Received: 18 April 2022, Accepted: 04 June 2022

Original Research

Journal of Advanced Veterinary Research
(2022) Volume 12, Issue 3, 221-226

Trehalose as an Alternative of Egg Yolk in Ram Semen Extender

Abdelghany A. El-Shereif *, Ahmed Z. Fath El-Bab, Adel N.M. Nour El-Din, Mohamed H. Salem

Animal and Fish Production Department, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria, 21545, Egypt

***Correspondence**
Abdelghany Awad El-Shereif, Animal and Fish Production Department, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria, 21545, Egypt.
E-mail: abdelghany.awad@alexu.edu.eg

Abstract

The aim of this study was to investigate the influence of replacing egg yolk with trehalose in Tris-citric-yolk extender on post-thawing of Barki and Rahmani ram spermatozoa parameters namely, progressive motility, viability, abnormalities, membrane integrity, and acrosome integrity. Fifteen ejaculates from 3 rams presenting each breed were collected by using the artificial vagina. Accepted semen samples were pooled and kept under 37°C then were immediately distributed on extenders with replaced egg yolk levels of 5, 4, 3, 2, 1and 0 % by respective levels 0, 25, 50, 75, 100, and 125 mM of trehalose. After an equilibration period, cooled semen was aspirated into 0.5mL straws and were frozen and stored in the liquid nitrogen. After 24 h, straws were thawed in a 37°C water bath for the 60s. Results revealed no significant differences between the two studied breeds of ram for all post-thawing spermatozoa parameters. The extender containing 50 mM trehalose and 3% egg yolk gave the highest post-thawing sperm motility and membrane integrity (41.43% and 44.47 %), while the lowest values (34.29 % and 37.40 %) were obtained from the extender containing 125 mM trehalose and 0% EY and was highly significant in total sperm abnormalities. Post-thawing sperm viability and acrosome integrity were not significantly affected by substituting different levels of trehalose for egg yolk. In conclusion, reducing egg yolk dawn to 1 % versus using high concentrations of trehalose (up to 100 mM) did not affect sperm characteristics, but complete elimination of egg yolk from the extender reduced post-thawing characteristics.

KEYWORDS
Trehalose, Ram semen, Egg yolk, Tris-Citric-Yolk, Cryopreservation

INTRODUCTION

Egg yolk is the major constituent of extender used for domestic animals' semen storage and cryopreservation. The benefits of egg yolk utilization in semen extenders were first reported by Phillips and Lardy (1940). Therefore, egg yolk was used in the formation of semen freezing extender as a cryoprotective agent (Bogart and Mayer, 1950) at a percentage ranging between 2.0 to 20 % (Khlifaoui *et al.*, 2005; Ali Al Ahmad *et al.*, 2008; Tonieto *et al.*, 2010). Egg yolk has a variety of useful components but the low-density lipoprotein (LDL) is the main effective component that prevents the spermatozoa membrane damage during the freezing-thawing process (Moussa *et al.*, 2002; Amirat *et al.*, 2004).

Nevertheless, using egg yolk as a cryoprotectant agent in semen freezing extenders exhibited some disadvantages, among which: Egg yolk is an animal originated biological medium usually susceptible to contamination, difficulty of manual isolation of egg yolk to eliminate traces of albumen. Furthermore, it contains granules of the same size and shape as spermatozoa which that prevent the metabolic exchanges of the spermatozoa or reduce their motility and may interfere with microscopic observations or biochemical assays (Pace and Graham, 1974; Watson and Martin, 1975). Also, egg yolk is affiliated with sanitary risks, including the production of harmful metabolites and toxins and the hazards of infection that all result in reducing semen quality (Amirat *et al.*, 2004; Yildiz *et al.*, 2007). In fact, the risk of introducing exotic diseases such as avian influenza through the transportation of extenders containing egg yolk is a widespread concern (Amirat *et al.*, 2004). It would therefore be advantageous to replace egg yolk with another molecule that has a cryoprotective effect.

