Turk. J. Fish.& Aquat. Sci. 23(4), TRJFAS22300
https://doi.org/10.4194/TRJFAS22300

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Effect of Antioxidants on Cryopreserved Turbot (*Scophthalmus maximus*) Spermatozoa Quality and DNA Damage

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How to Cite

Polat, H., Kurtoğlu, İ.Z. (2023). Effect of Antioxidants on Cryopreserved Turbot (*Scophthalmus maximus*) Spermatozoa Quality and DNA Damage. *Turkish Journal of Fisheries and Aquatic Sciences*, *23(4)*, *TRJFAS22300.* https://doi.org/10.4194/TRJFAS22300

Article History
Received 21 July 2022
Accepted 30 September 2022
First Online 04 October 2022

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Keywords
Antioxidant
Cryopreservation
DNA damage
Sperm quality
Turbot

Abstract

The effects of several antioxidants added to cryopreservation extenders on turbot (*Scophthalmus maximus*) sperm post-thaw motility, fertilization, sperm characteristics, and DNA damage were investigated. The following antioxidants were used in conjunction with the conventional extenders: rosmarinic acid (RA) (25, 50, and 100 µg/ml); uric acid (UA) (25, 50, and 100 µg/ml); and taurine (TA) (25, 50, and 100 µg/ml). On sperm samples that had been diluted by the extenders at a 1:9 ratio, cryopreservation was carried out. The results demonstrated that the post-thaw motility of sperm was higher in 25 µg/ml RA (77.41±2.90%), followed by 50 µg/ml RA (74.86±3.2%), 50 and 100 µg/ml UA (69.56±3.30% and 70.74±2.51%, respectively) which significantly differentiated from conventional extender. The highest hatching rate (70.67±4.67%) was attained at UA 50 µg/ml, while the highest fertilization rate (81.67±2.52%) was observed in RA 25 µg/ml. At UA 50 µg/ml, the DNA fragmentation rate was lowest (5.07±1.57%). In conclusion, antioxidants had a protective impact on sperm of turbot that had been frozen and thawed.

Introduction

Cryopreserving fish sperm is frequently used to improve aquaculture programs. In aquaculture, it is preferred to store superior sperm for a long period of time and have them available for usage as needed (Sterbenc et al., 2019). Since it enhances artificial insemination for genetic manipulation and breeding selection studies, the technique has been utilized (Routary, 2020). By keeping their gametes in GenBank, cryopreservation is a useful technique for saving the gametes of endangered species. Fish sperm cryopreservation has lately gained popularity as a way to maintain aquaculture output, maximize sperm utilization, and preserve genetic variability (Cabrita et al., 2010; Kutluyer et al., 2014). Techniques for sperm cryopreservation are used with marine fish, but notably with freshwater species. Studies to enhance sperm quality after freezing and thawing sperm of many species have had favorable results (Chen et al., 2004; Hatipolu et al., 2010; Ciereszko et al., 2014; Zidni et al., 2020).

Despite the fact that sperm cryopreservation has successful applications, routine procedures have been shown to seriously impair sperm function through cold shock, osmotic stress, intracellular ice crystal formation, extenders, cryoprotectant agents, equilibration time, freezing speed, and thawing temperatures (Amidi et al., 2016; Ahn et al., 2018). The composition of membrane lipids, sperm motility and viability, acrosome state, and

sperm DNA damage may all be negatively impacted by these impairments (Zribi et al., 2010; Cabrita et al., 2011; Sandoval-Vargas et al., 2021). The main factors contributing to sperm damage after frozen-thawed storage include intracellular ice crystal formation, changes in antioxidant defense mechanisms, cold shock, osmotic stress, excessive reactive oxygen species (ROS) production, and the elements of these conditions (Figueroa et al., 2019; Bozkurt et al., 2021; Félix et al., 2021). Since sperm's natural antioxidant defenses are insufficient to stop lipid peroxidation, antioxidants must be added to extenders. Antioxidants have been suggested in numerous studies as a way to counteract the damaging effects of ROS on spermatozoa during the freezing process (Kirilova et al., 2015; Amidi et al., 2016; Kutluyer et al., 2016).

In recent years, a number of research have been carried out to evaluate the impact of cryopreservation on the motility, fertilization, and hatching rate of turbot sperm under various conditions and storage times (Dreanno et al., 1997; Chereguini et al., 2003; Chen et al., 2004). When larval and young turbot were intentionally produce using fresh and cryopreserved sperm, growth and survival rates were examined, and it was shown that they were comparable (Chereguini et al., 2001). Cryopreserved turbot sperm had a decreased rate of fertilization, according to Dreanno et al. (1997), while fresh and cryopreserved turbot sperm had identical fertilization and hatching rates (Chereguini et al., 2003). Aydin et al. (2021) have conducted a study on the value of cryogenic freezing procedures in the cryopreservation of turbot sperm in more recent years. More thorough researches are required because it is believed that freezing procedures have a considerable impact on sperm parameters after thawing.

