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EFFECT OF ANTIOXIDANT AND ITS CONCENTRATION ON THE SPERM QUALITY OF WALKING CATFISH *Clarias batrachus* POST CRYOPRESERVATION

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ABSTRACT

The walking catfish *Clarias batrachus* is popular and affordable in Southeast Asian countries. The development of breeding technology for this species is ongoing, with sperm cryopreservation being a key aspect of study that supports its breeding program. Cryopreservation is an essential method in fish breeding, particularly for sustaining breeding initiatives that supply larvae throughout the years. However, this process can lead to oxidative stress, which may decrease sperm quality due to structural and physiological damage, including DNA fragmentation. Adding an antioxidant to the freezing medium could help mitigate these negative effects and maintain sperm quality. This study aims to evaluate the effectiveness of various types and concentrations of antioxidant on the quality of *C. batrachus* sperm post-cryopreservation. Four types of antioxidants were tested: ascorbic acid, Myoinositol, alpha-tocopherol, and Butylated Hydroxytoluene, each at concentrations of 0, 20, 40, and 60 mg/L. Ringer solution, 5% DMSO and 5% egg yolk were used as extender and cryoprotectants, respectively. Sperm samples were stored in liquid nitrogen for 2 weeks, after which sperm quality was analyzed. The results showed that both the type and concentration of antioxidant had a significant effect ($p<0.05$) on all measured sperm quality parameters. Ascorbic acid at a concentration of 40 mg/L produced the best results, achieving 62.33% motility, 66.00% viability, and 61.67% fertility. Electrophoresis analysis showed no DNA fragmentation in any of the treatments, indicating that the genetic structure of sperm remained intact. Ascorbic acid at 40 mg/L is the most effective antioxidant for cryopreservation of sperm *C. batrachus*.

INTRODUCTION

Walking catfish *Clarias batrachus*, also referred to as *sengko* in Acehnese, is an economically important freshwater fish species cultured in Southeast Asian countries (Handayani *et al.*, 2023; Handayani *et al.*, 2024). This fish is preferred by the market over the African catfish *Clarias gariepinus* due to its savory flavor and reasonable price. The availability of high-quality larvae is essential for ensuring optimal fish growth and profitability. In general, sperm cryopreservation is a crucial

method for enhancing larval production efficiency (Bozkurt, 2019; Judycka *et al.*, 2019). This method enables sperm to be stored at extremely low temperatures in liquid nitrogen for extended periods, allowing access to cryopreserved frozen stock whenever needed. Consequently, artificial breeding can be performed in the year without relying on the fish spawning season (Khan *et al.*, 2021).

Cryopreservation plays a crucial role in genetic conservation and fish breeding programs, including genetic engineering, crossbreeding, gynogenesis, and androgenesis, aimed at producing high-quality fish larvae. However, the freezing and thawing processes often lead to structural damage and physiological disorders in the frozen sperm (Mayer and Pšenička, 2024and Parihar *et al.*, 2023). This damage is frequently caused by the presence of free radicals, particularly reactive oxygen species (ROS), which arise from oxidative stress due to an imbalance between free radical production and the cellular antioxidant system capacity (Zhu *et al.*, 2023). ROS can impair crucial components, such as the plasma membrane structure of sperm, DNA, and mitochondria, ultimately resulting in decreased sperm motility, viability, and fertilization capacity after cryopreservation (Len *et al.*, 2019). Therefore, one effective way to minimize the negative impact of oxidative stress is to incorporate antioxidant compounds into the freezing medium (Sandoval-Vargas *et al.*, 2021).

Some antioxidants have been used in the cryopreservation of fish sperm, for instance, Ascorbic acid or vitamin C is a water-soluble antioxidant effective in neutralizing free radicals and preventing sperm DNA damage (Agarwal and Majzoub, 2017). Butylated hydroxytoluene (BHT), a synthetic antioxidant often used in cryopreservation, has been shown to improve sperm quality by preventing damage to the plasma membrane of spermatozoa caused by temperature shock, and protecting changes in the structure of membrane proteins, specifically phospholipids, during the freezing process (Öğretmen and Inanan, 2014). Alpha-tocopherol, which is a lipophilic antioxidant, has been reported to protect lipid membranes from peroxidation and increase sperm viability post-cryopreservation (Almeida and Ball, 2005). Myoinositol is a natural compound that helps stabilize cell membranes and maintain sperm motility through osmotic and enzymatic mechanisms. This antioxidant has an osmo-protective effect that protects cells from osmotic stress due to a lack of water or excess minerals (Ahanger *et al.*, 2024).

