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Effect of Antioxidants at varying Concentrations on Sperm Quality of Naleh Fish (*Barbonymus* sp.) Post-cryopreservation

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Abstract

Cryopreservation can generate oxidative stress, which in turn may harm cellular structures and diminish sperm quality upon thawing. Several studies highlight that antioxidants are pivotal in reducing the harmful effects of Reactive Oxygen Species (ROS). However, the ideal type and concentration depend on the fish species.#Thus, the goal of this research is to determine the most effective type and concentration of antioxidants in maintaining the quality of post-cryopreservation sperm of naleh fish (*Barbonymus* sp.). The procedures were conducted in 2 stages using a Completely Randomized Design (CRD) that included 5 treatments with 4 replications for each. In the first stage, 5 types of antioxidants, namely ascorbic acid, β-carotene, glutathione, butylated hydroxytoluene (BHT), and myo-inositol, were tested at a concentration of 10%. The most effective antioxidant from this stage was then tested again at 5 different concentrations (0%, 5%, 10%, 15%, and 20%) in the second stage. Ringer's solution, 10% dimethyl sulfoxide (DMSO), and 5% egg yolk served as extenders and cryoprotectants. The sperm were stored at -196°C in liquid nitrogen for a duration of 14 days. The results indicated that both the antioxidant type and concentration had a significant impact on sperm motility, motility duration, viability, abnormalities, and fertilization rate ($P < 0.05$). Glutathione showed the highest effectiveness in maintaining sperm quality. Further test results showed that glutathione at a concentration of 10% could better maintain sperm quality. The analysis of naleh fish sperm DNA integrity following antioxidant supplementation demonstrated the absence of DNA fragmentation across all samples, these findings indicate that the sperm DNA remained undamaged in both fresh and post-cryopreservation conditions. Therefore, 10% glutathione was recommended for use in the cryopreservation process of naleh fish sperm.

Keywords: Naleh Fish, Antioxidant, Glutathione, DMSO, Ringer

1. Introduction

As a native freshwater species of Indonesia, the naleh fish (*Barbonymus* sp.) represents a valuable resource with high economic value and potential for development as a target for cultivation, both for food and ornamental purposes (Muchlisin *et al.*, 2015). The natural population of the species is found in the inland waters of the Southwest Aceh region, specifically in Nagan Raya Regency (Batubara *et al.*, 2018). However, high levels of exploitation without regard for catch time and size have led to population decline or overfishing (Batubara *et al.*, 2019). This shows that commercial cultivation is necessary to meet increasing market demand and maintain the sustainability of its natural stock.

One challenge in cultivating naleh fish is the limited availability of superior seeds due to the lack of development of seeding technology. This is partly due to the scarcity of quality broodstock, which is dependent on the natural spawning season that lasts from March to June (Efizon *et al.*, 2021). During this period, the broodstock of naleh fish becomes mature, making artificial spawning impossible throughout the year. To overcome the challenge, sperm cryopreservation represents a strategic solution, particularly for species with short and seasonal reproductive periods such as naleh fish (Maulida *et al*., 2021). This technology ensures the year-round availability of viable male gametes for artificial fertilization (Muthmainnah *et al.*, 2019). In addition, sperm available through cryopreservation can also be used to support breeding and genetic conservation programs, as well as gamete exchange between regions (Muchlisin, 2005; Zairin *et al.*, 2005; Maulida *et al.*, 2020).

The effectiveness of sperm cryopreservation is highly influenced by the correct blend of storage media, which involves the choice of extenders, dilution ratios, cryoprotectants, and the inclusion of antioxidants (Lestari *et al.*, 2013; Maulida *et al.*, 2020; Maulida *et al.*, 2023). Extenders function to prolong viability and prevent premature activation, while cryoprotectants such as DMSO, glycerol, and extracellular substances, including egg yolk or skim milk, protect cells from damage due to freezing (Sieme *et al.*, 2016; Muthmainnah *et al.*, 2019). During fish sperm cryopreservation, increased ROS production commonly occurs from the cooling phase through thawing, inducing oxidative stress that may compromise sperm quality (Sandoval-Vargas *et al*., 2021).

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Because many fish spermatozoa, particularly those of cyprinid species, are rich in polyunsaturated fatty acids, they are highly prone to oxidative lipid damage, making antioxidant supplementation a critical component for preserving post-thaw sperm quality (Castro *et al*., 2025). Antioxidants, including glutathione, vitamins C, and E, play an essential role in neutralizing ROS and minimizing oxidative damage to sperm membranes, mitochondria, and DNA during cryopreservation (Feradis, 2009; Kaltsas, 2023).