In Türkiye, one of the most expensive commercial flatfish species is the turbot, *Scophthalmus maximus* (also known as *Psetta maxima*). Attention is drawn to turbot as a potential source of high market value, stock expansion, and aquaculture in Türkiye (Aydın, 2021). Since 1998, the Central Fisheries Research Institute (SUMAE) has successfully cultured this species. The production of high-quality juveniles, gamete synchronization, safeguarding the sperm of superior individuals, and gene banking all depend on the successful cryopreservation of turbot sperm (Aydin et al., 2020).

In this study, it was aimed to develop the cryopreservation technique for turbot sperm by examining the effects of different antioxidants on post-thaw sperm quality, fertilization rate, and DNA damage.

Material and Methods

Fish and Gamete Collection

Experiments were conducted in May 2021 using 5-year-old hatchery-reared turbot (average length and weight of 47.67±1.8 cm, 2100.7±341.4 g) in the Marine Fish Hatchery of the Central Fisheries Research Institute (SUMAE), Türkiye. Fish were kept in 15 m^3 circular three tanks (stock density 4-6 kg/m^2) during the study period and supplied with flow-through seawater (average seawater temperature of 12.7±0.97°C) and under natural photoperiod. Turbots were fed *ad libitum* with chopped wild fish such as red mullet, whiting, horse mackerel, and anchovy once a day. After delicately washing and drying the genital zone, sperm was obtained by stripping ripe males. After drying the genital area, sperm was collected into a syringe with extreme caution to minimize fecal and urine contamination. Before activation, sperm-containing syringes were maintained at 4°C. Eggs were collected by abdominal massage of females (Polat et al., 2018).

Assessment of Sperm Motility and Quality Parameters

A computer-assisted sperm analysis system (CASA, SCA, Sperm Class Analyser, V.5.6. Microptic, SL, Barcelona, Spain) was used to determine the motion parameters of the sperm samples. For sperm imaging, 10 µl deep Makler chamber was used. The sperm motility parameters: percentage of motile sperm (MOT, %), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), linearity index (LIN, %), amplitude of lateral head displacement (ALH, µm), straightness index (STR, %), oscillation index (WOB, %), and beat frequency (BCF, Hz) were determined (Beken et al., 2022). The percentage of motile spermatozoa was evaluated at 10 s and 60 s post activation. Spermatozoa were activated by seawater and the percentage of motile spermatozoa was measured three times for each sample. Sperm concentration was determined with a hemocytometer under a light microscope at 400× magnification from non-activating medium diluted samples (1:1000), and expressed as a billion of spermatozoa per ml (10^9/ml) (Şahin et al., 2012). Microhematocrit capillary tubes (75x1.1-1.2 mm) were filled with sperm and one end of the tubes was locked with clay. The tubes were then centrifuged at 12. 000 rpm for 10 minutes to determine spermatocrit. The volume of white packed material divided by the total volume of sperm multiplied by 100 is the definition of a spermatocyte (Rurangwa et al., 2004). The pH indicator papers (Merck pH 5.5-9.0) were used to determine the pH of the sperm. To determine the osmolality of fresh sperm, 50 µl of sperm sample was put into an Eppendorf tube and measured in mOsmol/l with Gonotec Osmomat 3000 (Gonotec GmbH, Berlin, Germany).

Sperm Cryopreservation

Sperm was collected from 10 males for cryopreservation and a pool (motility score > 90% for each male). Ringer medium was used for cryopreservation of turbot semen. The ringer medium consists of 150 nM NaCl, 2.5 mM KCl, 3.5 mM $CaCl_2$, 1

mM $MgCl_2$, 7.0 mM $NaHCO_3$, 2.8 mM glukoz and 10 mM hepes. The pH of the Ringer medium was maintained at 7.4 (Multi pH meter, Mettler Toledo). Semen was diluted with extender at a volume ratio of 1:9 and 10% DMSO was used as a cryoprotectant (Dreanno et al., 1997). The antioxidants of rosmarinic acid (RA), uric acid (UA) and taurine (TA) were added to the extender separately at 25, 50 and 100 µg/ml concentrations. The concentrations of the antioxidants were selected based on Pereira et al., (2005), Liu et al. (2014), Luño et al. (2014), Kutluyer et al. (2016), Zidni et al., (2022) studies. The diluted semen was sucked into 0.5 ml straws (IMV Technologies, L'Agile, France) and placed on a tray in nitrogen vapour at 6.5 cm above the surface of liquid nitrogen in a Styrofoam box. After 10 minutes, the straw was immersed in liquid nitrogen and stored at −196°C. After a week, the frozen sperm was thawed in a water bath at 40°C for 5 s for quality analysis in CASA and fertilization experiments (Ciereszko, 2014). Analyses were performed in triplicates.

