FULL ARTICLE

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Effect of cryoprotectant on the motility, viability, fertilization, and DNA integrity of naleh fish *Barbonymus* sp. (Cyprinidae) sperm

Efeito do crioprotetor na motilidade, viabilidade, fertilização e integridade do DNA no sêmen de peixes naleh Barbonymus sp. (Cyprinidae)

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ABSTRACT

Naleh fish *Barbonymus* sp. is a commercial freshwater fish, which is indigenous to Aceh, Indonesia. The population of this species has declined over the years as a result of habitat perturbations and overfishing. Hence, the crucial need to develop a cryopreservation method to support breeding programs. This involved the use of a cryoprotectant as an important component. The objective of this study, therefore, was to explore the best cryoprotectant for naleh fish spermatozoa, and a total of five types were tested. These include the DMSO, Methanol, Ethanol, Glycerol, and Ethylene Glycol at a similar concentration of 10%, which were individually combined with 15% egg yolk, and every treatment was performed in three replications. Conversely, Ringer's solution was adopted as an extender, and the sperm was cryopreserved in liquid nitrogen for 15 days. The results showed significant influence on sperm motility and viability, as well as egg fertility of naleh fish ($P < 0.05$), although the DMSO provided the best outcome, compared to others at 47.17%, 50.13%, and 45.67%, respectively. Furthermore, DNA fragmentation had not occurred in the fresh and cryopreserved sperm samples, indicating the protective effect of tested cryoprotectants. It is concluded that the 10% DMSO and 15% egg yolk is the best cryoprotectant for naleh fish spermatozoa.

Keywords: Dimethylsulfoxide. Ringer. DNA fragmentation. Cryoprotectant.

RESUMO

O peixe naleh *Barbonymus* sp. é um peixe comercial de água doce, originário de Aceh, Indonésia. Durante vários anos, as perturbações provocadas no seu habitat e a pesca predatória determinaram o declínio da sua população, cuja preservação deve apoiar-se em um programa de reprodução controlada, com o emprego de espermatozoides criopreservados. O presente trabalho realizou um estudo comparativo de cinco crioprotetores: dimetilsultóxido, metanol, etanol, glicerol e etileno glicol. Todos os crioprotetores foram testados na concentração de 10%, combinados a 15% de gema de ovo. Cada tratamento foi efetuado em triplicatas. A solução de ringer foi utilizada como extensor e o esperma foi criopreservado em nitrogênio líquido por 15 dias. Os resultados obtidos revelaram a existência de influência significante ($P<0,05$) na viabilidade e motilidade espermática bem como na fertilidade dos ovos do peixe naleh, em que o dimetilsulfóxido apresentou o melhor resultado com os valores de 47,17%, 50,13% e 45,67%, respectivamente. Por outro lado, a fragmentação do DNA não ocorreu nas amostras de esperma fresco e criopreservado, indicando o efeito protetor dos crioprotetores testados. A conclusão obtida foi que o dimetilsulfóxido e 15% de gema de ovo foram o melhor crioprotetor para os espermatozoides do peixe naleh.

Palavras-chave: Dimetilsulfóxido. Solução de Ringer. Fragmentação de DNA. Crioprotetor.

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Introduction

Naleh fish *Barbonymus* sp. is one of the native and commercial freshwater fish in the Nagan Raya waters, Aceh Province, Indonesia (Muchlisin et al., 2015a), this species has potency for intensive cultivation industry (Muchlisin, 2013). The naleh fish possess morphological similarities with *B. gonionotus* (Figure 1). However, both species are known to differ at a higher genetic distance, thus presumed as cryptic species, endemic to the region (Batubara et al., 2021; Batubara, 2019a). Presently, the naleh fish is known as one of the main fishing targets of local fishermen, and overfishing has resulted in a declining wild population (Batubara et al., 2019b,). This incidence has been linked with habitat perturbation, due to deforestation and illegal gold mining, and also the introduction of alien fish species, which subject a wild population to additional pressure (Batubara et al., 2018; Muchlisin, 2008, 2013). These concerns prompt the crucial need for a naleh fish conservation program. Sperm cryopreservation is one of the alternatives ways for conservating the germplasm of the fish and is one of the important methods in fish breeding.

