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# The effect of cryoprotectant on the viability and motility of goldfish *Carassius auratus* sperm after 48 hours refrigerated

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ABSTRACT

The principle of sperm preservation is to prolong the viability of spermatozoa by reducing the degree of metabolism during storage at low temperatures, and one method involved was a cryoprotectant as a sperm protector. This study aimed to determine the best type of cryoprotectant for refrigeration storage of goldfish *Carrasius auratus* sperm. A completely randomized design was used, and five types of cryoprotectant were tested, namely Methanol, Ethanol, Ethylene Glycol, Glycerol, and DMSO at a 10% concentration combined with 15% egg yolks. The sperm was diluted with Ringer's solution and stored at 4ºC for 48 hours. The ANOVA test revealed that cryoprotectant significantly affected the motility and viability of sperm after 48 hours of refrigeration ($P<0.05$). The results showed that 10% DMSO combined with 15% egg yolk could maintain sperm motility and viability better than other treatments, with values of 85.80% and 86.50%, respectively, which were significantly different from the other treatments. Glycerol produced the second-best sperm quality with motility and viability values of 71.13% and 74.13%, respectively. Therefore, DMSO is the most effective cryoprotectant for refrigeration storage of goldfish sperm.

## Introduction

Blaxter (1953) was the first researcher studied the sperm preservation on herring fish *Clupea harengus* sperm with carbon dioxide and 15% glycerol at -79ºC, this technique was previously developed by Polge and Rowson (1952) for bull sperm. Presently, many species of freshwater and marine fish are being studied for sperm storage techniques (Afriani *et al.,* 2021). The principle of low-temperature sperm preservation is to maintain and extend the life span of spermatozoa by reducing the metabolic rate of sperm cells during storage (Lemma, 2011).

The main obstacle of sperm preservation is spermatozoa damage due to temperature shock, particularly at freezing temperatures. To overcome this problem, sperm diluents must be added to cryoprotectant (Bhattacharya *et al.,* 2018). Cryoprotectant is chemical compounds that prevent cell damage caused by the freezing process (Bhattacharya and Prajapati, 2016), primarily due to the formation of micro ice crystals in sperm cells and the diluent (Shaluei *et al.,* 2013). Therefore, the application of a suitable cryoprotectant is crucial to minimize mechanical cell damage as the temperature decreases (Tambing *et al.*, 2000; Kurniawan *et al.,* 2013; Bhattacharya, 2018).

Cryoprotectant is classified as intracellular and extracellular. Intracellular cryoprotectant has a small molecular weight and size that allows them to pass through the permeable sperm cell membrane (Tiersch, 2011; Maulida *et al.,* 2020) and protect the cell by preventing the formation of micro ice crystals. Meanwhile, extracellular cryoprotectant has

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a large molecular weight and size that prevents it from passing through the sperm cell membrane, thereby protecting the cell from outside, particularly from temperature shock and the toxic effects of diluent and cryoprotectant (De-Andrade *et al.,* 2014; Muchlisin *et al.,* 2015a; Muchlisin *et al.,* 2015b; Maulida *et al.,* 2020). The commonly used intracellular cryoprotectant includes Dimethyl sulfoxide (DMSO), Glycerol, Methanol, Ethanol, and Ethylene Glycol (Abinawanto *et al.,* 2012; Abinawanto *et al.,* 2013; Muchlisin *et al.,* 2015a; Maulida *et al.,* 2021). Meanwhile, the popular extracellular cryoprotectant includes honey, glucose, skim milk, egg yolk, coconut water, soybean milk, and lactose (Abinawanto *et al.,* 2013; Bozkurt *et al.*, 2014; Eriani *et al.,* 2017; Muchlisin *et al.,* 2020; Abinawanto *et al.*, 2021).

The goldfish has attractive color patterns and fin shapes, and requires minimal maintenance, making it a popular ornamental fish in Indonesia and worldwide (Rosid *et al.,* 2019; Izzah *et al.,* 2020). The beautiful color and fins were commonly produced by cross-breeding (Kumar *et al.,* 2017; Dhewantara *et al.,* 2018) through artificial or semi-artificial methods. Artificially induced breeding involves collecting sperm and eggs from the broodfish and fertilizing them in a separate container using dry or wet methods. The sperm is then diluted with an extender and stored at a cold temperature (4°C). Due to its short life span, the fertilization process must be completed quickly to avoid a reduction in sperm quality.