Evaluation of Fertility and Hatching

Fertility tests were carried out using cryopreservation sperm whilst fresh sperm was used as a control. Fertilization was performed on 10, 000 :1 sperm to egg ratio. The fresh sperm sample was diluted with an extender prior to fertilization to get the same sperm concentration as that of frozen sperm (Cabrita et al., 2011). Eggs were obtained from three females (average length and weight of 56.33±0.46 cm, 3896.7±100.81 g) and an egg pool was prepared for fertilization trials. Exactly 2 g of eggs were taken from the pool was placed into beakers (1000 ml). Sperm samples were added immediately and activated with seawater. Upon 5 minutes post activation, the eggs were rinsed with hatchery water and incubated in a temperature-controlled incubator (at 14°C, 18‰ salinity). The daily water renewal rate was 50%. The fertilization success of each antioxidant dose and fresh sperm was assessed in triplicates. The number of fertilized eggs was determined by microscopic examination at 2-64 blastomere stages. Fertilized and unfertilized eggs were pronounced depending on whether cell division was visible or not. Fertilization rate was calculated by dividing fertilized egg number to the total number of eggs and hatching rate by yolk-sac larvae number to the fertilized number of eggs (Polat et al., 2018).

Assessment of Sperm DNA Damage

DNA damage Sperm Chromatin Dispersion, SCD (Fernandez et al., 2003) was determined using the GoldCyto Sperm®Kit (Goldcyto Biotech Corp.). After the frozen semen straws were thawed, they were taken into Eppendorf tubes, diluted 1:1 with phosphate buffer solution (PBS) that did not contain Ca^{+2} and Mg^{+2}, and washed by centrifugation at 10,000 rpm for 10 minutes. Then, it was diluted with PBS to have 5-10 million sperm per ml, and Eppendorf containing agarose at 37ºC was taken and 30 µl of the sample was fixed on the slide. The slide was acid denatured for 7 minutes at room temperature (22ºC) in a horizontal position and then it was incubated for 25 minutes in 5 ml of lysis solution. Lysis solution is removed by distilled water treatment for 5 min in a tray. Then it was treated with a series of ethanol (70%, 90%, and 100%) for 2 minutes per treatment. The slides were dried at 25 ℃ and incubated with SpermBlue® stain solution for 10 min. The stain solution is removed using distilled water and dried at room temperature. DNA damage of the samples were examined in CASA.

Data Analysis

The data were statistically analyzed using one-way analysis of variance (ANOVA) following Duncan post-hoc in Statistical Package for the Social Sciences (SPSS) 16.0 for Windows. The results were given as means and standard deviations (SD). The confidence level was set at 95% for all statistical analyses.

Results

Sperm Characteristics of Hatchery-reared Turbot

The sperm characteristics of the 5-year-old turbot (n=40) originating from the hatchery are given in Table 1. The average percentage of motile cells was 92.58±2.93% in fresh sperm after activation. Curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity index (LIN), straightness index (STR), oscillation index (WOB), amplitude of lateral head displacement (ALH), and beat frequency (BCF) were determined as 91.78±26.85µm/s, 48.22±15.61µm/s, 71.19±22.93µm/s, 44.16±6.38%, 58.67±5.17%, 70.93±5.41%, 2.75±0.59 µm and 4.95±0.76 Hz, respectively.

Table 1. Sperm quantity parameters of hatchery reared Black Sea turbot

Parameters	Mean (±SD)
pH	7.10±0.20
Volume (ml)	2.09±0.50
Spermatocrit (%)	37.80±5.20
Concentration (x10^9 spz/ml)	1.98±0.30
Viability duration (dk)	19.78±2.70
Osmotic pressure (mOsmol/l)	308.33±4.40

The values were given as mean ± SD (N=3)

Effect of Antioxidants on Post-Thawed Turbot Sperm Motility

Motility rates of frozen-thawed samples are illustrated in Figure 1. At 10 seconds of post activation, RA 25 and 50 µg/ml, UA 50 and 100 µg/ml enhanced the post-thaw sperm motility in comparison with the standard extender. Motility values after post-thaw at other antioxidant doses added to the supplementation were similar to those of the standard extender (Figure 1). The highest motility values observed at 60 seconds post activation were 25 and 50 µg/ml doses of RA at 76.07±2.07% and 71.99±2,80% respectively. There were significant differences in the motility rates of frozen-thawed sperm among groups (P<0.05) (Figure 1).

The effect of antioxidants on other motility parameters of frozen-thawed sperm was presented at 10 seconds (Table 2) and 60 seconds (Table 3) post activation. Except for 25 µg/ml RA treatment at 10

