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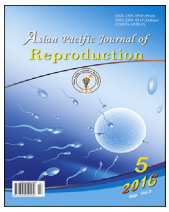

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Freezability of buffalo semen with TRIS extender enriched with disaccharides (trehalose or sucrose) and different glycerol concentrations

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ABSTRACT

Objective: To display the effect of different concentrations of glycerol, as a cryopreservative, on the quality of the frozen-thawed buffalo semen extended in TRIS extender enriched with disaccharides (trehalose or sucrose).

Methods: Semen samples were extended in Tris-Citric acid-Fructose-Egg yolk without addition of trehalose/sucrose and with 6.4% glycerol as a control (TFEG-C) and with the addition of Trehalose/Sucrose and different concentrations of glycerol to ensure 60 million motile spermatozoa mL^{-1}. Semen cooled slowly up to 5 and equilibrated for 4 h. Semen was packed into 0.25 mL polyvinyl French straws. The straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen (LN2) for 10 min then dipped in liquid LN2. Frozen straws were thawed at 37 °C for 1 min. The parameters studied were sperm motility, sperm viability, sperm abnormality, sperm membrane integrity (HOST), percent of normal intact acrosome and DNA fragmentation.

Results: The best sperm motility, sperm liveability, sperm abnormality, sperm cell membrane and DNA integrities appeared with TFES-G 5.5% (41.00 ± 2.08%, 70.40 ± 2.27%, 7.80 ± 1.19%, 68.10 ± 1.55%, 98.90 ± 0.50%, respectively) and TFES-G 7.3% (41.50 ± 1.98%, 70.70 ± 2.03%, 10.80 ± 0.88%, 69.30 ± 1.85% and 96.40 ± 0.88%, respectively).

Conclusion: From the present study, it can be concluded that addition of glycerol (5.5% or 7.3%) to Tris-Fructose-Egg yolk-Sucrose extender might help in improvement of the post-thawed characteristics of buffalo frozen semen.

1. Introduction

Artificial insemination with frozen semen is the leading and promising applied tools to improve buffalo breeding. Unfortunately, the unsatisfactory resulted conception rates of the buffalo frozen semen, is challenge, withhold its suitability in the field. During standard cryopreservation technique sperm cells are subjected to temperature alterations, cellular dehydration and freezing-thawing [1]. Moreover, cryopreservation processes is a major source for reactive oxygen species (ROS) generation that is accompanied with reduction in antioxidant systems resulting in compromised post-thawing semen quality and sperm and fertilizing ability [2–5].

Extenders composition and cooling, freezing and thawing programs are important factors affecting buffalo semen freezability and fertility [6]. Tris-based extenders are commonly used for semen preservation in most farm animals [7] including buffaloes. Cryoprotectants are either penetrating the sperm cells like glycerol or non penetrating like sugars [8]. Although the high toxicity of glycerol, it is still the best penetrating cryoprotectant in extenders used for semen freezing. [9–10] and the optimization of glycerol levels in the extender can achieved by a balance between the toxic and protecting consequences [8].

In the latest years, trehalose was added to the extenders of bull [11–12], buffalo bulls [13–14], ram [15–18], goat [19], to improve post thawing semen quality. Trehalose may be serving as a cryoprotectant [20–21], as it supports cell dehydration, resulting in decreased water flow across the sperm membrane during cryopreservation [22] and prevent ice crystals production [23–24]. Trehalose interacts with and modifies sperm membrane phospholipids and proteins resulting in improved membrane flexibility that withstand cryo-injuries [15–23]. Inclusion of sucrose as non-permeating cryoprotectant sugar in extenders seems to provide more protection to the sperm cells. Sucrose promotes cell dehydration before freezing and prevents injury caused by intracellular ice formation [23–24].

Coupling of more than one cryoprotectants with different mode of action in semen freezing extender could be excellent than that of sole one [26]. Earlier studies postulated the synergistic protective action between trehalose and glycerol in bull and boar semen freezing extenders [27–28]. Although the postulated synergic effects between glycerol and trehalose in few experiments have assess this hypothesis [29–31].

