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The Effect of Cryopreservation on *Cytochrome Oxidase1* (CO1) Gene and the Relationship with Spermatozoa Motility of Albino *Pangasius catfish* (*Pangasionodon hypophthalmus*)

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ABSTRACT

Cryopreservation is a technique for storing cells and tissues at very low temperatures for the possible usage of the stored cells and tissues throughout the year. Sperm cell cryopreservation in some species causes a decrease in sperm quality and DNA damage. Inappropriate cryopreservation protocols can cause changes in sperm physiology. Mitochondria are organelles that play a role in producing energy for sperm motility. Mitochondria have DNA molecules with small sizes compared to structures from nuclear DNA. This study analyzed the effect of cryopreservation on sperm motility and the *Cytochrome Oxidase1* (COI) gene. The CO1 gene in mitochondrial DNA plays a role in energy production for spermatozoa motility. The cryopreservation was performed using skim milk and 10% methanol cryoprotectant, and the temperature in the equilibration process was 4-5°C for 10 minutes. Cryopreservation took place for 14 days in the freezer at -80°C. In addition, the thawing process was performed for 1-2 minutes at 40°C. This study found that the number of lesions per 10 kb in the CO1 gene in post-equilibration spermatozoa was (9.24±3.74), and post-thawing spermatozoa (was 10.26±7.54). Spermatozoa motility was obtained in fresh spermatozoa, i.e., 87±1.5%, post-equilibration spermatozoa 79±4.5%, and post-thawing spermatozoa 30±3.2%. This study concluded that cryopreservation of spermatozoa causes CO1 gene lesions and that in cryopreserved spermatozoa, there is a decrease in spermatozoa motility compared to fresh spermatozoa.

1. Introduction

Sperm cryopreservation aims to produce quality gametes that can be used throughout the year (Cabrita *et al.* 2010). The cryopreservation process involves various extreme factors that can stress cells, and these conditions differ depending on the cell type and the procedure performed (Kopeika *et al.* 2005). Different cryopreservation protocols between species, such as the type of cryoprotectant, equilibration time, and freezing and thawing processes, can cause sperm structure and physiology changes (Tiersch *et al.* 2007). During the cryopreservation process, both lethal and sub-lethal cell cryoinjury can occur. The mechanism of cryoinjury can be in the form of damage to cell structures and changes in cell biochemistry. Inappropriate cryopreservation techniques can lead to increased reactive oxygen species (ROS) production, which is highly destructive and results in strand breakage and base modification. The DNA of mitochondria (mtDNA) is more reactive to ROS than the DNA of the nucleus. Higher mtDNA mutation rates are associated with the increasing vulnerability of mtDNA to mutations because of the protection of histone, limited DNA repertoire, and lack of chromatin structure (Kopeika *et al.* 2005).

Mitochondria are organelles that play a role in producing energy for cells. The mitochondrial genome is more vulnerable to damage than the nuclear genome (Carton-Garcia *et al.* 2013).

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Mitochondria have their DNA molecules with small sizes and are different in structure from nuclear DNA. Mitochondrial DNA molecules are compact and relatively short in size, are gene sequences easily determined, and have a higher mutation rate than nuclear DNA (DeSalle *et al.* 2017). Mitochondrial DNA (mtDNA) encodes several genes whose products play an essential role in oxidative phosphorylation to produce adenosine triphosphate (ATP) molecules as cellular energy for sperm motility and viability.

The *Cytochrome Oxidase1* (CO1 gene) is one of the genes in mitochondrial DNA that plays a role in energy production. The CO1 gene is associated with mitochondrial function related to the electron chain, ATP production, and sperm motility. The CO1 gene marker was also used to identify the breeding results of almost all animals, both intraspecies and interspecies. The CO1 gene has been widely studied because it has advantages such as studying genetic characteristics. The gene has few deletions and insertions in its sequence and many conserved parts (Hebert *et al.* 2003).

Cryopreservation techniques can cause lesions on DNA due to the excessive production of free radicals. ROS are free radicals in the form of oxygen and their highly reactive derivatives. The imbalance between ROS production and the ability of cells to detoxify reactive substances to repair the resulting damage is referred to as oxidative stress. ROS can cause changes in plasma membranes and metabolic pathways related to cell motility and viability, leading to decreased fertilization and the ability to produce offspring (Figueroa *et al.* 2015).

