EFFECTS OF OXYTETRACYCLINE SUPPLEMENTATION ON CRYOPRESERVED SPERM QUALITY OF SHABOUT (*BARBUS GRYPUS* HECKEL 1843): APOPTOTIC ANALYSIS, DNA DAMAGE AND OXIDATIVE STRESS

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Abstract. The study aimed to examine the effects of oxytetracycline supplementation to glucose extender on the Oxidative Status, Glutathione (GSH), Malondialdehyde (MDA), 8-Hydroxydeoxyguanosine (8-OHdG) and Apoptotic Spermatozoa of *Barbus grypus* post-thaw sperm. The semen was frozen in diluents containing three different oxytetracycline concentrations (0.78, 1.56 and 3.12 mg). All the comparable levels of oxytetracycline linearly improved the post-thaw sperm motility rate and duration significantly ($p < 0.0001$). MDA and Total Oxidative Status (TOS) were linearly and quadratically decreased, however, Total Antioxidative Status (TAS) quadratically and GSH linearly and quadratically increased with oxytetracycline ($p < 0.001$). The increasing oxytetracycline levels typically resulted in a linear decline in DNA damage in the 8-OHdG assay. The determined percentage of apoptotic spermatozoa were linearly and quadratically reduced with Oxytetracycline (OTC) ($p < 0.0001$). These results showed that oxytetracycline can be used as an antibiotic additive in semen extender, providing better sperm freezing-thawing, without decreasing semen quality and antioxidant and increasing the oxidant, DNA damage and apoptotic sperm level of the *Barbus grypus*.
Keywords: *freezing, oxidative status, apoptosis, freshwater, Barbus grypus*

Introduction

Under poor environmental conditions, the uncontrolled production of ROS and capable microorganisms typically outstrips the antioxidant capacity of the diluted provided seminal plasma, resulting in oxidative stress (Brown and Mims, 1995; Boonthai et al., 2016; Vickham et al., 2017). However, antibiotics supplementation in different amounts to extenders, could increase the fertilization rate of semen freezing media and the motility duration of the sperm.

The biggest problem with antibiotics is bacterial resistance. Bacterial resistance to antibiotics is the biggest challenge in the treatment of fish diseases and semen extender, antibiotic choice. Antibiotics have become standard additions to the extender in the sperm reconstitution process. Antibiotics are also toxic chemicals for spermatozoa. Oxytetracycline (OTC) is a broad-spectrum and low-cost antibiotic used in the

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treatment of many bacterial fish diseases (Long et al., 1989). It is routinely administered orally through direct addition to feeds moreover it is influential in strengthening the immune system (Rickers et al., 1980; Lunden et al., 2002; Serezli et al., 2005).

Sperm and eggs obtained from aquaculture with the desired characteristics are important in terms of breeding, as they affect fertilization. Sperm freezing allows the use of semen from these fish in the present and future. One of the factors affecting sperm quality is oxidative stress during sperm freezing and thawing (Bailey et al., 2008). However, excessive amounts of oxidative stress seem to typically represent the primary reason for the damage on polyunsaturated fatty acids (PUFA) in semen membrane structure of viable sperm. Researchers have revealed that oxidative stress reduces many sperm quality parameters such as sperm motility and DNA structure (Aramli et al., 2005). Hence, detection of DNA damage in cryopreservation studies conducted in fish semen today is among the most important criteria as it gives precise and sensitive results in determining sperm quality (Dhawan et al., 2009; Çavaş, 2011; Factori et al., 2014).

Current studies for the optimization of breeding and conservation of gene resources have focused on minimizing the molecular damage that occurs during freezing and thawing of semen (Li et al., 2010; Perez-Cerezales et al., 2010). Because both osmotic stress and oxidative stress caused by reactive oxygen species negatively affect sperm quality (Klaiwattana et al., 2016; Figueroa et al., 2019). From this point of view, it is thought that antibiotics supplementations to semen extenders may reduce cryoinjury damage (Cabrita et al., 2010; Figueroa et al., 2019). In addition, antioxidants can also control the production of intracellular reactive oxygen species (ROS), which occur in living organisms as a direct result of the possible effects of some essential metal and chemical oxidants (Wink et al., 1996; Ercal et al., 2001). Previous studies have typically suggested that these fertile cells produce a low profile, in terms of motility, morphology and DNA status, to viable sperm extended with some preventive antibiotics (Jasco et al., 1993; Hargreaves et al., 1998; Ercal et al., 2001; Li et al., 2007; Castillo et al., 2015).