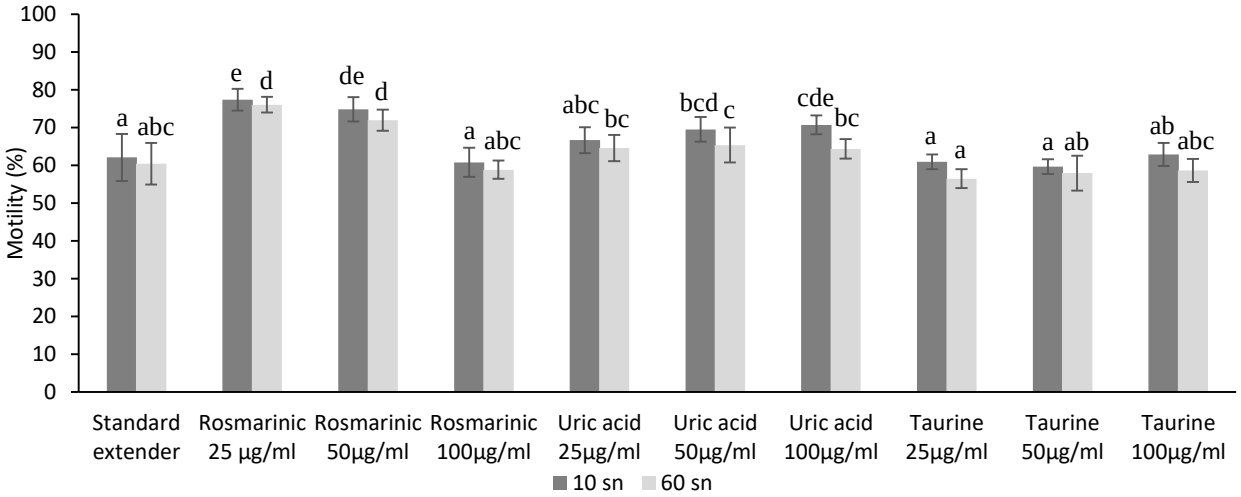

Figure 1. Effect of antioxidants at different concentrations on the motility rate of frozen-thawed turbot sperm. Different letters show the differences between treatments (P<0.05) (N=3).

Table 2. Effect of antioxidants on values of speed and other parameters at 10 s of frozen-thawed turbot sperm

Extenders	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Standard extender	42.12±7.67	20.82±3.69	29.32±6.10	34.29±2.86	51.55±2.49	56.50±2.05	1.68±0.19	3.87±0.21
Rosmarinic 25µg/ml	57.29±5.84	33.86±11.07	44.46±8.25	46.63±5.69	61.51±3.69	68.51±1.95	1.94±0.25	4.98±1.35
Rosmarinic 50µg/ml	55.03±8.72	28.547±3.87	41.03±6.58	40.17±6.61	56.01±5.18	64.63±5.93	2.00±031	4.27±0.77
Rosmarinic 100µg/ml	48.46±8.59	27.59±11.59	37.00±10.07	41.54±13.02	57.23±11.02	63.67±10.06	1.71±0.11	4.56±1.76
Uric acid 25µg/ml	48.90±5.11	25.45±4.34	35.72±4.66	37.19±7.86	53.62±6.83	60.82±7.72	1.84±0.11	4.26±0.50
Uric acid 50µg/ml	52.43±4.63	29.00±8.11	39.57±7.37	42.74±8.32	57.86±6.36	65.62±6.48	1.88±0.17	4.06±1.21
Uric acid 100µg/ml	55.10±2.19	29.47±3.29	40.77±1.47	38.18±6.30	54.64±5.68	60.40±3.65	1.94±0.12	4.53±0.90
Taurine 25µg/ml	52.22±5.74	32.48±7.62	41.66±7.31	47.80±8.05	62.50±6.23	68.95±6.04	1.72±0.06	4.89±1.38
Taurine 50µg/ml	46.01±5.4	22.79±7.62	32.69±7.31	34.14±8.05	50.53±6.23	57.69±6.04	1.76±0.06	3.81±1.38
Taurine 100µg/ml	51.06±7.76	28.50±0.38	39.12±4.40	43.42±4.48	58.57±4.00	66.90±1.88	1.83±0.31	4.32±0.77

The values were given as mean ± SD. (N=3). VCL: Curvilinear velocity (µm/s), VSL: Straight line velocity (µm/s), VAP: Average path velocity (µm/s), LIN: linearity index (%), STR: Straightness index (%), WOB: Oscillation index (%), ALH: Amplitude of lateral head displacement (µm), BCF: beat frequency (Hz).

Table 3. Effect of antioxidants on values of speed and other parameters at 60 s of frozen-thawed turbot sperm

Extenders	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Standard extender	39.04±5.28	17.06±3.97	25.76±5.08	31.14±4.60	48.70±4.21	54.92±4.85	1.68±0.14	3.68±0.15
Rosmarinic 25µg/ml	48.58±3.5	23.48±7.58	34.23±6.61	36.61±9.37	53.56±7.27	60.27±8.21	1.92±0.16	4.16±0.97
Rosmarinic 50µg/ml	45.44±3.65	21.28±1.13	31.45±3.59	36.26±1.84	53.19±1.17	60.24±4.42	1.90±0.11	3.78±0.21
Rosmarinic 100µg/ml	41.30±1.06	18.76±1.62	28.24±2.06	31.64±5.92	48.87±5.15	55.83±6.62	1.70±0.08	3.57±0.07
Uric acid 25µg/ml	46.61±6.66	22.05±2.66	32.26±4.77	35.92±2.84	53.43±3.13	58.89±2.83	1.88±0.25	3.74±0.22
Uric acid 50µg/ml	50.26±6.49	24.83±4.12	36.18±5.78	35.56±5.03	52.17±4.33	59.14±4.69	1.88±0.14	3.94±0.19
Uric acid 100µg/ml	48.24±4.39	23.06±2.02	34.67±2.35	35.03±5.57	51.36±4.04	59.9±7.63	1.7±0.07	3.64±0.12
Taurine 25µg/ml	47.30±7.79	25.39±7.23	35.14±8.76	38.29±8.81	54.54±7.01	61.14±8.92	1.72±0.13	3.96±0.57
Taurine 50µg/ml	42.85±5.41	20.91±2.42	30.75±3.23	37.01±5.26	53.50±4.66	61.67±5.97	1.73±0.12	3.86±0.68
Taurine 100µg/ml	40.96±8.82	16.71±9.53	25.34±12.10	29.39±13.27	48.21±9.73	51.47±16.18	1.80±0.17	3.42±0.23