Several studies showed that antioxidant can maintain the quality of fish sperm during cryopreservation process. For example, Ascorbic acid is effective in increasing post-thawing sperm motility and viability in rainbow trout (*Oncorhynchus mykiss)* (Kutluyer *et al.*, 2014), Atlantic salmon (*Salmo salar)* (Figueroa *et al.*, 2018), and zebrafish (*Danio rerio)* (Marques *et al.*, 2018). BHT improved sperm quality of common carp (*Cyprinus carpio)* (Öğretmen and Inanan, 2014), Russian sturgeon (*Acipenser guldenstadti)* (Osipova *et al.*, 2016), salmon masu (*Oncorhynchus masou)*

(Chelewani *et al.*, 2024), and beluga fish (*Huso huso)* (Osipova *et al.*, 2014). Alpha-tocopherol prevented lipid peroxidation during cryopreservation of Atlantic salmon (Figueroa *et al.*, 2018), and European seabass (*Dicentrarchus labrax)* (Martínez-Páramo *et al.*, 2012). Myoinositol is effectively used in the cryopreservation of sperm of climbing perch (*Anabas testudineus)* (Maulida *et al.*, 2024) and Mesopotamian catfish (*Silurus triostegus)* (Doğu *et al.*, 2021).

Although cryopreservation can maintain sperm quality, the method has biological risks. The process may lead to DNA fragmentation and the formation of free radicals due to reactive oxygen species (ROS). These free radicals are highly reactive with cellular components, including nucleic acids, resulting in DNA base oxidation, and single or double-strand breaks (Gualtieri *et al.*, 2021). Sperm cell nucleus is incapable of repairing DNA damage, making any resulting harm irreversible (Sharma and Agarwal, 2018). DNA fragmentation significantly diminishes sperm quality, adversely affecting fertilization capability, embryo development deformation, and even the survival and quality of offspring (Qiu *et al.*, 2020). Studies related to the suitability of the type and concentration of antioxidant in the cryopreservation process of walking catfish sperm have not been conducted. This is crucial because sperm cells from each fish species have different responses to the types and concentrations of antioxidants used (Villalobos *et al.*, 2025). Therefore, this study aimed to determine the best type and optimal concentration of antioxidants for the cryopreservation of walking catfish sperm.

MATERIALS AND METHODS

Broodstocks

This study was conducted from June to August 2024. Broodfish were sourced from collectors at Lambaro Central Market in Aceh Besar Regency, Indonesia, and then conditioned in a pond for 2 weeks. During this period, broodfish were fed mealworm larvae ad libitum, twice daily at 9:00 AM and 5:00 PM. Sperm cryopreservation process took place at the Hatchery and Breeding Laboratory of the Faculty of Marine and Fisheries at Syiah Kuala University in Banda Aceh, Indonesia. Additionally, DNA fragmentation analysis was performed at the Brackish Water Aquaculture Center laboratory in Ujung Batee, Aceh Besar, Indonesia.

Experimental design

A completely randomized factorial design was used to examine the sole and variation effects of the types and concentrations of antioxidants used. Four types of antioxidants were tested, namely Ascorbic acid (T1), Myo-inositol (T2), Alpha-tocopherol (T3), and BHT (T4). Each antioxidant type was evaluated at four concentrations: 0 mg/L (D1), 20 mg/L (D2), 40 mg/L (D3), and 60 mg/L (D4). In addition, each treatment was replicated three times, leading to a total of 16 treatment combinations or 48 units. The combinations were as follows: T1D1, T1D2, T1D3,

T1D4 - T2D1, T2D2, T2D3, T2D4 - T3D1, T3D2, T3D3, T3D4 - T4D1, T4D2, T4D3, T4D4. The Ringer's solution was used as an extender, following a previous study by L. S. Handayani et al. (2024), with a sperm-to-Ringer's solution dilution ratio of 1:40 (v/v). Samples of 5% DMSO and 5% egg yolk were used as intracellular and extracellular cryoprotectants (Handayani *et al.*, 2024c).