Research has explored using antioxidants in the cryopreservation media for fish sperm, such as ascorbic acid (Vitamin C) introduced in Atlantic salmon (*Salmo salar*) (Figueroa *et al.*, 2018), butylated hydroxytoluene (BHT) in depik fish (*Rasbora tawarensis)* (Muchlisin *et al*., 2023), as well as glutathione and myo-inositol in betok fish (*Anabas testudineus)* (Maulida *et al.*, 2024). Muthmainnah *et al.* (2019) reported that supplementation with glutathione maintained the percentage of sperm motility in seurukan fish (*Osteochilus vittatus*), while melatonin supplementation improved post-thaw motility and membrane integrity in the freshwater fish *Prochilodus lineatus* (Motta *et al*., 2022). In addition, inositol supplementation has been shown to significantly enhance post-thaw motility, membrane integrity, and overall sperm quality in Mesopotamian catfish (*Silurus triostegus*) (Doğu *et al*., 2021). The combination of β-carotene and α-tocopherol in cryopreservation media in trout pelangi fish (*Oncorhynchus mykiss*) helped protect sperm membrane from oxidative damage, thereby improving quality after freezing (Kutluyer *et al.*, 2014). Maulida *et al.* (2021) examined the suitability of cryoprotectant types and concentrations, and reported that DMSO was the best cryoprotectant for fish. Diani (2024) also concluded that a 10% DMSO concentration was the most effective for cryoprotecting naleh fish sperm. Despite the existing literature, research concerning the suitability of antioxidants and their concentrations for cryopreservation of naleh fish sperm have never been conducted. Consequently, the present study assessed various antioxidants and their concentrations to identify the most effective combination for supporting post-thaw sperm performance in naleh fish (*Barbonymus* sp.).

2. Materials and Methods

2.1. Time and Site

The present study was undertaken in April 2025 at Gampong Meunasah Krueng in the Beutong District of Nagan Raya Regency, Aceh Province, and at the Fish Hatchery and Breeding Laboratory of the Faculty of Marine and Fisheries, Syiah Kuala University.

2.2. Experimental Design

In this research, a Completely Randomized Design (CRD) was used as the basis for the experimental setup, comprising five treatment levels with four replications. The first stage tested the effectiveness of five types of antioxidants: ascorbic acid, β-carotene, glutathione, BHT, and myo-inositol. Each was used at a concentration of 10% to determine the most effective type of antioxidant. The second stage continued with testing 5 levels of concentration, namely 0%, 5%, 10%, 15%, and 20% of the best antioxidant obtained in the first stage, specifically glutathione.

2.3. First Experiment Procedure

2.3.1. Parent provision

Fish samples for the experiment were sourced from the Nagan River within the Nagan Raya Regency area. Approximately 20 male and 10 female broodstock of final gonad maturity were used, with males averaging 16 cm in length and 36 grams in weight. The broodstock individuals were kept in a pond for 14 days, receiving natural food, including silkworms, provided twice daily, in the morning (07:00 Western Indonesian Time) and afternoon (18:00 Western Indonesian Time).

2.3.2. Sperm retrieval

Ten mature male naleh fish broodstock were weighed and injected intramuscularly with Ovaprim (0.5 ml/kg) in the back at a 45° angle, then placed in a broodstock tank. Sperm were collected by gently massaging the abdomen 7 to 8 hours after injection and then aspirated using a separate syringe for each broodstock. This was collected in an ice box at 4°C, exhibiting ≥70% motility, and was used. Good-quality sperm were collected in a petri dish and temporarily stored at 4°C.

2.3.3. Preparation of extenders, cryoprotectants, and antioxidants

Ringer's solution served as the extender, with several compositions including 3.25 g NaCl, 0.125 g $CaCl_2$, 0.1 g $NaHCO_3$, which was then added with distilled water to 100 ml. The sperm was then combined with the extender in a 1:60 ratio (1.67 ml sperm: 100 ml Ringer). Furthermore, the cryoprotectant used consisted of 1% DMSO and 5% egg yolk, achieved by adding 10 mL of DMSO and 5 mL of egg yolk, respectively. Sperm mixture was divided into 20 cryotubes, each containing 1.5 mL. Subsequently, 0.15 mL of different antioxidants (ascorbic acid, β-carotene, glutathione, BHT, and myo-inositol) was added to achieve a concentration of 10% in each treatment, with 4 cryotubes serving as replicates.