The aim of the current study was to investigate the effect various glycerol concentrations addition to fixed concentrations of trehalose and sucrose within a Tris-based extender on buffalo sperm parameters after the freeze–thawing process. Is trehalose or sucrose supplementation enable to reduce the glycerol levels in the extender and resulted in increased semen quality?

2. Materials and methods

Semen was obtained from five mature buffalo-bulls maintained at Abbasia semen Freezing Center, General Organization for Veterinary Services, Ministry of agriculture, Egypt, by using an artificial vagina (42 °C). After initial evaluation, only semen samples with at least initial sperm motility of 70%, and normal sperm of 80% were used for further processing. Visual motility, sperm liveability, sperm abnormalities and sperm membrane integrity were assessed [32]. For extension, Tris-Fructose-Egg yolk-Glycerol

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(TFEG) was utilized as described by [33]. Semen samples were pooled to ovoid individual variations and divided into 7 aliquots; one extended with the basic extender (TFEG-C, control) and other aliquots were extended with TRIS-Fructose-Egg yolk-Trehalose/Sucrose (TFET/S) extender containing the different concentrations of glycerol according to Chaveiro et al. [34] with some modifications, providing a final concentration of 60×10^6sperm/ml, as shown in Table 1.

Table 1
TRIS extender supplemented with different concentrations of glycerol.

Ingredients (g/100 ml)	Experimental extenders					
	TFET-G3.68%	TFET-G5.50%	TFET-G7.30%	TFES-G3.68%	TFES-G5.50%	TFES-G7.30%
Tris	0.806	0.806	0.806	0.806	0.806	0.806
Citric acid	0.466	0.466	0.466	0.466	0.466	0.466
Fructose	0.330	0.330	0.330	0.330	0.330	0.330
Glycerol%	3.680	5.500	7.300	3.680	5.500	7.300
Trehalose	4.96	4.96	4.96	–	–	–
Sucrose	–	–	–	4.96	4.96	4.96
Egg yolk %99 (v/v)	20	20	20	20	20	20

All media contained 0.475 g/L sodium penicillin, 0.8 g/L streptomycin sulfate.
TFET: TRIS-Fructose-Egg yolk-Trehalose; TFES: TRIS-Fructose-Egg yolk-Sucrose.

After freezing as described by El Sheshtawy et al. [12] and thawing in water bath (37 °C) for 30 s, estimation of the post-thawed sperm motility, liveability, and abnormalities were done according to Campbell [35]. Sperm membrane integrity was evaluated by using the HOST [36] and DNA integrity by using Acridine orange staining technique [37] were adopted as described by Abdel Kader [38].

The obtained data were tabulated and computed for statistical analysis, where appropriate, according to the SAS® computerized program v. 9.2 [39]. Mean ± SEM, ANOVA and LSD were calculated to deduce the effect and the most efficient concentrations of glycerol on the post-thawed characteristics of buffalo spermatozoa.

3. Results

The present results (Table 2) revealed significant differences in the post-thawed buffalo semen quality parameters after addition of various concentration of glycerol to sucrose enriched Tris-extender. The best sperm motility, sperm liveability, sperm abnormality, sperm cell membrane and DNA integrities appeared with TFES-G 5.5% (41.00 ± 2.08%, 70.40 ± 2.27%, 7.80 ± 1.19%, 68.10 ± 1.55%, 98.90 ± 0.50%, respectively) and TFES-G 7.3% (41.50 ± 1.98%, 70.70 ± 2.03%, 10.80 ± 0.88%, 69.30 ± 1.85% and 96.40 ± 0.88%, respectively). Combination of glycerol and trehalose did not exert any significant affect on many of the studied post-thawing semen parameters except sperm membrane integrity that were significantly higher in all glycerol concentrations as compared with control. Based on the obtained results, it can be concluded that addition of glycerol (5.5% or 7.3%) to Tris-Fructose-Egg yolk-Sucrose enriched extender resulted in improvement of the post-thawed characteristics of buffalo frozen semen. Moreover, addition of different concentrations of glycerol to trehalose enriched extender provides better preservation for sperm membrane.