Trehalose, a disaccharide, and a non-permeating cryoprotectant is found in a number of plants and animals that can resist dehydration or freezing (Westh and Ramløv, 1991). It has been found to have a significant role in preventing deleterious effects on the sperm plasma membrane (Aboagla and Terada, 2003), by interacting with the plasma membrane phospholipids. Where it can form hydrogen bonds with the polar head groups of them, and consequently helps prevent fusion events of juxtaposed membranes, creating an osmotic pressure that induces cell dehydration before freezing, which decreases the extent of cell injury by intracellular ice formation (Liu *et al.*, 1998).

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
ISSN: 2090-6277/2090-6269/ © 2011-2022 Journal of Advanced Veterinary Research. All rights reserved.

Furthermore, studies have on trehalose supplementation to the freezing extenders of semen. Aisen *et al*. (2000) revealed that the combination of trehalose and EDTA in tris extender gave the highest post-thaw ram sperm motility and post-thaw sperm resistance. Usage a tris-based extender supplemented with 50 mM trehalose led to a significantly higher percentage of post-thawing ram sperm motility as compared with tris extender lacking trehalose (Bucak *et al*., 2007). Also, Tonieto *et al*. (2010) tested trehalose and LDL as cryoprotectants in extenders for frozen ram semen and found that the presence of glycerol, trehalose, or trehalose + LDL gave the highest post-thaw sperm motility. Moreover, Jafaroghli *et al*. (2011) reported that tris extender supplemented with 100 mM trehalose produced the highest post-thawing ram semen motility. Their results also indicated that extenders containing 70 or 100mM trehalose or raffinose significantly reduced acrosome abnormalities, total sperm abnormalities, and hypo-osmotic swelling after the freezing-thawing process. Recently, Rostami *et al*. (2019) indicated that a tris-egg yolk extender containing 100 mM trehalose + 2 mM vitamin E significantly improved frozen-thawed ram semen parameters. Furthermore, Öztürk *et al*. (2020) reported that the addition of 60 mM trehalose combined with 3 % glycerol to Tris-egg yolk extender enhanced post-thaw sperm parameters. To the best of the authors' knowledge, no reports were available for replacing egg yolk with trehalose in semen extenders.

Considering trehalose properties, it has been hypothesized that trehalose could be an alternative to egg yolk in ram semen extender without any deleterious effects on sperm parameters. Therefore, the objective of this study was to evaluate the influence of replacing different percentages of egg yolk with trehalose in tris extender on post-thawing spermatozoa parameters of Barki and Rahmani ram.

MATERIALS AND METHODS

Animals and Semen collection

Semen from healthy, free diseases three Barki and three Rahmani mature rams was collected using an artificial vagina. Rams which belonged to the Experimental Station of the faculty of Agriculture, Alexandria University were fed as one group on berseem *(Trifolium alexandrinum*), rice straw with supplemented concentrate ration containing 14% crude protein. During the experimental period, the rams were allowed to graze for a period of 2 to 3h at mid-day and to drink fresh water freely. All procedures and experimental protocols were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Semen processing and evaluation

Tris-based extender used in this study was formed as Tris 3.028g, citric acid 1.675g, egg yolk 5mL, glycerol 5mL, penicillin 100.000 IU, and streptomycin 100mg/100mL, pH 6.8. Then, each 1 % of egg yolk was replaced by 25mM trehalose as shown in Table 1. Five ejaculates from each ram in the two breeds were collected. Semen was evaluated immediately for progressive motility and then was pooled and processed under 37°C. Dilution took place in tested extenders 1:20 semen to the extender to reach a final concentration of approximately 2x108 spermatozoa per mL. The diluted semen was gradually cooled down to 5°C for 4 h. After an equilibration period, cooled semen was aspirated into 0.5 mL straws and frozen by exposure to liquid nitrogen vapor 4-5 cm above the liquid nitrogen surface for 20 min before dipping into liquid nitrogen for storage. After 24 h, straws were thawed in a water bath (37°C) for 60s.

Progressive motility

It was assessed subjectively as described by Evan and Maxwell (1987). Briefly, 5 µl semen was placed onto a pre-warmed slide (37°C) and then was diluted with drops of 2.9% sodium citrate solution of the same temperature, carefully mixed, and covered with a 22x22 mm, pre-warmed coverslip (37°C). At least 5 fields of sperm were viewed using phase-contrast microscopy under 200x and 400x magnification.

