Asian-Aust. J. Anim. Sci.
Vol. 21, No. 12 : 1721 - 1727
December 2008
www.ajas.info

Effects of Sucrose and Glycerol during the Freezing Step of Cryopreservation on the Viability of Goat Spermatozoa

A. Farshad* and S. Akhondzadeh
Department of Animal Science, College of Agriculture, University of Kurdistan, Iran

ABSTRACT : Four experiments were conducted to study the following: i) the influence of different concentrations of sucrose (0.15, 0.3 and 0.5 M with osmolality of 308, 500 and 760 mOsm/kg, respectively) in diluents and control diluent (370 mOsm/kg) on intensity of motility and progressive motility of goat sperm without rehydration and freezing step in four incubation periods (0, 0.5, 2 and 4 h after dilution); ii) the influence of gradual dilution (in 3 steps) on improvements in ascertained results of the first experiment; iii) cryoprotective effects of different concentrations of sucrose (0.15, 0.22, 0.29 and 0.37 M with osmolality of 450, 560, 740 and 920 mOsm/kg, respectively) plus 7% glycerol and 20% egg yolk in basic diluent (Tris-Citric acid-Fructose) and iv) the effect of two concentrations of sucrose (0.15 and 0.22 M) with and without glycerol (7%). In experiment 1, we obtained better results for control diluent, 0.15 and 0.3 M sucrose supplemented diluents with 0 and 0.5 h incubation periods. In experiment 2, apart from a slight improvement, similar tendencies to experiment 1 were observed. In experiment 3, we obtained the best result for diluent with 0.22 M sucrose with regard to intensity of motility, progressive motility, live sperm and normal acrosomes (40±4%, 3.1±0.2, 37±4% and 37±4%, repectively). In experiment 4, we obtained the best result for diluent with 0.22 M sucrose plus 7% glycerol in regard to intensity of motility, progressive motility and live sperm (39±3%, 3.6±0.4 and 41±4%, respectively). The characteristic normal acrosomes in diluents without glycerol, i.e. diluents with 0.15 and 0.22 M sucrose showed better results (39±8 and 42±6% respectively). With regard to the release of hyaluronidase enzyme there were no significant differences between diluents ($p>0.05$). The results of the diluents with 0.15 and 0.22 M sucrose without glycerol were slightly lower than those with glycerol (69±11 and 70±11 vs. 72±11 and 70±11 $\times 120\times 10^6$ units ml^{-1}, respectively). In conclusion, the use of concentrated sucrose solutions showed that goat sperm can tolerate osmolality up to 560 mOsm (0.22 M) in the freezing period. In addition, glycerol proved to be a necessary cryoprotective agent in the cryopreservation of goat sperm, particularly for intensity of motility, progressive motility and live sperm. (**Key Words :** Goat, Spermatozoa, Cryopreservation, Osmolality, Sucrose, Glycerol)

INTRODUCTION

Damage to sperm during cryopreservation has been attributed to cold shock, ice crystal formation, oxidative stress, membrane alteration, cryoprotectant toxicity and osmotic changes (Watson, 1995). Regarding the latter, osmotic changes may occur gradually or rapidly, depending upon the cooling rate, whether or not ice nucleation is induced, and the method of adding the cryoprotectants, and involve two confounded stress factors: the final osmolality reached by the cells and the osmotic difference between the intra- and extracellular osmolalities (Curry and Watson, 1994). Sperm sensitivity to these osmotic changes depends not only on membrane water permeability, but also on environment osmolality (Curry et al., 2000). Besides, dehydration and rehydration during cryopreservation have an important impact on sperm, because dehydration decreases the risk of intracellular ice crystal formation, but excessive dehydration is also detrimental too (Hammerstedt et al., 1990; Watson, 1995; Agca et al., 2002). Therefore, adjusting the osmolality of the diluent is of central importance, since it influences the flux of water at the different steps of cryopreservation (Curry and Watson, 1994; Iguer-Ouada and Verstegen, 2001; Aisen et al., 2002).

