Received: 2 November 2020 | Revised: 23 December 2020 | Accepted: 7 January 2021

DOI: 10.1111/andr.12974

ORIGINAL ARTICLE

ANDROLOGY WILEY

Combination of fetuin and trehalose in presence of low glycerol has beneficial effects on freeze-thawed ram spermatozoa

Mustafa Numan Bucak[1] | Pınar Peker Akalın[2] | Nazan Keskin[3] | Mustafa Bodu[1] | Ali Erdem Öztürk[4] | Pinar İli[5] | Hüseyin Özkan[6] | Tohid Rezaei Topraggaleh[7] | Halil Ozancan Arslan[8] | Nuri Başpınar[9] | Şükrü Dursun[10]

[1]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Sciences, Selcuk University, Konya, Turkey

[2]Department of Biochemistry, Faculty of Veterinary Sciences, Hatay Mustafa Kemal University, Hatay, Turkey

[3]Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Turkey

[4]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Sciences, Erciyes University, Kayseri, Turkey

[5]Department of Medical Services and Techniques, Denizli Vocational School of Health Services, Pamukkale University, Denizli, Turkey

[6]Department of Genetics, Faculty of Veterinary Sciences, Hatay Mustafa Kemal University, Hatay, Turkey

[7]Department of Anatomical Sciences, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran

[8]Republic of Turkey Ministry of Agriculture and Foresty International Center For Livestock Reseach And Training, Ankara, Turkey

[9]Department of Biochemistry, Faculty of Veterinary Sciences, Selcuk University, Konya, Turkey

[10]Department of Gynecology and Obstetrics, Faculty of Veterinary Sciences, Aksaray University, Aksaray, Turkey

Correspondence
Pınar Peker Akalın, Department of Biochemistry, Faculty of Veterinary Sciences, Hatay Mustafa Kemal University, Tayfur Sökmen Campus, 31001, Hatay, Turkey.
Email: pinarpekerakalin@gmail.com

Abstract

Background: Freeze-thawing process negatively affects ram spermatozoa in terms of sperm quality, DNA integrity and antioxidant defence system. Thus, antioxidant supplementation of spermatozoa during freeze-thawing is suggested to improve sperm parameters.

Objectives: The aim of this study was to determine the effects of fetuin and trehalose added into ram semen extender on sperm parameters, antioxidant parameters, antioxidant-related gene expressions and DNA integrity during the freeze-thawing process, in low glycerol concentration.

Methods: Semen samples collected from six mature rams were pooled and splitted into equal aliquots and diluted with a tris-based extender containing different concentrations of glycerol (G5; %5 and G3; %3), fetuin (F; 2.5, 5 and 15 mg/mL) and trehalose (60 mM) as eight groups (G5F0, G5F2.5, G5F5, G5F15, G3F0, G3F2.5, G3F5 and G3F15).

Results: G3F5 group resulted in the highest motility, mitochondrial activity and viability and the lowest DNA fragmentation and DNA damage ($p < 0.05$). Also, G3F0 displayed considerably more cryoprotective effect compared with G5F0 group ($p < 0.05$) in terms of motility, mitochondrial activity and viability rates. Lipid peroxidation levels decreased in G5F5 group compared with G5F0 group ($p < 0.05$). The levels of total glutathione increased in G3F2.5 group ($p < 0.05$) in comparison with the G5F0 group. *NQO1* gene levels were upregulated approximately twofold in G5F5, G5F15, G3F2.5, G3F5 and G3F15 groups compared with G5F0 group ($p < 0.05$). The levels of *GCLC* gene were approximately twofold higher in G3F0, G3F2.5, G3F5 and G3F15 groups compared with G5F0 group ($p < 0.05$). *GSTP1* gene levels were significantly higher with different levels in all treatment groups except for G5F2.5 and G3F0 groups in comparison with G5F0 group ($p < 0.05$).

Conclusions: Co-supplementation of tris-based extender having low glycerol (3%) with trehalose and fetuin to enhance the quality of ram spermatozoa after freeze-thawing process is recommended.

[Correction added on XX April 2021, after first online publication: the author contribution section has been updated in this version.]

Mustafa Numan Bucak, Department of Reproduction and Artificial Insemination, Faculty of Veterinary Sciences, Selcuk University, Alaaddin Keykubat Campus, 42003, Konya, Turkey.
Email: mnumanbucak@gmail.com

Funding information
This study was funded by the Scientific and Technological Research Council of Turkey (TUBITAK) (Project no: 114O642).

KEYWORDS
fetuin, freeze-thawing, glycerol, ram spermatozoa, trehalose

1 | INTRODUCTION

Application of artificial insemination (AI) carried out by using generally frozen-thawed semen or fresh is a well-developed technique that enhances genetic development, eradicates transmitted diseases, improves control of reproductive facilitates and the spread of precious conservation of genetic resources. However, the frozen-thawed ram semen fertility is lower than the intended success.[1-3] Semen cryopreservation proposes various benefits to the livestock industry which allows long-term storage of spermatozoa. However, it leads to cold shock, oxidative stress, the formation of ice crystal and osmotic changes within the cell membrane. Cryopreservation provokes decreased in vivo fertilizing ability, viability, motility and results in deterioration of plasma membrane integrity of spermatozoa.[4-7] The spermatozoa lipid content is a major predictive factor of lipid peroxidation, viability and cold shock. The mammalian sperm cells are sensitive to the damage of oxidative stress because of the high proportion of polyunsaturated fatty acids (PUFAs) of the plasma membrane.[8-10] The ram semen, subjected to cold shock coupled with oxygen radicals, are quite sensible to lipid peroxidation during cryopreservation. Cryopreservation induces oxidative stress by increasing reactive oxygen species (ROS) production which leads to a breakdown of sperm morphology, reduce motility, devastate membrane structure and decline in fertility.[11-13] In other respect, the low level of ROS participates in the hyperactivation, acrosome reaction, capacitation and sperm-oocyte binding processes.[5,11,14]

Ram spermatozoa membrane has a distinctive composition that makes the cryopreservation difficult; it includes a high amount of PUFAs in the membrane phospholipids; however, cholesterol-phospholipid ratio is low in the membrane.[15,16] Also, the seminal plasma of the ram is found to have a lower antioxidant potential compared with other species.[13,17] Furthermore, freezing negatively affect early capacitation of spermatozoa, and ram spermatozoa subjected to freeze and thawing are considerably more susceptible to the damages than other species.[17] Although laparoscopic insemination in sheep allows the use of frozen-thawed semen, the development of better extenders is needed for enhancing pregnancy.[18] To achieve convincing fertilization rates, a high percentage of motile and viable frozen-thawed sperm cells is vital in comparison with fresh spermatozoa.[19]

Several studies have deepened to improve sperm quality.[5,6,10,13,19-21] Cryoprotectants, which have high cell membrane permeability, low molecular weight and minimal toxicity, are used to conserve spermatozoa during cryodamage.[7] Besides the need for cryoprotectants, there is also a need to add cryo-supplement. These cryo-supplements have antioxidant effects and are used to improve sperm parameters.[7,22] Trehalose is a disaccharide as an energy source that consists of two D-glucose molecules. It takes part in sperm cryopreservation and has a protective effect against osmotic shocks and reduces cell damage caused by ice crystals. Trehalose also shows a direct interaction with phospholipid polar head groups, and because of this interaction, it stabilizes membrane phospholipids. However, the cracks of the inner and outer layer membrane (non-stabilized cell membrane) cause cell dehydration and osmotic imbalance.[16,23]