Sperm collection

A total of four mature male fish were injected with Ovaspec hormone at a dosage of 0.5 ml/kg of body weight and administered intramuscularly under the dorsal fin. After the injection, the broodstock was kept in a 200-liter aerated fiber tank for 8 hours. Following this, the males were placed in cold water (4°C) for anesthesia and then dissected to remove the gonads from the body cavity. The gonads were chopped to extract sperm, which was collected using a syringe and stored in a 1.25 ml tube kept in an icebox at 4°C. The fresh sperm was analyzed for the initial quality, and only samples with motility above 70% were selected for the experiment. The qualifying sperm samples were pooled in a glass beaker and stored at 4°C for further processing.

Cryopreservation process

According to Handayani *et al.* (2024b)The optimal extender for walking catfish sperm is Ringer solution at a 1:40 dilution (sperm:Ringer, v/v). A solution of 5% DMSO and 5% egg yolk were used as intracellular and extracellular cryoprotectants, respectively (Handayani *et al.*, 2024c). The tested antioxidants included Ascorbic acid, Myoinositol, BHT, and Alpha tocopherol, each evaluated at four concentration levels of 0, 20, 40, and 60 mg/L.

To achieve the specified dilution ratio, 50 mL of Ringer solution was mixed with 1.25 mL of sperm, resulting in a sperm-to-Ringer ratio of 1:40 (v/v). Subsequently, 5 mL of egg yolk and 5 mL of DMSO were added to the mixture, ensuring concentrations of 5% for both DMSO and egg yolk. Sperm dilution mixture was distributed into 48 cryotubes, each containing 1 mL of the sample. Each cryotube was supplemented with the respective tested antioxidant, including ascorbic acid (T1), myo-inositol (T2), alpha-tocopherol (T3), and BHT (T4). A total of 12 cryotubes were left without any antioxidant to serve as controls at a concentration of 0 mg/L (3 tubes for each respective treatment). Moreover, 12 cryotubes received 0.02 mL for a concentration of 20 mg/L, 0.04 mL for 40 mg/L, and 12 others were supplemented with 0.06 mL to achieve 60 mg/L.

All cryotubes were initially stored in an ice box and then equilibrated at 4°C for 5 minutes. Subsequently, the samples were evaporated at 5 cm above the surface of liquid nitrogen at -79°C for 5 minutes. Cryotubes were immersed in liquid nitrogen at -196°C for 2 weeks, and the thawing process was initiated by removing the cryotube containing the frozen sperm from the container and placing it in a water bath

at 30°C for 5 minutes. Both microscopic and macroscopic analyses of sperm were performed following the freezing process.

Sperm quality analysis

Fresh and cryopreserved sperm were analyzed for color, pH, sperm density, motility, viability, and fertility. The pH value was measured using a pH meter and sperm concentration using a hemocytometer.

Motility assessment was performed using a stereo microscope (Zeiss Primo Star, Switzerland). A total of 50µl sperm dilution sample was dropped on an object glass and 100µl of water was added to activate sperm. The sample was covered with glass and observed under stereo microscope at 400X magnification. The Optilab Viewer 3.0 application was used to record sperm movement for 5 minutes in five different fields of view. The rate of motility was determined by counting a minimum of 100 sperm cells that moved straight forward or progressively, randomly selected in each field of view. To determine the percentage of sperm motility, the formula was used: Sperm motility (%) = number of sperm moving straight forward/total number of sperm observed x 100.

Sperm viability assessment was carried out using a 0.2% eosin staining method. A total of 50 µl of sperm sample was mixed with 50 µl of 0.2% eosin solution (1:1 ratio) on a cover glass, then homogenized to make a thin smear. The preparation was dried and observed under a microscope at a magnification of 400X, in five random fields of view. Viability was assessed based on observations of at least 100 sperm cells per experimental unit. Live sperm is transparent and has round sperm heads, while dead sperm has opaque pink heads with irregular head morphology. To determine the percentage of sperm viability, the formula was used: Sperm viability (%) = number of live sperm/total number of observed sperm x 100.