One of the important factors for the efficacy of an extender is its supplementation with sugars, which have several functions in the extender: They provide energy substrate for sperm cells during incubation, maintain the osmotic pressure of the diluent and act as a cryoprotectant (Yildiz et al., 2000). Storage temperature, molecular weight of the sugar (Molinia et al., 1994) and the type of buffer (Abdelhakeam et al., 1991) used in the extender affect

* Corresponding Author: Abbas Farshad. Tel: +98-9121864426, Fax: +98-8716620550, E-mail: AFarshad@uok.ac.ir
Received March 11, 2008; Accepted June 8, 2008

cryoprotective ability of sugars. Although iso-osmotic diluents are commonly used as a semen extender, hyperosmotic diluents over a wide range of sugar concentrations are known to improve sperm integrity after freezing-thawing (Aisen et al., 2002). During dehydration and rehydration along cryopreservation, sugars may interact with phospholipid membranes at low hydration, stabilizing them. Furthermore, most sugars induce a depression in the membrane phase transition temperature of dry lipids and form a glass (vitrification) during drying (Fernandez-Santos et al., 2007).

Glycerol has been used successfully as a cryoprotectant in the freezing of diluted semen for many years (Fiser and Fairful, 1989). Despite the beneficial effects of glycerol on sperm cryopreservation, it can have direct effects on bioenergetic balance since increased membrane permeability for ions and any stimulation of membrane-associated ion pumps will increase ATP consumption (Hammerstedt and Graham, 1992). Addition of glycerol can induce osmotic damage to spermatozoa, but the extent of the damage varies according to the species. However, goat spermatozoa are reasonably tolerant to these osmotic conditions (Purdy, 2006). With such negative consequences, the use of glycerol is generally recommended only for cryopreservation of semen (Morrier et al., 2002). The post-freezing fertilization rate of ram and goat semen is known to be influenced by method, temperature and rate of addition of glycerolated or non-glycerolated extenders (Tuli and Holtz, 1994). The optimum level of glycerol varies in different extenders and under different sets of conditions that are followed for processing and freezing the semen (Deka and Rao, 1986).

When mammalian spermatozoa are frozen, cellular damage causes leakage of various enzymes as well as other materials. Hyaluronidase enzyme play an important role in the process of fertilization (McRorie and Williams, 1974) and its untimely leakage due to cellular injury is detrimental for the fertilization process. Therefore, estimates of hyaluronidase enzyme have been recommended as a marker for semen quality since this indicates sperm damage during the process of freezing and thawing (Dahami and Kodagali, 1990; Belorkar et al., 1991).

Within this study, an attempt was made to partially dehydrate goat spermatozoa before freezing by means of a concentrated sucrose solution. This reduces the damaging effects of the intracellular formation of ice crystals. In addition, the effects of glycerol as a cryoprotective agent on goat sperm and release of the hyaluronidase enzyme were examined.

MATERIALS AND METHODS

Collection and evaluation of semen

Seventy healthy mature goat bucks, 9 months to 10 years of age and 55-120 kg, were used in the study. Each buck was fed 1.5 kg of concentrate mixture consisting of equal parts of oats and dried sugar beet pulp, with straw, salt lick and fresh water supplied *ad libitum*. Semen was collected twice a week 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, mass activity (undiluted semen), consistency (density judged subjectively), motility (diluted with normal saline), progressive motility, live spermatozoa (eosin-nigrosin stain), normal acrosomes (by viewing wet mounts of diluted spermatozoa fixed in buffered Formalin-Citrate solution) and sperm concentration (sperm cells were counted in four squares of a hemocytometer after 1:200 dilution of semen with 0.5% eosin solution). Ejaculates with a volume of more than 1.5 ml and mass activity higher than 3 (0 to 5 score) were selected, pooled and processed further.