2.3.4. Sperm freezing and thawing

A total of 20 cryotubes filled with sperm samples were first stored in a chilled box (4℃) for 5 minutes, followed by exposure to the vapor phase of liquid nitrogen (-79℃) for 5 minutes, and frozen in liquid nitrogen (-196℃) for 2 weeks. After this procedure, the samples were thawed gradually according to concentration using a temperature-controlled bath maintained at 32℃ for 3 minutes, and then macroscopic as well as microscopic examination of sperm quality was carried out.

2.4. Second Experiment Procedure

2.4.1. Preparation of extenders, cryoprotectants, and antioxidants

The procedures for providing broodstock and sperm collection, preparing extenders, and adding cryoprotectants were similar to those used in the first phase of the study. Approximately 1.67 ml of sperm was combined with 100 ml of Ringer's solution (1:60 ratio), followed by 10% DMSO and 5% egg yolk as cryoprotectants. This mixture was divided into 20 cryotubes (1.5 ml each), and the best antioxidant from the first phase of the study, glutathione,

was added. One set of cryotubes was left without antioxidant addition, while the other four sets were added with 0.075 ml, 0.15 ml, 0.225 ml, and 0.3 ml of glutathione, respectively, to produce concentrations of 5%, 10%, 15%, and 20%, respectively.

2.4.2. Sperm freezing and thawing

In this study, 20 cryotubes with sperm samples inside were stored in a chilled box (4°C) for 5 minutes, then evaporated in the neck of a nitrogen tank (-79°C) for 5 minutes and frozen in liquid nitrogen (-196°C) for two weeks. Subsequently, the samples were thawed in stages according to concentration in a water bath (32°C) for 3 minutes, and then macroscopic and microscopic sperm quality examinations were carried out after freezing (Maulida *et al*., 2024).

2.5. Assessment of Sperm Quality Parameters

2.5.1. Macroscopic and Microscopic Observations of Sperm

Macroscopic observation: Macroscopic observations were conducted on fresh sperm, including assessment of color, pH, and consistency. Furthermore, its consistency was assessed based on the rate of sperm flow in the test tube. This was categorized as thin (rapid flow to the bottom of the tube), moderate (slow flow and some of sperm adhered to the walls of the tube), and thick (very slow flow and much of sperm remained at the edges of the tube) (Arifiantini, 2012). Sperm pH was evaluated by dropping a sample onto pH paper and then comparing the color change with a standard pH scale. The normal pH value for fish sperm was 6.5 (Muchlisin *et al.*, 2020). *Microscopic observation* an analysis was conducted on fresh and post-cryopreserved sperm, including observations of motility, viability, and abnormalities. Sperm motility was assessed by positioning a drop of the sample onto a glass slide, adding one to three drops of water to initiate sperm activation, and then observing it using a microscope and Spermvision software (Minitube, Germany). Sperm were categorized as motile when moving in a straight, progressive, agile, and non-rotating manner. Motility was calculated using the formula of Muchlisin *et al*. (2004):

$$\text{Motility (\%)} = \frac{\text{Number of sperm that move straight forward}}{\text{Number of observed sperm}} \times 100$$

Motility duration was assessed by recording the period between the beginning and the end of movement, using a microscope magnification of 100× or 400×. Viability was analyzed by mixing a drop of sperm with a drop of 2% eosin, then making a smear, drying it, and observing it under a microscope. Live sperm were transparent white, while dead sperm were red. To quantify viability, the formula outlined by Ax *et al*. (2000) was applied:

Viability (%) = (Number of live sperm / Number of observed sperm) × 100

Abnormality monitoring was performed on the same specimens as viability. Sperm were considered abnormal when abnormalities were observed in the head and tail. Abnormality was assessed based on the formula of Akbar *et al*. (2015).

Sperm abnormalities (%) = (Number of abnormal sperm / Number of observed sperm) × 100

2.5.2. Fertilization

Fertilization was performed using 10 mature female broodstock, which were administered Ovaprim at a dosage of 0.5 ml/kg body weight and allowed to undergo ovulation for 7 hours. Eggs were removed by stripping and collected in a petri dish. One ml of eggs (250 to 400 eggs) was mixed with 1 ml of sperm (1:1 ratio), then 2 to 5 drops of water were applied to trigger sperm motility, and the the preparation was gently stirred using sterilized chicken feather. The eggs were left for 4 minutes to fertilize sperm, then incubated at 28℃ for 48 hours. Successful fertilization was observed after 6 hours, as fertilized eggs appeared clear, while unfertilized eggs appeared cloudy white. The following equation, adapted from Muchlisin *et al*. (2015), was used to determine the fertilization rate:

Fertilization (%) = (Number of fertilized eggs / Number of eggs observed) × 100

2.5.3. DNA Fragmentation

DNA Extraction and Electrophoresis

Sterile microcentrifuge tubes were used to transfer the sperm samples, which were then spun at 14,000 rpm for 3 minutes to create a pellet. Around 100 µl of this pellet was placed in a new sterile tube, after which 300 µl of cell lysis buffer was added. The suspension was carefully inverted 5–6 times to ensure even consistency and was left to incubate at ambient temperature for 10 minutes. The solution was subjected to another spin (at 14,000 rpm for 20 seconds), and the supernatant fluid was removed. A 15-second vortexing step was performed to fully resuspend the pellet, followed by the addition of 100 µl of nuclei lysis solution and subsequent vortexing (Promega, 2017).

Next, 35 µl of a protein precipitation mixture was introduced and mixed on a vortex machine for 20 seconds, then spun in a centrifuge at 14,000 rpm for 3 minutes. The resulting clear supernatant was carefully transferred to a fresh tube preloaded with 100 µl of isopropanol and mixed by vortexing for 15 seconds. This was followed by another centrifuge step at 14,000 rpm for 1 minute at ambient temperature. The supernatant was thrown away, and a 70% ethanol solution (in a 1:1 ratio) was used to rinse the DNA pellet, then centrifuged for another minute. Following ethanol removal, the pellet was air-dried for 15 minutes, rehydrated with 35 µl of DNA rehydration solution, and subsequently incubated at 65 °C for 1 hour. The final measurement involved determining DNA concentration and purity using a Nanodrop C2000 spectrophotometer (Chen *et al.*, 2018).

For the electrophoresis procedure, 1.5 grams of agarose was completely dissolved in 100 milliliters of TBE buffer by heating clear and boiling. After cooling sightly, 2 microliters of DNA dye was pipetted to the mix, and transferred into a gel mold to solidify for about 30 minutes. The prepared agarose gel had a concentration of 1.5% and was placed in an electrophoresis chamber filled with 1× TBE buffer. Next, 10 microliters of DNA sample were blended with 22 microliters of loading dye and then loaded into the wells, alongside 5 microliters of DNA marker. The electrophoresis was conducted at 135 volts for 30 minutes, after which the DNA bands were visualized and captured using a UV-Doc transilluminator.

2.6. Data Analysis

Descriptive analysis was performed on sperm data, including pH, color, consistency, and DNA fragmentation, while data on percentage of motility, duration of motility, viability, abnormality, fertilization, and egg hatching were statistically analyzed using SPSS version 25. The data were initially tested for normality, and when normally distributed and homogeneous, a one-way ANOVA was performed. When the treatment had a significant effect, Duncan's test was applied to evaluate the most effective treatment.

3. Results

The fresh sperm of naleh fish appeared milky white, had a pH of 7, and exhibited a medium level of consistency. Furthermore, the average sperm density was 263 x 10^6 cells/mL, with percentages of motility, motility duration, viability, sperm abnormalities, and fertility of 98.2%, 464.58 seconds, 93%, 0.2% and 89.2%, respectively (Table 1). The results of observations on naleh fish sperm showed sperm morphology with indications of abnormal sperm marked with red arrows. Abnormalities detected included asymmetrical head shape and bent tail. For sperm viability, dead sperm were black, while live sperm were white or appeared transparent (Figure 1).

Table 1. Characteristics of fresh sperm quality of naleh fish (*Barbonymus sp.*)

Parameter	Result
Color	Milky White
Average pH	7
Average consistency	Medium
Average sperm concentration (cells/mL)	263 x 10^6
Average fresh sperm motility (%)	98.2±1.40
Average motility duration (seconds)	464.58±45.55
Average fresh sperm viability (%)	93±1.15
Average fresh sperm abnormality (%)	0.2±0.4
Average egg fertility (%)	89.2±4.41

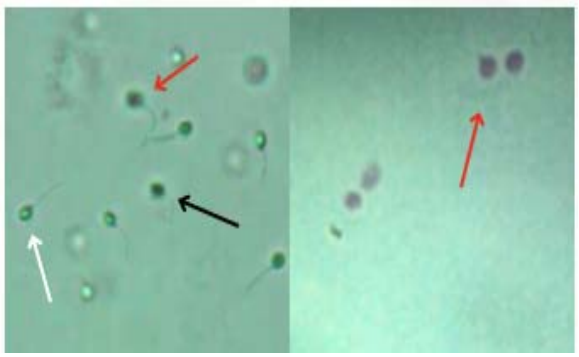

Figure 1. Sperm morphology of naleh fish observed under 400× microscopic magnification, with red arrows indicating abnormal sperm, black arrows indicating dead sperm, and white arrows indicating live sperm.