As the proper ratio of steady-state concentration of oxidants to antioxidant increases oxidative stress, cellular response to it prominently causes DNA damage, cell cycle arrest and apoptosis (Aprioku, 2013; Agarwal et al., 2018). Spermatozoa are equipped with antioxidant defense mechanisms and are likely to eliminate ROS. Thus, it protects gonadal cells and mature sperm from oxidative damage (Henkel, 2011).

The researches about the effect of oxytetracycline on the genetic material in sperm are sparse. Li et al. (2007) reported that amoxicillin induced DNA lesions at the going like that of intracellular reactive oxygen species induction. ROS was accumulated after quinolone removal from alive cultures co-treated with chloramphenicol to sufficiently emphasize DNA-break-mediated killing (Hong et al., 2020). It has been demonstrated that idarubicin at elevated concentrations in the expanded range of 0.001 to 10 μM causes DNA damage in normal human lymphocytes. It has been showed that idarubicin typically causes the genotoxicity (Blasiak et al., 2002).

In previous studies, semen morphology and functions were tested while evaluating the effect of some antibiotics (Rahimi et al., 2015; Boonthai et al., 2016). But, OTC supplementation to semen extender has not been tried yet in *Barbus grypus*. In this study, the effects of oxytetracycline supplementation to the semen extender on DNA damage, oxidative stress parameters and semen quality of *B. grypus* were investigated.

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Materials and Methods

Study area and semen collection

This study was carried out in the research laboratory of the Biochemistry Department of the Harran University Faculty of Medicine. Preliminary examinations of semen samples taken from the recently deceased Shabout (B. grypus) were carried out in the Fisheries Department laboratories of Harran University Bozova Vocational School, Şanlıurfa, Turkey.

In the study, the samples aged 4-5 years obtained from the fishermen in Atatürk Dam Lake, were used (n=~~9~~). Age estimates were obtained from the scales. The size of captive *B. grypus* changed between 2000.00 and 2850.00 g (mean 2400.00 ± 26.00 g) in body weight and 61.00 and 70.00 cm (mean 65.55 ± 3.59 cm) in total length. After, the urogenital papilla of the caught fish was carefully dried to prevent the contact of urea or feces to sperm. Then, semen was collected in 5 ml glass tubes by applying abdominal massage. As soon as the collection, they were placed in styrofoam containing ice (+4.00°C) and transferred to the laboratory without delay.

Chemicals

OTC, DMSO and all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sperm evaluation

Sperm motility, duration and concentration

For the examination of spermatological characteristics, 5 µL samples of fresh and frozen-thawed milt was activated with 25 µL of sperm activating solution (0.29% NaCl) and analysed under a light microscope (400X magnification) to confirm the duration of motility of the spermatozoa and was expressed as percentage of motile spermatozoa before and after freezing (Tekin et al., 2003). For further use, the frozen-thawed milt was stored in fridge (1-4°C). The sperm concentrations were determined using Thoma haemocytometer (TH100; Hecht-Assistent, Sondheim, Germany) and expressed as $x10^9$ spz/ mL (Tvedt et al., 2001). Following the assay, examples were excluded from the research if they gave an unexpected appearing or macroscopical pathalogical conditions, owned spermatozoan motility below 80% or lack to a sperm concentration of 9.00×10^9 spz/mL (Ansari et al., 2012; Wang and Dong, 2017). Approximately 1.5 mL of semen was taken from each sample and centrifuged at 2000 *g* for 30 minutes to measure seminal plasma osmotic pressure and pH. Subsequently, all suitable semen samples were mixed to avoid individual variation, and a semen pool was created.