Assessment of spermatozoa characters

The assessment of spermatozoa included intensity of motility, progressive motility, live sperm and normal acrosomes. To evaluate progressive motility, a sample of the diluted spermatozoa was placed under a cover slip in the centre of a pre-warmed (37°C) slide, transferred to a heated microscope stage set at 37°C and subjectively assessed by phase contrast microscopy (×100 magnification). The proportion of spermatozoa with progressive motility was determined using an arbitrary scale of 0 to 5 (0, 1, 2, 3, 4 or 5 = 0 to 10%, 10 to 25%, 25 to 50%, 50 to 70%, 70 to 90% or 90 to 100% of the motile spermatozoa showing progressive motility). The rate of live sperm was performed using a modification of the eosin-nigrosin stain procedure described by Chauhan and Anand (1990). A mixture of 10 μl of diluted spermatozoa and 10 μl eosin-nigrosin stain 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. Normal acrosomes were assessed by viewing wet mounts of diluted spermatozoa fixed in buffered Formalin-Citrate solution (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 100× oil immersion objective and white light. Spermatozoa (n = 200 per slide) were examined and the percentage with normal acrosomes determined.

Experimental procedure

The first experiment was designed to investigate the influence of different concentrations of sucrose (0.15, 0.3

Table 1. Composition of diluents used in experiment

Components	Control diluent	Diluent containing sucrose
Tris (g)	37.86	15
Citric acid (g)	21.13	9
Fructose (g)	10	3
Penicillin G (1,000 IU/ml) and streptomycin sulphate (1 mg/ml)	500	500
Sucrose (M)	0	0.15, 0.3 and 0.5
Distilled water (ml)	1,000	1,000
Osmotic pressure (mOsm/kg)	370	308, 500 and 760
pH	6.9	6.3, 6.3 and 6.5

and 0.5 M with osmolality of 308, 500 and 760 mOsm/kg, respectively) in diluents and control diluent (370 mOsm/kg) on intensity of motility and progressive motility of sperm without rehydration and freezing in four incubation periods (0, 0.5, 2 and 4 h after dilution). A modified solution (Tuli and Holtz, 1992) without egg yolk and glycerol (Table 1) was used as control diluent for this experiment; sperm dilution was performed in a one-step procedure at 37°C at 1:5 ratio and the diluted semen was stored in a water bath at 37°C.

In the second experiment, the effect of gradual dilution (in 3 steps) on improvement of the ascertained results of the first experiment was studied. In this experiment, semen and extender were mixed at the same ratio but in 3 steps extending over a period of 15 min. The procedure was as follows: one part semen and one part extender (both kept at 37°C) were mixed together. 10 min later twice the amount of extender was added. As a third step, 5 min later the last part of the extender was added to arrive at a ratio of one part semen to five parts extender (at 37°C). According to the first experiment, intensity of motility and progressive motility of sperm were assessed.

The third experiment was designed to investigate the cryoprotective effect of different concentrations of sucrose (0.15, 0.22, 0.29 and 0.37 M with osmolality of 450, 560, 740 and 920 mOsm/kg, respectively) plus glycerol (7%) and egg yolk (20%) in basic diluent. In this experiment, one part semen and 2.5 parts of basic extender containing 20% egg yolk were mixed at 37°C in one step. The extended semen was cooled to 5°C within 2 h. Thereafter, 7% glycerol was added to the extended semen and was permitted to equilibrate at 5°C for 1 h. As a next step, extender containing different concentrations of sucrose (in separate test tubes) was added in a 1:2.5 ratio in 3 parts at an interval of 5 min to provide a final extension of 1:5. The extended semen was then packaged in medium French straws (0.5 ml). The open end of the filled straw was sealed with polyvinyl chloride powder. Straws were kept at 5°C for 2 h before being exposed to the vapour of liquid nitrogen (4 cm from the LN_2 surface level) for 10 min. They were then stored in liquid nitrogen for 24 h. The straws were thawed in water at 37°C for 2 min and thereafter, in order to rehydrate, centrifuged at room temperature (800×g, 10 min) to discard the initial extender. The semen was diluted again in a 1:5 ratio and was assessed for intensity of motility, progressive motility, the percentage of live spermatozoa and normal acrosomes.

Because the extender which contained 0.22 M sucrose with 560 mOsm/kg was marked as the best extender in the third experiment, the fourth experiment was designed to investigate the effect of two concentrations of sucrose (0.15 and 0.22 M) with and without glycerol (7%). Semen samples were prepared, frozen and thawed as described in experiment 3. Intensity of motility, progressive motility, the percentage of live spermatozoa, normal acrosomes and hyaluronidase release (120×10^6 Units ml^{-1}) were measured after thawing. The method described by Foulkes and Watson (1995) was used to measure the concentration of released hyaluronidase enzyme.