Analysis of variance indicated that the type of antioxidants had a significant effect on motility, duration of motility, viability, sperm abnormalities, and fertility of naleh fish eggs ($P < 0.05$). According to Duncan's further test results, the highest motility and viability were found with the use of glutathione (98.5% and 92.8%, respectively). These rates were significantly different compared to β-carotene and BHT treatments, but not different from the ascorbic acid and myo-inositol treatments. The highest motility duration was also observed with the use of glutathione (428.75 seconds), a value different from those of the other treatments. Glutathione resulted in the minimal level of sperm abnormalities (0.2%) and achieved the highest fertilization success (88.6%). Nevertheless, these results were statistically comparable to those recorded for ascorbic acid, BHT, and myo-inositol (Table 2). Therefore, the best sperm quality was obtained with the use of glutathione.

In the Glutathione test with several concentration levels, based on the ANOVA test results, differences in glutathione concentration significantly influenced sperm motility, motility duration, viability, sperm abnormalities, and fertility of naleh fish eggs ($P < 0.05$). The Duncan test indicated that the 10% concentration resulted in the highest motility (98.7%) and viability (91.7%). This value was significantly distinct from the 20% and 0% treatments, but not significantly different from the 5% and 15% treatments. Furthermore, the highest motility duration was also found at the 10% concentration, specifically 452.5 seconds, which differed significantly from the other treatments. The lowest sperm abnormality value (0.2%) was observed at the 10% concentration treatment, although it did not differ significantly from the 5% and 15% treatments. Meanwhile, the highest fertilization rate was also found at the 10% Glutathione concentration (84.9%), a value that was significantly different from the other concentrations, except for 5% (Table 3). In general, the best quality was obtained when using 10% glutathione.

Gel electrophoresis results demonstrated that sperm DNA from all treatment groups, including the antioxidant treatments (Figure 2) and the various glutathione concentrations (Figure 3), remained intact, with no observable DNA fragmentation. These results indicate an absence of DNA damage or degradation in both fresh and thawed sperm, suggesting that neither the treatments nor the storage process compromised the DNA quality of naleh fish sperm.

Table 2. Percentage±SD of spermatozoa motility, motility duration, viability, sperm abnormality, and fertility of naleh fish (*Barbonymus sp.*) post cryopreservation with combination of ringer extender, 5% egg yolk, and 10% DMSO using different types of antioxidants. Values within the same column for each respective antioxidants followed by different superscripts indicate a significant difference (P<0.05).

Antioxidant	Motility (%)	Motility Duration (sec)	Viability (%)	Sperm Abnormality (%)	Fertility (%)
Ascorbic Acid	92.2±2.87^{bc}	228.75±54.5^{a}	89.3±2.49^{bc}	1.2±0.95^{ab}	82.6±4.33^{bc}
Beta Carotene	71.0±9.5^{a}	237.50±18.4^{a}	81.1±7.23^{a}	1.5±0.57^{b}	72.2±5.93^{a}
Glutathione	98.5±0.57^{c}	428.75±90.4^{b}	92.8±1.31^{c}	0.2±0.50^{a}	88.6±4.19^{c}
BHT	79.5±3.10^{a}	231.25±25.6^{a}	84.3±5.12^{ab}	2.0±0.81^{b}	76.7±3.16^{ab}
Myo-inositol	89.2±7.80^{b}	226.25±52.1^{a}	89.2±3.40^{bc}	1.2±0.5^{ab}	81.0±1.91^{b}

Table 3. Percentage results of motility, motility duration, viability, sperm abnormality, and fertility of naleh fish (*Barbonymus sp.*) post cryopreservation with combination of ringer extender, 5% egg yolk, and 10% DMSO using different concentrations of antioxidants. The values in the same column at respective concentration followed by a different superscript denote significant difference (P<0.05).