Figure 1 – The naleh fish *Barbonymus* sp. that occurred in Nagan Raya waters, Aceh, Indonesia (Batubara et al., 2018).

In addition, broodstock domestication is the crucial step in the fish breeding program. Presently, broodstock domestication has already been initiated by the Ichthyos Research Group, Faculty of Marine and Fisheries, Syiah Kuala University. The domesticated broodstock samples are premature and ready to spawn, as the mature variety only occurs during the early rainy season, which is similar to records from the wild population. In addition, an asynchronous maturation was detected between males and females (Batubara, 2019), hence cryopreservation is a possible method to overcome this challenge. Muchlisin et al. (2004) reported on the higher tendency to obtain sperm of better quality during the spawning season, which is then cryopreserved for use at any time of a breeding program.

Cryopreservation is a process to preserve the cells or tissues in low temperatures (-196ºC) (Agarwal, 2011). In fish, the first sperm cryopreservation was reported by Blaxter (1953) in herring *Clupea harengus*, and presently, more than 200 species of marine and freshwater fish sperm have been cryopreserved (Afriani et al., 2021; Hiemstra et al., 2005). In cryopreservation, cryoprotective agent (CPA) is known to play an important role during this process, and therefore, it must be known which type of CP is suitable for species to species, this is because the CPA is species dependent (Chao & Liao, 2001; Sarder et al., 2012). There are generally two types of CPA, intracellular (ICPA) and extracellular (ECPA) (Muchlisin, 2005; Muchlisin et al., 2020). The ICPA possesses the capacity to penetrate sperm cells and confer internal protection through the formation of ice crystals, while the latter confers an external shield from temperature shock, both during cryopreservation and thawing (Muchlisin et al., 2020). Some of the commonly used intracellular CPAs include DMSO (dimethylsulfoxide), glycerol, methanol, ethanol, and ethylene glycol, while egg yolk, honey, and glucose have been adopted as extracellular. Several studies demonstrated better output while using a combination of both types, compared to an individual application. For example, Abinawanto et al. (2013, 2018) used a combination of methanol and egg yolk for botia *Chrombotia macracanthus* sperm and Java barb *Barbonymus gonionotus*, while Muchlisin et al. (2020) used a combination of egg yolk and DMSO for depik fish *Rasbora tawarensis* spermatozoa. Currently, there is no information on the preferred combination of ICPA and ECPA for naleh fish spermatozoa. Therefore, this study is considered crucial, because every cryoprotectant has peculiar advantages and disadvantages, as each fish species requires specific types and optimal concentrations. This research involved the testing of common intracellular CPAs combined with egg

yolk to determine the best cryoprotectant for naleh fish *Barbonymus* sp. sperm.

Materials and Methods

Experimental design

This research involved a completely randomized experimental design. The tested ICPAs were DMSO, methanol, ethanol, glycerol, and ethylene glycol, each at a similar concentration of 10%. These were individually combined with 15% egg yolk, while the control treatment was sperm without CPAs. Every test was conducted with three replications.