The sperm membrane integrity

It was assessed by using a hypo-osmotic swelling test (HOST) (Buckett *et al*., 1997). The assay was performed by incubating 10 µL of semen with 200 µL of a 100mOsM hypo-osmotic solution (9 g fructose and 4.9 g sodium citrate/ 1-liter double distilled water) in a 1.5 mL Eppendorf tube at 37°C for 60 min. After incubation, 100 µL of the mixture was spread with a coverslip on a warm slide. A total of 200 sperm were counted in at least 5 different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

Sperm viability

Sperm viability assessment was performed using a live/dead staining technique according to Barth and Oko (1989) with some modifications. A small drop of semen was placed on a clean, pre-warmed slide, and a drop of Eosin-Nigrosine solution (3.3 g eosin Y, 20 g Nigrosine, 1.5 g sodium citrate in 300 mL of water, adjusted to pH 7.0) was mixed with the semen. Another clean

Table 1. Composition of extenders and experimental design

Extenders No.	Ingredients [1]				
	Tris (g)	Citric acid (g)	Glycerol (mL)	Egg yolk (mL)	Trehalose (mM)
1 (T_0-EY5)	3.029	1.675	5	5	-
2 (T_{25}-EY4)	3.029	1.675	5	4	25
3 (T_{50}-EY3)	3.029	1.675	5	3	50
4 (T_{75}-EY2)	3.029	1.675	5	2	75
5 (T_{100}-EY1)	3.029	1.675	5	1	100
6 (T_{125}-EY0)	3.029	1.675	5	-	125
Up to 100 mL double distilled water					

[1] all extenders contained 100,000 I.U. penicillin procaine and 100,000 µg streptomycin sulfate.

slide was placed on the first one drawn apart without pressure to make a smear. The smear was then dried on a heating stage (37°C) and examined under the microscope. About two hundred spermatozoa were scored per slide using light microscopy 1000x magnification and oil immersed object. Sperm-colored pink were considered non-viable (dead), and unstained (clear) cells were counted as viable (live).

Abnormal spermatozoa

For abnormal spermatozoa assessment, the same stained semen smears which were used in live sperm counts were also utilized in determining the percentage of morphologically abnormal spermatozoa.

Acrosome Integrity

The same thin semen smears stained with eosin-nigrosine stain which were used to evaluate the percentage of sperm viability were also used to evaluate the acrosome integrity of the sperm. Acrosomes were classified according to Spindler *et al*. (2004); acrosomes that had a smooth appearance and remained intact with the sperm head were classified as normal. Sperm with a shoulder visible in the equatorial region were classified as sperm with lost acrosomes (react acrosome).

Statistical analysis

Motility, viability, abnormalities, membrane, and acrosome integrity were statistically analyzed using the General Linear Model (GLM) procedure of SAS (2004). Measured sperm parameters illustrated as mean ± SEM per treatment group and a P-value ≤ 0.05 were used to determine significance throughout this study. Significant differences between means were detected using Duncan's Multiple Range Test.

RESULTS

The post-thawing Barki and Rahmani progressive motility, viability, sperm abnormalities, membrane integrity, and acrosome integrity as affected by replacing egg yolk with different levels of trehalose in tris-based extender are presented in Table 2. Results revealed no significant differences between the two breeds of rams in all studied sperm parameters post-thawing.

Post-thawing sperm progressive motility, membrane integrity, and total abnormalities were significantly affected by the replacement of egg yolk with different levels of trehalose in the tris-based extender. The highest post-thawing sperm motility percentage (41.43 %) was obtained for an extender that contained 50 mM trehalose and 3% egg yolk. On the other hand, the lowest post-thawing sperm motility percentage (34.29 %) belonged to the extender that contained 125 mM trehalose + 0% egg yolk.

Sperm post-thawing membrane integrity was significantly higher by reducing the percentages of egg yolk and increasing trehalose levels in the tris extender. However, the absence of egg yolk from the tris extender resulted in the lowest post-thawing percentage of sperm membrane integrity (37.40%) while the highest value (44.47%) was recorded for the extender that contained 50 mM trehalose and 3% egg yolk.