Cryopreservation protocols

Study design, extender composition, semen freezing and thawing

The pooled semen sample was divided into 5 portions of 5 groups. OTC was not added in the control and fresh semen group. Oxytetracycline supplemented extender groups were classified into 3 groups of 0.78, 1.56 and 3.12 mg of OTC (modified by Elia et al., 2014). Experimental and control group sperm were immediately diluted from each sperm samples with 1:3 v/v (sperm 1:diluents 3) with the Glucose extender (0.3 M Glucose) containing 10% dimethyl sulphoxide (DMSO).The extended semen was packaged in 0.25 ml straws, and equilibrated at 4ºC for 10 minutes. The straw was

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frozen in a styrofoam box at 5 cm above the liquid nitrogen surface for 12 minutes. The frozen semen was stored for 2 weeks in liquid nitrogen for further evaluations. The frozen semen straws were placed in a 40°C water bath for 8 second (Kopeika et al., 2007).

The pooled semen sample was divided into 4 portions of 3 freezing groups and 1 fresh semen group. Thus, for the male *B. grypus*, there were 5 sperm pool samples in each time and the application was repeated 3 times, 4 parallel samples in each time (repetitions (n)=60).

Preparation of sperm samples for biochemical analysis

In this study, equal amount of sperm samples was taken from all trial groups. Sperm samples were diluted 1/10 with PBS, homogenized with a homogenizer (Tissue Lyser LT, Qiagen) and centrifuged to obtain supernatants.

Oxidative stress analysis

The effects of extenders containing oxytetracycline on the oxidative stress indices of semen were evaluated by examining the Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Glutathione (GSH) and Malondialdehyde (MDA) parameters. GSH levels were assessed through reaction with OPA (1 mg/mL o-phthaldialdehyde in methanol) following to the modified technique of Kand'ar and Hajkova (2014) with GSH used as a standard. GSH samples were assessed via microplate reader (SpectraMax, M5, San Jose, California, USA), with excitation at 345 nm and emission at 425 nm. Results were expressed as nmol/g in sperm cell.

MDA levels in the sperm were assessed following the technique defined by Ohkawa et al. (1979). ELISA plates were read by a microplate reader (SpectraMax, M5, San Jose, California, USA), at 532 nm. The results were obtained as nmol/g in sperm.

Measurement of oxidative stress status

TOS and TAS were detected in sperm homogenates by using commercially available kits (Rel Assay®, Diagnostics kits, Mega Tıp, Gaziantep, Turkey) with an autoanalyzer (Cobas Integra 800, Roche Diagnostics, Indianapolis, IA, USA). TOS and TAS results were presented in mmol H_2O_2 equivalent/L (Erel, 2005) and mmol Trolox equivalent/L, respectively (Erel, 2004). The ratio of the TOS to the TAS revealed the Oxidative Stress Index (OSI), which is used as an indicator for total oxidative stress (Harma et al., 2003).

Apoptotic analysis by ELISA and DNA damage measurement

The protective effects of extenders containing oxytetracycline on spermatozoon DNA damage were investigated by intracellular 8-Hydroxydeoxyguanosine (8-OHdG) ELISA and comet assay. The results were obtained as ng/ml in sperm.

Apoptotic analysis by ELISA of fish sperm cells

Analysis of apoptosis in sperm samples was performed according to the protocol of the commercially available kit (Cell Death Detection ELISA^{PLUS}, Roche). The assay is based on the quantitative double-antibody sandwich enzyme immunoassay principle and uses monoclonal antibodies directed against DNA and histones, respectively. Anti-DNA POD antibody binds to single- and double-stranded DNA. Therefore, the ELISA allows

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detection of mono- and oligonucleosomes from various species and can be applied to measure apoptotic cell death in many different cell systems.

Measurement of 8-hydroxydeoxyguanosine (8-OHdG) in fish sperm

8-OHdG is one of the very significant signs of oxidant-induced DNA damage. Quantification of 8-OHdG was done by using Fish 8-OHdG ELISA kit (BT-LAB). Protocol was followed as described in the manufacturer instructions.

Statistical analysis

Minitab 17.0 was used for analysis. Normality and homogeneity of variance of the data were checked by Kolmogorov-Simirnow's tests before analyses. The data that did not yield normal distribution underwent logarithmic transformation. Percent motility and apoptotic cell data were arcsine transformed. The data generated were analyzed with the one-way ANOVA test and the differences in the data of the trial groups were revealed by using the Tukey multiple comparison test. Linear or quadratic trends on OTC comparable levels and sperm variables were determined using orthogonal polynomials (Rosales et al., 2017). All mean values represent mean ± SE from triplicate.

