10 min. They were then stored in liquid nitrogen for 24 h. The straws were thawed in water bath at 37°C for 2 min. In the second part of first experiment, semen and extender were mixed at the same ratio, but in 2 steps extending over a period of 2 h. The procedure was as follows: semen and extender (without glycerol), were mixed together. The extended semen was cooled to 5°C within 2 h. Thereafter, different concentrations of glycerol (1, 3, 5 and 7%, v/v) were added to the extended semen to arrive a ratio of 1:4 (semen/extender). The samples were packaged in French straws (0.25 mL). The open end of the filled straws was

sealed with polyvinyl chloride powder, were permitted to equilibrate at 5°C for 1 h. According to the first part of experiment 1, the semen was frozen and thawed. The thawed semen assessed to motility, progressive motility, viability and morphological normal acrosome.

The second experiment was designed to investigate the effect of differents concentrations of DMSO (1, 1.25, 1.5 and 1.75%) at two different temperatures. The preparation and procedures of this experiment were according to the first experiment. Because the extenders containing 7% glycerol and 1.75% DMSO were marked as the best extenders, third experiment was designed to compare the effects of best concentrations of glycerol (7%) and DMSO (1.75) added at 37 and 5°C. The preparation and procedures of this experiment were according to the experiment 1 and 2.

Statistical analysis: Data for each experiment were subjected to analysis of variance (ANOVA) after angular transformation of percentages. Analyses were performed using a General Linear Models (GLM) program of SAS (2001). Data are presented as Mean±Pooled Standard Error of Means (SEM). When significant differences were found, means were compared using the Duncan-test. Differences were considered to be statistically different at $P<0.05$.

RESULTS

The average of volume, sperm concentration (10^7 mL^{-1}), mass activity (1-5), motility (%), progressive motility (%), viability (%) and the rate of morphological normal acrosome (%) in the ejaculates of Markhoz goat spermatozoa were 1.67, 3.95, 4.07, 86.13, 80.35, 88.53 and 88.51, respectively (Table 1).

Table 2 shows the effect of the supplementation of Tris-citric acid-fructose-egg yolk extender with different concentrations of glycerol on the motility and progressive motility, viability and morphological normal acrosome of Markhoz goat after freezing and thawing. There was a significant differences in motility, progressive motility and viability of spermatozoa by increasing of glycerol concentrations ($p<0.05$), but the rate of morphological normal acrosome decreased significantly ($p<0.05$). Concerning the temperature, the results show that the temperatures of addition at 37°C in contrast to 5°C affected motility, progressive motility and viability of spermatozoa significantly positive ($p<0.05$). However, the characteristic morphological normal acrosome showed an opposite trend and have been decreased significantly ($p<0.05$) with the increasing of glycerol concentrations.

Table 3 shows the effect of different concentration of DMSO at two temperatures on the spermatozoal characteristics. There was a significant differences in motility, progressive motility and viability of spermatozoa by increasing of DMSO concentrations ($p<0.05$). The results of dilutents containing 1.75% DMSO in both temperatures were significantly higher ($p<0.05$) and 1% lower ($p<0.05$) than other concentrations (1.25 and 1.5%), but the rate of morphological normal acrosome decreased significantly ($p<0.05$) by increasing of DMSO

Table 1: Macroscopic and microscopic characters of seminal plasma Markhoz goat spermatozoa

Seminal characters	n	Mean	SEM	Min- Max
Volume (mL)	56	1.67	0.5	0.6-1.8
Sperm concentration(10^7 mL^{-1})	56	3.95	0.7	1.8-3.6
Mass activity (1-5)	56	4.07	0.6	3-5
Motility (%)	56	86.13	6.6	75-95
Progressive motility (%)	56	80.35	7.6	70-92
Viability (%)	56	88.53	7.3	82-95
Normal acrosome (%)	56	88.51	5.2	86-93

Table 2: Effects of different concentrations of glycerol at two different temperatures on freezed-thawed Markhoz goat sperm

	37°C				5°C			
	1	3	5	7	1	3	5	7
Parameters	(%)							
Motility	43.5±1.1^{cd}	47.6±1.5^{b}	54.4±1.5^{a}	58.5±1.4^{a}	30.9±0.9^{f}	34.6±1.3^{ef}	38.4±0.9^{de}	44.1±1.0^{bc}
Progressive motility	23.1±0.8^{de}	25.2±0.7^{bc}	28.7±1.0^{b}	34.7±1.5^{a}	18.0±0.7^{f}	20.2±0.5^{ef}	21.0±0.7^{def}	23.9±0.7^{cd}
Viability	50.0±1.1^{cd}	55.5±1.5^{b}	61.6±1.0^{a}	65.9±1.0^{a}	45.1±1.5^{d}	47.1±1.2^{d}	49.1±1.0^{cd}	53.2±1.2^{bc}
Normal acrosome	92.6±0.7^{a}	91.6±0.7^{ab}	89.1±0.9^{bc}	88.2±0.8^{c}	93.4±0.5^{a}	93.1±0.5^{a}	91.3±0.7^{ab}	87.9±0.6