Therefore, a cryoprotectant is added to the diluent to prolong the life span of the sperm. It is typically added in frozen storage or cryopreservation, while it is not commonly used in non-frozen storage (refrigeration) because micro-ice crystals do not form in sperm cells and diluents. In this study, extracellular (egg yolks) and intracellular cryoprotectants were combined and used in refrigeration storage temperature. This study aimed to analyze the role of cryoprotectant in refrigeration storage of the goldfish sperm and to determine the most suitable for short-term sperm preservation at refrigeration temperature (4°C).

## Materials and Methods

### Time and location

This study was conducted at the Marine Biology Laboratory, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Banda Aceh, from January to March 2022.

### Experimental design

A completely randomized design was used in this study. Five types of cryoprotectant were tested, namely Methanol, Ethanol, Ethyl Glycol, Glycerol, and DMSO at a 10% concentration in combination with 15% egg yolks. Each treatment was replicated three times. Furthermore, Ringer's solution was used to dilute the sperm and prepared based on a method by Muchlisin *et al.* (2004). The tested treatment was modified by Abinawanto *et al.* (2013) as follows:

T1: Sperm + Ringer's + 15% egg yolk (without cryoprotectant)
T2: Sperm + Ringer's + 15% egg yolk + 10% methanol
T3: Sperm + Ringer's + 15% egg yolk + 10% ethanol
T4: Sperm + Ringer's + 15% egg yolk + 10% Ethylene glycol
T5: Sperm + Ringer's + 15% egg yolk + 10% Glycerol
T6: Sperm + Ringer's + 15% egg yolk + 10% DMSO

### Broodfish preparation and sperm collection

A total of 10 male and 20 female broodfish were obtained from suppliers in Banda Aceh and allowed to acclimatize for 14 days in a fiber circular tank. The fish were fed a commercial diet (30% crude protein, and 3% crude lipid) twice daily at 08.00 AM and 6.00 PM ad libitum.

A total of three male goldfish were selected from the brood tank and injected with 0.3 ml/kg Ovaprim to stimulate sperm maturation. After 14 hours, sperm was collected by cleaning the genital area with a dry tissue to avoid contaminating with feces and water. The abdomen of the fish was gently pressed to the genital pore, and the expelled sperm was collected using a syringe and then placed in an ice box containing crushed ice (4°C).

### Extender and cryoprotectant preparation

The Ringer's solution was used as an extender, and 100 ml of the solution was prepared with 0.75 g NaCl, 0.02 g KCL, 0.2 g CaCl2, and 0.02 g NaHCO3 (Muchlisin *et al.,* 2004). The cryoprotectant tested was methanol, ethanol, ethylene glycol, glycerol, and DMSO at 10% concentration combined with 15% egg yolk. The egg yolk was prepared by washing the egg using the tap water, and then cleaning the shell using 70% alcohol. The eggshell was cracked and the albumin from the egg was separated. The egg yolk was then separated from the vitelline membrane in a cup using filter paper.

To obtain a 10% cryoprotectant test concentration, 2 ml of sperm was mixed with 120 ml of Ringer's (1:60, v/v), yielding 122 ml of sperm dilution. Subsequently, 18 ml of egg yolk was added to the sperm dilution to produce 140 ml of sperm solution with a 15% egg yolk concentration. The diluted sperm and egg yolk mixture were distributed into 18 cryotubes, with every cryoprotectant having three tubes, and each tube containing 1.25 ml of diluted sperm. Furthermore, 125 microlites of each test cryoprotectant were added to the tubes. The cryotubes were then placed in a refrigerator at 4°C for 48 hours.

**Viability and motility analysis**

The motility rate was measured by dripping 1-2 drops of fresh or preserved sperm onto a glass slide and then activating the sperm with 2 drops of tap water. The glass slide was then covered with a cover glass and observed under a stereo microscope (Zeiss Primo Star) at 400X magnification. According to Muchlisin *et al.* (2004), motility was calculated by randomly selecting at least 200 spermatozoa in each treatment and counting only forward-moving sperm as motile.

For the sperm viability analysis, 1-2 drops of fresh and preserved sperm were placed on the glass slide, followed by 1 drop of 0.2% eosin, and then flattened or smeared. The glass slide was then observed under a stereo microscope with 400X magnification. Sperm viability was calculated in five fields of view on average. Live sperm had spherical and transparent heads, while dead sperm had opaque pink heads due to absorbed eosin and an irregular-shaped head (Maulana *et al.,* 2014; Maulida *et al.,* 2022).

