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RESEARCH PAPER

The Positive Effect of Antioxidants in Maintaining the Post-Cryopreserved Sperm Quality of Rainbow Trout *Oncorhynchus mykiss* (Walbaum, 1792)

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Abstract

Rainbow trout aquaculture is challenging due to the restricted spawning seasons. Sperm cryopreservation technology can help ensure a successful spawning season, but its effectiveness is restricted by oxidative stress during freezing and thawing, which reduces the availability of male gametes. This study aimed to evaluate the effect of several types and concentrations of antioxidants on post-cryopreservation rainbow trout sperm quality. Subsequently, a factorial completely randomized design with three replications was applied, testing four types of antioxidants Ascorbic acid, Myo-inositol, Butylated hydroxytoluene (BHT), and Alpha-tocopherol at concentrations of 0, 20, 40, and 60 mM, respectively. The evaluation parameters included motility rate, sperm lifespan, and sperm motion characteristics using the Computer Assisted Sperm Analysis system. The results showed that the type and concentration of antioxidants significantly affected sperm motility and sperm lifespan ($P<0.05$). The treatment method carried out using 40 mM Ascorbic acid and 20 mM BHT led to the highest sperm motility of 46.67% and sperm lifespan of 34 seconds. Although both antioxidants showed similar effectiveness, BHT at a concentration of 20 mM is more recommended due to its lower toxicity and technical as well as economic efficiency considerations.

Introduction

Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792*)* is a species of freshwater fish native to watersheds in the Western Pacific region of North America and Canada. This species has high economic value and has been widely introduced and cultivated in various parts of the world, including Europe, Asia, and Australia (Habibnia et al., 2024). These fish are commonly found in countries such as Norway, Chile, Türkiye, and Mexico (Ortega and Valladares, 2017; Stanković et al., 2015). The farming of rainbow trout has developed in several countries in Europe and Asia. According to FAO (2023), the aquaculture production of this species reached 744.000 tonnes in 2021 and increased to 2.748 tonnes in 2023 (Escamilla-Rosales et al., 2024). Rainbow trout produced through aquaculture is exported in fillet from both developed and developing countries (Ortega and Valladares, 2017). Despite advancements in aquaculture, there is still a challenge, particularly in artificial breeding, as this fish species struggles to spawn outside its natural season (El Kamouh et al., 2023). As a result, seed availability is limited throughout the year, leading to fluctuations in

aquaculture production. To address this issue, the application of frozen sperm storage technology, known as cryopreservation, offers a potential solution.

Sperm cryopreservation is an appropriate technology that aims to maintain long-term sperm viability, making the sperm sustainably available and can be used when needed (Maulida et al., 2024). This method ensures a constant supply of sperm and can be applied to selective breeding and other hatchery technologies such as sex reversal or mono-sex (Ryu et al., 2022). The success of sperm cryopreservation is largely determined by the methods used, for example the suitability of extenders, the type and concentration of cryoprotectants such as antioxidants, antibiotics, and the freezing and thawing processes (Judycka et al., 2019).

During the storage process, there is still a possibility of oxidative stress on sperm cells that can damage cell membranes and sperm DNA (Kolyada et al., 2023). This oxidative stress is mainly caused by the formation of reactive oxygen species (ROS) or free radicals during the freezing and thawing process. Therefore, antioxidant substances are needed that can prevent the occurrence of ROS (Yang et al., 2021). Antioxidants play an important role in cryopreservation media by reducing oxidative stress, neutralizing ROS that may be formed during the cryopreservation process, and helping maintain sperm quality during storage (Zidni et al., 2022).