The values were given as mean ± SD. (N=3). VCL: Curvilinear velocity (µm/s), VSL: Straight line velocity (µm/s), VAP: Average path velocity (µm/s), LIN: linearity index (%), STR: Straightness index (%), WOB: Oscillation index (%), ALH: Amplitude of lateral head displacement (µm), BCF: beat frequency (Hz).

seconds post activation, no significant differences were observed among all treatments at 10 seconds and 60 seconds ($P>0.05$). Three different TA dosages were tested in this study, and their effects on post-thawed sperm motility were similar to that of the standard extender. The highest motility rate (62.90%) was determined at 100 µg/ml. Except for RA 100 µg/ml, this motility rate was lower than that of other antioxidant dosages (Figure 1).

Fertilization and Hatching Rates

The effects of antioxidants added to extenders on fertilization are presented in Table 4. he standard extender had a 70.67±1.50% fertilization rate. The highest fertilization rate in the antioxidant supplemented extender was 81.67±2.52% in RA 25 µg/ml. Fertilization rates after thawing were found to be higher in all of the antioxidant added extenders compared to the standard extenders. Fresh sperm had a greater fertilization rate than frozen sperm (Table 4). The lowest (55.67±5.10%) hatching rate was observed in the standard extender. The highest (70.67%±4.00%) and the lowest (59.00±3.00%) hatching rates in the antioxidant supplemented extender were achieved at UA 50 µg/ml and TA 50 µg/ml, respectively.

DNA Fragmentation

Based on CASA analysis, fragmented and unfragmented DNA images of the samples are shown in Figure 2. Table 5 shows the increasing in DNA fragmentation after cryopreservation. DNA fragmentation was found to be lower in all antioxidant added groups when compared to the standard extender. DNA fragmentation was higher at all doses of Taurine compared to other antioxidant groups. The lowest (5.07±1.57%) DNA fragmentation occurred in the group supplemented with 50 µg/ml of UA.

Discussion

Because of the nature of cryopreservation, procedures that are commonly used substantially damage sperm function. In this study, we discovered that adding antioxidants to the extender boosted post-thawed motility and reduced DNA damage, despite the fact that antioxidants had no impact on the fertilization rate.

The results of this investigation revealed parallels in terms of sperm volume to those of other studies on Atlantic turbot (Suquet et al., 1994) and Black Sea turbot (Aydın et al., 2021). However, turbot's high sperm motility rate and prolonged cell movement compensate for its low sperm output (Chauvaud et al., 1995). According to Suquet et al. (1998), the sperm density (average number of spermatozoa) of Atlantic turbot ranges between 2 and 9 (10^9 ml^{-1}) and that of Black sea turbot between 1.49 and 2.15 (10^9 ml^{-1}) (Aydın et al., 2021; Polat et al., 2021). The sperm density of 5-year-old hatchery-reared turbot in the current investigation fell within this range. When compared to other species, such as Atlantic halibut (11.9-37.2x10^9), seabass (10-40x10^9), Atlantic salmon (13.8-16.5x10^9), and rainbow trout (4.8-25.4x10^9) (Chauvaud et al., 1995), turbot has a low spermatozoa concentration. Additionally, the pH of the sperm as measured in the current study is consistent with earlier investigations (Dreanno et al., 1998; Polat et al., 2021).

In recent years, considerable improvements have been made in the cryopreservation of sperm. Cryodamage, however, are still a concern and have an effect on sperm quality during the freezing and thawing procedures (Liu et al., 2014; Hezavehei et al., 2018; Boryshpolets et al., 2020). ROS production must be inhibited and the seminal plasma must be protected with antioxidants (Öğretmen et al., 2015). The strength and stability of UA as a reducing agent allow it to have a positive impact on sperm motility. According to the findings, the turbot seminal plasma had 34.73±0.53 µg/ml more UA in November than spermatozoa (Han et al., 2015). UA (0.25 and 0.5 mmol/l) is the main antioxidant that affects sperm motility rate and it has a key role in the antioxidative protection of spermatozoa, according to in vivo research by Lahnsteiner and Mansour (2010). UA also significantly protects brown trout sperm from free radicals (Franz et al., 2010). Kutluyer et al. (2014) found that the motility value of the