Broodfish domestication and sperm collection

A total of 30 male and 10 female fish were sampled from Nagan Raya waters, Aceh, Indonesia using casting nets. Fish sampling was carried out at locations that were presumed to have fish based on information from local people. The sampled fish was placed in plastic bags that contained oxygen and then was transported to the laboratory in Syiah Kuala University, Banda Aceh, Indonesia. In The laboratory, the broodfish were acclimatized in a circular plastic tank (vol. 6000L) for 14 days. Also, feeding was performed to satiation with a commercial diet, two times daily. Ten late mature samples (total length 142.75 ± 1.16 cm, and body weight 10.98 ± 0.53 g) were taken at random, injected with 0.5 ml kg-1 body weight of Ovaprim, and then reserved in a different tank for 8 h. Subsequently, these fish were removed from the tank and water was cleaned from their genital pore using a tissue paper. This area of the abdomen was then subjected to gentle pressure, followed by sperm collection with a sterile syringe, which was then merged and mixed in a beaker. The beaker was placed in styrofoam with crushed ice (4°C). Thus, the fresh sperm were characterized based on pH, color, and consistency, and also for initial quality (motility and viability). Also, only sperm with minimum motility above 60% were used for cryopreservation (Muchlisin et al., 2020).

Extender preparation

The Ringer's solution used as an extender was prepared based on Muchlisin et al. (2004) as follows, 100 ml comprised of 0.75 g NaCl, 0.02 g KCL, 0.02 g $CaCl_2$, 0.02 g $NaHCO_3$. These materials were diluted in the distilled water to obtain 100 ml volume, which was then placed at 4°C in the crushed ice, before use in the experiment.

Cryoprotectant preparation

The ICPAs (DMSO, methanol, ethanol, glycerol, and ethylene glycol) were purchased from the supplier (Sigma-Aldrich), and tested at 10% concentration for each, while the egg yolk was extracted from chicken eggs purchased from a local market. A total of 100 Ringer's solutions were mixed with 5 ml sperm, resulting in 105 ml of sperm diluted at a ratio of 1: 20 (v/v, sperm: extender), followed by the addition of 10 ml egg yolk. This solution was distributed into 18 cryotubes (vol. 2 ml) with each filled with 1.5 ml solution (Ringer's + sperm + egg yolk). Then, 0.15 ml of the respective cryoprotectant was added to obtain a final concentration of 10% ICPAs and 15% egg yolk. Also, the control treatment was devoid of any ICPA, and all procedures were conducted at 4°C.

Equilibration and thawing

The equilibration process was initiated at 4°C in the crushed ice for 5 min, followed by the removal of tubes, which were placed 5 cm above liquid nitrogen (-79°C) for 5 min, and immersed subsequently, at -196°C for 15 days. This procedure is based on Muchlisin et al. (2015b), and after 14 days, the tubes were removed from the tank and thawed in a water bath (30°C) for 5 min. These sperm samples were then analyzed for quality, motility, viability, fertilization, and DNA integrity.

Motility analysis

One drop of cryopreserved sperm was activated using one drop of tap water on an object glass. This sample was then observed under a stereomicroscope, which was connected to a CCD camera, and monitored for live activity on the computer (400X magnification), as the motion was recorded as a videotape. Therefore, motility was calculated based on 50 randomly chosen spermatozoa previously recorded, which was then compared with the examination for fresh sperm (Muchlisin et al., 2020).

Viability analysis

One drop of cryopreserved sperm was mixed with one drop of 0.2% eosin dye on the glass object as a smear sample. This was then observed under a stereomicroscope at 400 magnification. Life was indicated by the presence of clarity and transparency, while rose-colored substance denoted death (Ax et al., 2000; Maulana et al., 2014). Therefore, the viability is calculated based on 200 randomly chosen spermatozoa, which was then compared with the examination for fresh sperm.

Eggs collection and fertility analysis

The eggs were collected from two late mature female fish by applying gentle pressure from the abdomen to the genital pore. The eggs were batched into a plastic basin and kept in crushed ice (4°C). A total of 0.25 ml thawed diluted sperm (approximately 0.68 x 109 sperm cells ml-1) was mixed with 1 ml of eggs (approximately 1,483 eggs), and then two drops of tap water were added and mixed homogeneously. This combination was allowed contact for 5 min, to ensure egg fertilization. Therefore, a total of 100 eggs were taken at random and incubated in the plastic jars at a water temperature of 27°C. A successful outcome is discernible at 2 h post-fertilization, based on the clear and transparent presentation of eggs, while opacity was used as an indicator for failure (Muchlisin et al., 2015b; Muthmainnah et al., 2018). The fertility rate was calculated as follows: Fertilization rate (%) = total of fertilized eggs/total of incubated eggs x 100.