Concerning post-thawing sperm viability and normal acrosome integrity, the results of the present experiment revealed that replacing different levels of egg yolk with respective levels of trehalose did not significantly affect any of these parameters.
Analysis of variance indicated that there were no significant interactions between breeds (Barki and Rahmani) and treatments (replacement of egg yolk with different levels of trehalose on the percentages of post-thawing semen characteristics Table 3.
It is worth noting that the complete absence of egg yolk resulted in the lowest post-thawing sperm membrane integrity (37.40%) and in the highest total sperm abnormalities (15%) as compared with the control extender and with any other extender under investigation.

DISCUSSION

The highest post-thawing sperm motility value (41.43%) obtained from an extender containing 50 mM trehalose and 3 % egg yolk was comparable with the 36.9% value reported by Valente *et al*. (2010) using a tris-based extender containing 4.5 % egg yolk and 2 mM trehalose but was higher than 31.2 and 32.5 % reported by Tonieto *et al*. (2010) when used tris extender containing 8 % LDL + 100 mM trehalose + 5 % glycerol or containing 20 % egg yolk

Table 2. Effect of replacement of egg yolk with different levels of trehalose in tris extender on the percentages of post-thawing Barki and Rahmani ram semen characteristics.

	Parameters[1]				
Factors	PMOT %	MI %	SV %	SAbs %	NAI %
Breed[2]:					
Barki	38.54±1.22	40.27±1.35	48.04±1.08	8.88±1.01	64.49±2.04
Rahmani	38.00±1.19	41.11±0.88	49.97±1.38	7.74±0.98	66.97±1.22
Treatments[3]:	**	**	NS	**	NS
T_0-EY5	38.13 ±2.49 [ab]	39.87 ±1.48 [ab]	46.56±1.56	6.94 ±0.65 [b]	61.48±1.99
T_{25}-EY4	38.13 ±2.30 [ab]	40.96 ±1.55 [ab]	53.09±2.78	6.28 ±0.53 [b]	64.02±1.81
T_{50}-EY3	41.43 ±1.43 [a]	44.47 ±1.06 [a]	48.83±2.12	5.26 ±0.96 [b]	66.30±1.82
T_{75}-EY2	38.50 ±1.67 [ab]	40.09 ±2.66 [ab]	48.85±1.97	9.01 ±1.67 [b]	69.03±1.60
T_{100}-EY1	38.89 ±2.00 [ab]	41.34 ±2.28 [ab]	48.06±1.08	8.78 ±1.99 [b]	64.08±5.46
T_{125}-EY0	34.29 ±2.30 [b]	37.40 ±2.10 [b]	48.30±2.56	15.00 ±2.24 [a]	69.50±2.70

[1] PMOT: progressive motility, MI: membrane integrity, SV: sperm viability, SAbs: sperm abnormalities, NAI: acrosome integrity.
[2] Breeds are not statistically different for all parameters.
[3] Different letters within the same column indicate significant differences among the groups (P≤ 0.05).
T0-EY5: control tris extender with 5% egg yolk and without trehalose, T25-EY4, T50-EY3, T75-EY2, T100-EY1 and T125-EY0: tris extenders with 25, 50, 75, 100 and 125mM trehalose and 4, 3, 2, 1 and 0 % egg yolk respectively.

Table 3. Effect of breed and replacement of egg yolk with different levels of trehalose in tris extender on the percentages of post-thawing semen characteristics

Items	Barki					Rahmani				
	PMOT %	MI %	SV %	SAbs %	NAI %	PMOT %	MI %	SV %	SAbs %	NAI %
T_0-EY5	38.75±2.54	40.82±1.79	47.06±2.73	6.94±0.83	60.46±2.48	37.50±2.43	38.92±1.17	45.95±1.39	6.94±1.09	62.49±1.33
T_{25}-EY4	38.75±2.38	41.85±1.50	52.87±4.38	6.54±0.81	64.28±2.22	37.50±2.22	40.07±1.60	53.31±3.93	5.85±0.50	63.75±3.14
T_{50}-EY3	41.25±1.40	45.46±1.11	46.66±2.11	6.12±1.43	68.42±2.11	41.67±1.46	43.48±1.01	50.99±3.65	3.84±0.29	64.18±2.86
T_{75}-EY2	40.00±1.58	38.76±2.73	49.68±1.60	9.99±2.50	68.03±2.89	37.00±1.76	41.42±2.59	48.03±3.82	7.38±1.74	70.02±1.61
T_{100}-EY1	39.70±2.11	39.85±2.66	46.96±1.82	10.24±3.72	58.00±10.61	38.09±1.91	42.83±1.90	48.94±1.34	7.31±1.69	70.16±1.78
T_{125}-EY0	30.62±2.63	34.89±1.88	44.81±1.12	16.49±2.20	67.77±4.57	37.97±1.99	39.90±2.31	51.78±4.70	13.89±3.77	71.22±3.24

