The effect of combination of methanol with several concentration of honey solution on sperm quality of botia *Chromobotia macracanthus* Bleeker 1852 after short-term storage at -80°C

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Abstract

Background: Clown loach or botia, *Chromobotia macracanthus* Bleeker 1852, is a popular freshwater ornamental fish. Lack of high quality broodstock and asynchronous gonad maturation are the main problem in breeding of this species. Cryopreservation is one of the methods to overcome this problem, and cryoprotectant plays a vital role in this process. Herein, we tested two types of cryoprotectants, namely methanol as a permeating cryoprotectant and honey solution as a natural non-permeating cryoprotectant. Hence, the objective of the present study was to determine best combination of honey solution and methanol for short-term storage of *C. macracanthus* sperm.

Methods: One level of methanol (10%) combined with six levels of honey solution (0%, 0.1%, 0.3%, 0.5%, 0.7% and 0.9%) were tested in this study. Ringer's solution was used as an extender. The diluted sperms were equilibrated for 25 min at 4 °C and then kept at -80 °C for 48 h. Sperms was thawed for 13 s at 40 °C. Spermatozoa viability, motility, and fertilization rates were then observed.

Results: The one-way ANOVA showed that combination of methanol with several concentrations of honey had a significant effect on sperm viability and fertilization rates ($P < 0.05$), but did have a significant effect on sperm motility ($P > 0.05$). The study revealed that the 0.1% honey solution combined with 10% methanol resulted in the highest rates of motility ($89.4 \pm 5.45\%$), viability ($85.75 \pm 4.79\%$), and fertilization ($98.55 \pm 1.69\%$).

Conclusion: A 0.1% honey solution combined with 10% methanol in Ringer's was the best cryoprotectant for *C. macracanthus* spermatozoa preserved at -80 °C.

Introduction

Botia *Chromobotia macracanthus* Bleeker 1852 is a popular ornamental freshwater fish originating from Sumatera and Borneo, Indonesia [1], and this is one of the sumptuous most popular aquarium fish in Indonesia. The wild botia has been intensively harvested over the years resulting in decreasing populations in the habitat [2–6]. Besides, the habitat degradation associated with illegal logging is also threatening botia fish populations [3,4,7–14]. Fortunately, the bioecology of the botia has been well documented [2,5,15–19], and also the breeding technology of botia has been developed successfully [2,18,20–25], and therefore currently, the supply has been swift from wild population to aquaculture production to meet the market demand.

However, botia hatcheries are still experiencing some obstacles, especially in developing high-quality broodstocks. This is because of long-lived gonad maturation and unsynchronized male and female gonad maturation [2,20,22,24]. Therefore, sperm cryopreservation is one of the potential solutions to overcome this problem [26–36]. According to Tsai and Lin [37], cryopreservation is a cell-storage technique that employs very low temperatures to maintain cell structure over time.

Several species of fish sperm have been successfully cryopreserved, for instance bagrid catfish *Mystus nemurus* [26,38], African catfish *Clarias gariepinus* [35,39–43], giant goramy *Osphronemus goramy* [44,45], lampam *Barbonymus gonionotus* [46,47], salmonid spermatozoa [48–52], common carp *Cyprinus carpio* [53–58], zebra fish, *Danio rerio* [59–63] Mozambique tilapia, *Sarotherodon mossambicus* [64,65], rainbow trout, *Oncorhynchus mykiss* [66–71], and seurukan *Osteochillus vittatus* [72]. However, cryopreservation of botia sperm has never been reported previously.

