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*Short Communication*

# Cryoprotectants for cryopreservation of spermatozoa of tropical oyster *Magallana bilineata* (Röding, 1798)♣

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**Abstract**

Cryopreservation is a freezing method specific for cells such as gametes and tissues in a sub-zero temperature. The role of cryoprotectant (cryoprotective agent) is essential to reduce the cryoinjury and retain the original biological function of gametes during cryopreservation. This study looked into the effectiveness of four types of cryoprotectants (dimethyl sulfoxide, sucrose, egg yolk and honey) at four concentrations (5%, 10%, 15%, and 20%) on the spermatozoa of tropical oyster, *Magallana bilineata*. Sperm exposed to DMSO 10% underwent freezing has recorded the highest fertilization rate, which was significantly higher than other treatments but showed no significant differences with the control (fresh sperm). The usage of sucrose is less effective compared to DMSO, but showed no significant differences with egg yolk. This study is important to determine the best cryoprotectant to be used for spermatozoa cryopreservation of *M. bilineata*, to provide a solution to oyster hatchery operators to secure all year-round spawning using cryopreserved sperms.

**Keywords**: cryopreservation, spermatozoa, fertilization rate, tropical oysters

## 1. Introduction

Tropical oysters spawn year round, triggered by the onset of rainy season. In Malaysia, rainy seasons vary amongst regions, therefore non-synchronized spawning occurs. The non-synchronized conditions affect eggs development, which results in spawning of eggs with variable maturity levels. The chances of fertilization and larval development greatly depend on the egg maturity and sperm quality (Paniagua-Chavez, Buchanan, Supan, & Tiersch, 2000; Paniagua-Chavez & Tiersch, 2001; Smith *et al.*, 2001). Coupled with predicted ocean acidification events, larval stages will not be able to develop normally, hence the livelihood and services surrounding the oyster industry will be jeopardized. Establishing a constant seed supply through cryopreservation on top of regulating physical and biological conditions at the culture area throughout the year might help to overcome losses to aquaculture production. Cryopreservation is the use of very low temperature to preserve structurally intact living cells and tissues (Jang *et al.*, 2017; Pegg, 2015).

Cryopreservation studies on oysters suggest that preservation of early larval stages are more tolerant of the cryoprotectants used and could yield higher larval survival compared to cryopreserved embryos, eggs or sperm.

However, freezing of cells in cryopreservation without any protection will cause cell mortality due to the cellular damage from the formation of intracellular ice crystals during the freezing process and the solution effect which causes cell dehydration (Bhattacharya, 2018). The cryoinjury of cryopreserved cells can be overcome by the presence of

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certain type molecules which act as an agent to protect the cells at low temperature, they are define as cryoprotectants (Best, 2015; Pegg, 2007).

Glycerol has been proven to be one of the first cryoprotectants used successfully in protecting freezing fowl spermatozoa (Polge, Smith, & Parkes, 1949). Dimethyl sulfoxide (DMSO) is considered the universal cryoprotectant for sperm of most species as it yields the highest fertilization rate of thawed sperm (Hassan, Qin, & Li, 2015; Muchlisin *et al.*, 2015). Yet, high concentrations of cryoprotectants are toxic and resulted in sperm mortality (Best, 2015; Muchlisin *et al.*, 2015). Cryoprotectant toxicity had become a limitation to a successful cryopreservation, hence many strategies have been pursued to solve this problem, such as optimize cooling rates during freezing or to optimize the time in adding the cryoprotectant during sperm cooling. However, none of the strategies was proven to be adequate (Best, 2015).

This study has provided an economical, affordable and effective method to cryopreserve the spermatozoa of tropical oyster, *Magallana bilineata*. By storing the frozen sperm in cryovials, placing them in a straw over liquid nitrogen before plunging them into sub-zero bath, the method can be accomplished by the local hatcheries and culture centre to conduct cryopreservation of tropical oysters regularly, especially during the raining seasons, where unsynchronized spawning would occurred.

Due to the limitation of current available studies about tropical oyster cryopreservation, this study will explore the suitability of cryopreservation and cryoprotectant on the spermatozoa of the commercial tropical oyster species, *M. bilineata*.

## 2. Materials and Methods

### 2.1 Oyster

The tropical oyster were collected from an oyster farm in Sungai Merbok, is a mangrove-fringed estuary situated in Kedah state, in Malaysia. Adult oysters with shell length of 6-8 cm were selected and brought back to School of Biological Sciences, Universiti Sains Malaysia. Oysters were cleaned to avoid any contamination. In this study, only gametes from matured oysters with ripe gonads, cream-colored and opaque, covering most of the visceral mass were used.