A study has been carried out on rainbow trout sperm cryopreservation by several investigation bodies, including Ubilla and Valdebenito (2011) who used antioxidant such as polyphenol, trolox C, plus trolox, and vitamin C with the same test concentration of 0.1 mM. The results showed that after seven days of storage, polyphenol and vitamin C yielded better outcomes for the sperm sperm lifespan,. Lahnsteiner et al. (2011) reported that the use of 0.1-0.25 mM catalase, 0.25-0.5 mM superoxide dismutase, 0.25-0.5 mM peroxidase, and 1.5-3.0 mM glutathione and 1.5-3.0 mM methionine did not improve sperm motility percentage, likely due to inappropriate concentrations percentage. Kocabaş et al. (2022) reported that the addition of antioxidants inhibited the negative effects of Arsenic use on rainbow trout sperm cryopreservation. Furthermore, Akça et al. (2021) and Kocabaş et al. (2022) reported that the use of 0.015 mM glyphosate and 0. 008 mM Aroclor 1254 had no effect on the fertilization of rainbow trout after cryopreservation. The studies that have been conducted generally produce low sperm quality (motility) ranging from 30-40%, thereby, there is every possibility to improve by exploring several other types and concentrations of antioxidants, including Ascorbic acid, Myo-inositol, BHT, and Alpha-tocopherol which have never been tested on rainbow trout.

Ascorbic acid is a water-soluble antioxidant, and this material prevent reactive oxygen species by donating electrons from its enediol group to neutralize the ROS effect. Through this mechanism, highly reactive free radicals, such as superoxide (O_2^-) and hydroxyl radicals (OH), are converted into more stable molecules that are less harmful to sperm cells. During this process, Ascorbic acid undergoes oxidation and forms a relatively stable ascorbyl radical (Agwu et al., 2023). BHT (Butylated Hydroxytoluene) is a lipophilic antioxidant that acts by neutralizing free radicals and terminating the chain reaction of lipid peroxidation in the sperm membrane (Khumran et al., 2015). Its phenolic group donates a hydrogen atom to stabilize peroxyl radicals, thereby preventing damage to phospholipids that are rich in polyunsaturated fatty acids. BTH is effective in several fish species such as Coho salmon *Oncorhynchus kisutch* (Merino et al., 2020), and Masu salmon *Oncorhynchus masou* (Chelewani et al., 2024). Myo-inositol acts as a regulator of cellular signaling and a stabilizer of the plasma membrane, and is involved in the formation of inositol triphosphate (IP_3), which regulates Ca^{2+} homeostasis and supports sperm motility as well as physiological function (Maulida et al., 2024). Alpha-tocopherol can prevent lipid peroxidation by donating hydrogen atoms to peroxyl radicals or lipids (Sandoval-Vargas et al., 2021). Therefore, the role of these antioxidants in rainbow trout sperm cryopreservation needs to be tested to find the type and concentration of antioxidants that are more effective. This is important because the sperm of each fish species respond differently to the type and concentration of antioxidants used (Sevastei et al., 2023).

Material and Methods

Experimental Design

This study used a factorial completely randomized design, and the factors tested were different types and concentrations of antioxidants, with 3 replicates each (Figure 1). The antioxidant types tested were Ascorbic acid (A1), Myo-inositol (A2), Butylated Hydroxytoluene (A3), and Alpha-tocopherol (A4). Each antioxidant type was tested at four concentration levels, namely 0 mM (C1), 20 mM (C2), 40 mM (C3), and 60 mM (C4), combined with 10% DMSO and 10% egg yolk as extracellular cryoprotectant. Each treatment with three replicates, resulting in the following treatment combinations; A1C1, A1C2, A1C3, A1C4; A2C1, A2C2, A2C3, A2C4; A3C1, A3C2, A3C3, A3C4; A4C1, A4C2, A4C3, A4C4.

Fish and Sperm Collection

This study was conducted from February to March 2024. The experimental protocols and handling of animals were approved by the Ethics Committee on Animal Experimentation of Karadeniz Technical University (Trabzon, Türkiye) (the protocol number 2023/14). The rainbow trout used as breeding stock were captured using an electroshock device from a stream in Uzungöl (Trabzon, Türkiye), where fish had

escaped from local production farms into the natural environment. A total of 15 captured male rainbow trout with a length of 25-35 cm and a weight of 450-550 grams were acclimatized for 15 minutes to reduce stress. Each fish was first anesthetized with Benzocaine (30 mg/L), after which gentle abdominal pressure was applied for stripping. To prevent sperm activation and contamination from mucus, water, urine, blood, or feces, the abdominal surface was carefully dried with paper towels. The sperm that came out was collected in a 50 ml tube. Sperm from each male was placed individually. The sperm samples obtained were stored in a styrofoam box for initial quality analysis, where sperm with motility above 90% were used for the freezing process. Then all sperm that met the criteria were pooled in one Falcon tube and stored at 4°C. The fish that had finished stripping were returned to their habitat. The cryopreservation process and observation of rainbow trout sperm were carried out at the Laboratory of the Department of Wildlife and Ecology, Forestry Faculty, Karadeniz Technical University, Trabzon, Türkiye.