The integrity of the mitochondrial genome is a prerequisite for functional mitochondria. The high concentration of ROS generated through the oxidative phosphorylation pathway makes mitochondria very susceptible to oxidative stress that causes mitochondrial DNA lesions. This study aims to analyze the effect of cryopreservation on the CO1 gene and its relation to spermatozoa motility. Several methods for detecting damaged mitochondrial DNA have been developed, but these techniques frequently necessitate large amounts of genomic DNA input. By amplifying DNA fragments of varying lengths, a rapid and quantitative method for assessing the extent of damage to mitochondrial DNA using qualitative PCR (qPCR) was developed (Rothfuss *et al.* 2010). The results were shown in CO1 gene lesions per 10 kb that were produced after equilibration and thawing processes in spermatozoa and their association with sperm motility of albino *Pangasius catfish*.

2. Materials and Methods

2.1. Study Time and Places

The research was carried out from September 2019 to February 2021. The study was conducted in several places: Depok Ornamental Fish Cultivation Research Center, albino catfish cultivation of Family Jaya Kekupu, Depok, and the Laboratory of Reproduction and Genetics of Aquatic Organisms, Faculty of Fisheries and Marine Sciences, IPB University.

2.2. Sample Collection Locations

The research sample consisted of 6 male albino catfish from the Family Jaya Kekupu albino catfish cultivator, Pasar Putih Village, Sawangan District, Depok City, West Java.

2.3. Stripping Process

Materials and tools consisted of spermatozoa of albino catfish, centrifuge tube, GnRH analogue hormone (Ovaprim) (dose = 0.5 ml/kg body weight), syringe, blue ice, gloves, ice box, and paper tissue. First, the fish were injected with a GnRH analogue hormone (0.5 ml/kg body weight), and a single injection was performed intramuscularly (Mosha 2018). Spermatozoa were collected by stripping 10-12 hours after induction. Before stripping, the outer side of the fish's urogenital opening was cleaned using a cloth to reduce contamination. The sorting procedure was done by pressing the fish's abdomen towards the urogenital opening, and the ejaculated sperm was placed in a centrifuge tube. After that, the cement was stored at 4°C for cryopreservation.

The study used three treatments: fresh spermatozoa, post-equilibration spermatozoa, and post-thawing spermatozoa. This research has obtained permission from the research ethics committee of the Faculty of Medicine, University of Indonesia, with the number: KET-294/UN2.F1/ETIK/PPM.00.02/2019.

2.4. Cryopreservation Process

The materials used were an activator solution composed of 0.2633 g NaCl, 0.0373 g KCl, and 0.4727 g Tris in 100 ml distilled water and a fish ringer extender solution with a composition of 0.65 g NaCl, 0.025 g KCl, 0.035 g $CaCl_2 \cdot 2H_2O$, 0.02 g $NaHCO_3$ in 100

ml of distilled water. Both solutions were stored in a refrigerator at 4-5°C. Intracellular cryoprotectant methanol was added with a concentration of 10%, and extracellular cryoprotectant was added with skim milk. The previously used skim milk was put in a glass container, then soaked in a water bath for 1 minute at 87-97°C. The diluent used for cryopreservation consisted of 10% methanol (50 µL), skim milk (50 µL), and Ringer's fish extender (350 µL). All ingredients are mixed in a cryotube that has been labelled.

The following procedure is the mixture of 50 µL semen of albino catfish with a diluent composition of 1:9. The equilibration process was carried out at a temperature of 4-5°C for 10 minutes. The deep freezing temperature was -80°C for 14 days. Thawing was accomplished by immersing cryotubes in a water bath at 40°C for 1-2 minutes (Horvath *et al.* 2003; Sularto *et al.* 2013; Hasanah *et al.* 2020).

2.5. DNA Extraction

Sperm DNA extraction was performed on fresh spermatozoa, post-equilibration spermatozoa, and post-thawing spermatozoa. DNA extraction using the exgene clinic sv protocol. First, 100 µL of sperm was placed in a 1.5 ml microcentrifuge tube. Afterwards, 100 µL of buffer H2 and 20 µL of proteinase K were added to the tube and vortexed. Samples were incubated at 56°C for 1 hour. Then, the authors added 200 µL of BL buffer, vortexed the mixture, and incubated the mixture for 10 minutes at 56°C. The following procedure was the addition of 200 µL of absolute ethanol to the sample, homogenized with a vortex, and then precipitated by spin down. The mixture was transferred into the sv column, and the mixture centrifugation was at >8,000 rpm for 1 minute. After which, the procedure was the replacement of the collection tube with a new one, adding 600 µL of BW buffer, and centrifuge the mixture at >8,000 rpm for 1 minute. The following procedure was the replacement of the collection tube with the new one and adding 700 µL of TW buffer. After that, the procedure was centrifuging the mixture at >8,000 rpm for 1 minute. Then, the procedure was discarding the supernatant and returning the sv column to the collection tube. Then, the procedure was centrifugation for 1 minute at >8,000 rpm to remove the remaining wash buffer, and then the procedure was placing the sv column into a new 1.5 ml tube. The AE buffer of as much as 100 µL was added and then incubated for 1 minute at room temperature. After that, the tube was centrifugated at >8,000 rpm (full speed) for 1 minute. The quantity and purity of DNA were determined by spectrophotometer. Only the DNA with a purity of A260/A280 >1.8 was used for PCR.