Table 2
Effects of different glycerol concentrations on sperm assessment parameters of frozen buffalo semen (Means ± SEM).

Extender	Sperm parameters (%)				
	Motility	Liveability	Abnormality	Membrane integrity	DNA integrity
TFEG-C	33.00 ± 2.26^b	71.50 ± 1.60^a	11.00 ± 1.45^c	64.00 ± 2.08^b	97.00 ± 1.06^a
TFET-G3.68%	33.00 ± 1.53^b	67.80 ± 1.63^a	12.60 ± 1.40^a	65.50 ± 2.63^a	96.10 ± 0.86^c
TFET-G5.50%	35.00 ± 3.50^b	68.70 ± 2.50^a	13.50 ± 1.44^b	66.70 ± 2.77^a	98.00 ± 0.74^a
TFET-G7.30%	37.00 ± 4.29^b	68.40 ± 3.14^b	16.30 ± 0.90^a	68.70 ± 1.94^a	96.00 ± 1.01^b
TFES-G3.68%	39.50 ± 1.57^a	69.70 ± 1.52^a	12.90 ± 1.20^b	66.80 ± 1.83^a	96.70 ± 0.96^a
TFES-G5.50%	41.00 ± 2.08^a	70.40 ± 2.27^a	7.80 ± 1.19^b	68.10 ± 1.55^a	98.90 ± 0.50^b
TFES-G7.30%	41.50 ± 1.98^a	70.70 ± 2.03^a	10.80 ± 0.88^a	69.30 ± 1.85^a	96.40 ± 0.88^a

Values within the same column with different letters differed significantly at least at $P < 0.05$.

4. Discussion

Reduction in motility, viability and fertility are the most common deleterious effects on sperm during cryopreservation owing to multifactorial cryo-damage [9]. Such cryodamage can be alleviated by optimizing the types and levels of cryoprotectants added to the extender to protect the sperm cells during freezing [34,40,41]. Although several reports have evaluated the efficiency of trehalose cryoprotection at various concentrations in extenders, the possible synergistic cryoprotective action of trehalose and glycerol has not been fully studied up to date in buffalo semen [29,31,42]. This experiment was designed to estimate the optimum levels of glycerol and trehalose or sucrose in a Tris-based extender that exert synergistic cryoprtotection and yielded better post-thawing semen quality when used for buffalo bull semen cryopreservation. It is a trial to reduce the glycerol levels in the extender by trehalose or sucrose supplementation that could resulted better semen cryopreservation. In this study, no extra-beneficial action on all post thawing semen parameters could be noticed by adding different levels of glycerol to Trehalose enriched extender as compared with control. The experimental reports belonging to the effect of inclusion of trehalose in semen extender on post-thawing semen quality are paradoxal. Some studies indicated that trehalose addition to extender was not beneficial as post-thawing semen quality was not significantly improved in bull [43,44] stallion [45], deer [46], and gazelle [47]. Moreover, the addition of sucrose to bull semen extender exhibits deleterious effect on post-thawing sperm motility [48]. In contrast, trehalose improved the post-thawing semen quality in ram [15,23,24], goat [49,50] and rabbit [51]. Contradictory to our results, addition of 50 mM trehalose to bull freezing extender improved post-thawing semen quality [12]. Najafi et al., [18] observed existence synergistic effect between both 5%glycerol and 100 mM trehalose and 7% glycerol with 50 mM trehalose in ram semen extender which offered excellent sperm protection that manifested by the better post-thawing sperm parameters than other combinations, or glycerol alone. Aisen *et al.* [23] and Jafaroghli et al. [25] noticed a better cryoprotective effects and a superior recovery of post-thawing ram spermatozoa with addition of 3% or 5%glycerol combined with 100 mM trehalose in the extender. Khalili et al. [41] observed that combining 200 mM of trehalose (198.24 mM) with 8% glycerol yielded the best protection and survival for goat semen during cryopreservation. These various reports clarify the differences between species regarding the optimum concentration of trehalose and glycerol in extender that offer the best protection of sperm cells during cryopreservation. Our findings revealed slight improvement in characteristics of buffalo frozen semen with sucrose and glycerol concentration of 5.5% and 7.3%, a finding which came in agreement with that observed in some earlier reports of Tambing et al. [52]; Farshad and Akhondzadeh [53] and Qureshi et al. [54] in buck semen; Corcuera et al. [55] in boar semen; Miclea et al. [56] in ram semen; Taşdemir et al. [57] in bull semen. Glycerol is the most common and efficient cryoprotective agent in extender and its concentration is crucial for the success of semen cryopreservation in farm animals [58,59]. The improvement of semen preservability in our results might be due to the link of glycerol to intracellular water of the spermatozoa that prevent ice crystals production which have a deleterious and damaging action on sperm membrane during cryopreservation [8,60,61].