Cryopreservation Procedure

The extender used was an immobilization solution for rainbow trout based on previous studies carried out by Kocabaş et al. (2022). This solution consisted of 0.3 M glucose, 10% DMSO, and 10% egg yolk. Furthermore, these materials were poured into an Erlenmeyer flask, and distilled water was added to reach a volume of 100 ml. Sperm was diluted with an extender solution with a ratio of 1: 3 (sperm: extender), and 10 ml of sperm was mixed with 30 ml of immobilization solution. Then 4 ml of DMSO was added to the sperm dilution to produce a 10% concentration and 4 ml of egg yolk to produce a 10% concentration as an intracellular and extracellular cryoprotectant.

The mixture of sperm dilution was distributed into 16 falcon tubes, where each contained 5 ml of the sperm extender solution and each of the four falcon tubes was added to each test antioxidant (Ascorbic acid, Myo-inositol, BHT, and Alpha-tocopherol), namely 0 ml to produce a test concentration of 0 mM, 0.02 ml to produce 20 mM, 0.04 ml to produce 40 mM, and 0.06 to produce 60 mM. Additionally, all falcon tubes were transferred into a 0.5 ml straw and put into a styrofoam box and continued with the equilibration process (initial freezing) at 4°C for 10 minutes, then the sample was evaporated at a distance of 3 cm above the surface of liquid nitrogen at -79°C for five minutes and put into liquid nitrogen at -196°C and stored for one week. After one week of thawing, the straw containing frozen sperm was taken from the liquid nitrogen tube and dipped in a 30°C water bath for 30 seconds and emptied into an Eppendorf, kept on ice for further analysis.

Macroscopic and Microscopic Analyses

Macroscopic analyses of color, pH, and sperm concentration were performed to assess fresh sperm quality, while microscopic analyses of motility were