Table 4. Effect of antioxidants on the fertilization and hatching of frozen-thawed turbot sperm

	Fertilization rate (%)	Hatching rate (%)
Fresh sperm	89.67±3.20^{a}	76.67±3.50^{a}
Standard extender	70.67±1.50^{c}	55.67±5.10^{d}
Rosmarinic 25 µg/ml	81.67±2.52^{b}	67.00±2.00^{bc}
Rosmarinic 50 µg/ml	78.33±4.51^{b}	70.00±4.36^{b}
Rosmarinic 100 µg/ml	75.00±3.61^{bc}	65.33±1.53^{bce}
Uric acid 25 µg/ml	75.67±3.50^{bc}	66.33±1.20^{bc}
Uric acid 50 µg/ml	78.33±3.20^{b}	70.67±4.00^{b}
Uric acid 100 µg/ml	80.33±3.50^{b}	68.33±3.20^{b}
Taurine 25 µg/ml	76.33±2.50^{bc}	61.33±2.50^{cde}
Taurine 50 µg/ml	75.67±1.50^{bc}	59.00±3.00^{de}
Taurine 100 µg/ml	78.56±3.50^{b}	65.33±5.50^{bc}

The values were given as mean ± SD. (N=3). Means, followed by different letters within columns indicate significant differences ($P\leq0.05$)

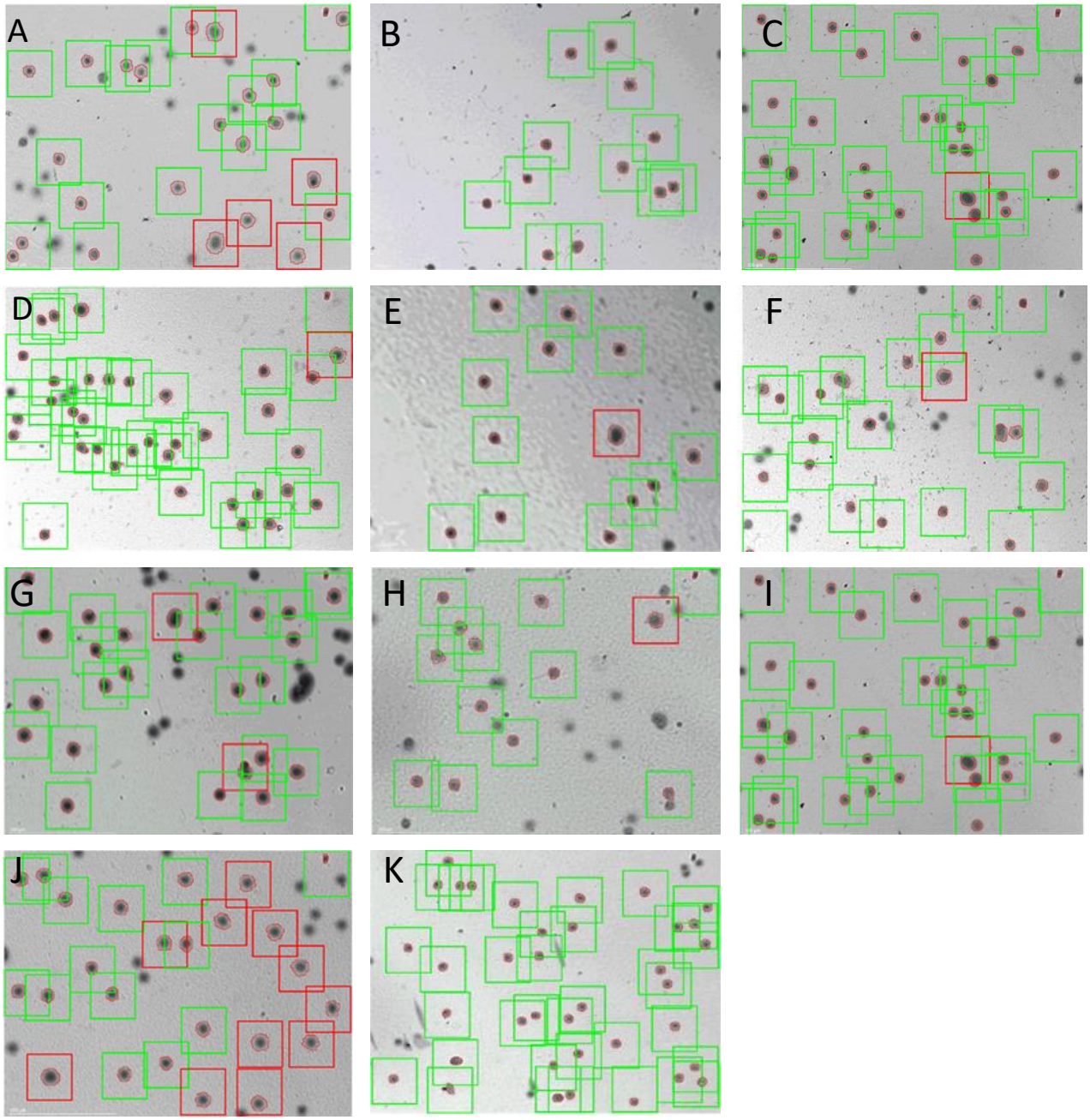

Figure 2. Fragmented and unfragmented DNA images of the samples. A) RA 25 µg/ml, B) RA 50 µg/ml, C) RA 100 µg/ml, D) UA 25 µg/ml, C) UA 50 µg/ml, F) UA 100 µg/ml, G) TA 25 µg/ml H) TA 50 µg/ml, I) TA 100 µg/ml, J) Standart extender, K) Fresh sperm (magnification ×2000, in the green frame spermatozoa not fragmented DNA, in the red frame spermatozoa fragmented DNA) N=3