2.6. Primer Design and Optimization

DNA sequences were obtained from GenBank. The gene markers obtained were forward and reverse primers in the order presented in Table 1.

Primer optimization was performed using gradient PCR. Genes were designed with long and short bases. Amplification was carried out at several melting temperatures. It was 55.3; 57.3; 59.3; 61.3; 63.3; and 65.3°C for the CO1 gene with a base length of 517 bp and 87 bp. The PCR program was stage 1, 95°C for 10 minutes for 1 cycle. Stage 2 of the PCR program was 15 seconds at 95°C, 10 seconds at 62.2°C, and 50 seconds at 72°C for 35 cycles. Stage 3 of the PCR was 72°C for 5 minutes for 1 cycle.

The PCR product obtained was then continued with electrophoresis. Gel electrophoresis consisted of 0.6 g of agarose mixed with 30 ml of TBE 1×. The prepared gel was placed horizontally on the electrophoresis box containing the electrode buffer solution. The PCR product was added as much as 2 µL per well. The electrophoresis was carried out at 100 V for 30 minutes. Staining using PEG green as much as 3 µL. The DNA ladders used were 100 bp.

2.7. Quantitative PCR (qPCR)

qPCR was performed using a Rotorgen 6,000 apparatus. DNA lesions were analyzed for the CO1 gene. Primers were designed into two fragments of different lengths (short and long fragments) located in the same gene to compare DNA lesions. Long and short fragment primers were analyzed using qPCR at the same time. The reaction blend comprised of 10 µL sensiFAST SYBR no-ROX kit, 2 µL5 µM forward

Table 1. The marker type and the sequence of primer base

Marker	Forward primer (5'-3')	Reverse primer (5'-3')	PCR Product (bp)	Source
CO1	GCTTGCTAATTCGGGCAGAGC	ATGCCAGCGGTTAGTACGGG	517	GenBank: EU871048.1
CO1	CGCCAGACATGGCATTTCCC	CCCCAGATGAGGCGAACAGT	87	GenBank: EU871048.1

primer, 2 µL 5 µM reverse primer, 4 µl DNA template (3 ng), and 2 µL nuclease-free water so that the final volume was 20 µL. Three repetitions were performed per sample, and negative control was entered for each primer set. The procedure was pre-incubation at 95°C for 10 minutes. Then, the process was 35 cycles of denaturation at 95°C for 15 seconds and annealing at 62.2°C for 10 seconds. Then, the procedure was the elongation at 72°C for 50 seconds and a threshold of 0.3. The process was the serial dilution of 1 g/µL of sperm DNA (1:10,000) and amplified with long and short fragment primers to determine the amplification efficiency and authenticate the qPCR outcomes (Carton-Garcia *et al.* 2013).

2.8. DNA Lesion Analysis

The number of DNA lesions per 10 kb was measured using the Rothfuss *et al.* (2010) formula:

$$\frac{\text{Lesion rate}}{\text{10 kb fr. (bp)}} = (1 - 2^{-(\Delta Ct\ long\ -\ \Delta Ct\ short)}) \times \frac{10{,}000\ (bp)}{\text{size of long}}$$

The variance in Ct values between samples (fresh, post-equilibration, and post-thawing spermatozoa) for short and long fragments was expressed and measured as lesions per 10 kb. The COI gene was analyzed, and the lesions acquired from each treatment were counted.

2.9. Spermatozoa Motility

Before calculating the motility, the albino catfish semen was first diluted. The dilution used for the assessment of spermatozoa motility was 100 times. Semen dilution in the observation of spermatozoa motility was performed by adding 10 µL of semen into 90 µL of extender, called the 10 times dilution. A further dilution is taken by taking 10 µL of the 10 times dilution and adding it to 90 µL of the activator solution, and the dilution is a 100 times dilution. Furthermore, 10 µL of the diluted semen was dripped onto the object glass and covered with a cover glass. The spermatozoa motility was observed under a microscope (40 × 10 magnifications). Spermatozoa motility calculations were started immediately after dilution, and spermatozoa movement was observed for 1-2 minutes (Verma *et al.* 2009). The spermatozoa motility was observed using a Bright field microscope with a magnification of 40 × 10 (objective x ocular).