DNA extraction

The DNA was extracted using the Genomic DNA Purification Kit (Promega, Malaysia), following the manufacturer's protocol. Approximately 100 µl of pellet sperm tissue was transferred into a sterile 1.5 ml tube, followed by the addition of 300 µl cell lysis solution, and then the sperm was vortexed for 30 sec. This mixture was subsequently incubated at room temperature for 10 min and centrifuged at 15,000 rpm for 20 sec. The supernatant was discarded and 100 µl of nuclei lysis solution was added into the pellet before a re-vortex, which resulted in the lysate. A total of 35 µl protein precipitation solution (RNase Solution) was then added before another round of re-vortex for 20 sec, followed by centrifugation at 15,000 rpm for 3 min. The supernatant was removed and placed into a new tube, and then 100 µl of isopropanol was added and centrifuged at 15,000 rpm for 1 min. The resulting supernatant was discarded and an equal volume of 70% ethanol was added into the pellet before subjecting to another recentrifugation for 1 min. The liquid was discarded and the pellet dried at room temperature for 15 min. This was followed by hydration with 35 µl DNA rehydration solution, which was then incubated at 65°C for 1 h, and the resulting medium was reserved at 2°C before use in electrophoresis.

Electrophoresis

A total of 2 g agarose was mixed with 100 ml of 1X TBE buffer, which was then heated with a microwave at 250°C for 2 min. Subsequently, 2 µl of DNA dye was added and cast for 30 min, followed by the removal of agar gel into the electrophoresis batch with a TBE buffer solution. A total of 10 µl DNA samples with 2 µl loading dye were inserted into the wells, while about 5 µl DNA marker was introduced into different wells. The process of electrophoresis was conducted on a 0.8% agarose gel at 135 Volts for 30 min, and on termination, the agar gel was placed onto the E-BOX VX2 gel documentation system to record the band image.

Data analysis

Data on sperm motility, viability, and fertility were subjected to a one-way ANOVA test, which was followed by Duncan's multiple range test at 95% confidence level, using the SPSS Ver. 20.0. Information obtained concerning pH, color, sperm consistency, and DNA integrity was descriptively analyzed.

Results

The fresh sperm possessed milky-white coloration, with pH 6.4, and sperm density of 3.1 x 10^9 cells ml^{-1}. Also, 74.67% sperm motility, 77.03% viability, and 72.67% fertility were recorded, and assumed to be significantly affected by the introduction of cryoprotectants, based on the ANOVA test ($P<0.05$). After 15 days of cryopreservation, the highest values of sperm motility, sperm viability, and fertility were recorded in samples with DMSO, at 47.17%, 50.13%, and 45.67%, respectively (Table 1), which were significantly different from methanol, ethanol, glycerol and ethylene glycol. However, lower values were recorded in the various cryoprotectants after treatment. Moreover, the analysis of

Table 1 – Sperm motility, viability, and fertility of naleh fish *Barbonymus* sp. after 15 days of cryopreservation using several types of cryoprotectants i.e. DMSO, methanol, ethanol, glycerol, and ethyl glycol. The experiment was performed with three replications. The mean values (±*SD*) with different superscript (a - f) in the same column are significantly different ($p < 0.05$).