Fetuin, belonging to the cystatin family, is expressed during embryogenesis in multiple tissues.[24,25] In addition, it is a part of the major glycoprotein of foetal calf serum (FCS), representing about 45% of the total protein and has a serine protease inhibitor activity.[26,27] The addition of FCS to semen extender had a positive effect on motility, acrosomal integrity and plasma membrane integrity of liquid stored rabbit semen up to 72 h at 5°C.[28] Fetuin which is also a commercially available protein is pronounced to enhance sperm characteristics in different animals species.[6,28,29]

Glycerol is widely used as a cryoprotectant during freeze-thawing process of spermatozoa.[7] Unfortunately, it may exhibit undesired toxic and osmotic effects in the spermatozoa during the freeze-thawing.[30] Hence, it is suggested to decrease the concentration of glycerol in the media by supplementation of high molecular weight cryoprotectants.[23] In ram spermatozoa, similar post-thawed sperm quality parameters were achieved by decreasing glycerol content from 5% to 3% by supplementation of 60 mM trehalose compared with 5% glycerol alone.[23] Herewith, in this study, in order to increase the quality of post-thawed ram semen, we added trehalose and fetuin in presence of low glycerol concentration (3%). In the different studies mentioned above, different fetuin concentrations were used, but up to now, there is no information available regarding the most effective dose of fetuin addition before cryopreservation on ram sperm characteristics. Moreover, we examined the effect of the fetuin concentration with the most positive impact on sperm quality parameters and gene expression patterns. Therefore, the objective of the current work was to investigate the effects of glycerol, fetuin and trehalose on frozen-thawed sperm microscopic parameters, levels of LPO, tGSH and AOP as well as expression levels of *NQO1*, *GCLC* and *GSTP1* genes.

2 | MATERIALS AND METHODS

2.1 | Material

2.1.1 | Chemicals and semen collection

The chemicals used (glycerol G2025, trehalose T0167 and fetuin T4512) and extender compositions were obtained from Sigma-Aldrich Chemical Co. The experimental study was carried out at Bahri Dagdas International Agricultural Research Institute in Konya, Turkey. The semen was collected by using artificial vagina from fertile and mature Merinos rams (n = 6 2–3 years of age). The Merino ram semen ejaculates were collected three times a week for 4 weeks (at least 12 ejaculates for each ram) on the breeding season. Ejaculates providing spermatozoa motility of >75%, sperm concentration >2.5 ± 0.2 × 10^9 spermatozoa/mL and mass activity with +4 scores were collected and pooled in the present study.

2.2 | Methods

2.2.1 | Experimental design

The tris-based extender (10.0 g/L fructose, 14.0 g/L citric acid, 27.1 g/L tris, 20% egg yolk and pH between 6.8 and 7.2) was used as cryopreservation diluent.[16] The pooled ejaculates were split into eight aliquots and diluted with the tris extender including 5% glycerol with different concentrations of fetuin as follows: 5% glycerol +0 mg/mL fetuin (G5F0), 5% glycerol +2.5 mg/mL fetuin (G5F2.5), 5% glycerol +5 mg/mL fetuin (G5F5) and 5% glycerol +15 mg/mL fetuin (G5F15) for the first four groups. The supplementation of 60 mM trehalose to semen extender were added in order to reduce the content of glycerol (from 5% to 3%) with different fetuin concentrations as follows: 3% glycerol +0 mg/mL fetuin (G3F0), 3% glycerol +2.5 mg/mL fetuin (G3F2.5), 3% glycerol +5 mg/mL fetuin (G3F5) and 3% glycerol +15 mg/mL fetuin (G3F15), for the second four groups. Each aliquots were adjusted to final concentrations of 400 × 10^6 spermatozoa/mL, loaded into 0.25 mL French straws and equlibrated at 4°C for 2 h. Thereafter, the straws were plunged into liquid nitrogen vapour during 15 min and stored at −196°C in the liquid nitrogen until the assays.

2.2.2 | Post-thawed semen evaluation

Microscopic parameters

Spermatozoon was thawed at 37°C for 30 s. After thawing, sperm motility parameters were analysed using a phase contrast microscope (200×). The extended semen samples (10 μL) were placed on a pre-warmed microscope slide and were counted in at least 5 randomly selected microscopic fields (around 200 sperm cell) and analysed for each sample.

Sperm viability was analysed with LIVE/DEAD™ viability kit (L 7011 ThermoFisher, SYBR-14/PI) which was described by Garner & Johnson.[31] Briefly, a solution of SYBR-14 was diluted with DMSO (Appli-chem A3006) as a ratio of 1:10, and thereafter, the diluted dye was split into equal aliquots (30 μL) in 500 μL Eppendorf tubes (Isolab LB.IS.078.03.021). The propidium iodide (PI) stain was solved in distilled water at 2 mg/mL, split into 30 μL aliquots and stored at −20°C. The frozen-thawed ram semen sample were diluted with phosphate-buffered solution (PBS) as a ratio of 1:3; then, 30 μL aliquots were mixed with 2.5 μL of PI and 6 μL of SYBR-14. The ram semen sample was properly mixed and incubated at 37°C for 15 min; afterwards, 10 μL of Hancock solution was added to stop the sperm act. The 2.5 μL drop of semen sample placed on microscope slide and then an amount of 200 spermatozoa were analysed using a fluorescence microscope (at 400× magnification) (Leica DM 3000 Microsystems GmbH; emission at 520 nm, excitation at 450–490 nm) to examine the sperm plasma membrane integrity (sperm viability). The red stained fluorescence of sperm head was accepted to be not viable spermatozoa (damaged spermatozoa), whereas the green stained fluorescence of sperm head was considered to be viable spermatozoa (non-damaged spermatozoa).

The assessment of the mitochondrial membrane potential of spermatozoa was performed using the JC-1/PI, which was described by Garner et al.[32] Cryopreserved ram spermatozoa were thawed at 37°C (in the water bath) for 30 s, and then, it was diluted as the ratio of 1:3 with PBS, and 300 μL diluted ram semen sample was quietly mixed with 2.5 μL of PI and JC-1. This mixture was incubated at 37°C for 15 min, and thereafter, 10 μL Hancock solution was added to a mixed sample to stop sperm act. For the assessment of mitochondrial membrane potential activity, 2.5 μL drop of semen sample was put on a microscope slide and then an amount of 200 spermatozoa were investigated using a fluorescence microscope (400× magnification). The stained orange or yellow fluorescence of sperm midpiece was considered to be high mitochondrial membrane potential activity, while the stained green of sperm midpiece was accepted to be low mitochondrial membrane potential activity.

The integrity of sperm acrosome was assigned by FITC-PNA (L7381, fluorescein isothiocyanate conjugated peanut agglutinin, Sigma-Aldrich Co.) and by propidium iodide (PI) staining as described by Nagy et al.[33], with some modifications. The frozen-thawed ram sperm samples were diluted with PBS as a ratio of 1:3, and thereafter, 60 μL diluted sample was mixed with 2.5 μL of PI and 10 μL of FITC-PNA. The sample was incubated at 37°C for 15 min and then, 10 μL Hancock solution was added to the diluted sperm sample and 2.5 μL of the sample was analysed. For the evaluation of sperm acrosome integrity, 2.5 μL ram semen sample (an amount of 200 spermatozoa) was investigated using a fluorescence microscope (at 400× magnification) (emission at 520 nm, excitation at 450–490 nm, Leica DM 3000 Microsystems GmbH). The stained green acrosome region of spermatozoa was considered to be non-intact acrosome (damaged acrosome), while the stained red fluorescence of acrosome region of spermatozoa was accepted intact acrosome (undamaged acrosome).