The following formula calculates the percentage of spermatozoa motility:

$$\%\ \text{motility} = \frac{\sum \text{motile spermatozoa} \times 100}{\sum \text{total spermatozoa}}$$

2.10. Data Analysis

Data analysis begins with a normality test to see whether the data is normally distributed and a homogeneity test to see whether the data is homogeneous. After that, the one-way ANOVA parametric test was performed with SPSS 23 software. The data expression was mean ± S.D and $P<0.05$ for the significant difference.

3. Results

3.1. DNA Extraction

DNA Extraction was performed on three treatments: fresh spermatozoa, post-equilibration, and post-thawing. PCR was only performed from high-purity DNA (A260/A280>1.8). The data are presented in Table 2.

DNA purity measurements showed that the spermatozoa DNA obtained from the extraction process had an excellent purity value and met the continued PCR requirements, ranging from 1.8 to 2.0. The concentration of spermatozoa found in fresh spermatozoa was high at 666 ng/µL, and there was a decrease in DNA concentration in post-equilibration spermatozoa, which was 350 ng/µL and post-thawing spermatozoa, which was 12 ng/µL compared to fresh spermatozoa. This condition is presumably due to DNA degradation during the freezing and thawing process by DNAse or other unknown factors that cause a decrease in DNA concentration.

3.2. DNA Amplification with PCR

The PCR amplification product was separated by agarose electrophoresis. The results showed that at TM 55.3, a band appeared at a position close to 100 bp; this was appropriate because the short base CO1 gene was designed at the 87 bp position. The 517 bp CO1 electrophoresis results showed that a

Table 2. Purity and concentration of extracted DNA

Treatment	260/280	Concentration (ng/µL)
Fresh spermatozoa	1,850	666
post-equilibration spermatozoa	1,862	350
post-thawing spermatozoa	2,000	12

band appeared at 500 bp. The results of 517 bp CO1 electrophoresis are presented in Figure 1.

3.3. CO1 Gene Lesions

Analysis of DNA lesions using qPCR first performed primer efficiency and qPCR validation with a serial dilution of 1: 10,000. Analysis of the CO1 gene lesions obtained results, as shown in Figure 2.

3.4. Spermatozoa Motility

Spermatozoa motility observations showed that fresh spermatozoa were 87±1.5%, post-equilibration spermatozoa were 79±4.5%, and post-thawing spermatozoa were 30±3.2%, which indicated a decrease in spermatozoa motility after freezing and thawing, as shown in Figure 3.

The ANOVA test results found a significant difference in spermatozoa motility between the 3 treatments ($p<0.05$). Fresh spermatozoa can move actively and quickly. This result shows that the high motility is due to the required nutrients' availability, so spermatozoa can utilize ATP to move. Meanwhile, spermatozoa motility decreased after thawing because the nutrient reserves in the spermatozoa began to run out.