In conclusion, the current study revealed a synergistic action of addition different concentration of glycerol to sucrose enriched extender on buffalo bull spermatozoa survivability during cryopreservation. This synergism could not be observed between trehalose and glycerol. The top post-thawing quality was noticed with combination of 5.5 and 7.3% glycerol and 4.96% sucrose in freezing extender. It seems being suggested to test other combinations of sucrose, trehalose and glycerol and any of other cryoprotectants and exploring other concentration ranges to formulate a suitable extender for buffalo bull semen.

Reference

[1] Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't better. *Theriogenology* 2002; **57**: 327-344.

[2] Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennigs Z, et al. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998; **59**: 1037-1046.

[3] Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev* 2000; **55**: 282-288.

[4] White IG. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod Fertil Dev* 1993; **5**: 639-658.

[5] Zhao Y, Buhr MM. Cryopreservation extenders affect calcium flux in bovine spermatozoa during a temperature challenge. *J Androl* 1995; **16**: 278-285.

[6] Andrabi S. Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod Dom Anim* 2009; **44**: 552-569.
[7] Purdy PH. A review on goat sperm cryopreservation. *Small Rum Res* 2006; **6**: 215-225.
[8] England GCW. Cryopreservation of dog semen: a review. *J Reprod Fertil* 1993; **47**: 43-55.
[9] Holt WV. Basic aspects of frozen storage of semen. *Anim Reprod Sci* 2000; **62**(1–3): 3-22.
[10] Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 2000; **61**: 481-492.
[11] Uysal O, Bucak MN. The role of different trehalose concentrations and cooling rates in freezing of ram semen. *Ankara Üniv Vet Fak Derg* 2009; **56**: 99-103.
[12] El-Sheshtawy RI, Sisy GA, El-Nattat WS. Effects of different concentrations of sucrose or trehalose on the post-thawing quality of cattle bull semen. *Asian Pac J Reprod* 2015; **4**(1): 26-31.
[13] Reddy NSS, Mohanarao GJ, Atreja SK. Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (*Bubalus bubalis*) sperm quality following cryopreservation. *Anim Reprod Sci* 2010; **119**: 183-190.
[14] Shahba MI, El-Sheshtawy RI, El-Azab AI. Improvement of buffalo semen freezability by using TRIS extender enriched with different concentrations of trehalose/sucrose. *J Innov Pharmac Biol Sci* 2016; **3**(1): 198-203.
[15] Bucak MN, Atessahin A, Varıslı O, Yuce A, Tekin N, Akcay A. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: microscopic and oxidative stress parameters after the freeze-thawing process. *Theriogenology* 2007; **67**: 1060-1067.
[16] Bucak MN, Atessahin A, Yuce A. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rum Res* 2008; **75**: 128-134.
[17] Cirit Ü, Bağiş H, Demir K, Agca C, Pabuccuoğlu S, Varişli Ö, et al. Comparison of cryoprotective effects of iodixanol, trehalose and cysteamine on ram semen. *Anim Reprod Sci* 2013; **139**: 38-44.
[18] Najafi A, Zhandi M, Towhidi A, Sharafi M, Sharif AA, Mahdi Khodaei Motlagh MK, et al. Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology* 2013; **66**: 275-282.
[19] Atessahin A, Bucak MN, Tuncer PB, Kızıl M. Effects of antioxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze-thawing process. *Small Rum Res* 2008; **77**: 38-44.
[20] Molinia FC, Evans G, Casares PIQ, Maxwell WMC. Effects of monosaccharides and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. *Anim Reprod Sci* 1994; **36**: 113-122.