Statistical analysis

Data for each experiment were subjected to analysis of variance (ANOVA) after angular transformation of percentages. Analyses were performed using the general

Table 2. Least squares means (Mean±SEM) of macroscopic and microscopic seminal characters of ejaculates of goat sperm

Seminal characters	n	Mean	SEM	Min	Max
Volume (ml)	230	1.5	0.6	0.3	0.4
Consistency (1-5)	230	3.4	0.4	2.5	4
Sperm density (10^9/ml)	230	2.9	0.9	1.1	5.6
Mass activity (0-5)	126	3.4	0.6	0.2	0.5
Motility (%)	242	74	7.3	60	90
Progressive motility (%)	242	4.2	0.5	0.3	0.5
Live sperm (%)	45*	79	8.4	64	95
Normal acrosome (%)	45*	87	4.4	77	98

* The semen samples were pooled.

Table 3. Effect of sucrose concentration in the diluent on the intensity of motility during incubation (one step dilution, n = 10)

Incubation period (h)	Control diluent (0)	0.15	0.3	0.5
After dilution (0)	70±4^{aA}	68±5^{aA}	66±4^{aA}	9±3^{bA}
0.5	71±5^{aA}	68±5^{aA}	65±3^{aA}	8±3^{bA}
2	69±5^{aA}	63±4^{abAB}	58±3^{bAB}	5±5^{cA}
4	63±5^{aA}	56±3^{abB}	54±4^{bB}	3±2^{cA}

Means in a row and column without a common small and capital letter, respectively, differ significantly (p<0.05, Tukey-test).

Table 4. Effect of sucrose concentration in the diluent on the progressive motility of sperm during incubation (one step dilution, n = 10)

Incubation period (h)	Sucrose (M)			
	Control diluent (0)	0.15	0.3	0.5
After dilution (0)	4.0±0.4^{aA}	3.8±0.5^{aA}	3.6±0.5^{aA}	1.3±0.1^{bA}
0.5	3.9±0.3^{aA}	4.0±0.6^{aA}	3.9±0.4^{aA}	1.0±0.0^{bB}
2	3.0±0.3^{aB}	3.0±0.7^{aB}	3.0±0.6^{aB}	1.0±0.0^{bB}
4	2.8±0.4^{aB}	2.8±0.4^{aB}	2.6±0.6^{aB}	1.0±0.0^{bB}

Means in a row and column without a common small and capital letter, respectively, differ significantly (p<0.05, Tukey-test).

linear models (GLM) program of SAS (SAS Institute Inc., 1999). Data are presented as means±pooled standard error of means (SEM). When significant differences were found, means were compared using the Tukey test. Differences were considered to be statistically different at p<0.05.

RESULTS

Macroscopic and microscopic seminal characteristics in primary evaluation are presented in Table 2. The average of consistency, sperm density (10^9/ml), mass activity, motility (%), progressive motility (%), live sperm (%) and normal acrosome (%) in the ejaculates of goat sperm were 3.4, 2.9, 3.4, 74, 4.2, 79 and 87, respectively.

In Experiment 1, the effect of sucrose concentrations in the diluents on the intensity of motility and progressive motility of sperm during incubation are presented in Table 3 and 4, respectively. An increasing concentration of sucrose and a prolongation of the incubation period reduced the intensity of motility as well as the progressive motility. The negative effect of the diluent with 0.5 M sucrose concentration was significantly higher (p>0.05) than other concentrations of sucrose. Concerning the incubation period, there were no significant differences in progressive motility (p>0.05) between 0, 0.5 and 2 h. Significant differences (p<0.05) appeared after 4 h of incubation in contrast to 0 and 0.5 h. The intensity of motility after 0 and 0.5 h of incubation was significantly (p<0.05) better than after 2 and 4 h.

Subsequently it was examined within experiment 2 whether a gradual dilution (in 3 steps) would lead to an improvement of the ascertained results. The results of this experiment are presented in Table 5 and 6. The results showed, apart from a slight improvement, similar tendencies to those observed in experiment 1.