Table 5. Effect of antioxidants on of frozen-thawed turbot sperm DNA fragmentation

	DNA Fragmentation	
	Not fragmented (%)	Fragmented (%)
Fresh sperm	99.67±26.83	0.33±0.38^{e}
Standard extender	71.13±19.01	28.87±11.26^{a}
Rosmarinic 25 µg/ml	89.73±19.19	10.27±2.94^{d}
Rosmarinic 50 µg/ml	94.80±5.51	5.20±1.11^{de}
Rosmarinic 100 µg/ml	90.33±11.02	9.67±2.79^{d}
Uric acid 25 µg/ml	90.27±9.97	9.73±2.41^{d}
Uric acid 50 µg/ml	94.93±9.39	5.07±1.57^{de}
Uric acid 100 µg/ml	93.33±5.21	6.67±1.61^{d}
Taurine 25 µg/ml	83.40±9.43	16.60±4.92^{bc}
Taurine 50 µg/ml	81.07±11.60	18.93±2.53^{b}
Taurine 100 µg/ml	88.80±14.41	11.20±4.76^{cd}

Different letters show significant differences between fresh and cryopreserved sperm (P<0.05). The data are presented as mean values with standard deviations (N = 3).

extender containing 0.25 mmol/l UA was higher than that of the conventional extender alone in their experiment on rainbow trout. According to the current findings, UA boosted post-thaw sperm motility more than the conventional extender.

It is well known that rosemary (*Rosmarinus officinalis*) has antioxidant properties. This plant extract has been shown to enhance sperm quality, lower lipid peroxidation, and boost fertilization when added to the freezing medium (Malo et al., 2010; Luo et al., 2014). Boar and deer sperm have been cryopreserved using RA, which has been proven to significantly enhance sperm motility (Zanganeh et al., 2013; Luo et al., 2014). The post-thaw motility in the cryopreservation of spotted halibut sperm was likewise greatly enhanced by the addition of 75 µM RA (Zidni et al., 2022). In comparison to the control group, the inclusion of RA significantly improved the vitality of boar sperm during 2 hours of incubation (Malo et al.,2011).

In this study, turbot sperm were cryopreserved for the first time using RA. At a dose of 25 µg/ml RA, the highest frozen-thawed sperm motility was found (77.41±2.90%), which significantly differed from other treatment groups ($P<0.05$). Importantly, it was discovered that the antioxidant dosages of 50 µg/ml RA and 100 µg/ml UA can be employed to effectively preserve turbot sperm. Prior research found that post-thawed turbot sperm motility was 70.3±15.6% (Dreanno et al., 1997) without antioxidant treatment. According to Luo et al. (2014), RA employed various doses (0 µM, 26.25 µM, 52.5 µM, and 105 µM) to cryopreserve boar sperm.

This study revealed that at 0 and 120 minutes post-thaw, the experimental extender with RA had a considerably higher rate of motility than the control ($P< 0.05$). The addition of RA increased motility, acrosome integrity, and viability while also having a positive impact on sperm quality metrics. Another study found that the addition of 4% rosemary extract to buck semen extender improved post-thawed sperm motility (Zanganeh et al., 2013). Based on the current study, natural antioxidants are essential for maintaining motility because they stop sperm from oxidizing during cryopreservation. In the current study, RA and UA also have a favorable impact on the motility of turbot sperm when they are frozen and thawed.

Sulfonated beta-amino acid TA has a variety of physiological and pharmacological properties, such as antioxidant, osmoregulation, and membrane stability (Spitze et al., 2003; Len et al., 2019). Different species-specific outcomes were recorded when taurine was added to the cryoprotectant solution. Although dogs and bulls' sperm motility was unaffected, taurine use improved the motility of cryopreserved sperm from goats, rams, and buffalo (Buff et al., 2001; Martins-Bessa et al., 2007). The optimal taurine concentration for cryopreserving sperm from Siberian sturgeons (*Acipenser baerii*) was reported to be 0.01 mmol/ml (Kovalev et al., 2021).