PMOT: progressive motility, MI: membrane integrity, SV: sperm viability, SAbs: sperm abnormalities, NAI: acrosome integrity.
T0-EY5: control tris extender with 5% egg yolk and without trehalose, T25-EY4, T50-EY3, T75-EY2, T100-EY1 and T125-EY0: tris extenders with 25, 50, 75, 100 and 125mM trehalose and 4, 3, 2, 1 and 0 % egg yolk respectively.

+ 100 mM trehalose but lacking glycerol, respectively. Generally, values of post-thawing motility of ram spermatozoa diluted with tris supplemented with trehalose varied greatly between 31.2 and 64.0 % (Aisen *et al*., 2000; Bucak *et al*., 2007; Tonieto *et al*., 2010; Valente *et al*., 2010). Rostami *et al*. (2019) reported that extenders containing 100 mM trehalose, 60 mM sucrose, and 10 mM raffinose increased progressive motility, and membrane integrity sperm viability and reduced the morphological abnormalities of frozen-thawed ram semen. However, the addition of 60 mM trehalose combine with 3 % glycerol to the tris-egg yolk extender enhanced post-thaw sperm parameters (Öztürk *et al*., 2020). Differences could be attributed to breeds and components of tris-based extenders especially levels of egg yolk, glycerol, and trehalose. It is worth noting that egg yolk, though added in different levels, was a basic common ingredient in these tris extenders.

The obtained results indicated the lowest post-thawing sperm motility in the extender without any egg yolk. Some reports indicated that LDL is the egg yolk component that provides the highest sperm protection during storage at 5 degrees and by deep freezing (Pace and Graham, 1974; Watson, 1981). However, the mechanism by which this protection is provided to sperm remains elusive. Some studies suggested different hypotheses for the protection mechanism of LDL. It was suggested that the phospholipid fraction present in LDL forms a protection film on the sperm surface (Quinn *et al*., 1980). Another hypothesis suggested that LDL replaces sperm membrane phospholipids that are lost or damaged during the cryopreservation process (Graham and Foote, 1987). Furthermore, Manjunath (2012) indicated that seminal plasma from many mammalian ungulates contains a family of a protein called Binder of Sperm (BSP) which bind to spermatozoa at ejaculation and modify the sperm membrane which may adversely affect the ability of sperm to be preserved. The same author indicated that LDL has a very high capacity for BSP protein which induces rapid, specific, and stable binding even after freeze-thawing of semen. Furthermore, LDL prevents lipid efflux from the sperm membrane and maintains sperm motility during sperm storage.

Similar to the results of many studies (Molinia *et al*., 1994; Bucak and Tekin, 2007; Tonieto *et al*., 2010; Jafaroghli *et al*., 2011), the tris-based extender containing both egg yolk and trehalose in this study slightly enhanced post-thawing sperm motility of rams. The exact mechanism by which sugars protect spermatozoa during freezing and thawing is not known, though it's of great concern to many investigators. Bakas and Disalvo (1991) and Pereira and Hünenberger (2008) proposed that the action of trehalose is connected with its ability to replace water at the membrane solution interface. Moreover, Crowe *et al*. (1984), and Patist and Zoerb (2005) suggested that trehalose interacts directly with polar head groups of phospholipids in the membrane during drying and freezing, reducing Van der Waals interaction among hydrocarbon chains. It has also been hypothesized that disaccharides penetrate the plasma membrane of the sperm and form hydrogen bonds with the polar head groups of phospholipids (Liu *et al*., 1998; Aboagla and Terada, 2003), thereby preventing deleterious lipid phase transition (Tada *et al*., 1990; Zuther *et al*., 2004). On the other hand, De Leeuw *et al*. (1993) and Zuther *et al*. (2004) hypothesized that non-penetrating sugars would be more effective in stabilizing the sperm membrane bilayer. Moreover, it was suggested that the addition of trehalose to the extender creates an osmotic pressure resulting in a hypertonic media and reducing the amount of freezable water within the cell by dehydration before freezing and preventing intracellular ice formation (Aisen *et al*., 2005). It was found that trehalose increased the fluidity of the sperm plasma membrane, through interactions with the phospholipids of the plasma membrane (Tonieto *et al*., 2010) and depresses the membrane transition temperature (Tuncer *et al*., 2013), therefore trehalose increases the resistance of sperm membrane during the freezing process.

