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Preliminary study on the cryopreservation of tropical bagrid catfish (*Mystus nemurus*) spermatozoa; the effect of extender and cryoprotectant on the motility after short-term storage

Z.A. Muchlisin[a,*], R. Hashim[b], A.S.C. Chong[b]

[a]*Department of Marine Sciences, Faculty of Sciences, Syiah Kuala University, Banda Aceh 23111-NAD, Indonesia*

[b]*Aquaculture Research Group, School of Biological Sciences, University Sains Malaysia, Penang, Malaysia*

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Abstract

The effects of different extenders, and cryoprotectants on the motility of tropical bagrid catfish (*Mystus nemurus*) spermatozoa were evaluated after short-term storage. Three extenders, physiological saline, Ringer or saline at three levels of sperm to extender dilutions (1:20, 1:30, or 1:40) and four cryoprotectants (DMSO, ethanol, glycerol or methanol) at three concentrations (5, 10, or 15%) were examined in two separate experiments. In the first experiment, milt was suspended in the respective extender at the three milt to extender dilution ratios in two sets of tubes. Extended milt in the first set of tubes was stored at −4 °C, and motility assessed after 24 h, while the second set was kept at 23 °C and sperm motility was assessed immediately and at 30-min intervals thereafter. Ringer retained sperm motility better than the other extenders at all dilution levels at temperatures of 23 and −4 °C respectively. At 23 °C, the sperm motility was almost completely lost after 150 min except for those in Ringer at 1:20 dilution level which still had a motility of 18% (compared to those kept at −4 °C for 24, which had motility from 39 to 71%, regardless of extender). In the second experiment, various cryoprotectants were added to solutions of milt (that was diluted in Ringer at 1:20 ratio and cryopreserved in liquid nitrogen for 15 days). Sperm cryopreserved in 10% methanol had the highest motility (58%) compared with those in the other cryoprotectants at all concentrations.
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Keywords: Tropical bagrid catfish; *Mystus nemurus*; Extenders; Cryoprotectant; Spermatozoa; Motility

* Corresponding author. Fax: +62-651-555-622.
E-mail address: icin@eudoramail.com (Z.A. Muchlisin).

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1. Introduction

Tropical bagrid catfish, *Mystus nemurus* is a popular species and forms a substantial part of Malaysia reservoir fisheries [1]. This species is also found in the rivers of Indonesia, especially in the Acheh province and in other Asian countries such as Thailand and Vietnam. It is a commercially important species for inland fisheries, sought for its taste, non-bony flesh and high protein content [2].

There are several reasons for the lack of aquaculture of the tropical bagrid catfish. This fish is difficult to spawn artificially, is sensitive to water quality changes and is easily stressed. In its natural habitat, it spawns twice a year from May to July and from September to November. Hatcheries are faced with problems in synchronizing maturity between male and female brood stock, and its slow growth further hinders efforts in finding high quality brood stock.

Sperm cryopreservation is one way to overcome the problems associated with brood stock supply. Sperm collection for cryopreservation should be carried out during the spawning seasons because the quality and quantity of spermatozoa is highest at this time. In seabass, for example, the concentration of spermatozoa decreased as the spawning season progressed [3]. Similarly, Suquet et al. [4] reported lower motility rates, fertilization rates and reduced short-term storage capacity at the end of the reproductive period of the fish.

Over the last decade, studies on the cryopreservation of mammalian spermatozoa, including livestock and human spermatozoa, have progressed substantially. However, cryopreservation of fish spermatozoa is still in its infancy and confined to aquatic species such as salmonid [5], small abalone [6], Asian catfish [7], rainbow trout [8,9], yellowtail flounder [10], European cyprinid [11], African catfish [12,13], European catfish [14,15], common carp [16], Japanese eel [17], ayu [18], and Japanese bitterling [19].