DNA fragmentation of the spermatozoa was analysed using the TUNEL assay described by Henkel et al.[34] The procedure of the in situ cell death Detection Kit (POD; ROCHE) was used for this assessment. The slides were counterstained with methyl green, randomly selected 100 spermatozoa from 10 different regions using a light microscope and counted to assess the percentage of TUNEL-positive cells with brown colour in their nuclear region representing DNA fragmentation.

DNA damage of spermatozoa was analysed using COMET (single cell gel electrophoresis) assay which is a reliable method to detect single- and/or double-strand breaks of DNA, base damages, cross-links and apoptotic nuclei.[35] The images of 200 randomly selected nuclei were examined using a light microscope (at 400× magnification, B-600Ti Optika) equipped with a 4083.B5 OptikamB5 digital camera (Optika Microscopes). DNA damage of the spermatozoa was observed by a tail of fragmented DNA that migrated from the sperm head, emerging like a 'comet' pattern, while the lack of a comet was considered to be undamaged (non-damage) DNA (Figure 1).

2.2.3 | Oxidative stress parameters

Preparation of supernatants

In brief, cryopreserved ram semen samples were thawed and centrifuged at 600 g × 10 min × 4°C to separate the extender. And then, the cellular pellet was washed three times with PBS at 600 g for 10 min. After the last centrifugation, the supernatant was discarded and the pellet was picked up in a 2-mL beaker in ice water and sonicated in PBS on a continuous basis by a sonicator (D-12207, Gerate-Typ: UW 2070) 5 times for 10 s with 30 s cooling period between.[36] For the assays of LPO, 10 µL of 0.5 mM butylhydroxytoluene (B1378 BHT, Sigma-Aldrich Co.) was added into 120 µL homogenate samples to prevent further peroxidation and stored at −80°C until analysis. For the analysis of tGSH and AOP levels, the remaining homogenate was centrifuged at 4°C (×8000 g) for 15 min, and the supernatant of the sample was stored at −80°C.

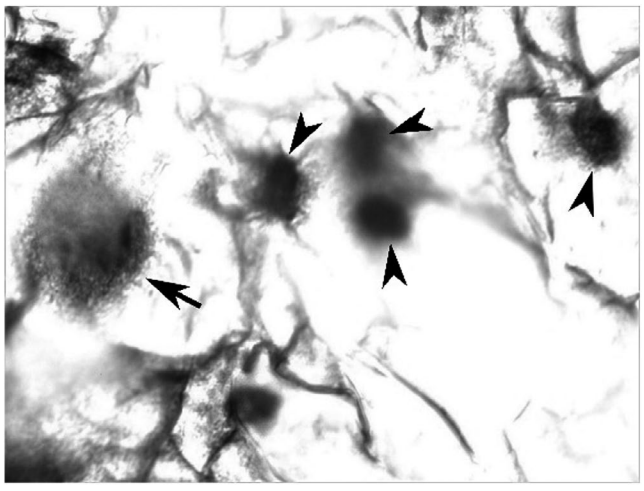

FIGURE 1 DNA damages in spermatozoa which were assessed by single cell gel electrophoresis (COMET assay). Spermatozoa without DNA damage (arrow head), and spermatozoa with damaged DNA (arrow). Original magnification ×400.

Levels of lipid peroxidation (LPO)

Levels of lipid peroxidation levels were examined spectrophotometrically (UV 2100 UV-VIS Recording Spectrophotometer; Shimadzu) by using LPO-586™ Oxis Research commercial kit (Oxis Research™; Bioxytech) which the assay was based on the reaction of N-methyl–2- phenylindole (chromogenic reagent) with 4-hydroxyalkenals and MDA at 45°C. The 4-hydroxyalkenal or MDA molecule reacts with 2 molecules of N-methyl-2-phenylindole in acetonitrile, to provide a fixed chromophore at 586 nm.

Levels of Total glutathione (tGSH)

The levels of total glutathione were analysed spectrophotometrically with GSH-420™ Oxis Research commercial kit. Briefly, tris phosphine was added to decrease any oxidized glutathione (GSSG) to the reduced state (GSH). The chromogen was used forming thioethers with all thiols in the present study sample.

Levels of antioxidant potential (AOP)

Antioxidant potential levels were analysed spectrophotometrically by using AOP-490™ Oxis Research commercial kit. The analyse of levels of antioxidant potential of the present study was based upon the reduction of Cu^{+2} to Cu^{+} by the consolidated effect of all antioxidants present in the sample. Briefly, Bathocuproine intently forms a 2:1 complex with Cu^{+} having a maximum absorbance at 490 nm.

2.2.4 | Gene expression analyses

Sperm cell separation from somatic cells

The solutions of BoviPure (40% and 80%; Nidacon International AB) were used to isolate sperm cell fractions. In brief, 0.5 mL volumes of BoviPure solution (40% and 80%) were added into centrifuge tubes. Thereafter, 0.25 mL of frozen-thawed ram semen sample was layered at the top of the tube and centrifuged at 600 × g for 20 min at 4°C. The supernatant of the sample was removed, and sperm pellet was washed twice with 0.5 mL of PBS. For analysing the existence of somatic cells, 0.1 mL PBS was added to sperm pellet and placed at 40× magnification stereo microscope; also, sperm solution was processed for RNA isolation.

Isolation of total RNA and cDNA synthesis

After separation of somatic cells, total RNA was isolated from sperm cells. 1 mL TRIzol Reagent (Invitrogen) was added to each sample for this purpose. Then, the samples were gently vortexed and kept for 10 min in room temperature. Chloroform was added as much as one in 4 of the TRIzol Reagent used in the samples. Samples were kept at room temperature for 10 min after gently mixed and were centrifuged at 4°C (12,000 g) for 15 min. Aqua phase of samples was collected to new sterile nuclease-free tubes, and isopropyl alcohol was added as much as half of the TRIzol Reagent used in the samples.

TABLE 1 Sperm quality parameters of Merino ram semen supplemented with different concentrations of glycerol, fetuin and trehalose after freeze-thawing process.

Groups	Motility %	JC1/PI %	SYBR−14/PI %	FITC-PNA/PI %
G5	31 ± 1.87[d]	31.00 ± 1.56[d]	41.35 ± 1.26[d]	52.99 ± 2.83
G5F2.5	38 ± 2.54[c]	38.70 ± 2.43[c]	48.94 ± 1.42[c]	53.55 ± 0.82
G5F5	45 ± 1.58[b]	45.44 ± 1.20[b]	54.47 ± 1.66[b]	52.34 ± 1.52
G5F15	46 ± 1.87[b]	46.46 ± 1.47[b]	56.15 ± 1.28[b]	52.95 ± 2.09
G3	48 ± 2.00[b]	48.59 ± 1.39[b]	57.04 ± 1.85[b]	52.47 ± 2.91
G3F2.5	48 ± 1.22[b]	49.21 ± 1.19[b]	58.36 ± 1.09[b]	55.62 ± 2.51
G3F5	54 ± 1.00[a]	54.67 ± 1.01[a]	63.51 ± 1.24[a]	56.44 ± 1.56
G3F15	48 ± 1.22[b]	47.71 ± 1.13[b]	57.17 ± 1.38[b]	57.41 ± 2.66
P	*	*	*	-

Abbreviations: FITC-PNA/PI, sperm integrity; G3, 3% glycerol +0 mg/mL fetuin +60 mM trehalose, G3F2.5, 3% glycerol +2.5 mg/mL fetuin +60 mM trehalose; G3F15, 3% glycerol +15 mg/mL fetuin +60 mM trehalose; G3F5, 3% glycerol +5 mg/mL fetuin +60 mM trehalose; G5 F 5, 5% glycerol +5 mg/mL fetuin; G5, 5% glycerol +0 mg/mL fetuin; G5F15, 5% glycerol +15 fetuin mg/mL; G5F2.5, % glycerol +2.5 mg/mL fetuin; JC1/PI, mitochondrial membrane potential; SYBR-14/PI, sperm viability.