Concentration (%)	Motility (%)	Motility Duration (sec)	Viability (%)	Sperm Abnormality (%)	Fertility (%)
0	73.5±12.2^{a}	265.0±17.7^{a}	78.8±7.43^{a}	2.5±1.29^{bc}	37.1±0.55^{a}
5	94.0±6.78^{b}	336.2±49.2^{b}	90.3±4.32^{b}	1.2±0.95^{ab}	79.4±1.38^{d}
10	98.7±0.50^{b}	452.5±56.7^{c}	91.7±1.75^{b}	0.2±0.50^{a}	84.9±4.40^{d}
15	91.7±6.39^{b}	335.0±30.0^{b}	86.7±2.62^{b}	1.5±1.29^{ab}	72.5±5.49^{c}
20	65.2±6.13^{a}	260.0±25.8^{a}	72.6±5.72^{a}	4.0±0.81^{c}	45.2±5.14^{b}

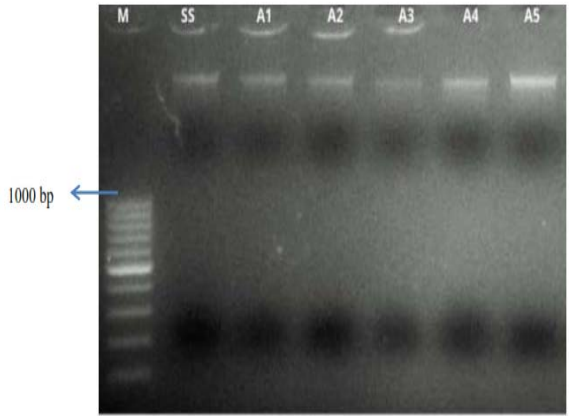

Figure 2. Gel electrophoresis results of naleh fish sperm samples from the first stage (comparison of antioxidant types). Note: M = Marker; SS = Fresh sperm; A1 = Ascorbic acid; A2 = Beta-carotene; A3 = Glutathione; A4 = BHT; A5 = Myo-inositol.

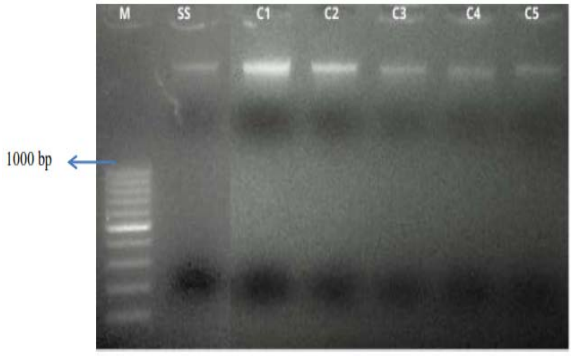

Figure 3. Gel electrophoresis results of naleh fish sperm samples from the second stage (comparison of glutathione concentrations). Note: M = Marker; SS = Fresh sperm; C1 = Glutathione (0%); C2 = Glutathione (5%); C3 = Glutathione (10%); C4 = Glutathione (15%); C5 = Glutathione (20%).

4. Discussion

According to the study findings, the fresh sperm presented a milky-white appearance, neutral pH (7), and moderate consistency. Concentration of fresh sperm from naleh fish was 263 x 10^6 cells/mL, with the percentage of motility, motility duration, viability, sperm abnormalities, and fertility, respectively, of 98.2%, 464.58 seconds, 93%, 0.2% and 89.2%. Based on these results, the quality of fresh sperm was suitable for use in the cryopreservation procedure (Di Santo *et al.*, 2012; Bier *et al.*, 2025). Motility was one important parameter in the study of sperm quality, both fresh and post-freezing (Sarosiek *et al.*, 2012). Whaley *et al.* (2021) reported that fresh sperm motility must exceed 70%.

The data suggested that the presence of antioxidants ascorbic acid and glutathione maintained post-freezing sperm motility equivalent to that of fresh sperm without freezing. However, the use of β-carotene, BHT, and myo-inositol resulted in slightly lower sperm quality compared to these 2. The results showed that all sperm treatments supplemented with antioxidants resulted in motility above 70%. This suggests that antioxidant supplementation could effectively maintain sperm quality, with the best sperm quality obtained with the addition of Glutathione. Differences in the suitability of the type of antioxidants and concentration used provided varying effectiveness, depending on the type and concentration suitable for use in naleh fish sperm. According to Li *et al.* (2017), each different type and concentration of antioxidant had different effects on the 3 sturgeon species tested. Further analysis showed that 10% glutathione was the optimal concentration, representing the most suitable antioxidant treatment for naleh fish sperm cryopreservation.