Cryoprotectants are among the factors that contribute to the success of cryopreservation program [27,35–37,43,73,74]. This is because cryoprotectants can protect spermatozoa from cold and heat shocks [27,35–37,43,71,73,74]. However, at high concentration cryoprotectants are toxic to cellular systems including spermatozoa [35,37,38,75–77]. Therefore, a non-toxic cryoprotectant is critical for the cryopreservation process [35,37,73,78,79]. In general, there are two types of cryoprotectant, namely permeating (intracellular) and non-permeating (extracellular) cryoprotectants. Several studies showed that the simultaneously used of both cryoprotectants types gave a better results. For instance, Abinawanto et al. [47,80] used a combination of skimmed milk and methanol for Java Barbs, *Barbonymus gonionotus*, sucrose and methanol for Indonesian Giant Goramy, *Osphronemus goramy* [44], honey solution, and Dimethyl Sulfoxide (DMSO) for Indonesian Giant Goramy, *Osphronemus goramy* and Indonesian shark minnow, *Osteochillus hasseltii* [45,81], and egg yolk and DMSO for depik, *Rasbora tawarensis* sperm [72]. In this study we tested the combination of honey solution as a natural non-permeating cryoprotectant and methanol as a permeating cryoprotectants with Ringer's solution as an extender. These materials have been successful in cryopreserving *O. goramy* spermatozoa [45,82], but for botia spermatozoa they have not been evaluated previously. Therefore, the objective of the present study was to determine the best concentration of honey solution combined with 10% methanol for botia spermatozoa storage at –80 °C.

Materials And Methods

Time, site and Broodfish preparation

The study was conducted from February 2017 to January 2018, at the hatchery of Research Institute for Ornamental Fish Culture, Depok, West Java Province, Indonesia. Forty mature males of botia with total body weight of 40 - 80 g were acclimated to laboratory conditions and fed on a commercial diet in 45 × 30 × 30 cm plastic containers for 15 days. Then the fish were grouped into four groups with average initial weights of 60.47 ± 10.34 g, and stocked in six plastic containers (45 x 30 x 30 cm). Each box was equipped with continuous aeration and a black plastic mesh lid to minimize disturbance and prevent fish from jumping out. Six experimental groups were assigned to four replicates in a completely randomised design. Fish were fed once daily to satiation at 09:00 AM.

Ethical approval

Preparation extender and cryoprotectant

The Ringer's solution and honey were used as extender and cryoprotectant, respectively. A stock of Ringers solution was prepared by dissolving 3.25 g NaCl; 0.125 g KCl; 0.175 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; and 0.1 g NaHCO_3 in distilled water up to 500 ml, and the solution was kept at 4 °C [45]. The pure honey was purchased from local market. Six concentrations of honey were tested, namely 0.1%, 0.3%, 0.5%, 0.7%, and 0.9%, for these purposes, the respective volume of honey of 0, 0.1, 0.3, 0.5, 0.7, and 0.9 ml were added into Fish Ringer's solution up to 100 ml [45].

Preparation of activator and eosin-Y solutions

The activator solution was prepared by diluting 0.263 g NaCl; 0.037 g KCl and 0.363 g Tris-HCl with aquabidest up to 100 ml. The solution was kept at 4 °C prior to use in the experiment [83]. The 0.5% of eosin-Y solution was prepared by diluting 0.5 g of the eosin-Y with distilled aquabidest up to 100 ml.

Sperm collection

Four males weighing 60.47 ± 10.34 g were treated intramuscularly with Ovaprim (Syndel Laboratories Ltd. Nanaimo, Canada) at dosage of 0.2 ml kg^{-1} body weight. After 18 h, sperms were collected from individual male donors by a gentle abdominal stripping method [39] and placed in 2 mL vials (Cryogenic storage vial, Nalgene Nunc International).

Sperm dilution

Fresh sperm was suspended in the diluent mixtures containing Ringers solution, 10% methanol, and the respective honey solution where applicable (Table 1). The composition of the solution was modified from [45]. The dilution ratio of the fresh sperm and diluent solution was 1:9 based on Sunarma et al. [81]. The compositions of each component of the diluent solution and the ejaculated sperm are presented in Table 1.

Equilibration, freezing and thawing

The diluted sperm was equilibrated at 4 – 5 °C in an ice box for 15 min then frozen at –80 °C in freezer for 48 h. Thereafter, the frozen sperm was thawed at 40 °C for 10 min in a water batch [45].

Sperm quality evaluation.

The fresh sperm was evaluated for colour and pH. The preserved sperm was analyzed for motility, viability, and abnormality rates using a Boeco Trinocular Microscope (Boeco, Germany) equipped with a digital eyepiece camera (MDCE-5a). The microscope was connected to a computer equipped with an image driving software (Scopephoto 2.0.4).