The basic objective of preserving spermatozoa is to reduce sperm motility during storage, resulting in high post-thaw motility; this is achieved with the use of appropriate extenders and cryoprotectants. The suitability of extenders and cryoprotectants differs among fish species. For example dimethyl-acetamide (DMA) has been used for rainbow trout spermatozoa [20], and dimethyl sulphoxide (DMSO) was found to be suitable for muskellunge spermatozoa [21], rainbow trout [22], penaeid shrimp [23] and Arctic charr [24]. In addition, methanol was reported to be suitable for Japanese bitterling [19], African catfish [13], European catfish testicular spermatozoa [15], and salmonid spermatozoa [25,26] with glycerol effective for ejaculated spermatozoa of European catfish [15]. To date, the type of extenders and cryoprotectants suitable for the tropical bagrid catfish spermatozoa is still unknown, despite the urgent need for cryopreserved spermatozoa for purposes of artificial spawning.

The objective of the present study was to determine the most suitable extender and cryoprotectant and their respective concentrations for tropical bagrid catfish spermatozoa dilution for cyropreservation.

2. Materials and methods

2.1. Male brood fish

One-year-old tropical bagrid catfish males were used in this study. They were raised in outdoor fiberglass tanks in the Aquaculture Research Center, Universiti Sains Malaysia

under natural light conditions (12D:12L). Males were injected with a GnRH analog (Ovaprim, Aqua Life, Syndel Int. Inc., Canada) at a dose of 0.1 ml kg^{-1} body weight, five times a week. During this time, the fish were fed 3% of their body weight daily of a 34% protein commercial diet (divided between two feedings, morning and evening).

2.2. Sperm motility analysis

Percentage of sperm motility was observed with a light microscope (Olympus, BH2, Japan) connected to 3 CCD color video camera (Model: KY-F55BE, JVC Japan) using Soft Imaging software analysis at 400× magnification. Percentage motility was determined by assessing the motility of at least 50 randomly chosen spermatozoa for each treatment.

2.3. Experiment 1: effect of extender and dilution ratio on sperm motility

2.3.1. Extenders

Three extender solutions were used: physiological saline, Ringer, and saline. Topical bagrid catfish milt was diluted in each of the three extenders at three dilution ratios of 1:20, 1:30, or 1:40. The chemical compositions of the extenders are shown in Table 1.

2.3.2. Collection and dilution of milt

Five male brood stock fish (weighing 784–886 g) were taken randomly from a group and anaesthetized in a solution of Tricaine methanesulfonate (MS 222), prepared by dissolving 4 g of MS 222 in 5 l tap water, and sacrificed by spinal transection. Testes were removed by dissection and perforated with a needle; milt was gently squeezed out and placed in the glass tube on crushed ice in an icebox. One drop of fresh milt was then placed on a slide and then activated with two drops of tap water, and the initial motility ratings recorded. Eighteen 5-ml clear vials (cryogenic storage vials (Cryogenic storage vials, Nalgene & Nunc Corp., New York, NY, USA) were placed in crushed ice and filled with 2-, 3- or 4-ml of the three extenders, followed by 0.1 ml of fresh inactivated milt. The vials were then labeled and heat-sealed. Nine of vials were then placed in a freezer (−4 °C) and after 24 h, thawed by placing the vials in a water bath (40 °C) for 5 min. Duplicate samples were then taken from each vial and analyzed for motility. The remaining nine vials were placed in a

Table 1
Chemical compositions and pH of the three extenders used in this experiment

Component ($g\ l^{-1}$)	Extender		
	Physiological saline	Ringer	Saline
NaCl	7.98	7.5	7
$NaHCO_3$	0.2	0.2	–
KCl	–	0.2	–
$CaCl_2{\cdot}2H_2O$	–	0.2	–
Glucose	5	5	–
pH	8.4	7.9	6.0

water bath at 23 °C, and duplicate samples were taken from each vial (every 30 min for 150 min) to determine sperm motility.

2.4. Experiment 2: effect of cryoprotectant and cryoprotectant concentration on sperm motility

Six male donors weighing 755–879 g were anaesthetized as described earlier and sacrificed by spinal transection. Testes were removed by dissection and perforated with a needle and gently squeezed into a glass tube (placed on crushed ice) to obtain the milt. Control samples were activated with tap water at a ratio of 1:20, and percentage motilities recorded immediately. Fresh milt diluted in Ringer at 1:20, was added to each of twelve 5-ml vials (Cryogenic storage vials, Nalgene & Nunc Corp.) containing DMSO, ethanol, methanol or glycerol at concentrations of 5, 10 or 15%. The vials which were placed in crushed ice, labeled, heat-sealed and allowed to equilibrate for 5 min (to allow time for the milt to be exposed to the cryoprotectant before freezing). The vials were then placed in an icebox (containing dry ice) for 5 min and then plunged into a container containing liquid nitrogen (34HC Cryogenic equipment Union Carbide Co. USA). After 15 days, samples were thawed in a water bath at 40 °C for 5 min and the percentage motility of preserved spermatozoa was recorded. The motility was estimated as described earlier, i.e., twice for each treatment.