[a,b,c,d]Means with different letters in the same colomn are significantly different from each other ($p < 0.05$).

The samples were briefly mixed and stored up at room temperature for 10 min. For precipitation RNA, samples were centrifuged at 4°C (12,000 ×g) for 10 min. Following the centrifugation, supernatants of samples were discarded and RNA pellets were washed with 70% ethyl alcohol twice. For this purpose, 1 mL ethyl alcohol was added and samples were centrifuged at 4°C (7500 ×g) for 5 min. Supernatants of samples were discarded, and samples were centrifuged for the last time with 1 mL 96% ethyl alcohol. Finally, pellets were kept at room temperature for 10 min then dissolved with 40 µL nuclease-free water. Concentrations and purity (A260/280) of samples were analysed with a nucleic acid spectrophotometer (Colibri Titertek Berthold). Genomic DNA digestion was carried out to the samples for eliminating possible contamination (Arcticzymes). The cDNA synthesis kit (Vivantis) was used for cDNA conversion, and the condition of thermal cycler was as follows: 10 min at 25°C, 60 min at 42°C and 5 min at 85°C, respectively.

Quantitative real-time PCR application and gene expression analyses

Expression levels of glutamate-cysteine ligase catalytic subunit (*GCLC*), glutathione S-transferase pi 1 (*GSTP1*) and NAD(P)H quinone dehydrogenase 1 (*NQO1*) genes were determined with qPCR.[37-39] Amplification of genes was carried out using SYBR Green I dye-containing kit (Diagen). The qPCR protocol was as follows: 10 min at 95°C, followed by 10 s at 95°C, 30 s at 60°C, 5 s at 72°C and 40 cycles in qPCR (ABI 7500 Fast), while each cDNA sample was performed as duplicate, Protamine 1 (*PRM1*) gene was used as a reference gene.

2.2.5 | Statistical analysis

All data were defined as mean ± SE. For microscopic and oxidative stress parameters, means were analysed with Duncan's post hoc test and ANOVA to adjust considerable differences. The values of LPO, tGSH and AOP were log10 transformed. For calculations of the gene expressions, the $2^{-\Delta\Delta Ct}$ method was used, and the results were explained as fold change. Statistical analyses were carried out with SPSS 13.0 (SPSS Inc.), and statistical relevance was adjusted at $p < 0.05$.

3 | RESULTS

3.1 | Sperm parameters

As shown in Table 1, fetuin at 2.5, 5 and 15 mg/mL doses with and without trehalose showed increased rates of motility, mitochondrial activity and viability rates compared with G5F0 group ($p < 0.05$). The highest motility, mitochondrial activity and viability rates were obtained in the group G3F5 in comparison with the other groups ($p < 0.05$). Also, %3 glycerol +trehalose 60 mM (G3F0 group) displayed considerably more cryoprotective effect compared with %5 glycerol group (G5F0) ($p < 0.05$) in terms of motility, mitochondrial activity and viability rates. No extender including fetuin and trehalose significantly improved acrosome integrity compared with G5F0 and G3F0 groups. These results revealed that fetuin and fetuin +trehalose could have positive effects on microscopic sperm parameters during the freeze-thawing process.

As shown in Figure 2A, the lowest value of DNA fragmentation (TUNEL analysis) was obtained in the G3F5 group in comparison with the other groups while the statistical significance was only determined between the G5F0, G5F2.5 and G5F5 groups ($p < 0.05$). Furthermore, the considerably least DNA damage (COMET analysis) was observed in the group G3F5 compared with the other groups while the statistical significance was only determined between the G5F0, G5F5 and G5F15 groups ($p < 0.05$) (Figure 2B).

3.2 | Oxidative stress parameters

The results of oxidative stress parameters are shown in Figure 3. The LPO levels decreased in G5F5 compared with G5F0 group ($p < 0.05$). The levels of tGSH increased in G3F2.5 group ($p < 0.05$) in comparison with the control group. There were no significant differences between all groups, as AOP levels increased in all of them ($p > 0.05$).

3.3 | Gene expressions analysis

As compared with G5F0 group, the level of *NQO1* gene expression was approximately twofold higher in G5F5, G5F15, G3F25, G3F5 and G3F15 groups compared with G5F0 group ($p < 0.05$). On the contrary, there were no considerable differences between G5F0 and the other groups (Figure 4A). While expression levels of *GCLC* gene approximately twofold more in G3F0, G3F2.5, G3F5 and G3F15 groups, this gene was expressed almost threefold more in G5F15 group ($p < 0.05$) (Figure 4B). As shown in Figure 4C, the level of *GSTP1* gene was significantly higher in all treatment groups except for G5F2.5 and G3F0 groups in comparison with G5F0 group ($p < 0.05$).

4 | DISCUSSION

Foetal calf serum (FCS) comprises a variety of proteins and is a component of most media used for maintaining the cells in the culture in animals.[40] Fetuin, a major glycoprotein of FCS, consists of two homologous proteins as fetuin-A and fetuin B.[27,41] In the present study, fetuin was added (as an antioxidant) into a freezing extender with and without trehalose and observed that fetuin and fetuin+trehalose supplementation into freezing extender at different doses showed some protective effects on the forzen-thawed ram semen. Fetuin was reported as a powerful antioxidant to improve sperm motility significantly in Brown Swiss bull spermatozoa (at a dose of 10 mg/mL) and rabbit spermatozoa (FCS at a dose of %10) compared with tris-based extender without fetuin.[28,42] On the other hand, in Holstein bull spermatozoa, fetuin administration into base extender did not enhance sperm motility at a dose of 2.5 mg/mL.[6] Jaiswal et al.[29] demonstrated that fetuin at a dose of 800 µg/mL improved forward goat sperm motility. It can be issued that in bull spermatozoa, fetuin at lower doses (2.5 mg/mL) is inadequate to improve sperm motility whereas, in ram and/or goat spermatozoa, lower doses are also effective in terms of sperm motility improvement, as in this study fetuin administration even at 2.5 mg/mL dose increased motility compared with %5 glycerol group significantly.[6]

In the present study, the addition of fetuin (the doses of 2.5, 5 and 15 mg/mL fetuin) to semen extender did not enhance acrosomal integrity of spermatozoa in comparison with tris-based extender without fetuin. On the contrary, tris-based media containing %10 FCS increased plasma and acrosomal integrity compared with control group during 72 h of the liquid storage in rabbit semen.[28] One possible reason for acrosomal integrity differences can be that the addition of different dosages of fetuin to semen extender and using different animal species. Also, plasma membrane integrity and mitochondrial membrane potential were increased by adding fetuin to semen extender, and these findings are in accordance with study in which supplementation of exogenous fetuin improved plasma membrane integrity.[28] Because the present study was the first one investigating the effect of fetuin on the mitochondrial membrane potential of cryopreserved ram semen,

