Journal of Livestock Science and Technologies, **2017, 5 (2): 29-34** ***http://lst.uk.ac.ir***

DOI: 10.22103/jlst.2017.9423.1169

Effects of storage duration at 5 ºC and type of cryoprotectant on cooled and frozen-thawed ram epididymal spermatozoa

A. Farshad*, F. Zarei and A. Farzinpour

Department of Animal Science, College of Agriculture, University of Kurdistan, Sanandaj-Iran.

*Corresponding author, E-mail address: afarshad@uok.ac.ir

Abstract Freezing of ejaculated and epididymal spermatozoa is currently a subject of interest with the purpose of establishing an efficient gene banking model for valuable animals or endangered species. Therefore, the present study evaluated the influence of different storage duration (0, 1.5 and 5 h) at 5 ºC and type of cryoprotectant on freezability of ram epididymal spermatozoa. With increasing the storage duration from 0 to 3 h at 5 ºC, the motility, progressive motility, viability and recovery rate of stored spermatozoa decreased ($P<0.05$), but acrosome integrity and hyaluronidase activity were not affected ($P>0.05$). Addition of trehalose and sucrose to the basic diluent improved ($P<0.05$) the quality of frozen-thawed spermatozoa. Sperm motility, progressive motility, viability and hyaluronidase activity were higher in the present of DMSO than the control and BSA. There was no difference in the acrosomal integrity between extenders. In conclusion, quality of froze-thawed spermatozoa was not improved when the extender was supplemented with sugar. However, further studies are needed to determine the fertility of frozen-thawed epididymal spermatozoa.

Keywords: epididymis, spermatozoa, ram, freezing, antioxidant

Received: 09 Nov. 2016, *accepted:* 14 Jul. 2017, *published online:* 25 Dec. 2017

Introduction

Freezing and thawing cause partial irreversible damage to sperm membrane, which decrease sperm quality including the motility and viability (Watson, 1995, 2000). Disruption of plasma membrane and acrosomal reaction are some of the main forms of damage induced by cryopreservation (Drobnis et al., 1993; Purdy et al., 2005).

Foote (2000) reported that the recovered motile sperm from the epididymides of bulls, boars, rams and stallions were widely used for artificial insemination after freezing and thawing process. Johnson et al. (1980) and Varisli et al. (2009) hypothesized that epididymal spermatozoa are more resistant to cold shock and should be more suitable for cryopreservation than ejaculated spermatozoa. Foote (2000) reported that epididymal sperm of bulls remain fully functional for long times at 5 ºC. Also, Kikuchi et al., 1998 and Kishikawa et al., 1999 suggested that temporary storage of epididymides at 4 ºC may be helpful in preserving the genome of different species.

Many studies have been carried out to study the effect of cryopreservation process on epididymal spermatozoa in several species (Nagai et al. 1988; Nakagata, 1992; Hishinuma et al., 2003; Kaabi et al. 2003; Monteiro et al., 2011; Alvarez et al., 2012; Karja et al., 2010). However, there are few available reports on the effects of cooling duration or type of cryoprotectant on epididymal spermatozoa. Therefore, in this study, an attempt was made to determine the effect of storage length (0, 1.5 and 3 h) at 5 ºC and several cryoprotectants such as trehalose, sucrose, bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) on post-thaw characteristics of ram epididymal spermatozoa.

Material and methods

Animal and location

Testes from 6 healthy mature rams aged between 3 to 4 years were obtained from a local abattoir in Sanandaj (35° 20' N latitude and 47° E longitude) and transported in a cool insulated container to the laboratory within 30 min.

Preparation medium and freezing diluent

The preparation medium for sperm isolation from the epididymis was Tyrode's solution containing 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.29 mM $NaH2PO_4$, 21.6 mM Na lactate, 2.1 mM $CaCl_2$ $2H_2O$, 0.4 mM $MgCl_2$ 6H2O, 10 mM HEPES buffer, 0.006 g/ml

BSA, 1 mM sodium pyruvate, 25 mg/mL gentamycin, 2 mg/L phenol red and pH= 7.4 (Parrish et al., 1988).