Egg Collection fertilization

The eggs were collected from the mature female by gentle abdominal pressure then put in the plastic basin and kept at 5 °C prior to use for fertilization. A total of 2 ml of eggs were mixed with 0.6 ml of thawed sperm (1:3 v/v) and two drops of tap water was added then mixed with a soft feather and left in contact for 5 min. A total of 100 eggs were taken randomly and incubated in a plastic basin. The fertilization rate was observed two hours after incubation. The fertilized egg was transparent, while the unfertilized egg was opaque. The fertilization rate was calculated using the following formula: Fertilization rate (%) = Fertilized eggs/ Total number of incubated eggs x 100 [75].

Statistical Analysis

The percentage data were arcsine transformed prior to analysis [26]. The data of sperm motility, viability and fertilization were analyzed using one-way ANOVA then followed by the *Duncan's multiple range test to determine the best treatment. The analysis was conducted using SPSS 14.* (SPSS, Chicago, IL, USA). The qualitative data such as semen color, volume, pH, and spermatozoa abnormality were analyzed descriptively.

Results

The fresh sperm was milky white, and pH was 7.9 (Table 2). The average diameter of the spermatozoa head was 3.5 µm, and the mean spermatozoa tail length was 32.81 µm. Viable sperm showed a green colour on the sperm head (Fig. 1a, Fig. 1b), whereas the non-viable sperm showed a pink or red colour on the sperm head (Fig. 1dc). In general, the quality of fresh sperm was higher than cryopreserved sperm. The motility, viability, and fertilization rates of fresh sperm were 97.75±2.63%, 83±2.45%, and 100±0.0%, respectively. However, the sperm quality was decreased gradually depending on the honey solution

concentration after 48 h preservation. The ANOVA test showed that the application of honey solution in the diluent gave the significant effect on the sperm viability and fertilization rates ($P < 0.05$), but did not give a significant effect on the sperm motility ($P > 0.05$).

In general, the sperm quality was decreased with increases of honey solution concentrations. The Duncan's multiple range test showed that the higher sperm motility was found at 0.1% honey solution ($89.4 \pm 5.45\%$), but it was not different significantly with other treatments. The higher sperm viability was also recorded at 0.1% honey solution (85.75 ± 4.78), and this value was significantly higher than other treatments except the control (74.5 ± 7.89). In addition, the higher fertilization rate was recorded at the 0.1% honey solution, and this value was significantly higher than for honey concentrations of 0.5%, 0.7% and 0.9%, but not significantly different to the different to the 0.3% treatment and the control (Table 2).

Discussion

The application of 0.1% honey with 10% methanol in the Ringer's solution gave the best results on spermatozoa quality of botia 48 h after freezing. The study revealed that sperm quality in the control (without honey) was lower than in the 0.1% honey treatment. However, the quality of cryopreserved sperm decreased gradually with higher concentrations of honey above 0.1%. This might be due to an increase viscosity of diluent when honey concentration was increased [84], and thereby preventing methanol entering the cell thereby reducing the protective effect of this permeating cryoprotectant inside the cell. Honey is known as natural non-permeating cryoprotectant. In general, the natural cryoprotectants are less toxic, inexpensive, and environmentally friendly [35,43]. Therefore, utilization of natural cryoprotectants as alternatives is highly recommended; however, it must be applied at the optimum concentration as recorded in this study.

The sperm motility rate in the best treatment (10% methanol + 0.1% honey) of this study is higher than others investigating combinations of cryoprotectants, such as the combinations of 20% skim milk + 5% methanol, 83.23% motility [47], 0.5% sucrose + 10% methanol, 81.62 % motility [44], 15% skim milk + 10% methanol, 80.98% motility [80], 0.7% honey solution + 10% DMSO, 80.48% motility [45], DMSO + honey solution, 63.33% motility [81] and DMSO + Fish Ringer's solution, 58% motility [26,85]. Therefore, we assumed that the combination of methanol and honey at concentration of 10% and 0.1% is an effective cryoprotectant to maintain sperm quality of botia during cryopreservation.