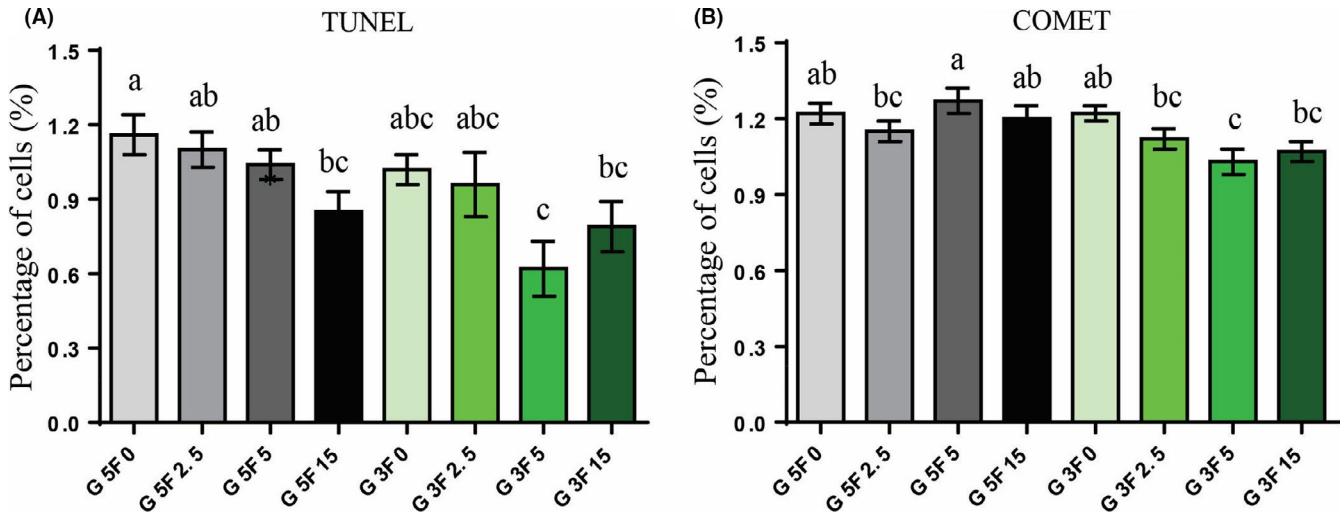

FIGURE 2 Graphs of DNA fragmentation (A) and DNA damage (B) of Merino ram semen (Mean ± SE) supplemented with different concentrations of glycerol, fetuin and trehalose after freeze-thaw process. Means with different letters in each graph are significantly different from each other ($p < 0.05$). Abbreviations: G5F0, 5% glycerol +0 mg/mL fetuin; G5F2.5, % glycerol +2.5 mg/mL fetuin; G5 F 5, 5% glycerol +5 mg/mL fetuin; G5F15, 5% glycerol +15 fetuin mg/mL; G3F0, 3% glycerol +0 mg/mL fetuin +60 mM trehalose, G3F2.5, 3% glycerol +2.5 mg/mL fetuin +60 mM trehalose; G3F5, 3% glycerol +5 mg/mL fetuin +60 mM trehalose; G3F15, 3% glycerol +15 mg/mL fetuin +60 mM trehalose, TUNEL, DNA fragmentation test; COMET, DNA damage test.

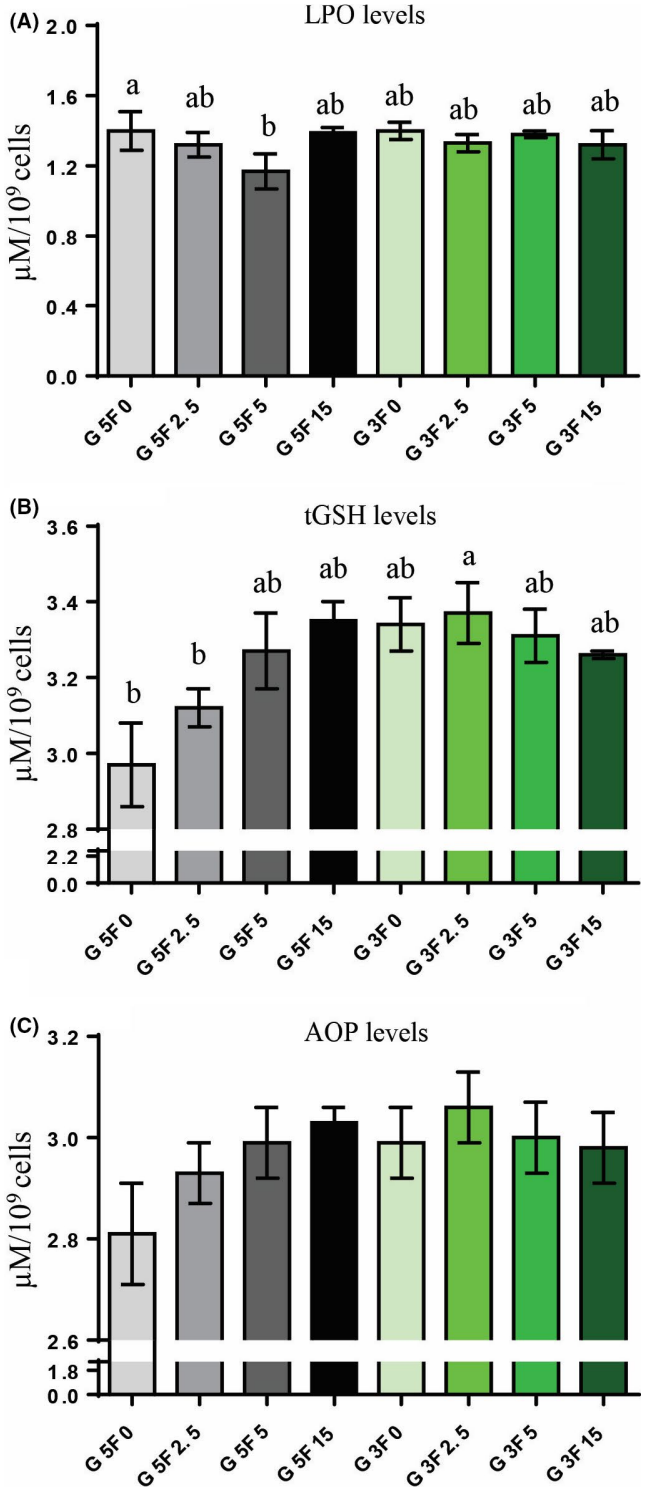

FIGURE 3 Graphs of LPO (A), tGSH (B) and AOP (C) levels (Mean ± SE) of Merino ram semen supplemented with different concentrations of glycerol, fetuin and trehalose after freeze-thaw process. Means with different letters in each graph are significantly different from each other ($p < 0.05$). Abbreviations: G5F0, 5% glycerol +0 mg/mL fetuin; G5F2.5, % glycerol +2.5 mg/mL fetuin; G5 F 5, 5% glycerol +5 mg/mL fetuin; G5F15, 5% glycerol +15 fetuin mg/mL; G3F0, 3% glycerol +0 mg/mL fetuin +60 mM trehalose, G3F2.5, 3% glycerol +2.5 mg/mL fetuin +60 mM trehalose; G3F5, 3% glycerol +5 mg/mL fetuin +60 mM trehalose; G3F15, 3% glycerol +15 mg/mL fetuin +60 mM trehalose; LPO, lipid peroxidation; tGSH, total glutatione; AOP, antioxidant potential.

we could not compare our findings with those of other studies. Most probably, these findings could be explained that fetuin could inhibit protease enzymes on membrane activity, thus mitochondrial membrane potential could be increased.[43]

Sperm DNA integrity is mightily suggested to be an objective marker for sperm quality, and the intact sperm DNA is crucial for sperm function. As freeze-thawing process and overproduction of ROS provoke DNA damage and fragmentation in spermatozoa, DNA integrity protection by antioxidants becomes crucial in order to provide high sperm quality.[4,28,42,44] Our findings are in accordance with Sarıözkan et al. [6,28] in which supplementation of exogenous fetuin protected DNA fragmentation in bull and rabbit spermatozoa. In addition to these, as FCS is supposed to stabilize the expanding cumulus extracellular matrix, it is widely used in the COC's (cumulus-oocyte complexes) culture.[45] Fetuin has been reported to inhibit zona pellucida hardening during the in vitro maturation equine oocytes[46] and of the mouse[27] and allow normal fertilization rates in oocytes cultured in the absence of FCS.[27,46]