2.5. Statistics

A one-way analysis of variance (ANOVA) and Duncan's multiple rage tests were used to determine if there were significant differences among treatments. A two-way ANOVA was conducted to determine the effect of extenders, dilution ratios, and the interaction, as well as cryoprotectants, cryoprotectant concentrations, and the interaction on the sperm motility. Percentage data were arcsine transformed prior to analysis.

3. Results

3.1. Experiment 1

The initial motility of fresh spermatozoa ranged from 90–94%, compared to 80–94% for extended sperm. When sperm was diluted with the different extenders at various dilutions, and stored at 23 °C, motility decreased with time (Fig. 1). In general, no motile spermatozoa were present after 150 min (except for those in Ringer at 1:20 dilution, that still had a motility of 18%). Initially, motility was numerically highest (92–94%) for spermatozoa in saline (at all dilution levels), but was not significantly different from those extended in physiological saline or Ringer. However, after 1 h, the motilities in the saline or physiological saline declined at a faster rate to levels below that of spermatozoa in Ringer.

Twenty-four hours after dilution and storage in the freezer at −4 °C, there was a decreasing trend in motility as milt dilution increased, irrespective of the extender used (Table 2). However, sperm motility at 1:20 was not statistically higher than sperm diluted at

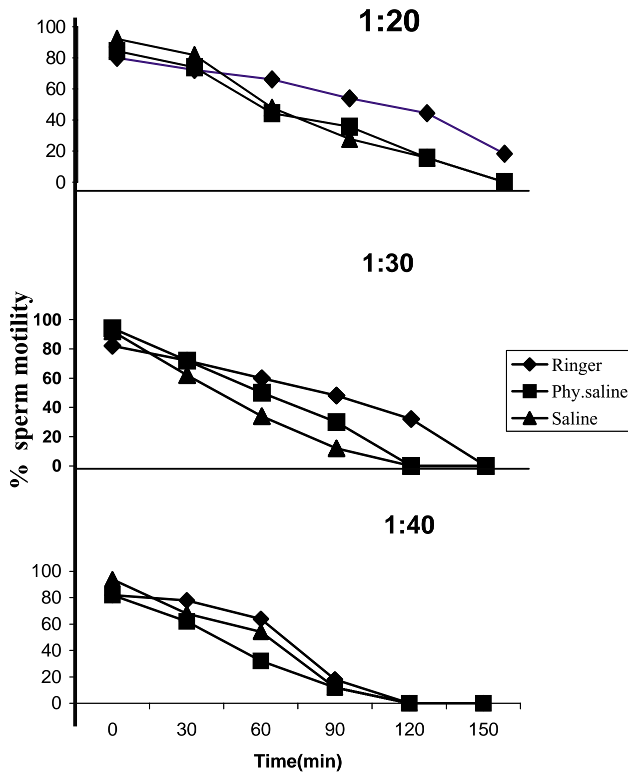

Fig. 1. Percentage of sperm motility of tropical bagrid catfish in various extenders and dilution ratios kept at 23 °C.

Table 2
Mean (±S.D.) sperm motility after 24 h storage (at −4 °C) in different extenders and dilution ratios (sperm:extender)

Extender	Sperm:extender	Motile (%)
Physiological saline	1:20	65 ± 1.4 a
	1:30	61 ± 7.1 a,b
	1:40	39 ± 7.1 c
Ringer	1:20	71 ± 7.1 a
	1:30	63 ± 4.2 a,b
	1:40	44 ± 8.5 c
Saline	1:20	49 ± 9.9 b,c
	1:30	49 ± 1.4 b,c
	1:40	40 ± 5.7 c

(a–c) Means without a common alphabet are different ($P < 0.05$).