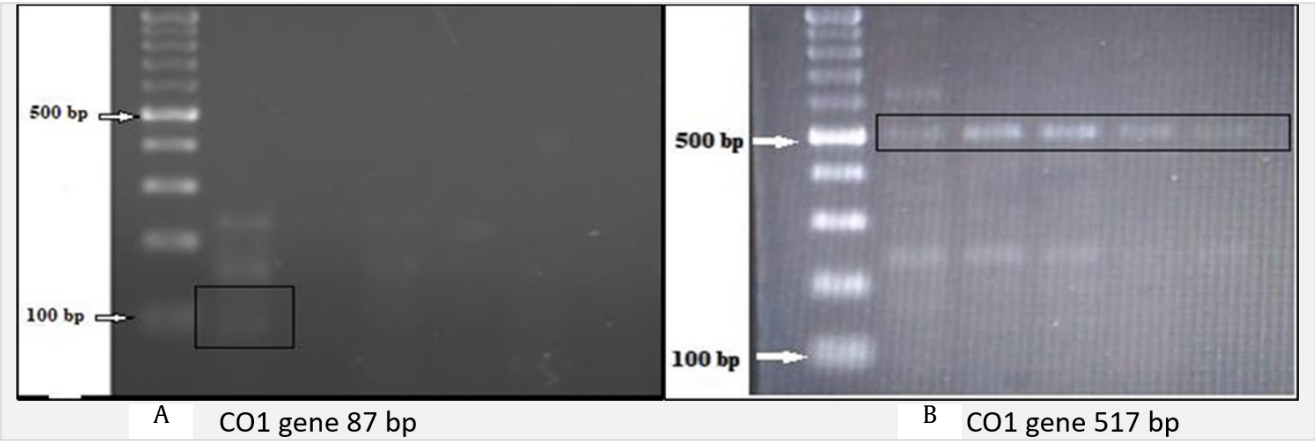

Figure 1. The electrophoresis of PCR products, genes CO1, was designed by long and short bases. CO1 gene results in 87 bp and 517 bp. 100 kb DNA ladder marker

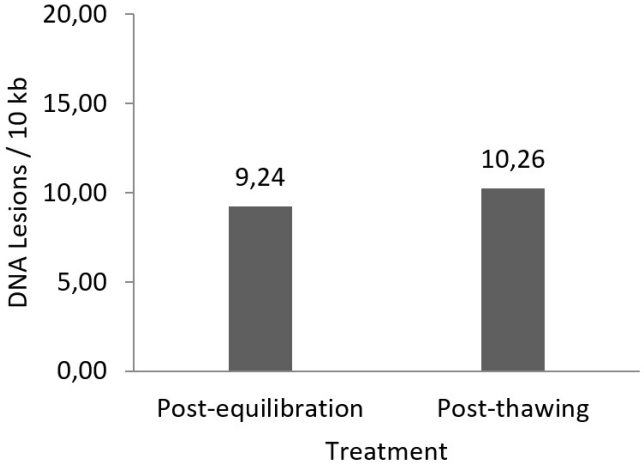

Figure 2. CO1 gene lesions per 10 kb

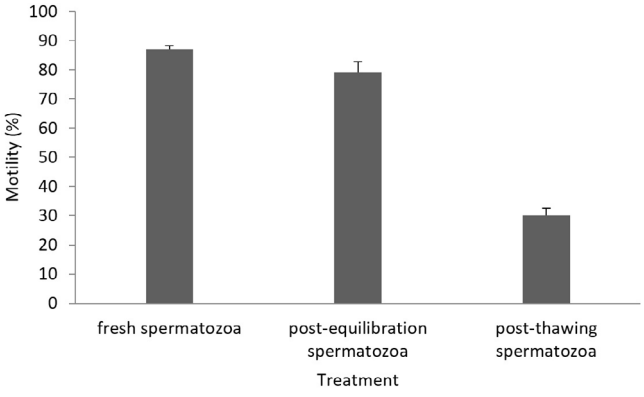

Figure 3. Spermatozoa motility of albino catfish after cryopreservation

4. Discussion

4.1. CO1 Gene Lesions

This study tried to analyze the effect of cryopreservation on the CO1 gene by measuring the number of lesions produced after equilibration and thawing. Analysis of gene lesions in post-cryopreservation albino *Pangasius catfish* spermatozoa found in CO1 gene lesions 9.24/10 kb in post-equilibration spermatozoa and 10.26/10 kb in post-freezing and thawing spermatozoa. The CO1 gene of post-melting spermatozoa showed a higher number of lesions than post-equilibration spermatozoa, although statistically, the difference was insignificant (P>0.05).

The basic principle of DNA lesion analysis is the qPCR-based amplification rate of two mtDNA fragments of different lengths. The long fragment amplification was used as an experimental probe to assess the extent of lesions caused by oxidative stress conditions during the cryopreservation process. Meanwhile, the fragment with a short base length of 87 bp is an internal normalization control. Amplification of short or small fragment nucleotide sequences under normal cellular and physiological conditions directs that ROS does not simply harm mtDNA due to the low possibility of lesion formation. With the assumption of a randomly generated distribution of lesions along the mitochondrial genome, the frequency of mitochondrial lesions could be calculated as the ratio of lesion incidence per nucleotide size amplification. Depending on the length of the sequence, the frequency of mitochondrial lesions is inversely related to amplification (Rothfuss *et al.* 2010).