[21] Sanchez-Partida LG, Setchell BP, Maxwell WMC. Effect of compatible solutes and diluent composition on the post-thaw motility of ram spermatozoa. *Reprod Fertil Dev* 1998; **10**: 347-357.
[22] Yildiz C, Kaya A, Aksoy M, Tekeli T. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology* 2000; **54**: 579-585.
[23] Aisen EG, Medina VH, Venturino A. Cryopreservation and post thawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology* 2002; **57**: 1801-1808.
[24] Aisen EG, Quintana M, Medina V, Morello H, Venturino A. Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. *Cryobiology* 2005; **50**: 239-249.
[25] Jafaroghli M, Khalil B, Frasad A, Zamiri MJ. The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. *Small Rum Res* 2011; **96**: 58-63.
[26] Blanco JM, Long JA, Gee G, Wildt DE, Donoghue AM. Comparative cryopreservation of avian spermatozoa: benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. *Anim Reprod Sci* 2011; **123**: 242-248.
[27] Hu JH, Li QW, Jiang ZL, Yang H, Zhang SS, Zhao HW. The cryoprotective effect of trehalose supplementation on boar spermatozoa quality. *Reprod Domest Anim* 2009; **44**: 571-575.
[28] Liu Z, Foote RH, Brockett CC. Survival of bull sperm frozen at different rates in media varying in osmolarity. *Cryobiology* 1998; **37**: 219-230.
[29] Gutierrez-Perez O, Juarez-Mosqueda Mde L, Carvajal SU, Ortega ME. Boar spermatozoa cryopreservation in low glycerol/trehalose enriched freezing media improves cellular integrity. *Cryobiology* 2009; **58**: 287-292.
[30] Kong D, Shang H, Guo K, Liu Y, Zhang J, Wei H. A study on optimizing the cryopreservation methods for bama miniature pig semen. *Exp Anim* 2012; **61**: 533-542.
[31] Storey BT, Noiles EE, Thompson KA. Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. *Cryobiology* 1998; **37**: 46-58.
[32] Graham EF, Schmehl MKL, Maki-Laurila M. Some physical and chemical methods of evaluating semen. In: *Proc. 3rd NAAB Tech Conf Artif Insemin Reprod, 12–14 April Milwaukee, WI*. Columbia, MO: National Association of Animal Breeders; 1970, p. 44-48.
[33] Foote RH. Fertility of bull semen at high extension rates in tris buffered extenders. *J Dairy Sci* 1970; **53**: 1475-1477.
[34] Chaveiro A, Machado L, Frijters A, Engel B, Woelders H. Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic supports. *Theriogenology* 2006; **65**: 1875-1890.
[35] Campbell RC, Dott HM, Glover TD. Nigrosin-eosin as a stain for differentiating live and dead spermatozoa. *J Agric Sci* 1956; **48**: 1-8.
[36] Jeyendran RS, Van Derv Ven HH, Perez-Pelaes M, Crabo BG, Zaneveld LJD. Development of an assay the functional integrity of human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 1984; **70**: 219-228.
[37] Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril* 1984; **42**: 87-91.
[38] Abd El-Kader MI. *Study of some factors affecting efficiency of frozen buffalo semen*. M.V.Sc. Thesis. Egypt: Theriogenology, Fac. Vet. Med., Banha University; 2010.
[39] SAS. *User's guide release 9.2 edition*. Cary: SAS Institute Inc.; 2008.
[40] Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl* 1990; **11**: 73-88.
[41] Khalili B, Farshad A, Zamiri MJ, Rashidi A, Fazeli P. Effects of sucrose and trehalose on the freezability of Markhoz goat spermatozoa. *Asian Aust J Anim Sci* 2009; **22**(12): 1614-1619.
[42] Kozdrowski R. The effect of trehalose on post-thaw viability and fertility of European brown hare (*Lepus europaeus* Pallas, 1778): spermatozoa. *Anim Reprod Sci* 2009; **116**: 326-334.