The present results of replacing different levels of egg yolk with levels of trehalose which did not significantly affect post-thawing sperm viability, and normal acrosome integrity disagree with those of Jafaroghli *et al*. (2011) who reported that the addition of 100 mM trehalose to tris extender containing 5 % egg yolk significantly increased post-thawing ram sperm viability and acrosome and membrane integrity and decreased total sperm abnormalities. Present results partially disagree also with those of Khalili *et al*. (2009) who worked on goats which indicates that the addition of 50, 75, or 100mM trehalose to tris extender containing 2.5 % egg yolk significantly improved all evaluated sperm characteristics following freezing and thawing. However, the present results are in agreement with Bucak *et al*. (2007) who found that the addition of 50 mM or 100 mM trehalose to tris extender containing 10 % egg yolk + 0.5 g fructose resulted in no significant effects on post-thawing ram sperm normal acrosome integrity. And, also, are in agreement with Aisen *et al*. (2000) who evaluated the effect of adding 2.0 mM trehalose to a tris-based extender containing 10 % egg yolk and 1% fructose (weight/volume) on post-thawing normal acrosome integrity and reported that trehalose treatment had no significant effect on the percentage of normal acrosome integrity.

The diversity of results could, partially, be attributed to differences in levels of trehalose added to the basic extender, other components of the extenders such as levels of egg yolk and glycerol, methods of freezing, and thawing, and species differences. It is worth noting that the results of the present experiment indicated no significant differences were detected between Rahmani and Barki breeds in any of the post-thawing spermatozoa parameters. From a biological point of view, the insignificant differences may be attributed to the two breeds belonging to the same species and were reared under the same managerial conditions, climatic effects, and feeding protocol.

CONCLUSION

Trehalose can be used to partially replace the high levels of egg yolk normally used in conventional tris extender, but the later must contain a low level of egg yolk (1%). Therefore, a synergistic effect may be found between egg yolk and trehalose in protect-

ing ram spermatozoa storage in liquid or frozen form. Further research is required to find out the best combination of levels of egg yolk or LDL and trehalose that give better ram sperm protection during semen storage at 5°C or by deep-freezing.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Amr M.A. Rashad for his help in statistical analysis and data curation. The authors also would like to thank the staff members of the Artificial Insemination Laboratory at The Faculty of Agriculture, Alexandria University Experimental station.

CONFLICT OF INTEREST

Authors declares that they have no conflict of interests.

REFERENCES

Aboagla, E.M.-E., Terada, T., 2003. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Biology of Reproduction 69, 1245-1250. doi:https://doi.org/10.1095/biolreprod.103.017889.

Aisen, E., Alvarez, H., Venturino, A., Garde, J., 2000. Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology 53, 1053-1061. doi:https://doi.org/10.1016/s0093-691x(00)00251-x.

Aisen, E., Quintana, M., Medina, V., Morello, H., Venturino, A., 2005. Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. Cryobiology 50, 239-249. doi:https://doi.org/10.1016/j.cryobiol.2005.02.002.

Ali Al Ahmad, M., Chatagnon, G., Amirat-Briand, L., Moussa, M., Tainturier, D., Anton, M., Fieni, F., 2008. Use of glutamine and low density lipoproteins isolated from egg yolk to improve buck semen freezing. Reproduction in Domestic Animals 43, 429-436. doi:https://doi.org/10.1111/j.1439-0531.2007.00930.x.

Amirat, L., Tainturier, D., Jeanneau, L., Thorin, C., Gérard, O., Courtens, J.L., Anton, M., 2004. Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl®, a commercial egg yolk extender. Theriogenology 61, 895-907. doi:https://doi.org/10.1016/s0093-691x(03)00259-0.