Results

Evaluation of sperm parameters, oxidant and antioxidant status, DNA damage

In fresh sperm, spermatozoa motility rate (%), motility duration (s), concentration ($x10^9$ spz/mL) and ph were 87.86±1.28, 118.33±2.77, 11.92±0.12 and 8.14±0.12, respectively (*Table 1*). As shown in *Table 1*, *Table 2*, *Table 3 and Table 4*, motility rate and duration, oxidant and antioxidant status, DNA damage rates and the percentage of apoptotic spermatozoa were significantly changed through the use of a glucose extender with OTC ($p<0.0001$).

Table 1. *Post-thaw spermatological parameters of B. grypus semen frozen in diluents containing three different oxytetracycline concentrations*

Dose of Oxytetracycline (Groups)		Parameters	
		Motility Rate (%)	Motility Duration (s)
Fresh		87.86±1.28[a]	118.33±2.77[a]
Control		31.12±3.42[d]	36.50±4.86[d]
0.78 mg		50.83±3.58[b]	63.33±2.61[c]
1.56 mg		52.50±3.05[b]	71.08±2.99[b]
3.12 mg		59.17±2.60[b]	76.50±2.00[b]
Pr >F[1]	ANOVA	0.0001	0.0001
	Linear trend	0.001	0.0001
	Quatratic trend	0.0001	0.0001

Data are presented Mean ± SE of values. Different letters as a,b,c show differences between groups ($p< 0.001$), *post hoc* comparisons with Tukey multiple comparison test

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Effect of oxytetracycline on post-thaw sperm motility and motility duration

Spermatozoa motility rate and motility duration are shown in *Table 1*. The freezing process typically had a significant negative effect on the motility and typical duration of it ($p<0.0001$). Increasing the levels of OTC significantly affected the spermatozoa motility rate and motility duration ($p< 0.05$). There were significant linear and quadratic trend levels of OTC for the spermatozoa motility rate and duration of motility ($p< 0.05$).

There were significant linear and quadratic trend levels of OTC for the spermatozoa motility rate and motility duration ($p< 0.001$). The spermatozoa motility rate and motility duration increased with the inclusion of OTC to extender compared with control ($p< 0.05$).

Effect of oxytetracycline on post-thaw antioxidant and oxidant status

The freezing process had a significant negative effect on both post-thaw antioxidant and oxidant status of sperm ($p<0.0001$). The post-thaw antioxidant level decreased markedly between 41.4 to 47.3% ($p<0.0001$). TAS and GSH were increased after thawing in high OTC doses compared to in no treatment group (*Table 2*). The post-thaw oxidant level in OTC proportionally raised with 9.0 to 14.0% after cryopreservation compared with in no treatment group ($p<0.05$).

Table 2. *Post-thaw oxidative stress parameters of B. grypus semen frozen in diluents containing three different oxytetracycline concentrations*

Dose of Oxytetracycline (Groups)		**Parameters**				
		TAS (mmol Trolox equiv/L)	**TOS (µmol H_2O_2 equiv/L)**	**OSI (AU)**	**GSH (nmol/g)**	**MDA (nmol/g)**
Fresh		1.88±0.07^{a}	12.11±1.09^{b}	1.02±0.01^{b}	44.90±0.60^{a}	16.28± 0.09^{a}
Control		0.99±0.08^{d}	13.85±0.08^{a}	1.23±0.01^{a}	26.29±0.34^{c}	15.42±0.42^{b}
0.78 mg		1.11±0.04^{cd}	10.97±0.62^{c}	0.81±0.03^{d}	27.63±0.08^{c}	13.10±0.35^{b}
1.56 mg		1.26±0.03c	11.34±0.06^{c}	0.90±0.01^{c}	36.29±0.19^{b}	8.66±0.33^{c}
3.12 mg		1.33±0.03^{b}	11.22±0.46^{c}	0.83±0.01^{cd}	37.56±0.23^{b}	8.27± 0.28^{d}
Pr >F[1]	ANOVA	0.0001	0.0001	0.0001	0.0001	0.0001
	Lineartrend	0.84	0.26	0.41	0.0001	0.0001
	Quatratic trend	0.01	0.05	0.05	0.0001	0.0001