This study demonstrated glutathione's superior effectiveness in maintaining naleh fish sperm quality compared to other antioxidants and doses. Beyond naleh fish, glutathione has proven successful in cryopreserving sperm from other species, including betok fish (*Anabas testudineus*) (Maulida *et al.*, 2024), depik fish (*Rasbora tawarensis*) (Muchlisin *et al.*, 2023), and seurukan fish

(*Osteochilus jeruk*) (Muthmainnah *et al.*, 2018). As a non-enzymatic antioxidant, glutathione's efficiency in cryopreservation stems from its multifaceted mechanism against oxidative stress. Its main role is to alleviate oxidative stress by neutralizing reactive oxygen species (ROS), particularly superoxide anions (O_2^-), and converting them into less harmful molecules such as water (H_2O_2) (Panday *et al.*, 2020). This action is vital because lipid peroxidation can damage sperm membrane structure, mitochondria, and acrosome integrity, all critical for successful fertilization (Smits *et al.*, 2019; Félix *et al.*, 2020).

Glutathione protected DNA and sperm chromatin structure from oxidative damage, while maintaining mitochondrial function, which was crucial for energy production (ATP) and sperm motility (Ribeiro *et al.*, 2022). Another advantage of glutathione was its ability to be regenerated from its oxidized form (GSSG) to the active form (GSH) by the enzyme glutathione reductase, making it resistant to long-term oxidative damage (Halliwell dan Gutteridge, 2015). In the context of fish sperm cryopreservation, Yang *et al.* (2025) on mandarin fish (*Siniperca chuatsi*) and Muthmainnah *et al.* (2018) on seurukan fish (*Osteochilus jeruk*) noted that the addition of glutathione in the freezing medium significantly maintained sperm motility, viability, and fertility compared to other antioxidants, specifically at concentration of 10%.

In addition to glutathione, the use of 10% ascorbic acid produced results that were not significantly different from those of glutathione. Therefore, ascorbic acid was also able to maintain the quality of naleh fish sperm after thawing. Ascorbic acid or vitamin C, with its antioxidants properties, was known to be effective in neutralizing free radicals, specifically ROS, through the mechanism of electron donation to pro-oxidant compounds, thereby preventing the chain reaction of lipid peroxidation in sperm cell membranes and maintaining their quality and viability (Li *et al.*, 2010). Muthmainnah *et al.* (2018) also stated that ascorbic acid could increase sperm motility and viability post-cryopreservation in seurukan fish (*Osteochilus vittatus)*, although the effect was less significant than glutathione. The effectiveness of ascorbic acid has been previously reported, for instance by Maulida *et al.* (2024) in betok fish (*Anabas testudineus)* and in the sperm of atlantic salmon (*Salmo salar)* (Figueroa *et al.*, 2018).

Although its value was lower than that of glutathione and ascorbic acid, the use of myo-inositol, β-carotene, and BHT also yielded satisfactory results, as sperm motility and viability were above 70%. Myo-inositol, with its antioxidants properties, increased osmotic pressure in sperm, enhanced sperm mitochondrial function by modulating intracellular calcium ions, and improved redox homeostasis during the cryopreservation process (Doğu *et al.*, 2021). The effectiveness of myo-inositol had also been reported by several authors in betok fish (*Anabas testudineus*) (Maulida *et al.*, 2024) and catfish (*Silurus triostegus*) (Doğu *et al.*, 2021).

BHT was reported to be effective in curbing oxidative stress by donating hydrogen atoms to free radicals, thereby breaking the chain of lipid peroxidation in cell membranes during the cryopreservation process, and its effectiveness was dose-dependent (Mostafa *et al.*, 2019). β-carotene played an important role in safeguarding cell membrane integrity and reproductive function, enabling this compound to integrate into phospholipid membranes and provided direct protection against lipid peroxidation (Martín-San Juan *et al.*, 2025; Sereti *et al.*, 2025). Therefore, all types of antioxidants used in this study yielded positive results for the quality of naleh fish sperm after freezing, but glutathione provided the greatest efficacy.

Sperm motility and viability improved with glutathione up to a 10% concentration, but decreased slightly at 15% and more sharply at 20%. Moreover, although all antioxidant treatments maintained post-thaw fertility above 70%, the consistently superior motility, viability, and fertility observed at 10% glutathione clearly demonstrate that this concentration provides the highest level of cryoprotection. These results indicate that 10% glutathione is not only adequate but optimal, as it yields post-thaw fertility values closest to those of fresh sperm and outperforms all other antioxidants tested. The marked decline in sperm quality at concentrations above 10% further highlights the importance of this threshold. While other antioxidants produced acceptable fertility levels, none achieved the stability or post-thaw functional integrity afforded by 10% glutathione. Collectively, these results justify the recommendation of 10% glutathione as the most effective and biologically appropriate concentration for naleh fish sperm cryopreservation.