Bakas, L., Disalvo, E., 1991. Effect of Ca2+ on the cryoprotective action of trehalose. Cryobiology 28, 347-353. doi:https://doi.org/10.1016/0011-2240(91)90041-l.

Barth, A.D., Oko, R., 1989. Abnormal morphology of bovine spermatozoa. First edition ed. Iowa University Press, Ames. Iowa. .

Bogart, R., Mayer, D.T., 1950. The effects of egg yolk on the various physical and chemical factors detrimental to spermatozoan viability. Journal of Animal Science 9, 143-152. doi:https://doi.org/10.2527/jas1950.92143x.

Bucak, M.N., Ateşşahin, A., Varışlı, Ö., Yüce, A., Tekin, N., Akçay, A., 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: microscopic and oxidative stress parameters after freeze–thawing process. Theriogenology 67, 1060-1067. doi:https://doi.org/10.1016/j.theriogenology.2006.12.004.

Bucak, M.N., Tekin, N., 2007. Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. Small Ruminant Research 73, 103-108. doi:https://doi.org/10.1016/j.smallrumres.2006.12.001.

Buckett, W.M., Luckas, M.J., Aird, I.A., Farquharson, R.G., Kingsland, C.R., Lewis-Jones, D.I., 1997. The hypo-osmotic swelling test in recurrent miscarriage. Fertility and Sterility 68, 506-509. doi:https://doi.org/10.1016/s0015-0282(97)00241-0.

Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A., Womersley, C., 1984. Effects of carbohydrates on membrane stability at low water activities. Biochim. Biophys. Acta - Biomembr 769, 141-150. doi:https://doi.org/10.1016/0005-2736(84)90017-8.

De Leeuw, F.E., De Leeuw, A.M., Den Daas, J.H., Colenbrander, B., Verkleij, A.J., 1993. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. Cryobiology 30, 32-44. doi:https://doi.org/10.1006/cryo.1993.1005.

Evan, G., Maxwell, W., 1987. Salamon's Artificial Insemination of sheep and goat. Butter woth, , Sydney, Australia.

FASS, 2010. Federation of Animal Science Societies. Guide for the care and use of agricultural 414
animals in research and teaching. 3rd ed. Champaigh, IL.

Graham, J., Foote, R., 1987. Effect of several lipids, fatty acyl chain length, and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. Cryobiology 24, 42-52. doi:https://doi.org/10.1016/0011-2240(87)90005-8.

Jafaroghli, M., Khalili, B., Farshad, A., Zamiri, M., 2011. The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. Small Ruminant Research 96, 58-63. doi:https://doi.org/10.1016/j.smallrumres.2010.11.010.

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. doi:https://doi.org/10.5713/ajas.2009.90286.

Khlifaoui, M., Battut, I., Bruyas, J.F., Chatagnon, G., Trimeche, A., Tainturier, D., 2005. Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level. Theriogenology 63, 138-149. doi:https://doi.org/10.1016/j.theriogenology.2004.04.012.

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. doi:https://doi.org/10.1006/cryo.1998.2117.

Manjunath, P., 2012. New insights into the understanding of the mechanism of sperm protection by extender components, Animal Reproduction pp. 809-815.

Molinia, F., Evans, G., Casares, P.Q., Maxwell, W., 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, 113-122. doi:https://doi.org/10.1016/0378-4320(94)90058-2.

Moussa, M., Martinet, V., Trimeche, A., Tainturier, D., Anton, M., 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen–thawed bull semen. Theriogenology 57, 1695-1706. doi:https://doi.org/10.1016/s0093-691x(02)00682-9.

Öztürk, A.E., Bodu, M., Bucak, M.N., Ağir, V., Özcan, A., Keskin, N., Ili, P., Topraggaleh, T.R., Sidal, H., Başpinar, N., 2020. The synergistic effect of trehalose and low concentrations of cryoprotectants can improve post-thaw ram sperm parameters. Cryobiology. doi:https://doi.org/10.1016/j.cryobiol.2020.03.008.

Pace, M., Graham, E., 1974. Components in egg yolk which protect bovine spermatozoa during freezing. Journal of Animal Science 39, 1144-1149. doi:https://doi.org/10.2527/jas1974.3961144x.