**Data analysis**

The qualitative data, which included pH, color, and consistency of sperm were presented in a table and then descriptively analyzed. Meanwhile, the motility and viability rates were subjected to a normality test, followed by an analysis of variance (ANOVA). When there is a significant effect, the data was subjected to Duncan's multi-range test at a 95% confidence level. These analyses were performed using SPSS software version 21.0.

## Results

The results showed that the fresh sperm of goldfish had a motility value of 87.5%, viability of 89%, pH 6.0 - 6.5, milky white color, and medium consistency (Table 1). Sperm morphology showed that the head of the sperm that absorbs eosin indicates dead sperm, while the white and transparent head indicates live sperm (Figure 1).

The ANOVA test revealed that cryoprotectant had a significant effect on the motility and viability of sperm after 48 hours post preservation at 4°C ($P<0.05$). DMSO produced the highest motility rate (85.80%), which was significantly different from other treatments, including controls. This was followed by Glycerol with a motility rate of 73.13%, which was significantly different from ethanol, methanol, and controls. DMSO also had the highest sperm viability (86.50%), which was significantly different from all other treatments. It was then followed by glycerol (74.13 ± 0.20%), which was not significantly different from Ethylene glycol. Meanwhile, the lowest sperm motility and viability were recorded in the control treatment (without cryoprotectant) but were not significantly different from the Methanol and Ethanol treatment (Table 2).

**Table 1.** The characteristic of fresh sperm of goldfish *Carassius auratus.*

| No. | Parameters | Description |
|---|---|---|
| 1. | Average volume | 0.33 ml |
| 2. | Colour | Milky white |
| 3. | pH | 6.0-6.5 |
| 4. | Consistency | Moderate |
| 5. | Sperm density (cells/ml) | $175.3x\ 10^6$ |
| 6. | Motility (%) | 87.46 ± 1.00 |
| 7. | Viability (%) | 88.80 ± 0.72 |
| 8. | Life span (seconds) | 217 |

**Table 2.** The motility and viability of goldfish sperm *Carassius auratus* post refrigerated at 4 °C for 48 hours. Mean value±SD at the same column with different superscripts are significantly different ($P<0.05$).

| Treatment | Motility (%) | Viability (%) |
|---|---|---|
| Control | $69.96 \pm 0.68^{a}$ | $71.33 \pm 0.61^{a}$ |
| Methanol | $71.20 \pm 0.72^{ab}$ | $72.46 \pm 0.94^{a}$ |
| Ethanol | $71.30 \pm 1.15^{ab}$ | $72.36 \pm 1.25^{a}$ |
| Ethylene Glycol | $72.10 \pm 0.36^{bc}$ | $73.90 \pm 0.62^{b}$ |
| Glycerol | $73.13 \pm 0.90^{c}$ | $74.13 \pm 0.20^{b}$ |
| DMSO | $85.80 \pm 0.52^{d}$ | $86.50 \pm 0.70^{c}$ |

## Discussion

The macroscopic and microscopic analysis showed that the fresh sperm of goldfish was of good quality and suitable for preservation. According to several studies, sperm used for preservation must have motility above 70% and be slightly acidic (Tiersch, 2011; Mangkunegara *et al.,* 2019; Maulida *et al.,* 2020). This is because the success of sperm preservation is strongly determined by the quality of fresh sperm before preservation (Horokhovatskyi *et al.,* 2018).