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

Table 3: Effects of different concentrations of DMSO at two different temperatures on freezed-thawed Markhoz goat sperm

	37°C				5°C			
	1	1.25	1.5	1.75	1	1.25	1.5	1.75
Parameters	(%)							
Motility	28.7±1.1^{c}	31.1±0.6^{bc}	35.0±1.5^{b}	40.1±1.6^{a}	31.0±1.7^{bc}	34.6±1.4^{b}	35.0±0.7^{b}	41.2±0.5^{a}
Progressive motility	17.0±0.8^{b}	17.1±1.2^{b}	20.4±1.1^{ab}	21.8±1.9^{a}	17.8±1.7^{b}	19.4±1.0^{ab}	21.7±0.4^{a}	22.1±0.4^{a}
Viability	41.2±0.9^{c}	42.9±0.6^{bc}	45.6±2.0^{abc}	51.0±1.4^{a}	42.7±1.1^{bc}	44.7±1.6^{bc}	47.6±2.4^{ab}	50.6±0.7^{a}
Normal acrosome	93.6±0.5^{a}	93.2±0.4^{a}	89.8±0.5^{b}	88.0±1.0^{b}	92.9±0.5^{a}	92.5±0.5^{a}	94.1±0.3^{a}	88.4±0.7

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

Table 4: Effects of glycerol and DMSO at two different temperatures on freezed-thawed Markhoz goat spermatozoa

	37°C		5°C	
Parameters	Glycerol(7%)	DMSO(1.75%)	Glycerol(7%)	DMSO (1.75%)
Motility (%)	54.6±1.7[a]	49.3±1.0[b]	50.4±1.1[b]	46.6±0.6[c]
Progressive motility (%)	37.5±0.5[a]	34.5±0.9[b]	33.0±1.3[b]	31.8±1.0[c]
Viability (%)	58.5±1.3[a]	53.0±0.7[b]	52.9±1.4[b]	49.6±1.2[c]
Normal acrosome (%)	78.6±0.9[c]	80.3±1.3[b]	82.0±0.9[a]	83.5±0.9

Values (Mean±SEM) with different letter(s) in the same row are significantly different ($p<0.05$, Duncan1-test)

concentrations. In addition, comparison of the data of two addition temperatures of DMSO showed significantly the same tendency ($p<0.5$).

Table 4 shows the effect 7% glycerol and 1.75% of DMSO added at 37 and 5°C on the different characteristics of sperm. In regard to motility, progressive motility, viability and acrosome integrity of sperm, the obtained results showed that the extender containing 7% glycerol added at 37°C was significantly ($p<0.05$) better (54.6±1.7; 37.5±0.5 and 58.5±1.3, respectively) than diluent containing 7% glycerol added at 5°C (50.4±1.1, 33.0±1.3 and 52.9±0.4, respectively) and diluents with 1.75% DMSO concentration added at 37°C (49.3±1.0, 34.5±0.9 and 53.0±0.7, respectively) and 5°C (46.6±0.6, 31.8±1.0 and 49.6±1.2, respectively). However, the characteristic normal acrosomes showed an opposite trend, i.e. the results of both diluents containing 7% glycerol (82.0±0.9) and 1.75% DMSO were at 5°C (83.5±0.9) significantly ($p<0.05$) better than glycerol and DMSO added at 37°C (78.6±0.9 and 80.3±1.3, respectively).

DISCUSSION

The survival of sperm during freezing and thawing is dependent on a number of interrelated factors amongst which are freezing techniques, the composition of cryoprotective diluents, dilution methods, cooling rates and thawing methods (Eiman and Terada, 2004). A cryoprotectant, either penetrating or non-penetrating, is included in a medium to minimize the physical and chemical stresses resulting from the freezing and thawing of spermatozoa (Purdy, 2006) and it also seems to be most effective when they can penetrate the cell and delay intracellular freezing and minimize the solution effects (Farrant, 1980).

The present study investigated the effects of different concentrations of glycerol and DMSO added at 37 and 5°C on the motility, progressive motility, viability and the rate of normal acrosome. The obtained results demonstrated that with increasing of glycerol concentrations, added to extenders at both temperatures, the motility, progressive motility and viability of post-thawed sperm have been improved significantly. These observations were in agreement with the findings of other studies which used diluents containing 6-7% glycerol, resulting in higher spermatozoa motility than freezing in extenders containing glycerol lower than 7% (Watson and Martin, 1975; Deka and Rao, 1986; Garcia and Graham, 1987; Evans and Maxwell, 1989; Tuli and Holtz, 1994; Kundu *et al*., 2000; Biswas *et al*., 2002; Bittencourt *et al*., 2004; Rasul *et al*., 2007; Farshad and Akhondzadeh, 2008). Furthermore, the obtained DMSO results in the present study showed similar trend. In regard to motility, progressive motility and viability of freeze-thawed sperm, 1.75% DMSO has been regarded as the optimal concentration and was significantly better ($p<0.05$) than other levels (1, 1.25 and 1.5%). These observations were in agreement with the findings by Vicente and Viudes-de-Castro (1996) and Moce and Vicente, (2002) in which freezing of semen with 1.75% DMSO extender resulted in higher sperm motility. Izadyar *et al*. (2002) observed that the concentrations of 0.07 and 0.14 M DMSO in diluents improved the freezability of sperm.