Treatment	Motility (%)	Viability (%)	Fertility (%)
15% egg yolk (control)	38.17 ± 0.28^{c}	42.00 ± 0.26^{c}	36.33 ± 0.57^{c}
10% DMSO + 15% egg yolk	47.17 ± 0.28^{a}	50.13 ± 0.45^{a}	45.67 ± 0.57^{a}
10% Methanol + 15% egg yolk	44.83 ± 0.28^{b}	49.10 ± 0.34^{b}	42.67 ± 0.57^{b}
10% Ethanol + 15% egg yolk	36.83 ± 0.28^{d}	39.03 ± 0.58^{e}	35.00 ± 1.00^{d}
10% Glycerol + 15% egg yolk	30.17 ± 0.28^{f}	33.83 ± 0.47^{f}	25.67 ± 0.57^{e}
10% Ethyl Glycol + 15% egg yolk	35.83 ± 0.28^{e}	40.37 ± 0.23^{d}	34.00 ± 1.00^{d}

DNA integrity showed the detection of no smear bands in all treatments, including the fresh samples, which indicates that the DNA fragmentation was not present in fresh and cryopreserved sperms (Figure 2).

Discussion

The fresh sperm of naleh was of good quality, based on the milky-white coloration, pH of 6.4, 74.61% motility, the viability of 77.03%, and fertility of 72.67%. This outcome was congruent with Melo & Godinho (2006), which reported on the characteristic milky-white color, slightly acidic pH of between pH 5.0-7.0 (Setyono, 2009), and the initial motility of above 70% (Christensen & Tiersch, 2005). The study revealed a reduction in sperm quality after treatment, although significantly higher values were reported with the DMSO cryoprotectant. Hence, this is the basis for its selection as the best for naleh fish sperm.

DMSO was also suitable for the sperm of depik fish *Rasbora tawarensis* (Muchlisin et al., 2020), seurukan fish *Osteochillus vittatus* (Muthmainnah et al., 2018), African catfish *Clarias gariepinus* (Omitogun et al., 2010), *Mugil cephalus* (Balamurugan et al., 2019), *Barbus grypus* (Dogu, 2012), common carp *Cyprinus carpio* (Irawan et al., 2010), and mahseers *Tor tambroides* and *T. dauronensis* (Chew et al., 2010). However, the concentration of DMSO used is species-dependent. For example, the best dilution for *R. tawarensis* was 5% (Muchlisin et al., 2020), 10% for *C. carpio* (Irawan et al., 2010), and 10% for *B. grypus* sperm (Dogu, 2012). This study showed the use of a single concentration across all CPAs. Further investigations need to determine the best concentration of DMSO specific to naleh fish spermatozoa.

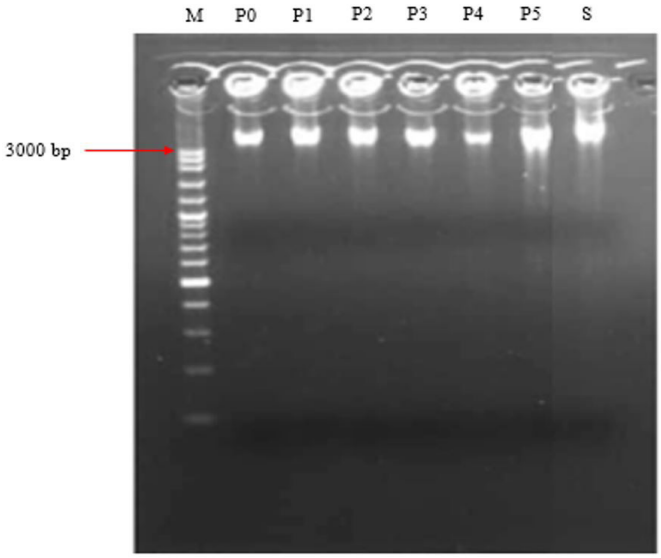

Figure 2 – The electrophoresis analysis of fresh and cryopreserved sperm of naleh fish *Barbonymus* sp. M=marker; P0=15 egg yolk (control); P1=DMSO; P2=methanol; P3=ethanol; P4=glycerol; P5=ethyl glycol, S= fresh sperm.