### 2.2 Gamete collection

Sperm collection was done using the stripping techniques because of suitability for a large volume sperm collection (Adam *et al.*, 2004; Hassan *et al.*, 2015). A total of 100 mL of 25 ppt seawater that had been filtered through 1μm pores was used to suspend the gametes before diluting with extender. The concentrated sperm suspension was sieved through 65 μm, 35 μm, followed by 20 μm sieve to separate the chunks of gonad tissue from the sperm. A hemocytometer was used to estimate the concentration of sperm. The final sperm concentration in every cryovial (2 mL) is expected to be around 25 million sperms, where 100 mL of the initial concentrated sperm was mixed with 500 mL of extender for a 1:5 ratio dilution. A range of dilution ratio has been used in cryopreservation and studies have shown that low motility of sperm is observed at higher dilution (Hassan *et al.*, 2015). Hence, a lower dilution ratio of sperm and extender, 1:5 was used in this study. The mixture was stirred using a glass rod to achieve an equal dispersion of sperm in the diluted suspension. The sperm suspension was left to condition for 10 minutes before being mixed with cryoprotectants prior to cooling.

Oocytes from the mature oysters were collected using the same method as the sperm, which was via strip spawning. The oocytes were sieved through a 65 μm and 48 μm sieves to separate the chunks of gonad tissues and remove the debris, followed by a 20 μm sieve to collect the retained oocytes. The oocytes were suspended in 25 ppt seawater that had been filtered through 1 μm pores (Liu & Li, 2015) and leave to condition for 10 min before being used to fertilize with cryopreserved sperms.

#### 2.2.1 Extender

An extender is liquid diluents, which contains salts, osmotic agents, pH buffers and sugars that used to suspend sperm during the preparation of mixture for cryopreservation. The extender was prepared based on Fish-Ringer Extender (FRE) formula, which consists of sodium chloride, NaCl (0.75 g), potassium chloride, KCl (0.10 g), calcium chloride, $CaCl_2$ (0.016 g), magnesium sulfate, $MgSO_4$ (0.023 g), monosodium phosphate, $NaH_2PO_4$ (0.041 g), and glucose (0.10 g) per 100 mL. The presence of phosphate in the mixture adjusts the pH to about 7.0 and inhibits the lethal damage to erythrocytes (Carleton, 1957). Neutral buffered formalin is a hypotonic solution in buffer ions and has a pH value within a range of 6.8 to 7.0.

### 2.3 Cryoprotectants

Four types of cryoprotectants were used in this experiment, namely Dimethyl sulfoxide (DMSO), sucrose, egg yolk and honey. For each cryoprotectant, four concentrations were examined, with distilled water being used to prepare the varied concentration of 5%, 10%, 15%, and 20%. DMSO and sucrose are classified as additive cryoprotectants while egg yolk and honey as natural cryoprotectants. A total of 100 μL sperm was pipetted into each cryovials filled with 100 μL cryoprotectants prior to freezing, a 1:1 ratio was used. These were the optimum dilution showing most effective cryopreservation of oyster sperms and to standardize with other research for comparison (Dong, Rodenburg, Hill, & VandeVoort, 2011; Paniagua-Chavez & Tiersch, 2001; Parades *et al.*, 2012; Tervit *et al.*, 2005).

#### 2.3.1 Cryopreservation process

Four types of cryoprotectants, DMSO, sucrose, egg yolk and honey at four concentrations (5%, 10%, 15%, and 20%) were tested within 24 hours freezing durations by calculating the fertilized eggs by the cryopreserved sperms. Four replicates for each treatment and concentrations were done in this experiment. Filtered seawater, 25 ppt was used as the control in place of cryoprotectant. The cryovial containing sperm and cryoprotectant mixture was left to equilibrate for 10 min before freezing process (Urbanyi, Horvath, Varga, &

Hirvath, 1999). Liquid nitrogen method was used for freezing of oyster sperm. The cryovials were held in a cryobox and stacked in a metal rack, fastened and placed 5 cm above the liquid nitrogen surface for 10 min before being immersed in liquid nitrogen for 24 hours. After storage for 24 hours in liquid nitrogen, the frozen sperm samples were promptly thawed at 40 °C in a water bath (Urbanyi *et al.*, 1999) until the solid state of gametes samples had melted to liquid state. This process will take approximately 10 to 15 seconds. A total of four control replicates were left at room temperature to undergo fertilization. The control for this study was prepared by directly fertilizing fresh oyster sperm and oocytes in the cryovials and left at room temperature.