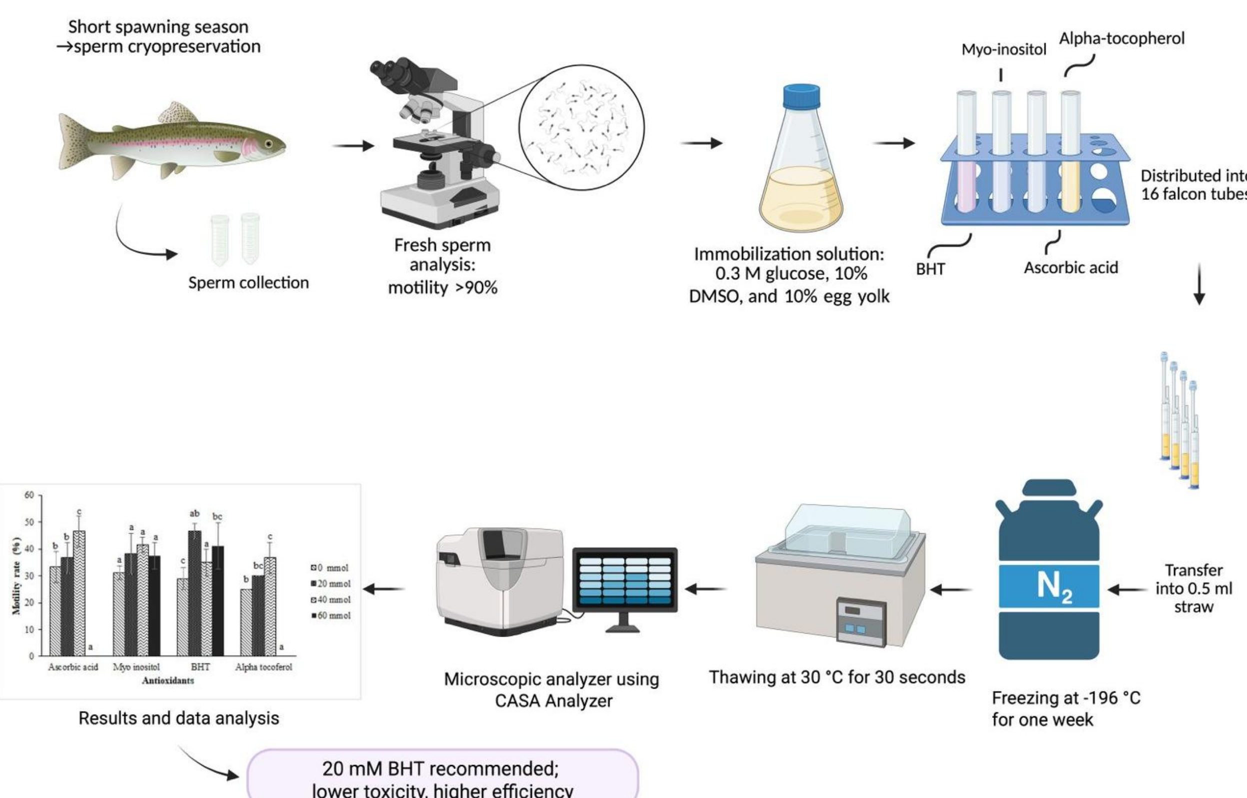

Figure 1. Graphical abstract of the study.

used to compare fresh and post-cryopreservation. Sperm pH was determined using a pH meter, sperm concentration was evaluated using a hemocytometer, and spermatocrit was assessed based on the method proposed by Handayani et al. (2024). Sperm consistency was determined based on the sperm flow rate observed inside the collection tube, with the following evaluation criteria: thin consistency (rapid flow rate), medium consistency (slightly reduced flow rate with some sperm adhering to the tube wall), and thick consistency (slow flow rate, leaving residual sperm along the edge of the tube). Sperm motility was determined as the progressive movement of sperm. Motility observations of fresh and frozen sperm were made using a Computer Assisted Sperm Analyzer (CASA) application connected to a Nikon E50i microscope (Nikon CI, Tokyo, Japan) with 400X magnification and a Basler Ace digital camera (ACA1300-200UC, Made in Barcelona, Spain). The sperm lifespan was measured from the initiation of sperm activation to the point at which forward movement ceased of almost 90% of the spermatozoa. In addition, some additional characteristics that determine the fertility level of the broodstock were also calculated, namely VCL (curvilinear velocity), VSL (straight linear velocity), VAP (average path velocity), LIN (linearity index), STR (straightness index), WOB (wobble index), ALH (amplitude of lateral head displacement), and BCF (beat cross frequency) also using CASA analyzer.

Statistical Analyses

The qualitative data, including pH, color, and sperm concentration, were analysed descriptively. While the average of quantitative data were subjected to statistical analyses. The percentage of motility and sperm lifespan were tested for normality using the Shapiro-Wilk test. If the data were normally distributed, it was subjected to a one-way ANOVA test, and when significant effects were detected (at P<0.05), the Duncan's multiple range test was applied at the 95% significance level (P<0.05) using SPSS 22.0.

Results

The results showed that fresh rainbow trout sperm was milky white, with a pH of 7.6 and a very thick consistency, with a density of 6.69×10^9 cells/mL, a mean motility percentage of 96.67±2.89%, and a fresh sperm sperm lifespan of 76.67±7.09 seconds (Table 1), thereby the quality of fresh sperm met the criteria for using it in the freezing process. This study shows that antioxidant supplementation significantly enhances post-thaw sperm quality over the control. Sperm motility reached 46.67% with 40 mM Ascorbic acid, which was significantly higher than the control at 33.33%. At 20 mM BHT, motility was maintained at 46.67%, which is significantly higher than the control at 30.00%. Furthermore, the VCL and VSL values in both Myo-inositol and BHT treatments increased, as indicated in Table 2. In the Myo-inositol treatment, VCL and VSL values were significantly higher at 40 mM. In contrast, the BHT treatment showed significantly higher VCL and VSL values at 60 mM concentration. These findings indicate that antioxidants play a critical role in preserving sperm quality during cryopreservation.