Data are presented Mean ± SE of values. Different letters as a,b,c show differences between groups ($p< 0.001$), *post hoc* comparisons with Tukey multiple comparison test

Levels of TAS, GSH, MDA, TOS and Oxidative Stress Index (OSI) were significantly affected by the OTC supplementation ($p< 0.0001$). Significant linear and quadratic trends were found between the increasing levels of OTC and the levels of GSH and MDA ($p< 0.0001$). Moreover, a quadratic trend was found between the increasing levels of the OTC and levels of TAS, TOS and OSI ($p< 0.05$). (*Table 2*).

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Changes in DNA damage paremeters and apoptosis

A significant increase in the level of 8-OHdG was observed in the control group and the fresh semen ($p<0.05$) (*Table 3*). And also, significant increase in the apoptotic spermatozoa rate was detected in the control groups ($p<0.0001$). As shown in *Table 4*, DNA damage in 8-OHdG was significantly decreased by the elevated OTC levels ($p<0.05$). All OTC doses had lower apoptosis levels ($p<0.0001$) (*Table 3*). A significant linear trend was found between the increasing OTC and the DNA damage levels ($p<0.0001$). Significant linear and quadratic trends were found between the increasing OTC levels and the percentage of apoptotic spermatozoa ($p< 0.0001$).

Table 3. *Post-thaw apoptosis analysis results of B. grypus semen frozen in diluents containing three different oxytetracycline concentrations*

Dose of Oxytetracycline (Groups)		**Parameters**
		Apoptozis (AR %)
Fresh		1.89±0.10[b]
Control		2.50±0.07[a]
0.78 mg		1.57±0.06[c]
1.56 mg		1.28±0.01[d]
3.12 mg		1.27±0.03[d]
Pr>F[1]	ANOVA	0.0001
	Linear trend	0.0001
	Quatratic trend	0.0001

Data are presented Mean ± SE of values. Different letters as a,b,c show differences between groups ($p< 0.001$), *post hoc* comparisons with Tukey multiple comparison test

Table 4. *Post-thaw DNA damage results of B. grypus semen frozen in diluents containing three different oxytetracycline concentrations (using by 8-Hydroxydeoxyguanosine (8-OHdG))*

Dose of Oxytetracycline (Groups)		**Parameters**
		8-OHdG (ng/mL)
Fresh		98.74±0.64[a]
Control		96.23±0.41[a]
0.78 mg		86.72±0.72[b]
1.56 mg		62.72±0.18[c]
3.12 mg		53.98±0.23[d]
Pr>F[1]	ANOVA	0.0001
	Linear trend	0.0001
	Quatratic trend	0.89

Data are presented Mean ± SE of values. Different letters as a,b,c show differences between groups ($p< 0.001$), *post hoc* comparisons with Tukey multiple comparison test

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Discussion

Studies have properly shown different protocols for each properly recognized species in semen cryopreservation (Huang et al., 2009; Liu et al., 2018). Handling the protocol steps separately is important for the standardization process. Because, each stage is related to each other and affects the quality of sperm (Yang and Tiersch, 2009; Yang et al., 2018). From this point of view, we evaluated the effects of antibiotics, on semen quality after long-term storage by considering several different parameters. In our study, we investigated the effect of oxytetracycline supplementation to semen extenders at different rates on semen quality, oxidative stress status, DNA damage and apoptotic cell rates after sperm freezing-thawing process.