The basic extender (tris-citric acid-fructose) contained: tris (3.634 g), fructose (0.50 g), citric acid (1.99 g), egg yolk (14%, v/v), streptomycin (100 ml/g), and penicillin G (100000 IU), pH=7 (Evans and Maxwell,1987). The freezing extender was supplemented with 5% (v/v) glycerol.

Experimental procedure

In the first part of study, the effects of incubation duration (0, 1.5 and 3 h) at 5 ºC on ram epididymal sperm characteristics were determined. The cauda epididymis was dissected free from each testis and sliced repeatedly with a scalpel blade (Parrish et al., 1988). To release the epididymal spermatozoa, the tissues were placed in Tyrode's medium and centrifuged at 700 g for 6 min. The supernatant was removed and spermatozoa were evaluated for motility, progressive motility, viability, acrosomal integrity, recovery rate and released hyaluronidase enzyme.

The second part of the study compared the effects of addition of sucrose (80 mM), trehalose (100 mM), bovine serum albumin (BSA, 5%) and dimethyl sulfoxide (DMSO, 1.75%) to basic tris–citric acid–fructose–yolk medium on post-thaw epididymal spermatozoa. The dilution process was performed in one-step at 37 ºC in 1:4 ratio (Evans and Maxwell, 1987), sperm packaged in French straws (0.5 ml) and the open end of the filled straws sealed with polyvinyl chloride powder. The straws were then kept at 5 ºC for 3.5 h before being held at 4 cm from liquid nitrogen surface level for 10 min. After 24 h, the frozen samples were thawed in a water bath at 37 ºC for 30 seconds and assessed for motility, progressive motility and viability. Acrosomal integrity and hyaluronidase activity ($120x10^6$ Units ml^{-1}) were measured according to the methods described by Foulkes and Watson (1995). Sperm recovery rate was computed using the following equation:

Recovery rate = (motility after freezing/motility before freezing) x 100

Statistical analysis

The experiments were conducted as a completely randomized design, and data subjected to analysis of variance (ANOVA) after angular transformation of percentages. Statistical analysis of data was performed by the GLM procedure of SAS (1996). All percentage data were presented as mean ± pooled standard error of the means (SEM). Means were compared using the Duncan's multiple range test at ($P<0.05$).

Results

The average macroscopic and microscopic characteristics of ram epididymal sperm are presented in Table 1. Figures 1-3 present the results of storage duration on motility, progressive motility, viability, acrosomal integrity, recovery rate and hyaluronidase activity of ram epididymis spermatozoa. Increasing the storage duration up to 3 h decreased ($P<0.05$) the rates of motility, progressive motility, viability and recovery rate, with no differences between 1.5 up to 3 h. Storage duration up to 3 h did not influence acrosomal integrity and hyaluronidase activity.

Table 3 shows the effects of trehalose, sucrose, BSA and DMSO in the extender on frozen-thawed ram epididymal spermatozoa. Trehalose and sucrose resulted in higher motility, progressive motility, viability, recovery rate and lower hyaluronidase activity compared to extenders containing BSA and DMSO. Acrosomal integrity was superior in the presence of trehalose compared to other diluents

Discussion

Motility, progressive motility, viability and normal acrosome in epididymal spermatozoa of ram were almost the same as that for semen collected using artificial vagina (Khalili et al., 2010). The results are in agreement with those of Amann et al. (1982), Kaabi et al. (2003) and Karja et al. (2010).