One-way ANOVA results showed that the type and concentration of antioxidants significantly affected rainbow trout sperm motility (P<0.05). Overall, the highest motility values were observed in the 40 mM Ascorbic acid and 20 mM BHT treatments, each yielding 46.67%. When analyzed separately, Ascorbic acid at 40 mM resulted in a significantly higher motility value (46.67%) compared to other Ascorbic acid concentrations. For Myo-inositol, motility at 40 mM (41.67%) was significantly higher than at other concentrations, but not statistically different from 20 mM and 60 mM (38.33% and 37.50%, respectively). In the case of BHT, motility at 20 mM (46.67%) was significantly higher than at other concentrations, although not significantly different from 40 mM (35.00%). For Alpha-tocopherol, motility at 40 mM (36.67%) was significantly higher than at other concentrations, but not significantly different from 20 mM (30.00%).

ANOVA results indicated that both the type and concentration of antioxidants had a significant impact on sperm lifespan (P<0.05). Overall, sperm lifespan was significantly higher in the 40 mM ascorbic acid and 40 mM myo-inositol treatments, each yielding 34.00 seconds. When evaluated individually, ascorbic acid at 40 mM showed a significantly higher sperm lifespan compared to its other concentrations. For Myo-inositol, the sperm lifespan at 40 mM (34.00 seconds) was significantly higher than the other concentrations; however, it was not statistically different from the values at 0 mM and 20 mM (33.50 seconds and 33.67

Table 1. Assessment results of the fresh sperm quality of rainbow trout *O. mykiss*

No	Parameters	Characteristic
1.	Color	Milky white
2.	Volume (ml)	5.18±1.07
3.	pH	7.60±0.13
4.	Consistency	Very thick
5.	Spermatocrit (%)	21.92±1.89
6.	Sperm concentration (cell/mL)	6.69±0.54
7.	Fresh sperm motility (%)	96.67±2.89
8.	Fresh sperm motility lifespan (seconds)	76,67±7,09

Table 2. Sperm motility characteristics of rainbow trout in presence or absence of antioxidants. Values±SD with different superscript in the same column are significantly different (P<0.05) by Duncan's multiple range test, and the bold letters represent the highest mean value for each parameter.