Fertilization analysis

Two mature female broodstock with an average size of 400 g, were injected with 0.5 ml/kg body weight of Ovaspec hormone. The samples were then placed in a 200-liter fiber tank for 8 hours. After this period, the fish were gently pressed to release the eggs, which were collected in a plastic basin and stored in an icebox at 4°C. The post-preservation sperm was mixed with the eggs in a 1:1 ratio (v/v). To activate sperm, five drops of tap water were added, and the mixture was gently stirred with soft feathers. The mixture was left undisturbed for 5 minutes to allow sperm to fertilize the eggs.

A total of 100 eggs were randomly selected and incubated in a container equipped with aeration at a temperature of 27°C. The success of fertilization was assessed after 2 hours of incubation. Fertilized eggs appeared transparent, while unfertilized ones were pale milky white. The percentage of fertilization was calculated using the formula: Fertilization rate (%) = (number of fertilized eggs/total number of eggs incubated) x 100.

DNA integrity analysis

DNA extraction was performed using the Genomic DNA Purification Kit (Promega, 2017). Sperm samples were centrifuged at 11.000 rpm for 15 minutes to obtain pellets. The pellets were then mixed with cell lysis solution and incubated at 60°C for 10 minutes. After this incubation, the samples were centrifuged, and the supernatant was discarded. The pellet was suspended in absolute ethanol and nuclei lysis solution. Protein precipitation solution was added, then the mixture was vortexed, and centrifuged once more. The supernatant was mixed with isopropanol, centrifuged again, and washed with 70% ethanol. DNA sample was dried and incubated at 65°C for 1 hour and was stored at 4°C. The quality of DNA was measured using a Nanodrop 2000c. After calibrating the instrument with DNA rehydration solution, a total of 1 µl of DNA was measured, and the results were displayed on the screen.

For agarose gel preparation, 2 g of agarose was dissolved in 100 ml of TBE buffer, heated until the mixture was clear. About 2 µl of DNA dye was added, and the solution was poured into a mold until the solution cooled. Electrophoresis was conducted by loading the gel with 10 µl of sample DNA, 2 µl of loading dye, and 5 µl of DNA marker. The process was run at 135 V for 30 minutes, or until the dye reached halfway or two-thirds of the gel. The results were read and documented using a Uvidoc transilluminator (UVIdoc-HD6, UVITEC Cambridge).

Data analysis

Data on pH, color, and sperm concentration were analyzed descriptively. In contrast, data on motility, viability, and fertility were first tested for normality before being subjected to analysis of variance (ANOVA). The Duncan post hoc test was performed at a significance level of 95% to identify differences among treatments and determine the most effective treatment. The analysis was conducted using SPSS version 22.0.

RESULTS AND DISCUSSION

Fresh sperm from walking catfish was milky white in color, with an average pH of 7.26, a density of 32.00 x 10^9 cells/mL, and a very thick consistency. Table (1) shows the average motility of the fresh sperm was 85.33%, viability was 86.50%, and fertility was 83.67%.. Therefore, the quality of the fresh sperm meets the criteria for cryopreservation.

Table (1): The fresh sperm characteristics of fresh sperm of *Clarias batrachus.*

No.	Parameters	Characteristics
1.	Volume (mL/fish)	1.25
2.	Color	Milky white
3.	pH	7.26±0.12
4.	Consistency	Very thickness
5.	Density (cells/mL)	32.00 x 10^9
6.	Motility (%)	85.33±0.76
7.	Viability (%)	86.50±1.32
8.	Fertility (%)	83.67±1.04

The analysis of variance (ANOVA) test indicated that both the type and concentration of antioxidants significantly influenced the motility, viability, and fertility of walking catfish sperm post-cryopreservation ($p<0.05$). Myoinositol showed the best results at a concentration of 40 mg/L, achieving motility, viability, and fertility values of 58.33%, 60.67%, and 57.50%, respectively. These results were significantly different from those obtained with other concentrations of Myoinositol. Similarly, Alpha-tocopherol also produced optimal results at a concentration of 40 mg/L, with motility, viability, and fertility values of 57.17%, 59.50%, and 56.33%, respectively. These values were significantly different from those at other Alpha-tocopherol concentrations, although the viability values for the 0 mg/L and 60 mg/L concentrations (49.50% and 48.00%, respectively) did not differ markedly.