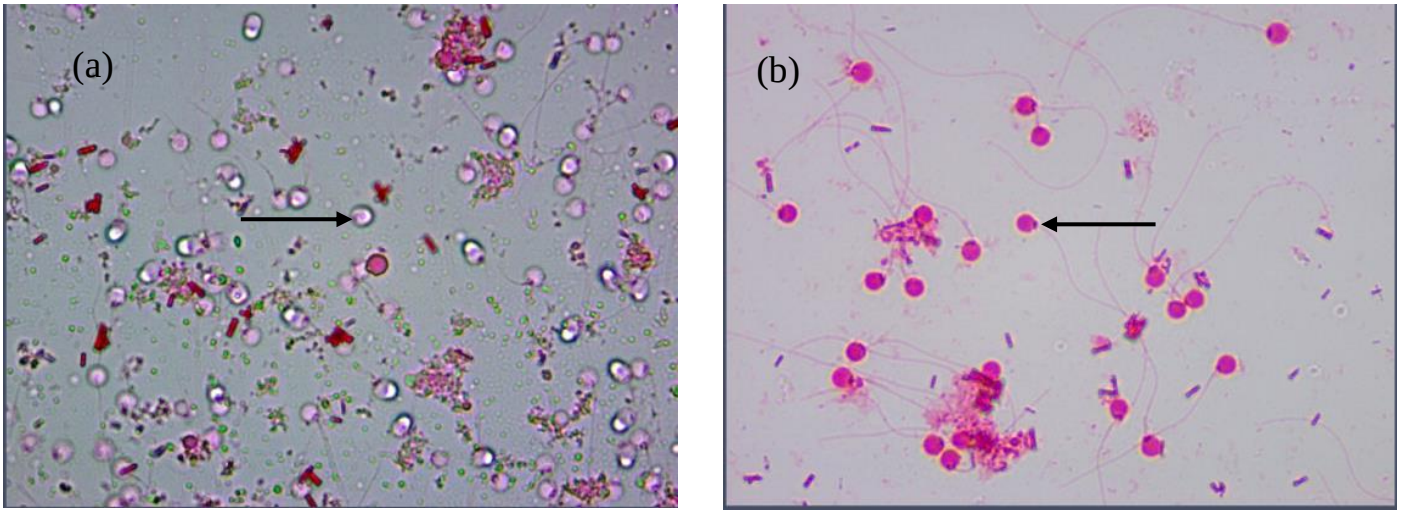


**Figure 1.** The live sperm (a), and dead sperm (b) of goldfish *Carassius auratus* refrigerated at 4°C for 48 hours.

The results showed that sperm motility after refrigeration using the cryoprotectant was better than without. Furthermore, all cryoprotectant treatments produced sperm motility above 70%, while treatments without it was below this value, and DMSO producing the best result. These results proved that apart from being crucial in cryopreservation, cryoprotectant also plays an important role in non-frozen sperm preservation (refrigeration).

The effectiveness of DMSO as a cryoprotectant has been reported in several studies. Maulida *et al.* (2021) reported that it was effective for preserving naleh fish *Barbonymus* sp. sperm. This compound was also suitable for bocachico *Prochilodus magdalena* (Martinez *et al.,* 2012), and kawan fish *Poropuntius tawarensis* (Muthmainnah *et al.,* 2019). The effectiveness of DMSO may be due to its lightweight molecule (78.13 g/mol) (Hine *et al.,* 2019), which makes it easier to enter the cell and protect from within quickly and effectively. It is a common intracellular cryoprotectant that has been widely used in fish sperm preservation (Muchlisin *et al.,* 2020). According to Shahverdi *et al.* (2018), DMSO can increase fluid viscosity and prevent the formation of micro ice crystals in cells, and it can enter into cells quickly, allowing the cell to achieve equilibrium with solutions outside the cells (isotonic).

In addition, glycerol was also quite effective in protecting sperm cells with slightly lower quality than DMSO. This result was similar to Abinawanto *et al.* (2017) which stated that glycerol was suitable for preserving gourami sperm for two days at 4°C. Glycerol was also suitable for cryopreservation of grouper *Epinephelus akaara* sperm (Ahn *et al.,* 2018). It has the advantage by retaining water within the cell and reducing the concentration of intracellular electrolytes and damage to spermatozoa (Siddiqui *et al.*, 2016). It can also be used by spermatozoa as an energy source (Ihsan, 2013).

The control treatment, which only contained 15% yolk and no intracellular cryoprotectant, was still able to maintain the sperm motility of goldfish above 50%, despite being statistically no different from the Methanol and Ethanol treatments. Muchlisin (2005) stated that egg yolk acts as an external (extracellular) protector for sperm cells. Egg yolk protein and lecithin function to protect sperm from damage caused by temperature shock by maintaining the integrity of lipoproteins in the plasma membrane of spermatozoa (Zambrowicz *et al.*, 2014; Sunwoo and Gujral 2015). Therefore, the use of intracellular and extracellular cryoprotectants provides optimal protection as recorded in this study, the similar finding was also reported by several researchers (Muthmainnah *et al.*, 2018; Muchlisin *et al.*, 2020; Maulida *et al.*, 2021).

## Conclusion

Cryoprotectant had a significant effect on the sperm quality of goldfish after 48 hours of refrigerated preservation. Furthermore, the use of cryoprotectant produced better results than without using it, and DMSO being the most suitable cryoprotectant for refrigeration storage of goldfish *Carassius auratus* sperm. Therefore, the cryoprotectant is also needed to maintain sperm quality in fish sperm refrigerated preservation.

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