Irrespective of the extender used, conditions, temperature of storage, and extension rate, the quality of sp-

Table 1. Macroscopic and microscopic characteristics of ram epididymal spermatozoa

	n	Mean	Min-Max	SEM
Concentration ($x10^9 mL^{-1}$)	16	2.9	2.3-3.5	0.15
Motility (%)	16	84.9	70-80	1.14
Progressive motility (%)	16	70.9	70-71	1.83
Viability (%)	16	77.9	72-84	1.41
Acrosome integrity (%)	16	96.8	94-98	0.45

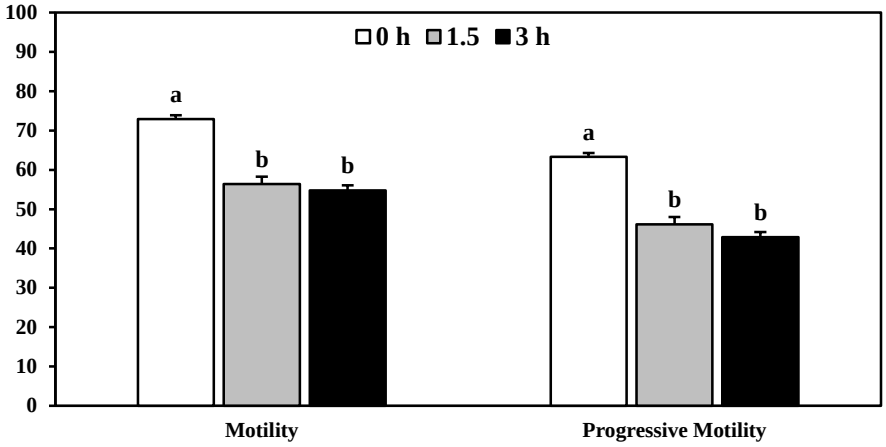

Figure 1. Effect of storage duration at 5 °C on motility (%) and progressive motility (%) of ram epididymal spermatozoa (Mean±SEM, n=16). Common letters (a, b) on bars indicate no significant difference (P>0.05, Duncant's multiple range test).

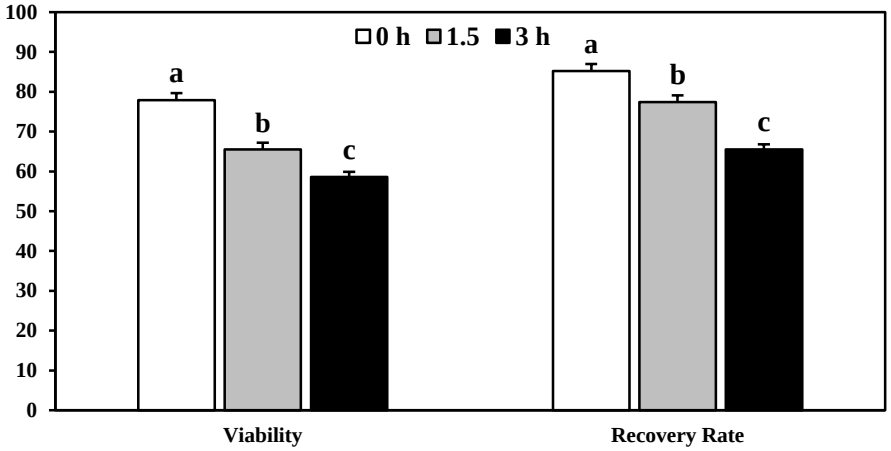

Figure 2. Effect of storage duration at 5 °C on viability (%) and recovery rate (%) of ram epididymal spermatozoa (Mean±SEM, n=16). Common letters (a, b) on bars indicate no significant difference (P>0.05, Duncant's multiple range test).