For BHT, the highest motility, viability, and fertility values were recorded at a concentration of 20 mg/L, with percentages of 55.67%, 57.00%, and 53.17%, respectively. While the motility value differed significantly from the other BHT concentrations, the viability values at 20 mg/L and 40 mg/L (57.00% and 56.33%, respectively) did not differ significantly. Additionally, the fertility values between the 0 mg/L and 40 mg/L concentrations (48.33% and 49.00%, respectively) were also comparable. Overall, the best motility, viability, and fertility values were observed with Ascorbic acid at 40 mg/L, achieving percentages of 62.33%, 66.00%, and 61.67%, respectively. These results were significantly different from those of all other antioxidants and concentrations tested in this study. The detailed data were presented in Table 2.

The analysis of DNA integrity using agarose gel electrophoresis demonstrated that no DNA damage was observed in both fresh sperm and sperm post-cryopreservation across all tested treatments. This indicates that the cryopreservation process did not adversely affect the stability of the sperm DNA structure. Figure 1 shows that all samples exhibited a single, clear, and intact DNA band at the top of the gel, with no smearing.

Table (2): Sperm motility, viability, and fertility of walking catfish *Clarias batrachus* according to type and concentration of antioxidant after 14 days cryopreservation.

Antioxidant	Exp. Code	Concentration (mg/L)	Motility (%)	Viability (%)	Fertility (%)
Ascorbic acid	T1D1	0	54.17 ± 1.04^{gh}	56.50 ± 0.87^{e}	52.33 ± 0.76^{ef}
	T1D2	20	57.00 ± 1.00^{ij}	59.83 ± 0.76^{f}	54.17 ± 1.04^{gh}
	T1D3	40	62.33 ± 0.76^{k}	66.00 ± 0.50^{g}	61.67 ± 0.58^{j}
	T1D4	60	52.50 ± 1.50^{ef}	54.17 ± 0.29^{d}	51.83 ± 0.58^{def}
Myoinositol	T2D1	0	51.00 ± 0.87^{de}	53.33 ± 1.04^{cd}	50.50 ± 0.50^{d}
	T2D2	20	53.83 ± 0.29^{fg}	54.00 ± 1.00^{d}	51.00 ± 0.87^{de}
	T2D3	40	58.33 ± 0.29^{j}	60.67 ± 1.15^{f}	57.50 ± 1.00^{i}
	T2D4	60	49.33 ± 1.53^{c}	51.17 ± 0.58^{b}	48.00 ± 0.87^{c}
Alpha tocopherol	T3D1	0	47.33 ± 1.04^{b}	49.50 ± 0.50^{a}	45.67 ± 1.15^{b}
	T3D2	20	55.17 ± 0.76^{gh}	56.17 ± 1.26^{e}	54.83 ± 0.29^{h}
	T3D3	40	57.17 ± 0.29^{ij}	59.50 ± 1.50^{f}	56.33 ± 1.15^{i}
	T3D4	60	45.50 ± 0.50^{a}	48.00 ± 1.32^{a}	42.17 ± 1.26^{a}
BHT	T4D1	0	51.33 ± 1.26^{de}	52.17 ± 1.53^{bc}	48.33 ± 0.58^{c}
	T4D2	20	55.67 ± 0.76^{hi}	57.00 ± 1.00^{e}	53.17 ± 0.76^{fg}
	T4D3	40	49.83 ± 0.76^{cd}	56.33 ± 0.29^{e}	49.00 ± 1.32^{c}
	T4D4	60	48.50 ± 0.87^{bc}	52.17 ± 1.15^{bc}	46.00 ± 0.50^{b}

Mean±SD in the same column with different superscripts are significantly different ($p<0.05$).

Figure (1): Electrographs of fresh and cryopreserved sperm of walking catfish *Clarias batrachus*. M= Marker; F= Fresh sperm; C= Positive control, T1D3= Ascorbic acid 40 mg/L, T1D4= Ascorbic acid 60 mg/L, T2D3= Myoinositol 40 mg/L, T2D4= Myoinositol 60 mg/L, T3D3= Alpha tocopherol 40 mg/L, T3D4= Alpha tocopherol 60 mg/L, T4D2= BHT 20 mg/L, T4D4= BHT 60 mg/L, N= Negative control.