Table 3
Mean (±S.D.) sperm motility after 15 days storage (in liquid nitrogen) in different crypoprotectants and different cryoprotectant concentrations

Treatment		Motile (%)
Cyoprotectant	Concentration (%)	
Methanol	5	30 ± 5.7 c
	10	58 ± 2.8 a
	15	18 ± 2.8 d
Ethanol	5	24 ± 2.8 c
	10	49 ± 4.2 b
	15	49 ± 1.4 b
Glycerol	5	11 ± 1.4 d
	10	14 ± 0.0 d
	15	30 ± 2.8 c
DMSO	5	48 ± 2.8 b
	10	32 ± 2.8 c
	15	28 ± 2.8 c

(a–d) Means without a common alphabet are different ($P < 0.05$).

1:30 for all extenders tested. Overall, sperm motility was highest in Ringer and lowest in Saline.

3.2. *Experiment 2*

Motility after 15 days of cryopreservation is shown in Table 3. The effect of cryoprotectants, cryoprotectant concentration and interaction between cryoprotectant and cryoprotectant concentration on the sperm motility of bagrid catfish were significant ($P < 0.05$). Cryopreservation resulted in a sharp drop in motility from 92% for fresh sperm to a highest of 58% for cryopreserved sperm in 10% methanol, followed by 10 and 15% ethanol and 5% DMSO (motilities of 49, 49 and 48%, respectively).

4. Discussion

Motility of tropical bagrid catfish spermatozoa varied with the extender and dilution ratio used, with the best motility in Ringer at 1:20. Motility decreased from 80 to 94% (for freshly activated spermatozoa) to between 39 and 71% after storage at −4 °C for 24 h (without cryoprotectants). Studies on dilution ratios to preserve sperm motility have been carried out extensively and appear to vary among fish species. Chao [27], reported that the acceptable dilution ratio of extenders are 1 part milt:1 part extender for the spermatozoa of grey mullet, black porgy, and tilapia; 1:4 for milkfish; and 1:20 for grouper, while Ritar and Campet [28] reported dilutions as high as 1:100 for the triped trumpeter.

In terms of extenders, our results concur with Yao et al. [29] who reported that Ringer gave highest motilities, however the dilution ratio of 1:30 reported was different from the 1:20 obtained in this study. In addition to the differences in the experimental design between our study and that of Yao et al. [29], it also noted that the differences in dilution

ratios could possibly be due to the different fertilization behavior between ocean pout (internal fertilization) and tropical bagrid catfish (external fertilization).

In our study, sperm motilities were initially low in Ringer (indicative of a degree of inhibition), but motility did not decreased as rapidly with time as did spermatozoa in physiological saline or saline. We suspect the low initial motility of spermatozoa in the Ringer, which may have reflected a low metabolic state of the spermatozoa, allowed the conservation of energy reserves (in the form of ATP), enabling them to maintain motility over a comparatively longer period of time. It has been suggested that ATP content of spermatozoa could be increased with the addition of certain cryoprotectants, e.g. dimethylacetamide for European catfish sperm [15].

Sperm immotility is said to be attributed to the presence of factors such as K^+, Na^+, Ca^{2+}, and Mg^{2+} ions. Morisawa et al. [30] reported that ionic concentrations in the seminal plasma of freshwater teleosts are higher than the environment, and spermatozoa became motile when diluted with hypotonic solution. Furthermore, they suggested that upon the release of spermatozoa into river water (which is hypotonic), there is both an increase in the concentration of free calcium ions and a decrease in the potassium ion concentration, resulting in sperm activation.

The ionic composition of Ringer consists of NaCl, KCl, $CaCl_2$ and $NaHCO_3$, but some ions such as K^+, and Ca^{2+} are lacking in the other extenders tested; this could explain the differences in sperm motilities observed. Fish seminal fluids generally have high concentrations of K^+, Na^+ and Cl^- ions, but low concentrations of Ca^{2+} and Mg^{2+} ions [5,7,30–32]. A review of previous reports indicates conflicting roles of these ions in inhibiting and activating fish spermatozoa, confirming that many characteristics of fish spermatozoa are species-specific. For example, K^+ ions inhibit the spermatozoa activation in rainbow trout [33], and ayu [34]. In addition, Baynes et al. [33] reported that Na^+ ions in a diluent, had an antagonistic effect on inhibition due to potassium ions, that was further exacerbated by the presence of Ca^{2+} and Mg^{2+} ions. However, contrary to these observations, Japanese eel spermatozoa were reported to be immotile when diluted in a K^+ free hypertonic solution [35]. Other ions in the seminal fluid (e.g. Na^+, Ca^{2+}, Mg^{2+} and Cl^-) are also reported to affect sperm motility [36].