The most notable protective impact on improving the post-thawed quality of red seabream (*Pagrus major*) sperm was TA (50 mM) (Liu et al., 2014). Extenders supplemented with 1 mM TA demonstrated enhanced overall motility (30.1±3.2%), velocity (18.1±2.6 lm/s), and linearity (46.0±4.8%) in contrast to the control extender in a study involving the cryopreservation of sperm from a European sea bass (*Dicentrarchus labrax*) (Martínez-Páramo et al., 2013). Additionally, it was noted that during cryopreservation of rainbow trout (*Oncorhynchus mykiss*), extenders treated with 50 mM taurine did not significantly differ from the control group in terms of sperm motility rate or motility duration (Ekici et al., 2012).

According to Kutluyer et al. (2016), goldfish (*Carassius auratus*) sperm cryopreserved at 4 mM TA concentration exhibited the highest post-thawed motility rate (72.50±3.54%) and time (17.50±0.71 s). TA treatments of 50 and 75 mM improved the fertility of scaly carp (*Cyprinus carpio*) cryopreserved semen (Yavaş et al., 2014). In this study, all treatment doses of TA showed a significant correlation to the conventional extender in terms of the motility of frozen-thawed turbot sperm ($P< 0.05$). Though in turbot sperm that had undergone cryopreservation, TA reduced DNA damage.

There are limited research on the rates of turbot sperm post-thawing fertilization and hatching (Chereguini et al., 2001; Chereguini, 2003; Chen et al., 2004). These study revealed that the post-thawing fertilization rates were quite similar to those of fresh sperm. The study's fertilization rates were higher than those from earlier turbot experiments (Dreanno et al., 1997; Chereguini, 2003; Chen et al., 2004). The higher fertilization rates can be attributed to antioxidants' beneficial influence on sperm motility after thawing. According to the examination of hatching rate trials, all antioxidant treatment groups surpassed the conventional extender (70.67±1.50%). However, it still couldn’t compete with fresh sperm. The ROS reducing effect of the antioxidants, and therefore less DNA damage, might have led to the successful completion of embryonic development and greater hatching rates than with the standard extender. The hatching rates obtained in this study were higher than those reported by Chen et al. (2004) and Chereguni et al. (2003).

DNA enables the transfer of incredibly densely packed genetic information to the egg. It also guarantees that the DNA is delivered in a physical and chemical state that allows the embryo to swiftly access genetic information. The major factor considered to cause sperm DNA damage is ROS (Shafik et al., 2006; Rex et al., 2017). The measurement of DNA damage during sperm cryopreservation and storage is necessary in order to avoid the probable loss of fertilization capacity and to explain any developmental failures throughout embryo development. The effect of cryopreservation on sperm DNA has been studied in a small number of marine and freshwater fish species and increased DNA fragmentation has been documented after freezing-

thawing (Cabrita et al., 2005) Other studies have showed that the use of antioxidant in sperm cryopreservation significantly reduces DNA fragmentation in fish species such as European sea bass, *Dicentrarchus labrax* (Martínez-Páramo et al., 2013), common carp, *Cyprinus carpio* (Öğretmen et al., 2015), gilthead seabream, *Sparus aurata* (Cabrita et al., 2011). In the current study, antioxidant use significantly lowered DNA damage in all groups in comparison with the standard extender alone. The lowest DNA damage was achieved in UA treatment of 50 µg/ml (5.07±1.57%) compared to the other groups. In this study, it has been reported for the first time that the used antioxidants significantly reduced the percentage of DNA damage of frozen thawed turbot sperm. This finding has suggest an increase in the quality of turbot cryopreserved sperm.

Conclusions

According to this study, introducing antioxidants to the extender during the cryopreservation of turbot sperm minimized adverse effects including sperm motility loss and DNA damage that typically occurs when sperm are frozen and thawed. Both UA 50 and 100 µg/ml and RA 25, 50 µg/ml significantly improved sperm motility and decreased DNA damage. However, when compared to the conventional extender, concentrations of 25, 50, and 100 µg/ml TA significantly reduced DNA damage. This might be a sign that adding 25 µg/ml RA and 100 µg/ml UA helps to preserve turbot sperm throughout cryopreservation.

Ethical Statement

All process and experimental protocols have been approved by Central Fisheries Research Institute (SUMAE), Animal Experiments Local Ethics Committee (No; 325.04.02-10) under the General Directorate of Agricultural Research and Policy surveillance.

Funding Information

This research which was a part of the Ph.D. thesis of Hamza POLAT and was funded by the General Directorate of Agricultural Research and Policies (Republic of Turkey Ministry of Agriculture and Forestry, Türkiye) (Project Number: TAGEM/HAYSÜD/B/21/ A6/P2/2452). The authors are acknowledged to Central Fisheries Research Institute-SUMAE (Trabzon, Türkiye) who provided the equipment and financial support.

Author Contribution

Hamza POLAT: Conceptualization, funding acquisition, project administration, methodology, formal analysis, investigation, writing-review & editing. İlker Zeki KURTOĞLU: Conceptualization, formal analysis, visualization, writing-original draft, writing-review & editing. Methodology, investigation.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Dr. Atife Tuba BEKEN for assisting us with the tests and Muhammed Doğan ÖZDEMİR for supporting the data visualization. We would also like to thank the personnel of the aquaculture unit of the Central Fisheries Research Institute for their contribution during experiments.

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