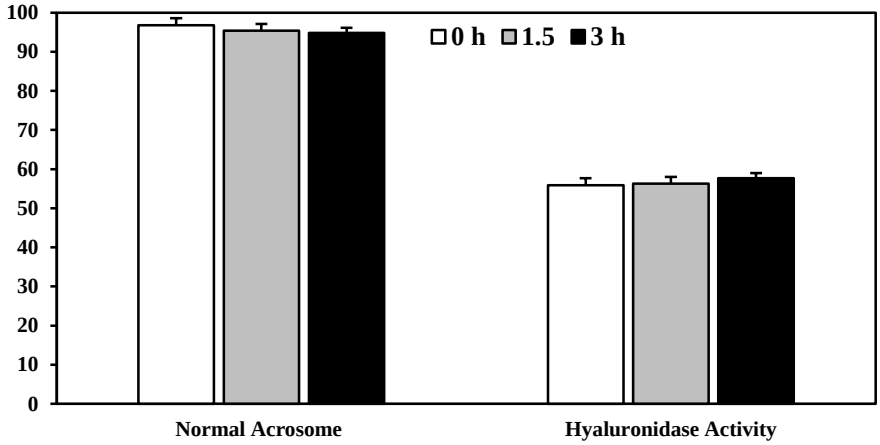

Figure 3. Effect of storage duration at 5 °C on normal acrosome (%) hyaluronidase activity (%) of ram epididymal spermatozoa (Mean±SEM, n=16). Common letters (a, b) on bars indicate no significant difference (P>0.05, Duncant's multiple range test).

Table 2. Effect of type of cryoprotectant on post-thaw ram epididymal sperm (n=16)

Cryoprotectant					
Characteristic	Control	Trehalose	Sucrose	BSA†	DMSO††
Motility (%)	58.8 ± 2.6^{b}	67.1 ± 1.7^{a}	65.7 ± 2.7^{a}	54.2 ± 2.5^{bc}	47.9 ± 3.5^{c}
Progressive motility (%)	66.5 ± 3.7^{ab}	73.1 ± 2.4^{a}	68.8 ± 3.9^{ab}	61.3 ± 4.6^{bc}	51.0 ± 3.5^{c}
Viability (%)	62.9 ± 2.2^{b}	70.9 ± 2.7^{a}	70.3 ± 1.2^{a}	60.1 ± 2.2^{b}	56.1 ± 3.7^{b}
Normal acrosome (%)	69.1 ± 1.1^{b}	75.5 ± 1.6^{a}	71.5 ± 1.3^{b}	71.0 ± 1.1^{b}	68.5 ± 1.5^{b}
Recovery rate (%)	63.0 ± 1.7^{b}	71.4 ± 2.1^{a}	66.7 ± 2.5^{ab}	56.2 ± 1.9^{c}	53.7 ± 2.6^{c}
Hyaluronidase activity (120 x 10^6 units/mL)	63.7 ± 2.4^{b}	57.7 ± 6.3^{a}	59.3 ± 4.3^{ab}	67.8 ± 2.6^{bc}	71.5 ± 4.5^{c}

Means with common letter (s) in the same row are not significantly different (P> 0.05; Duncan's multiple range test), † BSA= bovine serum albumin, †† DMSO= dimethyl sulfoxide

ermatozoa deteriorates as the duration of storage increases (Salamon and Maxwell, 2000). The results of this study indicated a negative effect on motility, progressive motility, viability, normal acrosome, recovery rate and hyaluronidase activity when the storage duration increased to 3 h at 5 ºC. These finding are similar to the results of O'Hara et al. (2010) and Aguado et al. (1994). However, Kaabi et al. (2003) showed that storage duration up to 48 h was detrimental to spermatozoa, but the storage at 5 ºC resulted in better quality compared to room temperature. Based on our observations and those of other researchers, we can hypothesize that ram epididymal spermatozoa could be used for artificial insemination within 24 h storage.