Experiment 3 examined the impact of different concentration of sucrose (added at 5°C) in basic diluent and the results are summarized in Table 7. Diluent with 0.22 M sucrose proved to be better than other diluents regarding intensity of motility and progressive motility. Some of the

Table 5. Effect of sucrose concentration in the diluent on the intensity of motility during incubation (gradual dilution, n = 10)

Incubation period (h)	Sucrose (M)			
	Control diluent (0)	0.15	0.3	0.5
After dilution (0)	73±3^{aA}	72±6^{aA}	72±3^{aA}	6±3^{bA}
0.5	71±3^{aA}	71±3^{aA}	67±4^{bB}	4±3^{cA}
2	70±2^{aAB}	69±3^{abAB}	66±3^{bBC}	2±2^{cB}
4	66±2^{aB}	66±4^{aB}	62±4^{bC}	2±1^{cB}

Means in a row and column without a common small and capital letter, respectively, differ significantly (p<0.05, Tukey-test).

Table 6. Effect of sucrose concentration in the diluent on the progressive motility of sperm during incubation (gradual dilution, n = 10)

Incubation period (h)	Sucrose (M)			
	Control diluent (0)	0.15	0.3	0.5
After dilution (0)	4.0±0.4^{aA}	4.0±0.6^{aA}	4.0±0.5^{aA}	1.0±0.0^{bA}
0.5	3.9±0.4^{abAB}	4.0±0.4^{aA}	3.6±0.5^{bA}	1.0±0.0^{cA}
2	3.4±0.3^{aBC}	3.4±0.4^{aBC}	3.0±0.4^{aB}	1.0±0.0^{bA}
4	3.0±0.3^{aC}	3.2±0.3^{aC}	2.9±0.3^{aB}	1.0±0.0^{bB}

Means in a row and column without a common small and capital letter, respectively, differ significantly (p<0.05, Tukey-test).

Table 7. Semen characteristics in different sucrose concentration after freezing-thawing (n = 10)

Sucrose (M)	Intensity of motility (%)	Progressive motility (0-5)	Live sperm (%)	Normal acrosome (%)
Control diluent (0)	34±6^{ab}	2.8±0.3^{a}	33±4^{ab}	28±7^{bc}
0.15	34±6^{ab}	2.4±0.6^{a}	33±4^{ab}	34±3^{ab}
0.22	40±4^{a}	3.1±0.2^{a}	37±4^{a}	37±4^{a}
0.29	29±36^{b}	2.7±0.4^{a}	29±4^{bc}	30±4^{abc}
0.37	14±3^{c}	1.9±0.4^{b}	25±5^{c}	26±7^{c}

Means in a column without a common letter differ significantly (p<0.05, Tukey test).

differences were significant (p<0.05). Diluent with 0.37 M sucrose also differed significantly (p<0.05) from the other diluents (control diluent, 0.15 and 0.29 M sucrose). This could also be ascertained with the intensity of motility and the percentage of live sperm. Concerning normal acrosomes, 0.22 M sucrose differed significantly (p<0.05) from the control diluent and 0.37 M sucrose,but only differed by tendency from 0.15 and 0.29 M sucrose concentration.

Experiment 4 dealt with the impact of the addition of glycerol to diluents with different sucrose concentrations and the results are summarized in Table 8. Diluent with 0.22 M plus glycerol proved to be significantly (p<0.05) better regarding the progressive motility than the other diluents. With regard to the intensity of motility and the percentage of live sperm similar results could be ascertained. However, the characteristic "normal acrosomes" showed an opposite trend, i.e. the diluents without glycerol showed better results (p<0.05). Effects of diluents on extracellular activity of hyaluronidase enzyme were not significant (p>0.05) at post-freeze stage, but the results of the diluents with 0.15 and 0.22 M sucrose without glycerol were slightly lower than those with glycerol.