Kolyada *et al.* (2023) explained that while antioxidants are beneficial, excessive concentrations can induce reductive stress, inhibiting crucial cellular signaling and negatively impacting sperm. Therefore, 10% Glutathione is ideal for naleh fish sperm. Other antioxidants show similar dose-dependent toxicity. Martín-San Juan *et al.* (2025) found that β-carotene at roughly 10% could reduce sperm motility and damage mitochondrial function, even acting as a pro-oxidant. Kutluyer *et al.* (2014) also reported no positive effect from β-carotene in rainbow trout (*Oncorhynchus mykiss*) sperm cryopreservation. According to Lahnsteiner dan Mansour (2010), correct antioxidant dosing improves frozen sperm quality, but incorrect doses cause harmful toxic effects.

A similar phenomenon was also reported by Banihani and Alawneh (2019), who stated that high antioxidants concentrations could cause reductive stress, negatively impacting mitochondrial function and sperm motility after thawing. The results of study by Yang *et al.* (2025) on mandarin fish (*Siniperca chuatsi*) at the 2 highest doses (4 and 8 mM) showed a decrease in sperm quality compared to the lower dose (2 mM). These results showed that excessively high doses caused redox stress, a disturbance in the balance within cells that disrupted normal body function due to excessive antioxidants levels or cellular toxicity. In addition to fish, the toxic effects of glutathione at high concentrations have also been noted in terrestrial animals, such as goat sperm (Zou *et al.*, 2021) and Murrah bull semen (Gangwar *et al.*, 2018).

DNA is a highly sensitive cellular component that is prone to damage during cryopreservation, making the preservation of its integrity crucial for maintaining sperm quality (Santi *et al.*, 2018). Sperm cells experience extreme environmental stress during freezing and thawing, leading to disruption of cellular structures and metabolic pathways. Such damage is typically characterized by

reduced motility and impaired plasma membrane integrity, ultimately decreasing fertilization potential (Khan *et al.*, 2021; Min *et al.*, 2024). During cryopreservation, spermatozoa experience osmotic, thermal, and oxidative stresses that predominantly affect the plasma membrane, leading to potential alterations in lipid composition and impairments in functional physiology (Sieme *et al.*, 2015; Castro *et al.*, 2025). Therefore, the inclusion of cryoprotectants and antioxidants is important for alleviating cryogenic damage and shielding sperm cells from reactive oxygen species (ROS).

Analysis of DNA integrity in naleh fish sperm following cryopreservation with antioxidant supplementation (Figures 2 and 3) revealed intact DNA bands across all treatments (A1–A5, C1–C5) as well as fresh sperm (SS), with no evidence of smearing or fragmentation. This suggests that DNA remained structurally intact, and that antioxidant supplementation contributed to maintaining DNA stability during the freezing process. These findings are consistent with Huang *et al.* (2022), who noted that cryoprotective agents (CPAs) incorporated into sperm diluents can lessen cellular injury by limiting ice crystal formation and preserving membrane integrity and cellular structure, critical aspects, particularly in slow-freezing methods that are prone to mechanical and osmotic stress. Similarly, Berean *et al.* (2024) highlighted the protective role of antioxidants in preserving motility, membrane integrity, and DNA structure, particularly in the context of small animal sperm cryopreservation. Hence, antioxidants represent an key factor in maintaining sperm DNA integrity throughout cryopreservation. It is noteworthy that gel electrophoresis, while widely applied in forensic and biological research to assess DNA degradation, primarily provides qualitative results (band patterns or smearing). Its sensitivity is limited, especially in detecting subtle or early-stage DNA damage (Bhoyar *et al.*, 2024).

5. Conclusion

In conclusion, variations in antioxidant type and concentration significantly affect the post-cryopreservation quality of naleh fish sperm ($P < 0.05$). Antioxidant supplementation helps preserve sperm quality, and glutathione is the most effective type of antioxidants. When glutathione was used, post-cryopreservation sperm quality increased up to the concentration of 10%, then decreases as concentration increases. Sperm DNA integrity analysis showed that none of the sperm DNA samples exhibited fragmentation, suggesting that no DNA damage was present in any of the samples evaluated. Consequently, 10% glutathione is recommended as an antioxidant for the cryopreservation of naleh fish sperm.

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Conflict of interests

The authors state that no conflicts of interest are associated with this publication.

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