It was observed that freezing-thawing process decreased sperm motility rate and motility duration of treatment groups compared to fresh *B. grypus* semen. Motility rate in cryopreserved sperm of Atlantic salmon (*Salmo salar*) displayed significant reductions compared to fresh spermatozoa (Figueroa et al., 2019). All the treatment groups had better results in specific terms of motility duration and motility rate ($P<0.05$). The sperm motility rate and duration of *B. grypus* significantly improved with the apparent increase in the elevated OTC levels because the consistent trends were linear and quadratic in this comparative study. On the other hand, in previous studies, there were different results of other antibiotics supplementation studies to the semen extender. Jasko et al. (1993) highlighted the deleterious results of gentamicin on the motility of stallion spermatozoa at concentrations greater than 1 mg/mL. Besides, Ericsson and Baker (1967) reported that tetracyclines are toxic to ejaculated semen and bind strongly to the sperm head. Nevertheless, there efficiently was no difference in motility after incubation of human semen at 37°C for 48 hours following freeze-thawing by adding a synthetic oxytetracycline, the doxycycline hyclate (2.6 and 260 µg/mL) to the extender (King et al., 1997). Tetracycline at small concentrations as low as 2.5 µg/mL on human semen in vitro caused inhibition of the determined percentage of motile spermatozoa. It was determined that all spermatozoa were immobilized by using 50 µg/mL tetracycline (Hargreaves et al., 1998). Researchers suggest that the harmful result of tetracycline is due to its ability to chelate Ca^{2+} and the effect of this antibiotic is mostly irreversible.

Cryopreservation protocols are very important for post-thaw sperm quality due to the oxidative stress it naturally results in semen. For the possible time of the sperm storage, free radicals are properly produced, the created formation of oxidative stress results in a gradual decrease in antioxidant capacity (Aprioku, 2013; Agarwal et al., 2018; Riesco et al., 2020). Oxidative stress is believed concerning the critical influences that damage spermatozoa (Cho and Agarwal, 2018; Selwam and Agarwal, 2018; Alahmar, 2018). However, the mechanisms of this phenomenon have not been adequately explained (Krzysciak et al., 2020). In this comparative study, properly compared to the fresh semen, a significant decrease in the TAS and GSH levels, marked increase MDA, TOS and OSI were accurately detected in control groups. The decrease in MDA, TOS and OSI levels after thawing may result to the obvious quadratic increase in the TAS and linear and quadratic rise within GSH measures to here caused via the elevation in the OTC doses. The levels of TOS and OSI in in patients with moxifloxacin combination therapy were significantly decreased, while the levels of TAS were significantly increased after treatment ($p < 0.05$) (Yang et al., 2017).

Chemotherapeutic drugs, some metallic component and chemical agent can as well be resulted in the reactive oxygen species (Wink et al., 1996; Ercal et al., 2001). In the

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preliminary examinations, controlled administration of penicillin, kanamycin, and gentamicin caused a marked reduction in ROS in rabbit semen (Duracka et al., 2019). On the contrary, tetracycline-induced testicular damage is associated with the induction of oxidative stress in testicular tissues as a result of a decrease in superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase, glutathione-S-transferase activities, as well as significant decrease in GSH levels (Farombi et al., 2008). While this indicates an important relationship between GSH and OTC, other researchers have also stated high GSH levels in normozoospermia ejaculates (Krzysciak et al., 2020). The study findings show that high OTC dose contributes to the maintenance of GSH levels. Semen with high MDA values are associated with decreased sperm motility and higher percentages of necrotic and apoptotic sperm (Sahnoun et al., 2017; Hassanin et al., 2018). In addition, the present study is similar to the consistent findings of motility, oxidant and antioxidant levels of *B. grypus* viable sperm in observed treatment groups. The source of free radicals containing lipid peroxidation products such as MDA is non-mitochondrial sources found in mammalian spermatozoa (Aitken et al., 1989). Tetracycline-induced testicular damage has been reported to result from the development of MDA with the increased effect of γ-glutamyltranspeptidase (Farombi et al., 2008). Thus, the decrease in MDA levels as a result of its inclusion in a high OTC may be due to its effect on seminal plasma, spermatozoa or mitochondrial enzymes (Aitken et al., 1989). Oxidative stress shows the disproportion between the production of reactive oxygen species and antioxidant protections that buffer oxidative damage (Halliwell, 1994). Decreased antioxidative structures maintain the balance and buffer with increased oxidative structures (Baysal et al., 2009; Gülüm et al., 2011).