DISCUSSION

The purpose of this study was to investigate whether presence of sucrose with and without glycerol would improve the intensity of motility, progressive motility, live sperm and normal acrosomes of buck sperm during freezing and thawing in basic diluent. The results showed that extender osmolality influenced sperm physiology and characters. Trehalose and sucrose probably play a key role in preventing deleterious alteration to the membrane during reduced water states (Aboagla and Terada, 2003). Moreover, disaccharides have been reported to be effective in stabilizing membrane bilayers (De Leeuw et al., 1993). A beneficial effect of sugar supplementation of the extender on the post-thaw viability of mammalian sperm cells has been reported in many studies (Garcia and Graham, 1989; Aslam et al., 1992). Our results showed that hypertonic extender (up to 560 mOsm/kg) improved the intensity of motility, progressive motility, live sperm and normal acrosomes. In agreement with our finding, Abdelhakeam et al. (1991) reported that although maximum survival of ram spermatozoa before freezing was obtained with an isotonic extender, a hypertonic extender gave the highest post-thaw motility. Joshi et al. (2006) found out that ram spermatozoa could tolerate a wide osmolality range for dilution. Also, these results agree with Fiser and Langford (1980) and Fiser et al. (1982) who reported that hypertonic diluents are superior to isotonic diluents. Increases in the permeability of the plasma membrane to water may allow formation of intercellular ice crystals during freezing which could damage the sperm structure during thawing. Freezing in hypertonic solution reduces freezable water within the cell aiding in prevention of intercellular ice formation (Fiser et al., 1981). Other studies indicated that sugars could interact directly with membrane lipids and proteins, altering their phase transition behaviour and hydration state (Aboagla and Terada, 2003). Many authors have shown that sugars also protect against the damage occurring to sperm cells during freezing-thawing, although this protection depends on many factors, such as storage temperature (Lapwood and Martin, 1966), molecular weight of sugar (Molinia et al., 1994) and the type of buffer used (Abdelhakeam et al., 1991). Environment osmolality is a significant factor when semen is cryopreserved. Not only do anisoosmotic solutions induce cellular stress, but they also influence the outcome of the cryopreservation process. However, this influence can be positive, since the osmolality of the environment

Table 8. Semen characteristics in different sucrose concentration after freezing-thawing (n = 10)

Diluent	Intensity of motility (%)	Progressive motility (0-5)	Live sperm (%)	Normal acrosome (%)	Hyaluronidase (120×10^{6} units ml^{-1})
Control diluent (0 M)+glycerol (7%)	31±4^{b}	2.7±0.3^{b}	32±2^{b}	29±3^{c}	79±11^{a}
Sucrose (0.15 M)	28±5^{b}	2.8±0.3^{b}	32±4^{b}	39±8^{ab}	69±11^{a}
Sucrose (0.15 M)+glycerol (7%)	33±4^{b}	3.1±0.3^{b}	32±5^{b}	33±5^{bc}	72±11^{a}
Sucrose (0.22 M)	30±3^{b}	2.9±0.2^{b}	35±3^{b}	42±6^{a}	70±11^{a}
Sucrose (0.22 M)+glycerol (7%)	39±3^{a}	3.6±0.4^{a}	41±4^{a}	36±6^{b}	73±10^{a}

Means in a column without a common letter differ significantly (p<0.05, Tukey test).

modifies water flux through the membrane and other events during the freezing and thawing. Indeed, it is well known that there are important osmotic changes along the freezing-thawing processes, due to the differential freezing of the medium, that produce water flux through the cell membrane (Curry and Watson, 1994). The initial extender osmolality influences the course of these osmotic changes (Liu et al., 1998). The results of this study revealed that although sucrose plus glycerol proved better with regard to intensity of motility, progressive motility and live sperm, the diluents without glycerol produced better results concerning normal acrosomes. In agreement with our findings, Molinia et al. (1994) obtained better post-thawing recovery of ram spermatozoa in diluents without glycerol that contained sucrose or trehalose. They reported that glycerol had probably masked the cryoprotective action of disaccharides. This preferential croprotective action of glycerol may be attributable to its ability to penetrate the ram sperm membrane more readily than the high molecular weight disaccharides. Although glycerol is widely used as a cryoprotectant for a variety of mammalian cells, its mechanism of action is still not clear. The main cause of cell damage is the formation of intra- and extacellular ice crystals during freezing. Glycerol is believed to protect the cells by minimizing ice crystal formation. Glycerol (CH_2OH-$CHOH$-CH_2OH) has three OH groups and hydrogen atoms of these OH groups are likely to form H-bonding with the oxygen atoms of the phosphate groups of membrane phospholipids (Kundu et al., 2000). Fiser and Fairfull (1986) stated that glycerol provides cryopreservation partly on a colligative basis by reducing the amount of ice formation and partly kinetically by increasing the time for water to leave the cell in response to the decreased vapor pressure of adjacent ice. The results of this study showed that diluents without glycerol give the better results concerning release of hyaluronidase, akin similar to normal acrosomes. Hyaluronidase release into seminal plasma from the acrosome is an early and sensitive indicator of acrosome damage during processing (Sirat et al., 1996) and freezing (Foulkes and Watson., 1995).