There was an increase in CO1 gene lesions in spermatozoa that were cryopreserved because the cryopreservation process produced ROS that could affect DNA integrity. ROS is an unavoidable process in most aerobic organisms. However, ROS are important in host defence against harmful microorganisms; excessive ROS causes oxidative stress and significant cellular damage to macromolecules, including lipids, proteins, and DNA. The formation of lesions in the CO1 gene during cryopreservation is associated with oxidative stress (Thomson *et al.* 2009). During cryopreservation, ROS production increases, forming oxidative products such as 8-oxodG, 8-oxodA, basic site, and thymidine dimer, inhibiting DNA polymerase development and ultimately causing a delay in Ct (Sikorsky *et al.* 2004). In addition, it is known that mitochondrial DNA chromatin is very susceptible to cryoinjuries. The low efficiency of chromatin remodelling during spermiogenesis could lead to susceptibility conditions that allow ROS to be generated during cryopreservation. This technique may lead to changes in sperm structure and function and DNA fragmentation (González-Marín *et al.* 2012).

Mitochondria are involved in the initiation of apoptosis in response to oxidative stress. Nuclear DNA encodes most mitochondrial proteins. Mitochondria have their genome engaged in producing intracellular ATP by the electron transport chain that reduces oxygen to water. As a by-product of this process, ROS are produced. ROS molecules can be converted to H_2O_2 spontaneously or by superoxide dismutase. Mitochondrial chromatin structure is also condensed lower than nuclear chromatin, making mtDNA more susceptible to oxidative stress. The most common mtDNA mutations are point mutations and nucleic acid modifications that lead to mitochondrial dysfunction and cell apoptosis (Rothfuss *et al.* 2010).

Carton-Garcia *et al.* (2013) used quantitative PCR on a snapper (*Sparus aurata*) and found some specific lesions due to cryopreservation in 2 *Cyt b* and COI genes that showed susceptibility to damage from mtDNA. This study analyzed the lesions after 14 days of storage at -80°C. The results obtained on the DNA of spermatozoa of albino *Pangasius catfish* with lesions per 10 kb is 10.26. Compared with the study of gene lesions in *Sparus aurata*, the results on the CO1 gene of spermatozoa of *Sparus aurata* fish lesions per 10 kb are 8.43, not much different. There are differences in several things that can affect the study's results, such as the method and temperature of storage, the type of cryoprotectant used, and the addition of antioxidants. The effect of a more extended storage period on spermatozoa of albino Pangasius catfish is also thought to cause an increase in DNA lesions.

The qPCR-based approach is vital for studying the susceptibility of certain DNA regions (nuclear and mitochondrial), particularly those that play a role in sperm quality. Several studies have reported the potential importance of genes as markers of sperm quality. Therefore, evaluating specific gene lesions after cryopreservation is critical, and traditional techniques such as DNA fragmentation cannot provide such information. qPCR-based assays are becoming a valuable method for analyzing lesions after cryopreservation in many genes allowing for

the enhancement of cryopreservation protocols to minimize damage (Carton-Garcia *et al.* 2013). Analysis of the mitochondrial CO1 gene in spermatozoa is critical because mitochondrial function must be maintained to ensure good motility.

The quantitative PCR method allows the amplification of up to 25 kb of DNA fragments using only a tiny amount (a few nanograms) of the total DNA. Although qPCR can assess nuclear and mitochondrial DNA damage, the multi-step procedure is time-consuming and requires optimization and high accuracy to obtain good experimental results. The qPCR-based application detects DNA variations that interfere with polymerase-based DNA amplification. The products of DNA damage caused by ROS are strand breakage, base modification, and the formation of DNA blocks that can inhibit primer elongation or block polymerase synthesis from complementary DNA strands. DNA damage quantification methods based on qPCR can provide specific target or gene information, thereby providing a more global assessment of sperm DNA damage. This analysis is based on the ability of typical DNA lesions to reduce and block the development of polymerases in template DNA which ultimately leads to decreased template amplification and Ct delay (Rothfuss *et al.* 2010).

According to Carton-Garcia *et al.* (2013), qPCR can analyze lesions caused by cryopreservation in specific genes even when DNA fragmentation and telomere shortening are not detected. The qPCR technique can complement traditional tests such as DNA integrity testing and offer detailed information regarding specific genomic regions. The CO1 gene under study plays an essential role in cellular energy. Further studies should be conducted to determine the acceptable range of typical gene lesions associated with cellular energy and oxidative stress conditions.

4.2. Spermatozoa Motility

Spermatozoa used for cryopreservation had initial motility >70%. Fish spermatozoa generally have a more curved movement trajectory than mammalian spermatozoa (Verma *et al.* 2009). Sperm is diluted 100 times in an extender solution with a high osmolality (immobilizing medium) to keep the spermatozoa stationary. After that, a second dilution is made in an activator medium or solution of low osmolality to activate the motility. Spermatozoa do not move in the seminal fluid of the plasma and will move actively when in contact with water, so to observe the motility of fish spermatozoa, drops of activator solution must be added to activate spermatozoa. In freshwater fish, spermatozoa can only survive for less than 2 minutes. Whereas the spermatozoa of marine fish can survive longer after meeting water and generally exhibit good resistance to freezing (Cosson *et al.* 2008).