Addition of trehalose to the basic diluent had a beneficial effect on sperm motility, progressive motility, viability, recovery rate and hyaluronidase activity compared to the control extender and the diluents supplemented with DMSO and BSA with no differences between trehalose and sucrose, except acrosomal integrity. Trehalose was superior to sucrose in preserving the acrosomal integrity. Previous studies also demonstrated the positive effects of trehalose and sucrose on sperm quality in goats (Farshad and Akhondzadeh, 2008; Aboagla and Terada, 2003; Khalili et al., 2009), bull (Foote et al., 1993; Chen et al., 1993; Woelders et al. (1997), mouse (McGinnis et al (2005), dog (Yildiz et al., 2000) and rabbit (Vicente and Viudes-de-Castro, 1996). It is noteworthy that the extenders used in these studies as well the one used in the present work were hypertonic diluents that are known to be superior to isotonic diluents (Abdelhakem et al., 1991, Molinia et al., 1994, Aisen et al., 2002, Bucak and Necmettin, 2007).

The exact mechanism by which hypertonic diluents preserve the integrity of the sperm membrane is still not clearly understood. However, it is hypothesized that trehalose and/or sucrose penetrate into the sperm cell membrane and form hydrogen bonds with the polar head groups of the membrane phospholipids, reorganize the sperm membrane with increasing its fluidity and decreasing the membrane phase transition temperature and form a glass drying (Liu et al., 1998, Molinia et al., 1994, Aisen et al., 2002, Aboagla and Terada, 2003). In addition, Aisen et al. (2002); Aboagla and Terada (2003) and Purdy (2006) suggested that hypertonic extenders play a key role in protecting the sperm membrane from deleterious effects of cryopreservation by creating an osmotic pressure resulting in cell dehydration and decreasing the detrimental effect of intracellular ice formation.

Conclusions

The results of this study showed that ram epididymal spermatozoa can be isolated, stored and cryopreserved successfully. However, the increasing in storage duration decreased the quality of spermatozoa. Moreover, the results showed clearly the tolerance of frozen-thawed epididymal spermatozoa in hypertonic diluents supplemented with trehalose and/or sucrose compared to isotonic extenders containing BSA and DMSO. In particular, acrosomal integrity and hyaluronidase activity, which play important roles in fertilization, were better maintained. However, further studies are needed to determine the fertility of processed spermatozoa.

References

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

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

Aguado, M.J., Garde, J., Madriadano, J.M., Perez, S., Garrido, D., Montoro V., 1994. Congelacio´n post mortem de semen de epidıdimo de morueco. Proceedings of the 7th Jornadas Int Reprod Anim e IA, Murcia, Spain.

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

Alvareza, M., Tamayo-Canula, J., Martinez-Rodrigueza, C., Lopez-Uruena, E., Gomes-Alvesa, S., Anela, L., Martinez-Pastora, F., De Paza, P., 2012. Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). *Animal Reproduction Science* 132, 145–154.

Amann, R.P., Hay, S.R., Hammersted, R.H., 1982. Yield characteristics, motility and cAMP content of sperm isolated from seven regions of ram epididymis. *Biology of Reproduction* 27, 723-733.

Bucak, M.N., Necmettin, T., 2007. Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. *Small Ruminant Research* 73, 103-108.

Chen, Y., Foote, R.H., Brockett, C.C., 1993. Effect of sucrose, trehalose, hypotaurine, taurine and blood serum on survival of frozen bull sperm. *Cryobiology* 30, 423- 431.

Drobnis, E. Z., L. M. Crowe, T. Berger, T. J. Anchordoguy, J. W. Overstreert and J. H. Crowe. 1993. Cold shock damage is due to lipid phase-transitions in cell membranes demonstration using sperm as a model. *Journal of Experimental Zoology* 265, 432-437.

Evans, G., Maxwell, W.M.C., 1987. Salamon's Artificial Insemination of Sheep and Goats. University Sydney, NSW, Australia.

Farshad, A., Akhondzadeh, A., 2008. Effects of sucrose and glycerol during the freezing step of cryopreservation on the viability of goat spermatozoa. *Asian-Australasian Journal of Animal Sciences* 21, 1721-1727.

Foote, R.H., 2000. Letter to the Editor: Fertilizing ability of epididymal sperm from dead animals. Journal of *Andrology* 21, 355.