Antioxidants	Antioxidant concentration (mM)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Ascorbic acid	0	51.10±4.19^{abcd}	25.71±2.99^{abc}	35.23±3.37^{abcd}	51.92±0.66^{de}	72.88±1.53^{de}	60.91±1.16^{bc}	1.53±0.09^{ab}	5.74±1.49^{abcde}
	20	59.91±8.11^{abcd}	23.09±15.97^{abc}	23.46±2.41^{abc}	20.99±1.11^{a}	45.88±1.40^{a}	45.75±1.02^{a}	2.14±0.18^{abc}	2.90±0.28^{a}
	40	55.50±0.08^{abcd}	18.10±5.85^{abc}	29.63±3.27^{abcd}	39.13±1.40^{cd}	67.56±2.95^{cde}	57.92±0.46^{bc}	2.41±0.32^{abc}	3.65±0.06^{abc}
	60	-	-	-	-	-	-	-	-
Myo inositol	0	30.71±33.02^{ab}	16.44±16.09^{abc}	22.08±21.97^{abc}	31.18±9.63^{abc}	56.03±10.17^{abc}	64.92±6.56^{cd}	1.06±0.75^{a}	4.03±0.51^{abcd}
	20	40.05±19.39^{abc}	11.56±2.18^{ab}	21.33±7.77^{ab}	**60.11±12.24^{e}**	**75.67±2.41^{e}**	**69.52±0.05^{d}**	2.53±0.45^{abc}	**7.57±0.61^{e}**
	40	80.95±26.94^{d}	**35.82±17.27^{c}**	**54.20±25.65^{e}**	38.06±0.11^{bc}	65.94±0.65^{cde}	57.73±0.74^{bc}	**3.68±1.61^{c}**	4.49±2.93^{abcd}
	60	27.27±5.22^{a}	9.27±3.87^{a}	15.07±4.67^{a}	33.24±7.86^{abc}	60.44±6.97^{bcd}	62.15±3.96^{bcd}	2.78±0.45^{bc}	4.74±2.21^{abcde}
BHT	0	51.87±5.05^{abcd}	16.00±8.82^{abc}	26.85±7.21^{abcd}	26.40±8.75^{abc}	52.36±10.56^{ab}	56.03±2.22^{b}	2.34±0.22^{abc}	3.40±0.42^{ab}
	20	67.96±19.50^{bcd}	11.90±0.15^{ab}	33.31±11.51^{abcd}	24.31±3.58^{ab}	49.90±4.29^{ab}	48.59±2.99^{a}	2.49±0.30^{abc}	4.45±2.47^{abcd}
	40	72.70±10.08^{cd}	14.58±6.46^{abc}	47.08±16.56^{bcd}	33.60±6.20^{abc}	58.69±6.40^{abc}	57.01±4.35^{b}	2.33±0.09^{abc}	6.28±0.18^{bcde}
	60	**86.93±8.04^{d}**	29.29±1.08^{abc}	49.31±4.53^{cd}	33.78±1.87^{abc}	59.54±3.27^{bcd}	56.73±0.04^{b}	2.43±0.05^{abc}	6.78±0.21^{de}
Alpha Tocopherol	0	54.81±0.90^{abcd}	17.13±7.23^{abc}	28.56±4.79^{abcd}	39.46±0.94^{cd}	67.50±3.03^{cde}	58.48±1.24^{bc}	2.23±0.06^{abc}	3.44±0.36^{ab}
	20	82.82±24.30^{d}	32.53±2.62^{bc}	49.72±5.81^{cd}	33.58±6.24^{abc}	69.31±8.61^{cde}	64.79±6.64^{cd}	3.17±1.64^{bc}	6.69±0.15^{cde}
	40	39.63±12.26^{abc}	10.89±1.59^{a}	20.06±2.39^{ab}	38.39±0.57^{bc}	65.88±0.74^{cde}	58.28±1.52^{bc}	2.48±0.02^{abc}	3.14±0.57^{a}
	60	-	-	-	-	-	-	-	-
Significant (P<0.05)		0.032	0.122	0.049	0.001	0.003	0.000	0.177	0.030

seconds, respectively). For BHT, the sperm lifespan at 60 mM (29.50 seconds) was significantly higher than the other concentrations, although it was not significantly different from any of the BHT concentrations tested. For alpha-tocopherol, sperm lifespan at 40 mM (29.00 seconds) was significantly higher than the other concentrations, yet this value was not significantly different from the sperm lifespan at 0 mM and 20 mM (28.00 seconds and 28.33 seconds, respectively) (Table 3).

One-way ANOVA results showed that the type and concentration of antioxidants significantly affected the sperm motility characteristics of rainbow trout ($P<0.05$). The significantly higher values of VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF were found in the treatment with added antioxidants. These values were significantly different between treatments, but the VCL parameter was not significantly different from the other treatments (Table 2).

Discussion

The results showed that antioxidant supplementation positively influenced the quality of rainbow trout sperm. In the use of Ascorbic acid and Alpha-tocopherol, the motility and duration of sperm motility or sperm lifespan increased as the concentration of antioxidants increased from 0 to 40 mM. Although if the concentration was increased beyond 40 all observed sperm died. This is presumably because at excessively high concentrations which may cause hypertonic environment for sperm. Additionally, ascorbic acid may shift to a pro-oxidant role by reacting with metal ions such as Fe^{2+} or Cu^{2+}, thereby generating highly reactive hydroxyl radicals, while excessive accumulation of Alpha-tocopherol may lead to the formation of tocopheroxyl radicals that damage membrane lipids (Figueroa et al., 2018). Moreover, the increased osmotic pressure creating a hypertonic induces water efflux from the cells, resulting in cell volume shrinkage, pH alterations that destabilize cellular homeostasis, and axonemal structural damage (Öztürk et al., 2019). While in the use of Myo-inositol and BHT, motility tended to increase as the concentration of Myo-inositol and BHT increased, but the quantity decreased slightly at concentrations above 40 mM. Therefore, in general, the best sperm motility and sperm lifespan values were obtained at a concentration of 40 mM Ascorbic acid and 20 mM Myo-inositol.