### 2.3.2 Fertilization of cryopreserved sperm and fresh oocytes

Fertilization was conducted in cryovials, with 1 mL of 1 μm filtered seawater and 15 μL of 0.25 M sucrose (sucrose provides energy to reactivate the sperms). An approximate of 200 fresh oocytes was pipetted in each cryovials for fertilization to take place with the cryopreserved sperms at room temperature. The sperm to egg ratio was about 400 to 500 sperm per egg. The fertilization was monitored under the microscope every 15 min for 2 hours, until one or two polar bodies were observed in the fertilized eggs. Two drops of 10% neutral buffered formalin were added in each vial to pause the fertilization once the calculations started to synchronize all the development of fertilized eggs. The presence of at least one polar body in the oocyte was considered as fertilized eggs, and the percentage based on the total eggs was used to calculate fertilization rate.

### 2.3.3 Statistical analysis

The fertilized eggs were counted using a microscope (Digi Sense *i*1300). The data was arcsine square root transformed before being used for ANOVA analysis with the Statistical Package for the Social Sciences (SPSS) version 20.0. One-way ANOVA was conducted to compare the effects if different cryoprotectant treatments on frozen sperms' fertilization ability. Tukey's post hoc test was used to identify the significant differences between all treatments. The end results are displayed as average percentage fertilized and standard deviation from experiments with different cryoprotectant.

## 3. Results

Figure 1 shows the fertilization rate of cryopreserved sperms using different cryoprotectants and concentrations for the oyster species *Magallana bilineata* compared to the control. DMSO seemed to be the best cryoprotectant compared to the other three cryoprotectants (sucrose, egg yolk and honey). Frozen sperms in DMSO at 10% concentration showed similar rate of fertilization (47.50 ± 2.08%) with the control (47.92 ± 1.53%). The second highest post-thawing fertilization rate was yielded by DMSO 5% with 40.59 ± 2.08% which is significantly lower than the control and DMSO 10%. However the fertilization rate of DMSO 5% was not significantly different from DMSO 15% (34.68 ± 3.21%). DMSO 20% showed the lowest fertilization rate with 13.60 ± 3.79%.

For the sucrose as cryoprotectant, sucrose 10% was the optimal concentration compared to the other concentrations of sucrose used in the experiment, with fertilization rate of 34.43 ± 5.57%. There is no significant difference between sucrose 5% (29.37 ± 3.06%) with sucrose 10%, but there was significant differences of these two concentrations with sucrose 15% (23.27 ± 1.73%) and sucrose 20% (18.29 ± 3.06%). The usage of sucrose was less effective compared to DMSO, but did not show any significant differences in fertilization rate with egg yolk as cryoprotectant. However, sucrose performed better than honey in this experiment. Similar to sucrose, egg yolk 10% showed the highest post-thawing fertilization rate with 31.10 ± 6.66%, which was significantly different from the three other concentrations of egg yolk as cryoprotectant. The second highest post-thawing fertilization rate was recorded in egg yolk 15% (21.42 ± 2.65%), which was also significantly

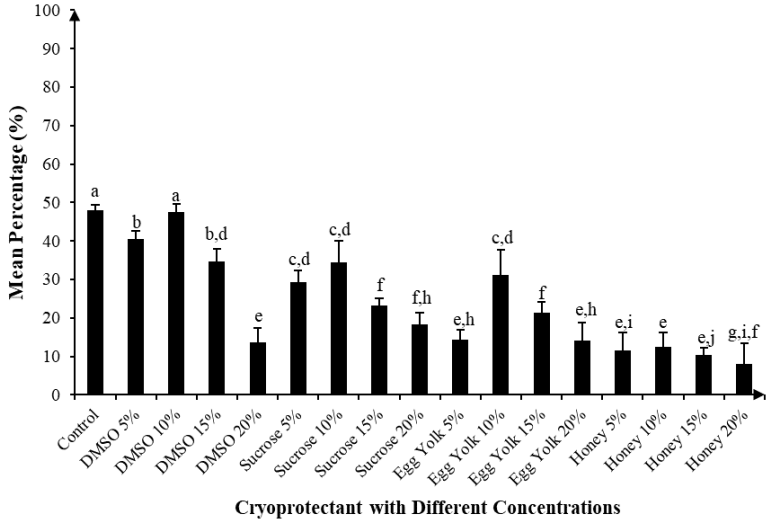


Figure 1. Fertilization rate of cryopreserved sperms using different cryoprotectants and concentrations for the oyster species *Magallana bilineata.* Different lowercase letters above graph bars indicate significant differences among treatments at $P < 0.05$ in Tukey's test.

higher than egg yolk 5% (14.23 ± 2.65%) and egg yolk 20% (14.07 ± 4.62%). Overall, egg yolk was less efficient as a cryoprotectant compared to DMSO, but better than honey and no significant differences with sucrose. Honey showed the least effectiveness as cryoprotectant compared to the other three cryoprotectants. However, honey 10% showed the highest post-thawing fertilization rate (12.44 ± 3.79%) among all the other concentrations of honey tested.