Lipid peroxidation (LPO) level, which is reported to increase during the freeze-thawing process in spermatozoa, is a well known parameter to indicate cell oxidative stress response.[10,11,44] Cellular antioxidants such as glutathione (GSH) and antioxidant potential (AOP) levels also reflect the cellular oxidative stress response against oxidative damage during freeze-thawing of spermatozoa.[20,47,48] Fetuin administration into freezing extender at a dose of 10 mg/mL in bull spermatozoa increased sperm antioxidant enzymes such as SOD and GPx activities.[6] Foetal calf serum has been shown to have partially inhibiting effect on 4-hydroxynonenal (the production of LPO) toxicity in Jurkat human lymphoma T cells in vitro.[49] Sarıözkan et al.[6] demonstrated reduced MDA levels after the freezing-thawing process of bull spermatozoa which was subjected to fetuin at a dose of 2.5 mg/mL. Similar with above results, the addition of fetuin displayed tremendous effect on the LPO and tGSH in spermatozoa compared with spermatozoa without fetuin while antioxidant potential also showed an insignificant increase in all fetuin and fetuin+trehalose administered groups. Therefore, it can be suggested that the decrease of LPO levels and increase of tGSH levels with 5 mg fetuin and 2.5 mg fetuin+trehalose administration may have some antioxidant effect during cryopreservation of ram semen. To explain this astonishing result, it should be emphasized that the combination of low glycerol and fetuin was the most protective effect on oxidative stress by increasing the antioxidant defence system.

The genes of glutamate-cysteine ligase catalytic subunit (*GCLC*), glutathione S-transferase pi 1 (*GSTP1*) and NAD(P)H quinone dehydrogenase 1 (*NQO1*) are powerful antioxidant response genes, which were reported to protect the cell from ROS.[50-53] The protein encoded by *NQO1* gene is induced under cellular stress responses notably oxidative stress and it reduces quinones to hydroquinones by catalysing two-electron mediated reaction.[52,54] Glutathione preserves the membrane lipids and proteins from

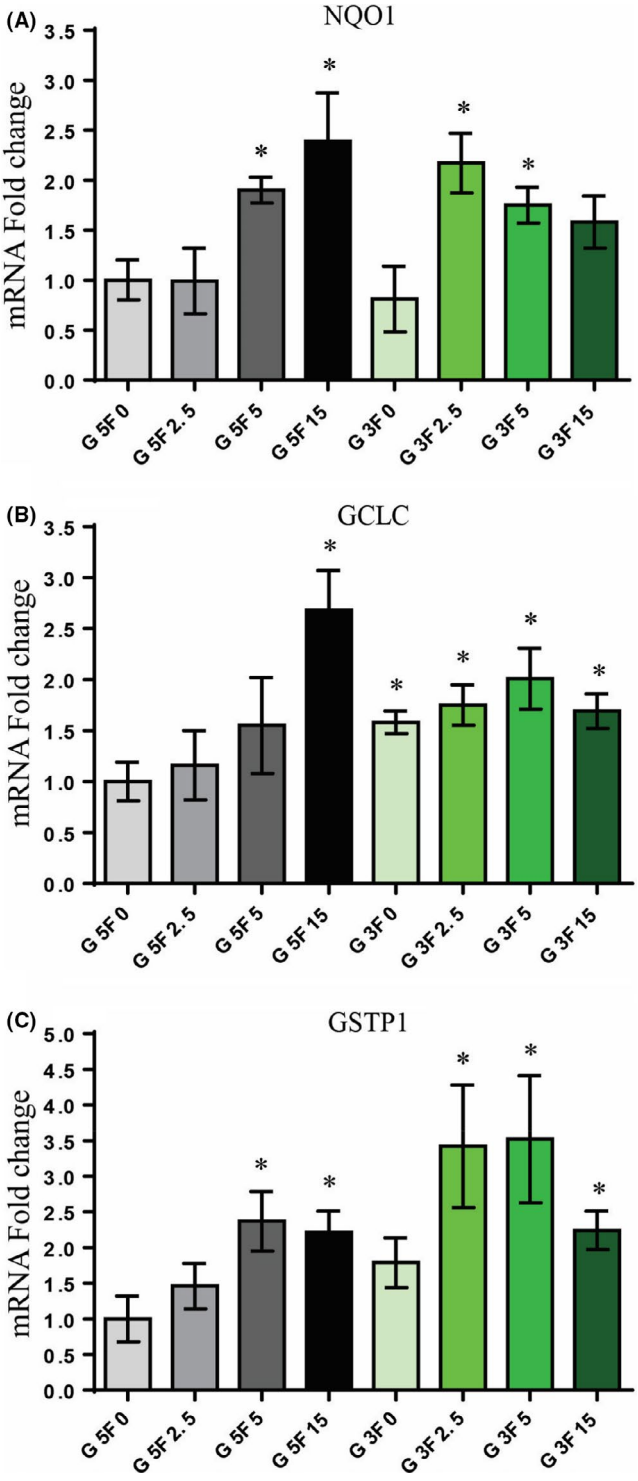

FIGURE 4 Graphs of NQO1 (A), GCLC (B) and (C) GSTP1 gene expression levels (Mean ± SE) in Merino ram semen supplemented with different concentrations of glycerol, fetuin and trehalose after freeze-thaw process compared with the group G5F0. *: ($p < 0.05$). Abbreviations: G5F0, 5% glycerol +0 mg/mL fetuin; G5F2.5, % glycerol +2.5 mg/mL fetuin; G5 F 5, 5% glycerol +5 mg/mL fetuin; G5F15, 5% glycerol +15 fetuin mg/mL; G3F0, 3% glycerol +0 mg/mL fetuin +60 mM trehalose, G3F2.5, 3% glycerol +2.5 mg/mL fetuin +60 mM trehalose; G3F5, 3% glycerol +5 mg/mL fetuin +60 mM trehalose; G3F15, 3% glycerol +15 mg/mL fetuin +60 mM trehalose; NQO1, NAD(P)H quinone dehydrogenase 1; GCLC, glutamate-cysteine ligase catalytic subunit; GSTP1, glutathione S-transferase pi 1.

reactive oxygen species (ROS) by radical-scavenging properties and is known to be the most abundant intracellular non-protein thiol that participates in cellular redox homeostasis.[20,47,48] *GCLC* gene, which is rate-limiting, is a part of GSH synthesis enzyme[55] and is supposed to be related to testicular antioxidant capacity during oxidative stress.[56,57] *GSTP1* protein is under consideration to have protecting roles against oxidative damage to spermatozoa.[47] *GSTP1* protein and the levels of GSH in seminal plasma of subfertile man were lower than in the fertile man.[58] In this study, higher level of the genes such as *NQO1*, *GCLC and GSTP1* can be responsible from decreased LPO and increased tGSH and AOP levels. First of all, *NQO1* protein which is encoded from *NQO1* gene is associated with NADPH dehydrogenase family and has vital antioxidant roles in the cell.[54] Induction of the *NQO1* protein to high levels in cells might be mediated by Ah receptor mechanisms or Nrf_2-mediated induction.[59] Moreover, cellular response to oxidative stress increases the *NQO1* gene expression level.[52,54] These findings accord with our study results, as the potential of antioxidant was increased by upregulating *NQO1*. Considering that *GCLC* is a part of the glutathione metabolic pathway,[55] the significant increase of tGSH levels and upregulation of *GCLC* gene expression by fetuin and fetuin+trehalose compared with %5 glycerol (without fetuin), it might be suggested that tGSH was regulated by this gene in ram spermatozoa during the freeze-thawing process. *GCLC* is supposed to be related to nuclear *Nrf2* pathway and increased testicular antioxidant capacity during cryopreservation.[56,57] Thus, it might be thought that the addition of fetuin can decrease oxidative stress damage by upregulating *GCLC* gene. The *GSTP1* protein was suggested to have protecting roles against oxidative damage to spermatozoa.[47] In addition to these, *GSTP1* was also reported to protect the human prostate tissue cells against oxidative stress and DNA damage.[58] In this study, fetuin and fetuin+trehalose, at some doses, preserved these antioxidant-related genes more efficiently. In ram spermatozoa, it can be speculated that fetuin may decrease LPO and increase tGSH and AOP levels by inducing Keap-1-Nrf2 pathway to increase upregulation of *NQO1* and *GCLC* genes. To our knowledge, this is the first study on the effects of fetuin on *NQO1*, *GCLC* and *GSTP1* genes and mitochondrial membrane potential of cryopreserved ram semen.