In this study, dilution was carried out using an extender and activator solution before the motility assessment. As is well known, seminal plasma molality plays a vital role in spermatozoa activation. Plasma seminal osmolality is used in developing extenders for semen in many fish species and reversibly suppresses spermatozoa activation. The glucose content in the seminal plasma is essential for protecting the spermatozoa membrane. In fish with external fertilization, the spermatozoa are released in a different environment where the spermatozoa become active and then survive for 1-2 minutes (Verma *et al.* 2009).

The use of a thawing temperature of 40°C is thought to cause the metabolism of spermatozoa to suddenly increase, which causes the duration of motility to be short because the energy source of spermatozoa is limited. The duration of motility and speed of spermatozoa also depends on temperature. If the temperature is high, the speed of movement and metabolism of spermatozoa will also be high so that the duration of motility will be short because the energy source of the spermatozoa is limited. On the contrary, if the temperature is low, the speed of movement and metabolism of spermatozoa will be slow so that motility can last longer (Alavi and Cosson 2005).

This study used an intracellular cryoprotectant, 10% methanol, and an extracellular cryoprotectant, skim milk. Both are good cryoprotectant combinations for the cryopreservation of fish spermatozoa. Several studies used methanol and skim milk, such as research by Abinawanto *et al.* (2012) using the cryoprotectant methanol and skim milk on gourami, the percentage value of motility 48 hours after cryopreservation is 80.98%.

Sperm motility and fertilization require energy supplied by ATP, which is synthesized through glycolysis in the cytoplasm or oxidative phosphorylation in the mitochondria. ATP generated in oxidative phosphorylation in the inner mitochondrial membrane is transferred to

microtubules to drive motility. Therefore, decreased sperm motility may be associated with mitochondrial damage. Reduced sperm motility can be caused by loss of mitochondrial function (O'Connell *et al.* 2002). This study found a decrease in functional mitochondria after cryopreservation which could be related to the decline in spermatozoa motility due to the role of mitochondria as ATP producers for the movement of spermatozoa.

Spermatozoa motility depends on dynein ATPase which hydrolyzes ATP to produce flagellar movement. Changes in motility are directly related to the ATP content during activation. If the supply of nutrients and energy is depleted, the contraction of the spermatozoa fibrils will stop, and the spermatozoa will not move. To move back, ATP and ADP must be regenerated by adding a phosphoryl group that requires an external energy source. Simple sugar metabolism through cellular respiration can produce ATP. The breakdown of ATP to ADP in the mitochondrial inner membrane has energy for the motility of spermatozoa. Spermatozoa motility occurs because of the movement of the flagellum, which is composed of microtubules. The ATPase enzyme activates the mitochondria's ATP to release phosphate bonds to form ADP and inorganic phosphate by removing energy for fibril contraction. If the supply of phosphate in ATP and ADP has been depleted, the contraction of the spermatozoa fibrils will stop, and movement will also stop. Spermatozoa motility can continue if ADP and ATP are regenerated by adding phosphate groups from energy sources in organic materials such as carbohydrates (Hidayaturrahmah 2007).

Oxidative stress conditions due to increased ROS during the cryopreservation process can damage mitochondria by inducing lipid peroxidation in the membrane, which causes changes in mitochondrial pore permeability. Mitochondrial damage disrupts cell metabolism to produce energy. The reduction of energy directly causes a decrease in cell motility. In salmon, spermatozoa motility decreases rapidly after thawing (Dziewulska *et al.* 2011).

References

Abinawanto, A., Nurman, K., Lestari, R., 2012. The effect of sucrose on sperm quality of *Osphronemus goramy* two days post-cryopreservation. *Journal of Agricultural Science and Technology B.* 2, 204-207.