CONCLUSION

In conclusion, substitution of Tris-Citric acid diluent with sucrose was found to significantly improve the freezability of goat spermatozoa, which were assessed for intensity of motility, progressive motility, live sperm and normal acrosomes. Furthermore, with the use of concentrated sucrose spermatozoa can tolerate osmolality up to 560 mOsm. A short equilibration (i.e. 30 min) proved to be optimal. In addition, glycerol proved to be a necessary cryoprotective agent in the cryopreservation of goat sperm, particularly for intensity of motility, progressive motility and live sperm.

REFERENCES

Abdelhakeam, A. A., E. F. Graham, I. A. Vazquez and K. M. Chaloner. 1991. Studies on the absence of glycerol in unfrozen and frozen ram semen: Development of an extender for freezing: Effects of osmotic pressure, egg yolk levels, type of sugars, and the method of dilution. Cryobiol. 28:43-49.

Aboagla, E. M. and T. Terada. 2003. Trehalose enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol. Reprod. 69:1245-1250.

Agca, Y., J. Gilmore, M. Byers, E. J. Woods, J. Liu and J. K. Critser. 2002. Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars. Biol. Reprod. 67:1493-1501.

Aisen, E. G., V. H. Medina and A. Venturino. 2002. Cryopreservation and post thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenol. 57:1801-1808.

Aslam, M., K. M. Ahmad, M. Ahmad and S. A. Gill. 1992. Additive effects of carbohydrates in Tris as bull semen extenders equilibrated for three or five hours. Pakistan Vet. J. 12:174-177.

Belorkar, P. M., A. J. Dhami and S. B. kodagali. 1991. Physiological norms and correlation of seminal characters, biochemical constituents, freezablity and fertility in crossbred bulls. Indian Vet. Med. J. 15:191-197.

Chauhan, M. S. and S. R. Anand. 1990. Effect of egg yolk lipids on the freezing of goat semen. Theriogenol. 34:1003-1013.

Curry, M. R. and P. F. Watson. 1994. Osmotic effects on ram and human sperm membranes in relation to thawing injury. Cryobiol. 31:39-46.

Curry, M. R., F. W. Kleinhans and P. F. Watson. 2000. Measurement of the water permeability of the membranes of boar, ram, and rabbit spermatozoa using concentration-dependent self-quenching of an entrapped fluorophore. Cryobiol. 41:167-173.

Deka, B. C. and A. R. Rao. 1986. Effect of glycerol level in Tris-based extender and equilibration period on quality of frozen goat semen. Theriogenol. 26:231-238.

De Leeuw, F. E., A. M. De Leeuw, J. H. Den Daas, B. Colenbrander and A. J. Verkleij. 1993. Effects of various cryoprotective agents and membrane- stabilizing compounds on bull sperm membrane integrity after cooling and freezing. Cryobiol. 30:32-44.

Dhami, A. J. and S. B. Kodagali. 1990. Freezability, enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. Theriogenol. 34:835-863.

Fernandez-Santos, M. R., F. Martinez-Pastor, V. Garcia-Macias, M. C. Esteso, A. J. Soler, P. de Paz, L. Anel and J. J. Garde. 2007. Extender osmolality and sugar supplementation exert a complex effect on the cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa. Theriogenol. 67:738-753.

Fiser, P. S. and G. A. Langford. 1980. Cryosurvival of ram sperm influenced by diluent osmolality. Fertil. Steril. 36:265-266.

Fiser, P. S., L. Ainsworth and G. A. Langford. 1981. Effect of osmolality of skim diluents and thawing rate on cryopreservation of ram spermatozoa. Cryobiol. 18:399-403.