Foote, R.H., Chen, Y., Brockett, C.C., 1993. Fertility of bull spermatozoa frozen in whole milk extender with trehalose, taurine or blood serum. *Journal of Dairy Science* 76, 1908-1913.

Foulkes, J.A., Watson, P.A. 1995. Hyaluronidase activity in seminal plasma as a method of assessing bull sperm integrity. *Journal of Reproduction and Infertility* 43, 349-353.

Hishinuma, M., Suzuki, K., Sekine, J., 2003. Recovery and cryopreservation of sika deer (*Cervus nippon*) spermatozoa from epididymides stored at 4ºC. *Theriogenology* 59, 813-820.

Johnson, L., Amann, R.P., Pickett, B.W., 1980. Maturation of equine epididymal spermatozoa. *American Journal of Veterinary Research* 41, 1190–1196.

Kaabi, M., Paz, P., Alvarez, M., Anel. E., 2003. Effect of epididymis handling conditions on the quality of ram spermatozoa recovered post-mortem. *Theriogenology* 60, 1249-1259.

Karja, N.W.K., Respat, E.M.A., Nuraini, I., Prihatno, S.A., Gustari, S., 2010. Characteristic of frozen-thawed epididymal spermatozoa and refrigerated storage of ram spermatozoa. *Journal of the Indonesian Tropical Animal Agriculture* 35, 63-67.

Khalili, B., Farshad, A., Zamiri, M.J., Rashidi, A., Fazeli, P., 2009. Effects of sucrose and trehalose on the freezability of Markhoz goat spermatozoa. *Asian-Australasian Journal of Animal Sciences* 22, 1614–1619.

Khalili, B., Jafaroghli, M., Farshad, A., Paresh-Khiavi, M., 2010. The effects of different concentrations of glycine and cysteine on the freezability of Moghani ram spermatozoa. *Asian-Australasian Journal of Animal Sciences* 23, 318–325.

Kikuchi, K., Nagai, T., Kashiwazaki, N., Ikeda, H., Noguch,i J., Shimada, A., 1998. Cryopreservation and ensuing in vitro fertilization ability of boar spermatozoa from epididymides stored at 4ºC. *Theriogenology* 50, 615–23.

Kishikawa, H., Tateno, H., Yanagimachi, R., 1999. Fertility of mouse spermatozoa retrieved from cadavers and maintained at 4 ºC. *Journal of Reproduction and Fertility* 116, 217–22.

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

McGinnis, L.K., Zhu, L., Lawitts, J.A., Bhowmick, S., Tone,r M., Biggers, J.D., 2005. Mouse sperm -desiccated and stored in trehalose medium without freezing. *Biology of Reproduction* 73, 627-633.

Molinia, F.C., Maxwell, W.M.C., 1994. Effect of monosaccharides and disaccharides in tris based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. *Animal Reproduction Science* 36, 113-22.

Monteiro, G.A., Papa, F.O., Zahn, F.S., Dellaqua, J.A., Melo, C.M., Maziero, R.R.D., Avanzi, B.R., Alvarenga, M.A., Guasti, P.N., 2011. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. *Animal Reproduction Scienc*e 127, 197-201.

Nagai, T., Takahashi, T., Masuda, H., Shioya, Y., Kuwayama, M., Fukushima, M., Iwasaki, S., Hanada, A., 1988. In-vitro fertilization of pig oocytes by frozen boar spermatozoa. *Journal of Reproduction and Fertility* 84, 585-591.

Nakagata, N., 1992. Production of normal young following insemination of frozen-thawed mouse spermatozoa into fallopian tubes of pseudopregnant female. *Experimental Animals* 41, 519-522.

Parrish, J.J., Susho-parrish, J., Winer, M.A., First, N.L., (1988). Capacitation of bovine sperm by heparin. *Biology of Reproduction* 38, 1171-1180.

Purdy, P.H., 2006. A review on goat sperm cryopreservation. *Small Ruminant Researc*h 63, 215-225.