In the present study, honey solution was applied as a non-permeating cryoprotectant, while methanol as a permeating cryoprotectant. The simultaneous application of both permeating and non-permeating cryoprotectants resulted in better cryoprotective effect because these cryoprotectants gave complementary effect outside and inside of the cells [85]. Besides being effective for botia sperm as recorded in this present study, the combination of methanol and honey have been successfully applied in several others species, for example in tropical bagrid catfish sperm *Mystus nemurus* [26], African catfish *Clarias gariepinus* [35], and Indonesian shark minnow, *Osteochilus hasseltii* Valenciennes, 1842 [81]. Although using the same combination of the materials, we found that the motility rate of the botia sperm

after cryopreservation was higher compared to previous studies in *M. nemurus*, *Osphronemus goramy*, and *O. hasseltii* [26,45,81]. Indeed, the fertilization rate in this present study was also higher than in *C. gariepinus* [35], *B. gonionotus* [47], *O. goramy* [45], *Oreochromis mossambicus* [63], *O. hasseltii* [81,24], *Mystus nemurus* [26], *C. carpio* [85], and *Lota lota* [51].

Conclusion

It is concluded that the combination of 0.1% of honey and 10% methanol are effective as cryoprotectant agents for botia fish *Chromobotia macranchanthus* sperm storage at -80 °C for 48 h.

Declarations

Declaration for ethical approval

We would like to declare that the study was approved by the Health Research Ethics Committee Faculty of Medicine University of Indonesia Cipto Mangunkusumo Hospital which Ethical approval number: KET-919/UN2.F1/ETIK/PPM.00.02/2019

Consent for publication

We would like to confirm that all of authors have approved the manuscript for submission

Availability of data and materials

We would like to declare that neither the manuscript nor any part of its content are currently under consideration or published in another journal, and no material submitted as part of a manuscript infringes existing copyrights, or the rights of a third party.

Competing interests

We would like to declare that we have no competing interest.

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Author's contributor

Abinawanto Abinawanto (ABI) was a major contributor in writing the manuscript. Siti Zuhriyah Mustopha (SZM) analyzed and interpreted the sperm motility and sperm viability before and after freezing. Retno Lestari (RLE) performed the sperm abnormality examination before and after freezing. Anom

Bowlaksono (ABO) evaluated fertilization rate before and after freezing. Martin Wilkes (MW) making English correction and proofreading the manuscript. Zainal Abidin Muchlisin (ZAM) read, criticized and providing the supporting data to the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Composition of diluent used for cryopreservation of botia fish *Chromobotia macranchanthus* perm

Composition	Experimental group					
	C	H 0.1%	H 0.3%	H 0.5%	H 0.7%	H 0.9%
Sperm (μL)	30	30	30	30	30	30
Methanol (μL)	30	30	30	30	30	30
Honey Solution (μL)	0	240	240	240	240	240
Fish Ringer (μL)	240	0	0	0	0	0

C = Control, without honey solution; H 0.1-0.9% = honey solution.

Table 2. Spermatozoa analyses of Botia's fish 48 h after freezing. Values are means $\pm$ SD of four replicates. Mean values having the same superscript are not significantly different ($p > 0.05$).

Experimental group	Sperm Quality Parameters		
	Motility (%)	Viability (%)	Fertilization (%)
Fresh sperm	97.75 ± 2.63^b	83 ± 2.45^b	100 ± 0.0^b
0% (control)	85.45 ± 11.99^a	74.5 ± 7.89^{ab}	91.61 ± 10.29^{ab}
H 0.1%	89.4 ± 5.45^a	85.75 ± 4.78^b	98.55 ± 1.69^b
H 0.3%	83.05 ± 4.18^a	67 ± 10.23^a	93.91 ± 9.33^{ab}
H 0.5%	81.15 ± 8.14^a	66.75 ± 9.56^a	89.67 ± 8.22^a
H 0.7%	81.9 ± 7.05^a	66.5 ± 14.07^a	84.46 ± 10.22^a
H 0.9%	82.27 ± 6.18^a	67 ± 14.07^a	82.39 ± 16.90^a

Figures

Figure 1

Viable sperm (a, b), and non-viable sperm (c). Magnification 1000X; Bar = 10 micrometer