5 | CONCLUSIONS

In conclusion, fetuin and/or trehalose could enhance post-thawed sperm characteristics, DNA integrity by decreasing oxidative stress and LPO level with increased antioxidant capacity and antioxidant associated gene expressions. To reduce glycerol concentration, we added trehalose into % 3 glycerol extender with fetuin and obtained similar results with fetuin + % 5 glycerol content.

G5F15 group represented higher motility, mitochondrial activity and viability rates, increased *NQO1*, *GCLC* and *GSTP1* gene levels and decreased DNA fragmentation compared with G5F0 group. In addition, G3F2.5 group had favourable effects on sperm quality parameters, tGSH levels and gene expression levels compared with G5F0 group. Beyond, the highest motility, mitochondrial activity and viability and the lowest DNA fragmentation and DNA damage results were obtained in glycerol 3% + trehalose 60 mM +Fetuin 5 mg (G3F5) group. G3F5 group also showed higher *NQO1*, *GCLC* and *GSTP1* gene levels compared with G5F0 group. Thus, co-supplementation of fetuin and trehalose in freezing extender is recommended to improve quality of frozen-thawed ram spermatozoa. Given the foregoing considerations of the in vitro data presented, it is suggested to evaluate the effect of fetuin and trehalose on fertility trials of frozen-thawed ram spermatozoa, iv vivo and in vitro.

ACKNOWLEDGEMENTS

The authors are thankful to Bahri Dagdas International Agricultural Research Institute, Konya, Turkey, for providing the rams.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR'S CONTRIBUTIONS

M.N.B., N.B. and P.P.A. designed the study. M.N.B., M.B., A.E.Ö., H.O.A. and Ş.D. collected, freezed sperm samples and performed sperm quality analysis. N.K. and P.İ. performed COMET and TUNNEL analysis. H.Ö. performed gene analysis. P.P.A. and N.B. performed oxidative stress parameters analysis. P.P.A. performed statistical analysis T.R.T. designed table and figures. M.N.B., P.P.A. and T.R.T. wrote and edited the manuscript.

ORCID

Pınar Peker Akalın https://orcid.org/0000-0002-6991-3727
Pinar İli https://orcid.org/0000-0002-3107-1798

REFERENCES

1. O'Hara L, Hanrahan JP, Richardson L, et al. Effect of storage duration, storage temperature and diluentson the viability and fertility of fresh ram sperm. *Theriogenology*. 2010;73:541-549.
2. Cesh S, Faigl V, Amirids GS. Semen processing and artificial insemination in health management of small ruminants. *Anim Reprod Sci*. 2012;130(3–4):187-192.
3. Casali R, Pinczak A, Cuadro F, Guillen-Mu˜noz JM, Mezzalira A, Menchaca A. Semen deposition by cervical, transcervical and intrauterine route for fixed-time artificial insemination (FTAI) in the Ewe. *Theriogenology*. 2017;103:30-35.
4. Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl*. 2000;21(1):1-7.
5. Bucak MN, Ateşşahin A, Yüce A. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process. *Small Rum Res*. 2008;75(2–3):128-134.
6. Sarıözkan S, Bucak MN, Tuncer PB, Büyükleblebici S, Eken A, Akay C. Influence of fetuin and hyaluronan on the post-thaw quality and fertilizing ability of Holstein bull semen. *Cryobiology*. 2015;71(1):119-124.
7. Martins AD, Agarwal A, Henkel R. *Sperm cryopreservation. In vitro fertilization*. Springer: Cham; 2019:625-642.
8. Aitken R, Fisher H. Reactive oxygen species generationby human spermatozoa: balance of benefit and risk. *BioEssays*. 1994;16(4):259-268.
9. Maxwell WMC, Watson PF. Recent progress in the preservation of ram semen. *Anim Reprod Sci*. 1996;42(1–4):55-65.
10. Bucak MN, Sarıözkan S, Tuncer PB, et al. The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. *Small Rum Res*. 2010;89:24-30.
11. Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MCG. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J Androl*. 2000;21(6):895-902.
12. Mukhopadhyay CS, Gupta AK, Yadav BR, et al. Effect of cryopreservation on sperm chromatin integrity and fertilizing potential in bovine semen. *Livest Sci*. 2011;136(2–3):114-121.
13. Motlagh MK, Sharafi M, Zhandi M, et al. Antioxidant effect of rosemary (Rosmarinus officinalis L.) extract in soybean lecithin-based semen extender following freeze–thawing process of ram sperm. *Cryobiology*. 2014;69(2):217-222.
14. Mislei B, Bucci D, Malama E, Bollwein H, Mari G. Seasonal changes in ROS concentrations and sperm quality in unfrozen and frozen-thawed stallion semen. *Theriogenology*. 2020;144:89-97.
15. Quinn PJ, Chow P, White IG. White Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. *J Reprod and Fertil*. 1980;60(2):403-407.
16. Aisen EG, Medina VH, Venturino A. Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology*. 2002;57(7):1801-1808.
17. Zalazar L, Iniesta-Cuerda M, Sánchez-Ajofrín I, Garde JJ, Valls AJS, Cesari A. Recombinant SPINK3 improves ram sperm quality and in vitro fertility after cryopreservation. *Theriogenology*. 2020;144:45-55.
18. Byrne GP, Lonergan P, Wade M, et al. Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and in vitro. *J Androl*. 2000;21:895-902.
19. Arslan HO, Herrera C, Malama E, Siuda M, Leiding C, Bollwein H. Effect of the addition of different catalase concentrations to a TRIS-egg yolk extender on quality and in vitro fertilization rate of frozen-thawed bull sperm. *Cryobiology*. 2019;91:40-52.
20. Gadea J, Selles E, Marco MA, et al. Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*. 2004;62:690-701.
21. Matsuoka T, Imai H, Kohno H, Fukui Y. Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. *J Reprod Develop*. 2006;52(5):675-683.
22. Soni Y, Talluri TR, Kumar A, Ravi SK, Mehta JS, Tripathi BN. Effects of different concentration and combinations of cryoprotectants on sperm quality, functional integrity in three Indian horse breeds. *Cryobiology*. 2019;86:52-57.
23. Öztürk AE, Bodu M, Bucak MN, et al. The synergistic effect of trehalose and low concentrations of cryoprotectants can improve post-thaw ram sperm parameters. *Cryobiology*. 2020;95:157-163.
24. Demetriou M, Binkert C, Sukhu B, Tenenbaum HC, Dennis JW. Fetuin/alpha2-HS glycoprotein is a transforming growth factor-beta type II receptor mimic and cytokine antagonist. *J Biol Chem*. 1996;271(22):12755-12761.
25. Dietzel E, Wessling J, Floehr J, et al. Fetuin-B, a liver-derived plasma protein is essential for fertilization. *Develop Cell*. 2013;25(1):106-112.