## 4. Discussion

This study showed that DMSO, as the universal cryoprotectant, has yielded the highest post-thawing fertilization rates among the other three tested cryoprotectants. The fertilization rate recorded in the cryopreserved sperm using DMSO 10% showed no significant difference with the fertilization rates of fresh sperm (the control). Hence, it is recommended that any commercial oyster hatchery should store the spermatozoa of oysters using DMSO 10% as cryoprotectant and freeze-store in liquid nitrogen for later fertilization when needed.

The post-thawing fertilization of cryopreserved sperm obtained in this study was similar with the results Ieropoli, Masullo, Do Espirito Santo, and Sansone (2004), where it was reported that the optimal sperm motility and 93% fertility was achieved using DMSO 10% for *Crassostrea gigas*. Yang, Supan, Guo, and Tiersch (2013) also reported that the most suitable cryoprotectant for sperm cryopreservation of *C. virginica* was DMSO 10%, yielding up to 77% fertility. This was reported that DMSO 10% concentration was optimal to cryopreserve spermatozoa with the lowest toxicity caused and the toxicity started to affect the natural biological property of the sperm once the concentrations rise above 10% (Riesco *et al.*, 2017; Santana, Cabrita, Eggen, & Beirao, 2020; Sandoval-Vargas *et al.*, 2021).

Sucrose, egg yolk and honey have been tested as spermatozoa cryoprotectant for various kinds of species, such as gourami (Abinawanto, Pratiwi, & Lestari, 2017), stallion (Consuegra *et al.*, 2018), sutchi catfish (Fanni & Santanumurti, 2019). However, these potential cryoprotectants have never been tested on tropical oysters.

Sucrose is a large polymer and non-penetrating cryoprotectant. It does not penetrate into cell membranes and act to improve the osmotic balance on the external membrane before freezing, which it commonly works in pair with penetrating cryoprotectants. Combination of penetrating cryoprotectants and non-penetrating cryoprotectants, such as mixing of DMSO and trehalose had yielded a better post-thaw fertility of sperm compared to using trehalose alone (Adam *et al.*, 2004). It is speculated that using sucrose alone is less effective as shown in this study, as it only function on the extracellular membrane without improving or maintain the stability of plasma membrane. According to Pegg (2007), sperm plasma membrane is an essential component to control the sperm cryosurvival. Hence, more study on the combined effect between penetrating and non-penetrating cryoprotectant in cryopreservation need to be conducted, as the non-penetrating cryoprotectant, like sucrose, can lower the volume of penetrating cryoprotectant needed in cryopreservation protocols.

Among the natural cryoprotectants, 5% and 10% of chicken egg yolk in this study had showed better fertilization rates in sperm cryopreservation. Similar results were shown in the fertilization rate of post-thawed spermatozoa of American catfish, *Clarias gariepinus* using egg yolk at 10% concentration as cryoprotectant (Muchlisin *et al.*, 2015). Egg yolk is a kind of non-penetrating cryoprotectant and one of the most preferred components in cryopreservation for domestic animals (Abdel-Aziz Swelum *et al.*, 2019; Dong *et al.*, 2011). Egg yolk is able to perform cell protection freezing because it contains low-density lipoprotein (LDL), which largely responsible for cold shock resistance (Orrego *et al.*, 2019). LDL adheres to sperm membrane and the phospholipids from LDL replaced the damaged phospholipids, stabilizing the spermatozoa membrane, thus providing protection for the sperm against ice formation, fluctuation of external environment and accumulation of harmful substances (Dong *et al.*, 2011; Muchlisin *et al.*, 2015; Neves, Heneine, & Henry, 2014).

However, the usage of egg yolk as cryoprotectant was less effective in spermatozoa cryopreservation of *M. bilineata* compared to DMSO because there maybe presence of granules that became the obstacles in metabolic exchange of spermatozoa and inhibit cell respiration, which increased their mortality (Akal, Kocyicit, & Selcuk, 2014). Further study is required to study the effect of LDL and phospholipids in cryopreservation of spermatozoa to reduce the weaknesses of egg yolk as cryoprotectant through the removal of noxious substances.

Overall, DMSO 10% is the most effective cryoprotectant for tropical oysters, *M. bilineata*. Egg yolk, which represents the natural cryoprotectant has shown the potential to become an alternative to DMSO due to its successfulness in preserving the oyster sperm although at lower percentage of fertility. Even though egg yolk is less effective as compared to DMSO, the usage of egg yolk as cryoprotectant is safer and at lower cost. Hence, more discovering of this cryoprotectant especially the presence and the role of its low-density lipoproteins in cryopreservation is highly recommended.

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