[43] Chen Y, Foote RH, Brockett CC. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. *Cryobiology* 1993; **30**: 423-431.
[44] Foote RH, Chen C, Brockett C, Kaproth MT. Fertility of bull frozen in whole milk extender with trehalose, taurine or blood serum. *J Dairy Sci* 1992; **76**: 1908-1913.
[45] Squires EL, Keith SL, Graham JK. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. *Theriogenology* 2004; **62**: 1056-1065.
[46] Fernández-Santos MR, Martínez-Pastor F, García-Macías V, Esteso MC, Soler AJ, de Paz P, et al. Extender osmolality and sugar supplementation exert a complex effect on the cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa. *Theriogenology* 2007; **67**(4): 738-753.
[47] Garde JJ, Soler AJ, Cassienello J, Crespo C, Malo AF, Espeso G, et al. Sperm cryopreservation in three species of endangered gazelles (*Gazella cuvieri*, *G. dama mhorr*, and *G. dorcas neglecta*). *Biol Reprod* 2002; **69**: 602-611.
[48] Woelders H, Matthijs A, Engel B. Effect of trehalose and sucrose, osmolality of the freezing medium and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology* 1997; **35**: 93-105.
[49] Aboagla EM, Terada T. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. *Biol Reprod* 2000; **69**: 1245-1250.
[50] Aboagla EM, Terada T. Effects of the supplementation of trehalose extender containing egg yolk with sodium dodecyl sulfate on the freezability of goat spermatozoa. *Theriogenology* 2004; **62**: 809-818.
[51] Dalimata AM, Graham JK. Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose. *Cryobiology* 1997; **48**: 831-841.
[52] Tambing SN, Toelihere MR, Yusuf TL, Sutama I-K. Effects of glycerol in tris extender on frozen semen quality of crossbred Etawah bucks. *J Ilmu Tern dan Veter* 2000; **5**(2): 84-99.
[53] Farshad A, Akhondzadeh S. Effects of sucrose and glycerol during the freezing step of cryopreservation on the viability of goat spermatozoa. *Asian Aust J Anim Sci* 2008; **21**(12): 1721-1727.
[54] Qureshi MS, Khan D, Mushtaq A, Afridi SS. Effect of extenders, post dilution intervals, and seasons on semen quality in dairy goats. *Turk J Vet Anim Sci* 2013; **37**: 147-152.
[55] Corcuera BD, Marigorta P, Sagüés AF, Saiz-Cidoncha F, Pérez-Gutiérrez JF. Effect of lactose and glycerol on the motility, normal apical ridge, chromatin condensation and chromatin stability of frozen boar spermatozoa. *Theriogenology* 2007; **67**: 1150-1157.
[56] Miclea V, Dascäl-ciornei A, Zăhan M, Dascäl-Ciornei V, Miclea I. The influence of the dilution rate and glycerol concentration on ram semen preservation. *Bull UASVM Anim Sci Biotechnol* 2010; **67**(1–2): 260-265.
[57] Taşdemir U, Büyükleblebici S, Tuncer PB, Coşkun E, Özgürtaş T, Aydın FN. Effects of various cryoprotectants on bull sperm quality, DNA integrity and oxidative stress parameters. *Cryobiology* 2013; **66**(1): 38-42.
[58] Morrier A, Castonguay F, Bailey JL. Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa. *Canad J Anim Sci* 2002; **82**: 347-356.
[59] Kulaksiz R, Ari UÇ, Daşkin A, Üner AG. The effect of different glycerol concentrations on freezability of semen from angora, kilis and saanen goats. *Slovak J Anim Sci* 2013; **46**: 39-44.
[60] Rodrigues BA. *Efeito do diluidor á base de albumina sérica bovina sobre a viabilidade in vitro do sêmen canino criopreservado*. Brazil: Veterinary Faculty, Federal University of Rio Grande do Sul; 1997, p. 176.
[61] Silva AR, Cardoso RCS, Uchoa DC, Silva LDM. Effect of tris-buffer, egg yolk and glycerol on canine semen freezing. *The Vet J* 2002; **164**: 244-246.