26. Lai PCW, Huang LL, Panrucker DE, Church RB, Lorscheider FL. Distribution of bovine fetuin and albumin in plasma, allantoic and amniotic fluids during development. *J Reprod Fertil*. 1981;63(1):53-60.
27. Schroeder AC, Schultz RM, Kopf GS, Taylor FR, Becker RB, Eppig JJ. Fetuin inhibits zona pellucida hardening and conversion of ZP2 to ZP2f during spontaneous mouse oocyte maturation in vitro in the absence of serum. *Biol Reprod*. 1990;43(5):891-897.
28. Sarıözkan S, Türk G, Cantürk F, Yay A, Eken A, Akcay A. The effect of bovine serum albumin and fetal calf serum on sperm quality, DNA fragmentation and lipid peroxidation of the liquid stored rabbit semen. *Cryobiology*. 2013;67:1-6.
29. Jaiswal BS, Das K, Saha S, Dungdung SR, Majumder GC. Purification and characterization of a motility initiating protein from caprine epididymal plasma. *J Cell Physiol*. 2010;222:254-263.
30. Swelum A, Mansour H, Elsayed A, Amer H. Comparing ethylene glycol with glycerol for cryopreservation of buffalo bull semen in egg-yolk containing extenders. *Theriogenology*. 2011;76(5):833-842.
31. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod*. 1995;53:276-284.
32. Garner DL, Thomas CA, Joerg HW, De Jarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biol Reprod*. 1997;57:1401-1406.
33. Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma-and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol Reprod*. 2003;68:1828-1835.
34. Henkel R, Hoogendijk C, Bouic P, Kruger T. TUNEL assay and SCSA determine different aspects of sperm DNA damage. *Andrologia*. 2010;42:305-313.
35. Duty S, Singh N, Ryan L, et al. Reliability of the comet assay in cryopreserved human sperm. *Human Reprod*. 2002;17:1274-1280.
36. Akalın PP, Başpinar N, Çoyan K, Bucak MN, Güngör Ş, Öztürk C. Effects of ultrasonication on damaged spermatozoa and mitochondrial activity rate. *Turk J Vet Anim Sci*. 2013;40:195-199.
37. Hamilton TRDS, Siqueira AFP, Castro LSD, et al. Effect of heat stress on sperm DNA: protamine assessment in ram spermatozoa and testicle. *Oxid Med Cell Longev*. 2018;3:1-14.
38. Şen G. Effects of embryo on endometrial antioxidant response in early pregnancy: Ovine model. Master Thesis. Selcuk University, Konya/Turkey; 2015.
39. Underwood SL, Bathgate R, Maxwell WMC, Evans G. Development of procedures for sex-sorting frozen-thawed bovine spermatozoa. *Reprod Domestic Anim*. 2009;44(3):460-466.
40. Hayman EG, Pierschbacher MD, Suzuki S, Ruoslahti E. Vitronectin-A major cell attachment-promoting protein infetal bovine serum. *Exp Cell Res*. 1985;160:245-258.
41. Olivier E, Soury E, Ruminy P, et al. Fetuin-B, a second member of the fetuin family in mammals. *Biochem J*. 2000;350(2):589-597.
42. Sarıözkan S, Tuncer PB, Büyükleblebici S, Bucak MN, Cantürk F, Eken A. Antioxidative effects of cysteamine, hyaluronan and fetuin on post-thaw semen quality, DNA integrity and oxidative stress parameters in the Brown Swiss bull. *Andrologia*. 2014;47(2):138-147.
43. Lee C, Bongcam-Rudloff E, Sollner C, Jahnen-Dechent W, Claesson-Welsh L. Type 3 cystatins: Fetuins, kininogen andhistidine-rich glycoprotein. *Front Biosci*. 2009;14:2911-2922.
44. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod*. 1989;41:183-197.
45. Simon CL, Mao SJT, Larsen WJ. Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. *J Biol Chem*. 1992;267(17):12380-12386.
46. Dell'Aquila ME, De Felici M, Massari S, Maritato F, Minoia P. Effects of fetuin on zona pellucida hardening and fertilizability of equine oocytes matured in vitro. *Biol Reprod*. 1999;61:533-540.
47. Raijmakers MT, Roelofs HM, Steegers EA, et al. Glutathione and glutathione S-transferases A1–1 and P1–1 in seminal plasma may play a role in protecting against oxidative damage to spermatozoa. *Fertil Steril*. 2003;79(1):169-172.
48. Seo YJ, Lee JW, Lee EH, Lee HK, Kim HW, Kim YH. Role of glutathione in the adaptive tolerance to H_2O_2. *Free Radic Biol Med*. 2004;37(8):1272-1281.
49. Larini A, Bianchi L, Bocci V. Effect of 4-hydroxynonenal on antioxidant capacity and apoptosis induction in Jurkat T cells. *Free Radic Res*. 2004;38(5):509-516.
50. Nebert DW, Roe AL, Vandale SE, Bingham E, Oakley GG. NAD(P)H:quinone oxidoreductase (NQO1) polymorphism, exposure to benzene, and predisposition to disease: a HuGE review. *Genet Med*. 2002;4(2):62-70.
51. Koide S, Kugiyama K, Sugiyama S, et al. Association of polymorphism in glutamate-cysteine ligase catalytic subunit gene with coronary vasomotor dysfunction and myocardial infarction. *J Am Coll Cardiol*. 2003;41(4):539-545.
52. Dinkova-Kostova AT, Talalay P. NAD (P) H: quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. *Arch Biochem Biophys*. 2010;501(1):116-123.
53. Henderson CJ, McLaren AW, Wolf CR. In vivo regulation of human glutathione transferase GSTP by chemopreventive agents. *Cancer Res*. 2014;74(16):4378-4387.
54. Ross D, Siegel D. Functions of NQO1 in cellular protection and CoQ10 metabolism and its potential role as a redox sensitive molecular switch. *Front Physiol*. 2017;8:595.
55. Chen X, Wang Y, Zhu H, et al. Comparative transcript profiling of gene expression of fresh and frozen–thawed bull sperm. *Theriogenology*. 2015;83(4):504-511.
56. Nakamura BN, Lawson G, Jefferson YC, et al. Knockout of the transcription factor Nrf2 disrupts spermatogenesis in an age-dependent manner. *Free Radic Biol Med*. 2010;49(9):1368-1379.
57. Somparn N, Prawan A, Senggunprai L, et al. Cellular adaptation mediated through Nrf2-induced glutamate cysteine ligase up-regulation against oxidative stress caused by iron overload in β-thalassemia/HbE patients. *Free Radic Res*. 2019;53(7):791-799.
58. Kanwal R, Pandey M, Bhaskaran N, et al. Protection against oxidative DNA damage and stress in human prostate by glutathione S-transferase P1. *Mol Carcinog*. 2014;53(1):8-18.
59. Vasiliou V, Theurer MJ, Puga A, Reuter SF, Nebert DW. Mouse dioxin-inducible NAD(P)H: menadione oxidoreductase: NMO1 cDNA sequence and genetic differences in mRNA levels. *Pharmacogenetics*. 1994;4(6):341-348.

How to cite this article: Bucak MN, Akalın PP, Keskin N, et al. Combination of fetuin and trehalose in presence of low glycerol has beneficial effects on freeze-thawed ram spermatozoa. *Andrology*. 2021;9:1000–1009. https://doi.org/10.1111/andr.12974