Fiser, P. S., L. Ainsworth and R. W. Fairfull. 1982. Cryosurvival of ram spermatozoa in hypertonic and isotonic diluents. Canad. J. Anim. Sci. 62:425-428.

Fiser, P. S. and R. W. Fairfull. 1986. Combined effects of glycerol concentration, cooling, velocity, and osmolality of skim milk diluents on cropreservation of ram spermatozoa. Theriogenol. 25:473-484.

Fiser, P. S. and R. W. Fairful. 1989. The effect of glycerol related osmotic changes on post thaw motility and acrosomal integrity of ram spermatozoa. Cryobiol. 26:64-69.

Foulkes, J. A. and P. A. Watson. 1995. Hyaluronidase activity in seminal plasma as a method of assessing bull sperm integrity. J. Reprod. Fert. 43:349-353.

Garcia, M. A. and E. F. Graham. 1989. Development of a buffer system for dialysis of bovine spermatozoa before freezing. II. Effects of sugars and sugar alcohols on post thaw motility. Theriogenol. 31:1029-1037.

Hammerstedt, R. H., J. K. Graham and J. P. Nolan. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J. Androl. 11:73-88.

Hammerstedt, R. H. and J. K. Graham. 1992. Cryopreservation of poultry sperm: The enigma of glycerol. Cryobiol. 29:26-38.

Iguer-Ouada, M. and J. P. Verstegen. 2001. Long-term preservation of chilled canine semen: effect of commercial and laboratory prepared extenders. Theriogenol. 55:671-684.

Joshi, A., A. K. Mathur, S. M. K. Naqvi and J. P. Mittal. 2006. Influence of osmolality of complete semen extender on motion characteristics of frozen-thawed ram spermatozoa. Asian-Aust. J. Anim. Sci. 19:1716-1721.

Kundu, C. N., J. Chakraborty, P. Dutta, D. Bhattacharyya, A. Ghosh and G. C. Majumder. 2000. Development of a simple sperm cryopreservation model using a chemically defined medium and goat cauda epididymal spermatozoa. Cryobiol. 40:117-125.

Lapwood, K. R. and I. C. Martin. 1966. The use of monosaccharides, disaccharides, and trisaccharides in synthetic dilutents for the storage of ram spermatozoa at 37°C and 5°C. Aust. J. Biol. Sci. 19:655-671.

Liu, Z., R. H. Foote and C. C. Brockett. 1998. Survival of bull sperm frozen at different rates in media varying in osmolarity. Cryobiol. 37:219-230.

McRorie, R. A. and W. L. Williams. 1974. Biochemistry of mammalian fertilization. Ann. Rev. Biochem. 43:777(Abstr.).

Molinia, F. C., G. Evans, P. I. Casares and W. M. C. Maxwell. 1994. Effect of monosaccharides and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. Anim. Reprod. Sci. 36:113-122.

Morrier, A., F. Castonguay and J. L. Bailey. 2002. Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa. Canad. J. Anim. Sci. 82:347-356.

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

Sirat, M. P., A. K. Sinha, B. K. Singh and R. L. Prasad. 1996. Effect of cryoprotectants on release of various enzymes from buck spermatozoa during freezing. Theriogenol. 45:405-416.

SAS Institute, Inc. 1999. SAS/STAT1 user's guide International. Version 8.02 TS level 02MO Cary NC: SAS Institute, USA.

Tuli, R. K. and W. Holtz. 1992. The effect of zwitterions buffers on the freezability of Boer goat semen. Theriogenol. 37:947-951.

Tuli, R. K. and W. Holtz. 1994. Effect of glycerolization procedure and removal of seminal plasma on post-thaw survival and GOT-release from Boer goat spermatozoa. Theriogenol. 42:547-555.

Watson, P. F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post thawing function. Reprod. Fertil. Dev. 7:871-891.

Weitze, K. F. 1977. Untersuchungen zur Tiefgefrierkonservierung von Kaninchensperma. Habil-Schr, Hannover, Tieraerztl. Hochschule.

Yildiz, C., A. Kaya, M. Aksoy and T. Tekeli. 2000. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. Theriogenol. 54:579-585.