Leibo & Pool (2011) observed that DMSO is a neutral solution, which acts as an intracellular CPA. This is known to protect against temperature shocks within the sperm cell during the freezing and thawing process. Meanwhile, the egg yolk confers external shields during cryopreservation, particularly from the loss or entry of fluids, which often results from differences in osmotic pressure on storage (Bozkurt et al., 2014; Laeni et al., 2020; Muchlisin, 2005). This phenomenon occurs because of the low-density lipoprotein content, which associates easily with the cell membrane, subsequently providing a protective mechanism (Babiak et al., 2002). Therefore, the egg yolk is commonly combined with other intracellular cryoprotectants.

Temperature shock triggers damage to cell membranes and intracellular organelles, as well as changes in permeability and the lipid components of the membrane. This causes a reduction in flagella activity, leading to a decline in sperm motility and viability (Ogbuewu et al., 2010). Gazali & Tambing (2002) reported on the tendency for the freezing process to form ice crystals with the capacity to physically injure sperm cells. Furthermore, there is also an increase in the amount of intracellular enzyme released, leading to the damage of lysosomes and mitochondria.

An interesting finding was recorded in sperm cryopreserved with egg yolk without ICPA, which showed higher motility, viability, and fertility, compared to the combination with ethanol, glycerol, and ethylene glycol. This outcome probably results from the toxic effect of these ICPAs at the concentration of use, which is higher than DMSO and methanol. Therefore, the sperm quality after cryopreservation was reduced, making them unsuitable, according to Muchlisin & Azizah (2009), due to the detrimental effect on the sperm cells of the tropical river catfish, *Mystus nemurus*.

Cryoprotectants play a crucial role in this preservation process, although toxicity has been recorded at higher concentrations, which possibly leads to sperm death. For example, the use of 15% methanol, N, N-dimethyl acetamide (DMA), and DMSO produced higher toxicity on Zebrafish *Danio rerio* than the 10% and 5% treatments. However, each cryoprotectant has a unique lethal level at the same concentration, e.g., glycerol was more toxic than DMA and DMSO on zebrafish sperm (Yang et al., 2007; Yang & Tiersch, 2009). Saragusty & Arav (2011) found that these effects were capable of disrupting the permeability of the

cell membrane, alongside changes in osmotic pressure, and ultimately damage sperm DNA.

The analysis of DNA fragmentation showed the absence of any smear band in the treatments and fresh sperm samples. This indicates similarity in DNA quality, as the DNA was unchanged during the cryopreservation process. This finding confirms the ability of all cryoprotectants tested to confer protective effects on DNA integrity. Bhattacharya (2018) proposed the capacity to ensure slow freezing as the damage preventive measure. Also, DNA fractionation during the process of cryopreservation possibly occurs because of increased calcium concentrations within the cells, which stimulate endonuclease activity. This leads to the formation of reactive oxygen species (ROS), subsequently elevating the incidence of peroxidation, and the formation of genotoxic components, which cause damage to inherent bonds (Cabrita et al., 2005; Leibo & Pool, 2011).

Besides, damage to the DNA does not always affect motility and fertility, as seen in brown-marbled grouper, *Epinephelus fuscoguttatus* (Yusoff et al., 2018). Upadhya et al. (2010) found that the affected sperm with good motility possess the capacity to fertilize an egg, followed by the development of potentially damaged genomes, and, consequently, causing larvae abnormality. Horvath & Urbanyi (2001) recorded higher amounts of abnormal larvae in eggs fertilized using cryopreserved sperm, of which some were haploid (Miskolczi et al., 2005). This particular outcome was, however, not observed in the current study.

Conclusion

We found that cryoprotectants significantly affect the motility, viability, and fertility of cryopreserved sperm, and no DNA fragmentation was detected in fresh and cryopreserved sperm. Also, a combination of 10% DMSO with 15% egg yolk was the best cryoprotectant for naleh fish sperm.

Conflict of Interest

No conflict of interest was declared.

Ethics Statement

The study complies with the guidelines of ethical animal use in research of Syiah Kuala University (Kode Etik Penelitian Universitas Syiah Kuala Tahun 2015).

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