The study demonstrated that the administration of antioxidants enhances the motility, viability, and fertility of walking catfish sperm after cryopreservation compared to without antioxidants. The optimal concentrations for Ascorbic acid, Myoinositol, and Alpha-tocopherol are 40 mg/L, while for BHT, it is 20 mg/L. Sperm quality declines when antioxidant concentrations exceed these optimal levels, potentially due to the toxic effects of the respective chemicals.

According to Dutta *et al.* (2019), administering antioxidants in appropriate doses improves sperm cell quality by reducing oxidative stress. However, excessive antioxidant concentrations can be detrimental due to their peroxidative properties, leading to cellular damage in sperm cells (Bansal and Bilaspuri, 2011). The findings indicated that 40 mg/L of Ascorbic acid yielded the best sperm quality after cryopreservation, making this specific type and concentration of antioxidant recommended for preserving walking catfish sperm. Several studies have also shown that 22 mg/L of Ascorbic acid is suitable for cryopreserving the sperm of gilthead seabream *Sparus aurata* and European seabass *Dicentrarchus labrax* (Cabrita *et al.*, 2011). Similarly, Kocabaş *et al.* (2022) reported that 32 mg/L of Ascorbic acid positively affected the sperm motility of rainbow trout after cryopreservation.

This effectiveness is attributed to Ascorbic acid, a water-soluble non-enzymatic antioxidant that plays a crucial role in neutralizing reactive oxygen species (ROS) produced during freezing and thawing (Mirzoyan *et al.*, 2006). Therefore,

Ascorbic acid is more effective in preventing lipid peroxidation and reducing oxidative damage, facilitating to maintain sperm quality and DNA integrity in post-cryopreservation sperm.

The study revealed that butylated hydroxytoluene (BHT) is effective at lower concentrations compared to other antioxidants tested, with an optimum concentration of only 20 mg/L. This suggests that BHT is more effective when used in lower concentrations, possibly because its toxicity and potential carcinogenic effects increase with higher concentrations. Merino *et al.* (2020) reported that a dose of 10 mg/L of BHT effectively maintained sperm quality in salmon *Oncorhynchus kisutch*. However, for Russian sturgeon *Acipenser guldenstadti*, the highest tolerable concentration was found to be only 22 mg/L (Osipova *et al.*, 2016). These differences in BHT effectiveness at various concentrations indicate that cellular sensitivity to this antioxidant varies significantly between fish species. This finding is supported by Li *et al.* (2018), who stated that different types and concentrations of antioxidants have varying effects on sperm quality among three sturgeon species: *Acipenser dabryanus*, *A. sinensis*, and *A. baerii*.

Furthermore, a DNA integrity analysis conducted on walking catfish sperm using the agarose gel electrophoresis method showed no DNA fragmentation in either fresh sperm or post-cryopreservation samples. This was indicated by the absence of smear formation on the electrophoresis gel and the presence of a single, intact DNA band, demonstrating that antioxidants in the cryopreservation medium can prevent degradation of DNA structure. Thus, the application of antioxidants has been shown to be effective in maintaining sperm DNA integrity. This aligns with previous findings by Muthmainnah *et al.* (2018) in seurukan fish *Osteochilus vittatus*, and Maulida *et al.* (2024) in climbing perch *Anabas testudineus*, who reported that antioxidants prevent DNA damage in the sperm of these fish.

CONCLUSION

In conclusion, the type and concentration of antioxidant significantly influence the quality of walking catfish sperm post-cryopreservation. Ascorbic acid, Myoinositol, and Alpha-tocopherol produced the best results at a concentration of 40 mg/L, while BHT yielded optimal results at a concentration of 20 mg/L. The use of 40 mg/L Ascorbic acid resulted in superior sperm quality compared to the other antioxidant and concentrations tested. Therefore, 40 mg/L Ascorbic acid is recommended as the preferred antioxidant for cryopreserving walking catfish sperm.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest with the publication of this work.

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