The use of Ascorbic acid as an antioxidant in sperm cryopreservation also produced positive results in several fish species, including *Salmo salar* and *Dicentrarchus labrax*, where its use at a concentration of 1.0 mM produced higher motility values compared to the control (Figueroa et al., 2018; Martínez-Páramo et al., 2012); in *Sparus aurata* with concentrations of 1 and 10 mM produced higher motility compared to the control (Cabrita et al., 2011). In addition, *Acipenser dabryanus*, *A. sinensis,* and *A. baerii* also had a positive effect on post-cryopreservation DNA integrity, with the best results obtained at concentrations of 2.5 - 10 mM (Li et al., 2018). This positive effect may be because Ascorbic acid is a water-soluble non-enzymatic antioxidant that is easily absorbed into cells, which plays an important role in neutralizing ROS generated during the freezing and thawing process (Cabrita et al., 2011; Gęgotek and Skrzydlewska, 2023; Sandoval-Vargas et al., 2021). In addition, Ascorbic acid can also prevent lipid peroxidation and reduce oxidative damage, thereby improving sperm quality and reducing DNA fragmentation in thawed spermatozoa (Abualreesh et al., 2021; Marques et al., 2018; Martínez-Páramo et al., 2012).

The results also showed that Ascorbic acid at concentrations of 20–40 mM improved sperm motility, but no motile sperm were observed at 60 mM. In contrast, BHT maintained sperm motility even at a

Table 3. Percentage of sperm motility and duration (sperm lifespan) using different types and concentrations of antioxidants in rainbow trout *Oncorhynchus mykiss* cryopreserved sperm after post thawing. Mean±standard deviation of motility and sperm lifespan values in antioxidants in the same column with different superscripts indicate significant differences ($P<0.05$) by Duncan's multiple range test, and the bold letters represent the highest mean value for each parameter.

Antioxidants	Code	Concentration (mM)	Motility rate(%)	Motility duration or Sperm lifespan (second)
Ascorbic acid	A1C1	0	33.33±5.77^{abc}	23.00±3.46^{a}
	A1C2	20	36.67±5.77^{bcd}	28.33±8.74^{abc}
	A1C3	**40**	**46.67±5.77^{e}**	**34.00±6.56^{c}**
	A1C4	60	-	-
Myoinositol	A2C1	0	31.67±2.89^{ab}	33.33±2.89^{bc}
	A2C2	20	38.33±7.64^{bcde}	33.75±4.03^{bc}
	A2C3	**40**	**41.67±2.89^{cde}**	**25.33±3.51^{ab}**
	A2C4	60	36.67±5.77^{bcd}	28.00±3.60^{abc}
BHT	A3C1	0	30.00±5.00^{ab}	27.75±4.11^{abc}
	A3C2	**20**	**46.67±2.89^{e}**	**34.00±2.65^{c}**
	A3C3	40	35.00±5.00^{bc}	28.40±5.55^{abc}
	A3C4	60	45.00±5.00^{de}	27.33±2.08^{abc}
Alpha-tocopherol	A4C1	0	25.00±0^{a}	30.00±4.55^{abc}
	A4C2	20	30.00±0^{ab}	28.33±2.89^{abc}
	A4C3	**40**	**36.67±5.77^{bcd}**	**29.00±2.65^{abc}**
	A4C4	60	-	-

concentration of 60 mM. This may be related to the nature of toxicity, where the toxicity of Ascorbic acid to rainbow trout sperm cells is believed to be higher than BHT, meaning that rainbow trout sperm cells are more sensitive to Ascorbic acid. Therefore, based on these findings, 20 mM BHT is recommended for the cryopreservation of rainbow trout sperm, as this concentration appears to be more compatible with the species' sperm physiology. This recommendation is also in line with the investigation carried out by Merino et al. (2020) who reported that a concentration of 2 mM BHT proved effective for *Oncorhynchus kisutch* sperm. However, in sturgeon *Acipenser dabryanus* sperm, the optimum concentration is lower, which is below 1 mM (Kolyada et al., 2023; Li et al., 2018; Osipova et al., 2016). This shows that the suitability of the type and concentration of each antioxidant is highly dependent on the fish species. This is because each sperm cell of different species responds differently to the antioxidant given, and if the antioxidant given exceeds the tolerance threshold it can have a negative effect on sperm, therefore it is important to know the concentration of the antioxidant.