DNA damage detection has been frequently used in recent years for the detection of post-thaw sperm damage of different living species (Irvine et al., 2000). New markers for sperm quality assessment can strengthen the optimization of existing sperm analysis methods. Nuclear and mitochondrial DNA damage are serious consequences of oxidative stress. Thus, reduction of reactive oxygen species can decrease DNA damage (Venkatesh et al., 2009). In addition, free radicals, specifically reactive oxygen species, cause DNA damage after that lead to cellular apoptosis (Aitken et al., 1998; Zobeiri et al., 2012). In this study, the effective OTC doses kept the DNA damage at under of the comparable level of fresh semen. A linear trend could explain of the typically decreased its destruction variation of elevated OTC levels for sperm DNA damage. This means that we have a linear trend in the DNA damage in the OTC levels that can be interpreted as a response to recovering DNA intact. Previous studies had opposite results to our research findings. Of these, Zobeiri et al. (2012) showed that after administration of Ciprofloxacin to mice, the percentage of sperm with single-stranded DNA raised considerably. A considerable increase in DNA fragmentation in rabbit semen in culture medium supplemented with penicillin, kanamycin and gentamicin was shown (Duracka et al., 2019). It has also been shown that mitochondrial dysfunction severely increases nuclear DNA fragmentation in sperm (Donnelly et al., 2000). The DNA lesions were induced by only Amoxicillin reported by Li et al. (2007). ROS had been accumulated after quinolone removal with cultures co-treated with chloramphenicol for sufficiently emphasize DNA-break-mediated killing (Castillo et al., 2015). Quinolones are attacked to DNA, thereby blocking DNA gyrase causing further causing permanent destruction to DNA (Yang et al., 2017). It has been shown in normal human lymphocytes that different concentrations of idarubicin (0.001 to 10 μM) can cause an increase in the

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percentage of damaged DNA. And also, this result of idarubicin shows that besides its various side effects, it is also important in terms of genotoxicity (Blasiak et al., 2002).

It is known that stress in cells leads to apoptosis. In our study, apoptotic cells were examined to determine the effects of sperm freezing stress. There was a significant difference in the comparable percentage of apoptotic cells ($p>0.05$) between fresh and cryopreserved sperm in control group. All the OTC levels resulted in significant ($p<0.0001$) decreases in apoptotic cells' percentage compared to determined values of control sperm. Therefore, OTC typically decreased the apoptosis of frozen sperm. This properly means that linear and quadratic trends typically has in the comparable percentage of apoptotic cells in the OTC levels that can be reasonably interpreted as a direct response to instantly recovering apoptosis. Antibiotics cause accumulation of DNA lesions and increased apoptotic response (Castillo et al., 2015). It has been reported that the negative consequences of doxorubicin occur due to increased DNA damage (L'Ecuyer et al., 2006).The decrease in the apoptosis after thawing may be due to the significant quadratic increase in the TAS, linear and quadratic rise GSH levels caused by the OTC levels. The essential fact that OTC increases TAS level, decreases DNA damage and meaningfully improves sperm criteria is evidence that it efficiently is a useful antibiotic. Because the possible formation of oxidative stress produce in a reduce in antioxidant capacity during the freezing-thawing process (Aprioku, 2013). Oxidative stress is also thought one of the primary variables that injury sperm (Selvam and Agarwal, 2018). In this manner, OTC may have slowed down apoptosis in viable sperm by sufficiently reducing oxidative stress during the freeze-thaw process.

Conclusions

As a result, spermatozoa motility rate and duration in post-thaw semen increased in all the OTC groups compared with control. The OTC supplementation to extender caused to increase TAS and GSH. In addition, it typically decreased TOS, OSI and MDA levels of post-thaw sperm. Considering the key findings of this comparative study, it was determined that the OTC not only decreased DNA damage but also reduced apoptosis. It was determined that the OTC supplementation to extender sufficiently protects the semen against pathophysiological events in post-thaw semen. On the other hand, we think that it would be useful to carry out more detailed studies in order to standardize the use of OTC in semen extenders.

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Institutional review board statement. All issues concerning the experimental methods and evaluation techniques were approved by the Scientific Ethical Committee, Harran University, Sanliurfa, Turkey (No: 11/02/2020-01/07).

Data availability statement. The datasets used and/or analyzed during in this study are available from the corresponding author upon reasonable request.

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