Purdy, P.H., Fox M.H., Graham J.K., 2005. The fluidity of Chinese hamster ovary cell and bull sperm membranes after cholesterol addition. *Cryobiology* 51, 102-112.

O'Hara, L., Hanrahan, J.P., Richardson, L., Donovan, A., Fair, S., Evens, A.C.O., Lonergan, P., 2010. The effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. *Theriogenology* 73, 541-549.

Salamon, S., Maxwell, W.M., 2000. Storage of ram semen. *Animal Reproduction Sciences* 62, 77-111.

SAS. 1996. SAS/STAT Software: Changes and enhancements through release 6. 12. SAS Institute Inc., Cary, NC, USA.

Varisli, O., Uguz, C., Agca, C., Agca, Y., 2009. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperatures. *Animal Reproduction Science* 110, 256-268.

Vicente, J.S., Viudes-de-Castro, M.P., 1996. A sucrose-DMSO extender for freezing rabbit semen. *Reproduction, Nutrition and Development* 36, 485-492.

Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thaw function. *Reproduction Fertility and Development* 7, 871-891.

Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Sciences* 60, 481-492.

Woelders, H., Matthijs, A., Engel, B., 1997. Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology* 35, 93-105.

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

Communicating editor: *Mohammad Javad Zamiri*

اثر نگهداری اسپرم اپیدیدیمیس قوچ در ۵ درجه سانتی گراد و در زمان های مختلف و استفاده از محافظت کننده ها بر انجماد پذیری اسپرم

ع. فرشاد، ف. زارعی و ا. فرزین پور

آدرس، آدرس، آدرس، آدرس، آدرس، آدرس، آدرس، آدرس، آدرس، آدرس، آدرس، آدرس، آدرس،

***نویسنده مسئول، پست الکترونیک:** afarshad@uok.ac.ir

چکیده امروزه یخ‌زدن اسپرم انزال شده و اسپرم موجود در اپیدیدیمیس دام‌های ارزشمند یا در حال انقراض برای ایجاد بانک ذخیره ژن مورد توجه قرار دارد. لذا انجام این طرح پژوهشی دو هدف مجزا را دنبال می کند: در بخش اول، اثر زمان های مختلف نگهداری اسپرم های جمع آوری شده از اپیدیدیمس در ۵ درجه سانتی‌گراد بررسی شد. در آزمایش دوم، اثر رقیق کننده های دارای تری هالوز، سوکروز، BSA و DMSO بر ویژگی های اسپرم های یخ زده اپیدیدیمیس بررسی شد. یافته های آزمایش اول نشان داد که با افزایش طول زمان نگهداری در ۵ درجه سانتی گراد ، جنبایی، جنبایی پیشرونده، زنده مانی و نسبت باز یافت اسپرم کاهش یافت ($p<0.05$)، هر چند یکپارچگی غشای آکروزم و میزان آزاد شدن آنزیم هیالورونیداز تغییر معنی داری نداشت ($P>0.05$). یافته های آزمایش دوم نشان داد که رقیق کننده های دارای تری هالوز و سوکروز در مقایسه با رقیق کننده های دارای BSA و DMSO کیفیت اسپرم های یخگشایی شده را بهبود بخشیدند. رقیق کننده دارای BSA و شاهد نیز نسبت به رقیق کننده دارای DMSO اثر بهتری داشتند رقیق کننده های شاهد، BSA و DMSO بر آکروزوم اثر معنی داری ندا شتند. در کل، این پژوهش نشان داد که افزایش زمان نگهداری کیفیت اسپرم های اپیدیدیمیس را در دمای ۵ درجه سانتی‌گراد کاهش داد و رقیق کننده دارای تری‌هالوز اثر معنی داری بر صفات کیفی اسپرم داشت، هر چند انجام آزمایش‌های تکمیلی دیگری برای ارزیابی باروری اسپرم ضروری است.