In addition to motility and its duration (sperm lifespan), the study also evaluated several key motility parameters, including VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF. Understanding these sperm motility characteristics is essential for gaining a comprehensive assessment of sperm quality following cryopreservation (Zilli et al., 2003). These parameters are related to the speed and strength of sperm movement towards and fertilization of the egg (Kutluyer et al., 2014). Antioxidants such as 40 mM myo-inositol and 20 mM α-tocopherol were found to be the most effective in enhancing sperm velocity parameters (VCL, VAP, and BCF). These three parameters cannot be separated because they collectively describe the physical and biomechanical qualities of spermatozoa movement. This is consistent with Gallego-Ríos et al. (2021), who stated that VCL reflects the intensity of movement, VAP indicates the efficiency of directional movement, and BCF describes the flagellar dynamics that drive both parameters. Good stability of flagellar motion increases the tendency for progressive movement, thereby elevating VAP as an indicator of swimming efficiency toward the egg.

Motility pattern parameters such as LIN, STR, and WOB also exhibited significant differences, indicating that the type and concentration of antioxidants influence the quality of spermatozoa movement patterns. This is consistent with Beken et al. (2025) who noted that these three parameters are interconnected, as enhanced flagellar stability reflected by higher WOB values improves directional consistency and increases STR, which subsequently contributes to higher LIN values as an indicator of trajectory linearity. Based on the results of observations and statistical tests, rainbow trout sperm motility characteristics showed significant differences between treatments except for the use of Ascorbic acid and Alpha-tocopherol at high concentrations (60 mM), where no sperm were detected alive or all sperm died at that concentration.

Although motility is a key parameter for sperm quality assessment (Tanga et al., 2021). The current study is limited by two main research gaps: a primary focus on sperm motility without assessing other indicators of sperm quality, and the scarcity of mature female broodstock, which prevented fertilization rate analysis. Therefore, future studies could include further analysis such as lipid peroxidation tests, DNA integrity evaluations, or sperm ultrastructural analyses etc. to provide a more comprehensive understanding of the sperm quality characteristics of cryopreserved sperm of this species (Dadras et al., 2022).

Conclusion

In conclusion, the type and concentration of antioxidants significantly affect the quality of rainbow trout sperm. In the use of Ascorbic acid, Myo-inositol, and Alpha-tocopherol, the best results were obtained at a dose of 40 mM, while with BHT the best results were at a dose of 20 mM. The use of 40 mM ascorbic acid and 20 mM BHT produced better sperm quality compared to other types and concentrations of antioxidants tested. Ascorbic acid and BHT can maintain sperm quality equally well, but based on technical and economic considerations, 20 mM BHT is recommended as an antioxidant in rainbow trout sperm cryopreservation.

Ethical Statement

The experimental protocols and handling of animals were approved by the Ethics Committee on Animal Experimentation of Karadeniz Technical University (Trabzon, Türkiye) (the protocol number 2023/14).

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Author Contribution

Luvi Syafrida Handayani: Responsible for conducting the experiment, data collection, data analysis, and first draft report; Sri Riska Rahayu: Responsible for sample collection, sample processing; Nanda Muhammad Razi: Responsible for sample collection, sample analysis; Elya Putri Pane: Responsible for sample processing, sperm motility analysis; Mehmet

Kocabas: Responsible for data analysis, final draft preparation and proofreading; Filiz Kocabas: Responsible for data analysis, first draf correction; Hafizuddin Hafizuddin: Responsible for data analysis, first draf preparation; Nur Fadli & Sulistiono Sulistiono: Responsible for developing research proposal and study design; Zainal Abidin Muchlisin: Responsible for developing research proposal, supervision and study design and approved the final draft of the paper.

Conflict of Interest

The authors 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.

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