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. *Cell Biology International* 29, 101-110. https://doi.org/10.1016/j.cellbi.2004.11.021

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P., 2010. Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology*. 26, 623-635. https://doi.org/10.1111/j.1439-0426.2010.01556.x

Carton-Garcia, F., Riesco, M.F., Cabrita, E., Herráez, M.P., Robles, V., 2013. Quantification of lesions in nuclear and mitochondrial genes of *Sparus aurata* cryopreserved sperm. *Aquaculture*. 402, 106-112. https://doi.org/10.1016/j.aquaculture.2013.03.034

Cosson, J., Groison, A. L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008. Studying sperm motility in marine fish: an overview on the state of the art. *Journal of Applied Ichthyology*. 24, 460-486. https://doi.org/10.1111/j.1439-0426.2008.01151.x

DeSalle, R., Schierwater, B., Hadrys, H., 2017. MtDNA: The small workhorse of evolutionary studies. *Frontiers in Bioscience-Landmark*. 22, 873-887. https://doi.org/10.2741/4522

Dziewulska, K., Rzemieniecki, A., Czerniawski, R., Domagała, J., 2011. Post-thawed motility and fertility from Atlantic salmon (*Salmo salar* L.) sperm frozen with four cryodiluents in straws or pellets. *Theriogenology*. 76, 300-311. https://doi.org/10.1016/j.theriogenology.2011.02.007

Figueroa, E., Merino, O., Risopatrón, J., Isachenko, V., Sánchez, R., Effer, B., Valdebenito, I., 2015. Effect of seminal plasma on Atlantic salmon (*Salmo salar*) sperm vitrification. *Theriogenology*, 83, 238-245. https://doi.org/10.1016/j.theriogenology.2014.09.015

González-Marín, C., Gosálvez, J., Roy, R., 2012. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. *International journal of molecular sciences*. 13, 14026-14052. https://doi.org/10.3390/ijms131114026

Hasanah, U., Alimuddin, A., Boediono, Abinawanto, 2020. Optimization of spermatozoa cryopreservation of *Albino pangasius* catfish: cryoprotectants with various concentrations and different equilibration times. *IOP Conference Series: Earth and Environmental Science*. 441, 012088. https://doi.org/10.1088/1755-1315/441/1/012088

Hebert, P.D., Ratnasingham, S., de Waard, R.D., 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biological Sciences*, 270, 96-99. https://doi.org/10.1098/rsbl.2003.0025

Hidayaturrahmah, 2007. Waktu motilitas dan viabilitas spermatozoa ikan mas (*Cyprinus Carpio* L.) pada beberapa konsentrasi larutan fruktosa. *Bioscientiae*. 4, 9-18.

Horvath, Á., Miskolczi, E., Urbányi, B., 2003. Cryopreservation of common carp sperm. *Aquatic Living Resources*. 16, 457-460. https://doi.org/10.1016/S0990-7440(03)00084-6

Kopeika, J., Zhang, T., Rawson, D.M., Elgar, G., 2005. Effect of cryopreservation on mitochondrial DNA of zebrafish (*Danio rerio*) blastomere cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 570, 49-61. https://doi.org/10.1016/j.mrfmmm.2004.09.007

Mosha, S.S., 2018. Recent comparative studies on the performance and survival rate of African catfish (*Clarias gariepinus*) larval produced under natural and synthetics hormones: a review. *Journal of Aquaculture Research and Development*. 9, 1-6. https://doi.org/10.4172/2155-9546.1000528

O'Connell, M., Mcclure, N., Lewis, S.E.M., 2002. The effects of cryopreservation on sperm morphology, motility, and mitochondrial function. *Human Reproduction.* 17, 704-709. https://doi.org/10.1093/humrep/17.3.704

Rothfuss, O., Gasser, T., Patenge, N., 2010. Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach. *Nucleic acids research*. 38, 24-24. https://doi.org/10.1093/nar/gkp1082

Sikorsky, J.A., Primerano, D.A., Fenger, T.W., Denvir, J., 2004. Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method. *Biochemical and Biophysical Research Communications*. 323, 823-830. https://doi.org/10.1016/j.bbrc.2004.08.168

Sularto, E., Tahapari, W., Hadie, Nurlaela, I., 2013. Penggunaan kuning telur bebek sebagai ekstender pada proses kriopreservasi sperma ikan patin jambal. *Konferensi Akuakultur indonesia*, 220-225.

Thomson, L.K., Fleming, S.D., Aitken, R.J., De Iuliis, G.N., Zieschang, J.A., Clark, A.M., 2009. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Human Reproduction.* 24, 2061-2070. https://doi.org/10.1093/humrep/dep214

Tiersch, T.R., Yang, H., Jenkins, J.A., Dong, Q., 2007. Sperm cryopreservation in fish and shellfish. *Society of Reproduction and Fertility supplement*. 65, 493.

Verma, D.K., Routray, P., Dash, C., Dasgupta, S., Jena, J.K., 2009. Physical and biochemical characteristics of semen and ultrastructure of spermatozoa in six carp species. *Turkish Journal of Fisheries and Aquatic Sciences.* 9, 67-76.