Patist, A., Zoerb, H., 2005. Preservation mechanisms of trehalose in food and biosystems. Colloids and Surfaces B: Biointerfaces 40, 107-113. doi:https://doi.org/10.1016/j.colsurfb.2004.05.003.

Pereira, C.S., Hünenberger, P.H., 2008. Effect of Trehalose on a Phospholipid Membrane under Mechanical Stress. Biophysical Journal 95, 3525-3534. doi:https://doi.org/10.1529/biophysj.108.131656.

Phillips, P.H., Lardy, H.A., 1940. A yolk-buffer pabulum for the preservation of bull semen. Journal of Dairy Science 23, 399-404. doi:https://doi.org/10.3168/jds.S0022-0302(40)95541-2.

Quinn, P., Chow, P., White, I., 1980. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. Reproduction 60, 403-407. doi:https://doi.org/10.1530/jrf.0.0600403.

Rostami, B., Ebrahimi, D., Sadeghipanah, H., Masoumi, R., Shahir, M.H., 2019. Effects of supplementation of tris-egg yolk extender with different sugars and antioxidants on freezability of ram semen. Cryobiology. doi:https://doi.org/10.1016/j.cryobiol.2019.10.198.

Spindler, R., Huang, Y., Howard, J., Wang, P., Zhang, H., Zhang, G., Wildt, D., 2004. Acrosomal integrity and capacitation are not influenced by sperm cryopreservation in the giant panda. Reproduction 127, 547-556. doi:https://doi.org/10.1530/rep.1.00034.

Tada, N., Sato, M., Yamanoi, J., Mizorogi, T., Kasai, K., Ogawa, S., 1990. Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. Journal of Reproduction and Infertility 89, 511-516. doi:https://doi.org/10.1530/rep.1.00034.

Tonieto, R., Goularte, K., Gastal, G.D.A., Schiavon, R.S., Deschamps, J.C., Lucia, T., 2010. Cryoprotectant effect of trehalose and low-density lipoprotein in extenders for frozen ram semen. Small Ruminant Research 93, 206-209. doi:https://doi.org/10.1016/j.smallrumres.2010.05.003.

Tuncer, P.B., Taşdemir, U., Büyükleblebici, S., Özgürtaş, T., Coşkun, E., Erol, H., Aydın, F.N., Gürcan, İ.S., 2013. Effects of different doses of trehalose supplementation in egg yolk extender in frozen–thawed Angora buck semen. Small Ruminant Research 113, 383-389. doi:https://doi.org/10.1016/j.smallrumres.2013.04.012.

Valente, S., Pereira, R., Baptista, M., Marques, C., Vasques, M., Pereira, M.S., Horta, A., Barbas, J., 2010. In vitro and in vivo fertility of ram

semen cryopreserved in different extenders. Animal Reproduction Science 117, 74-77. doi:https://doi.org/10.1016/j.anireprosci.2009.04.007.

Watson, P., 1981. The roles of lipid and protein in the protection of ram spermatozoa at 5 C by egg-yolk lipoprotein. Reproduction 62, 483-492. doi:https://doi.org/10.1530/jrf.0.0620483.

Watson, P., Martin, I., 1975. Effects of egg yolk, glycerol and the freezing rate on the viability and acrosomal structures of frozen ram spermatozoa. Australian journal of biological sciences 28, 153-160. doi:https://doi.org/10.1071/bi9750153.

Westh, P., Ramløv, H., 1991. Trehalose accumulation in the tardigrade Adorybiotus coronifer during anhydrobiosis. Journal of Experimental Zoology 258, 303-311. doi: https://doi.org/10.1002/jez.1402580305.

Yildiz, C., Ottaviani, P., Law, N., Ayearst, R., Liu, L., McKerlie, C., 2007. Effects of cryopreservation on sperm quality, nuclear DNA integrity, in vitro fertilization, and in vitro embryo development in the mouse. Reproduction 133, 585-595. doi:https://doi.org/10.1530/REP-06-0256.

Zuther, E., Büchel, K., Hundertmark, M., Stitt, M., Hincha, D.K., Heyer, A.G., 2004. The role of raffinose in the cold acclimation response of Arabidopsis thaliana. FEBS Letters 576, 169-173. doi:https://doi.org/10.1016/j.febslet.2004.09.006.