Glycerol and DMSO have since remained the most commonly used additives for the cryopreservation of the majority of cells, but the mechanism by which these two compounds protect spermatozoa during freezing-thawing process, has not been clearly understood and is not clear whether the protection of an extracellular or intracellular nature, or both (Almlid and Johnson, 1988). It only has been speculated that the action of glycerol and DMSO is mainly attributed to the colligative properties by reducing the salt concentration at a given temperature (Lovelock and Bishop, 1959; Watson and Martin, 1975; Purdy, 2006), the ability to prevent some of the phase transitions by increasing the water permeability and fluidity of sperm plasma membranes, any stimulation of membrane associated with ion pumps (Hammerstedt and Graham, 1992; Noiles *et al*., 1995; Holt, 2000; Rasul *et al*., 2007) decreasing of freezing point of cells (Watson and Martin, 1975; Purdy, 2006) and also dehydrate the sperm and minimize intracellular ice formation, which allow the cells to suffer less injury at that temperature (Watson and Martin, 1975; Vicente and Viudes-de-Castro, 1996; Kundu *et al*., 2002; Moce and Vicente, 2002; Purdy, 2006). However, this does not mean that glycerol and DMSO have no negative effects on sperm. In regard to acrosome integrity, the results of present study indicated that with

increasing of both cryoprotectants in extenders, the rate of intact acrosome decreased significantly ($p<0.05$). These observations were in agreement with findings by Almlid and Johnson (1988), Morrier *et al.* (2002), Sonmez and Demirci (2004) and Si *et al.* (2004), in which the increasing of glycerol the rate of normal acrosome significantly decreased ($p<0.05$), while the rate of motility of sperm increased. Dose-response studies of glycerol in the range of 0 to 7% have demonstrated that acrosomal integrity was negatively affected by increased glycerol concentrations, whereas the opposite was observed for sperm motility. However, there is no available information about the effect of DMSO on the acrosome integrity of goat sperm during freezing and thawing process. On the other hand Rasul *et al.* (2007) suggested that penetrating ability of DMSO into the cell is higher than glycerol, thereby also the lethal effect is attributed to its toxic effect rather than damage caused by osmotic effects. Based on this suggestion and the presented results in this study, DMSO eventually is more toxic than glycerol and therefore decreased the rate of motion characteristics of sperm after freezing and thawing. Against these results, the rates of intact acrosome were slightly better than glycerol, when DMSO used in extenders. The mechanism by which the glycerol and DMSO decrease the intact acrosome percentage cannot be clearly understood based on the results of this study. Purdy (2006) suggested that the decreasing of this characteristic induced by osmotics damage, but the extent of the damage varies according to species. Glycerol alters the plasma membrane bilayers of spermatozoa and interacts with bound proteins and glycoproteins (Hammerstedt and Graham, 1992; Buhr *et al.*, 2001). It also increases the bioenergetic requirements of spermatozoa (Hammerstedt and Graham, 1992) and alters the structural reorganization of sperm head plasma membranes after cryopreservation, which appears to disrupt the ability of the sperm to interact normally with cells of the female genital tract (Bailey *et al.*, 2000). Moreover, glycerol decreases the rate of NA^+-K^+-ATPase and also disrupts the ions balancing (Barenett, 1978) and toxic effects on metabolism of cryopreserved cells (Hammerstedt *et al.*, 1990).

Concerning the temperatures, the addition of glycerol may be performed at 1-, 2- or 3-step methodology at either 30, 37 or 5°C and the results have yielded variable (Salamon and Ritar, 1982; Tuli and Holtz, 1994; Leboeuf *et al.*, 2000). In the present study, the results showed that the single-step glycerolization at 37°C was significantly better than 2-step glycerolization at 5°C. These observations are consist with the findings by Salamon and Ritar (1982), Tuli and Holtz (1994), Sonmez and Dimirci (2004), Silva *et al.* (2006) and Rasul *et al.* (2007). The mechanism by which the glycerol increased the motility of spermatozoa at higher temperature cannot be clearly understood, but it has been speculated that the addition of glycerol at higher temperatures, within physiological limits, facilitates glycerol to across enough the plasma membrane before freezing at a lower temperature (Rasul *et al.*, 2007). Against obtained results in the present study, Morrier *et al.* (2002), Salamon and Mexwell (2000) and Pena *et al.* (1998) found no differences between addition of glycerol at different temperatures.

CONCLUSION

The results of presented study indicated that the increasing of glycerol (1-7%) and DMSO (1-1.75%) concentrations in extenders improved the motility, progressive motility and viability of Markhoz goat sperm after freezing and thawing, when added at 37°C. Concerning acrosome integrity, there was an opposite trend and diluents containing lower concentrations of both cryoprotectants showed better results. Furthermore, the presented results indicated, that glycerol was better than DMSO; particularly, when added to extenders by a single-step method at 37°C. Therefore, it can suggested, that glycerol is still the cryoprotectant of choice for freezing Markhoz goat sperm.

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