Several researchers have also reported the effect of pH on sperm motility. The differences in the pH of the extenders used appear to affect the potential motility of spermatozoa. In this study, the motility of thawed spermatozoa was highest in Ringer (pH 7.9), which had a pH similar to the seminal fluid of the tropical bagrid catfish (8.0). We speculated that motility of thawed spermatozoa would be highest (and retained for a longer interval) if the milt was extended in solution with a pH similar to the seminal plasma. In numerous species, pH is involved in the control of flagellar movement [37] and reports have cited species-dependent optimum pH values for sperm that ranged from 7.2 to 8.2. For example, optimal pH values were 7.5–8.0 for grouper, 8.0 for the Siberian sturgeon and ayu, and 8.2 for Asian catfish [7,18,38,39].

In addition to ions and pH, storage temperature also influences sperm motility. Our studies showed that at 23 °C, sperm motility was almost completely lost after 150 minutes (except for those in Ringer at 1:20 dilution level, which still had a motility of 18%) compared to those kept in freezer at −4 °C for 24 h, which had motility that ranged from 39 to 71%. The life span of spermatozoa is generally longer at lower temperatures.

Temperature is the most important environmental factor affecting the activity of poikilothermic animals such as fish, and in this context fish sperm, in particular the membranes [40]. According to Buda et al. [41] temperature affects cellular membranes and cells respond accordingly to achieve a new equilibrium between the environment and the physicochemical properties of their membranous structure. However, there is very little information on how cell metabolism might respond to in vitro exposure to various temperatures. Perhaps at low temperatures, spermatozoa become less motile due to a decreased metabolic rate (that conserves energy and minimizes the production of metabolic waste that may compromise sperm survival). Temperature dependence of sperm motility has been reported for Atlantic salmon and brown trout [42], rainbow trout [43], and Siberian sturgeon [38]. Perhaps there was a synergistic effect between the potassium ions in Ringer solution and the reduction of temperature that helped maintaining sperm integrity and motility.

Spermatozoa cryopreserved in 10% methanol had the highest motility. Similarly, Ohta et al. [19] reported that 10% methanol plus 90% foetal bovine serum gave a high percentage of motility for Japanese bitterling spermatozoa. In addition, Viveiros et al. [13] reported that 10% methanol was the most effective cryoprotectant for African catfish spermatozoa, as well as for salmonid sperm [25,26]. Perhaps methanol has a protective effect for testicular spermatozoa, as shown in the European catfish [15]. We found that sperm motility increased as concentrations of ethanol and glycerol increased, but decreased with increasing concentrations of DMSO. High levels of DMSO are known to be toxic to fish spermatozoa compared with other commonly used cryoprotectants such as methanol, ethanol and glycerol [44].

Cryoprotectants provide protection to labile enzymes (e.g. catalase), and stabilize proteins in unfrozen, aqueous solution, but alternatively can also induce protein denaturation at high temperatures and cause cryoprotectant toxicity in cellular systems [27]. During freezing, high concentrations of cryoprotectants, which may be lethal to unfrozen cells, can prevent ice formation and the concomitant freezing-thawing damage [27]. Nonetheless, very few spermatozoa of any species studied have been found to survive very low freezing temperatures without cryoprotectant. The addition of cryoprotectants to the milt greatly extends the tolerance of spermatozoa to freezing at slower rates and it is agreed that the optimal cooling rate depends on the nature and concentration of the cryoprotectant used [27]. It has also been reported that cryoprotectants are most effective when they can rapidly penetrate the cell during freezing, and delay intracellular freezing and minimize the solution effect [44].

The preliminary observations reported here (due to the limited supply of wild broodstock and the small volume of milt produced) suggested that Ringer solution at a dilution ratio of 1:20 together with 10% methanol are potential diluents for preservation of tropical bagrid catfish spermatozoa. Further confirmatory studies are currently underway, as well as determination of the optimum conditions for the long-term cryopreservation of sperm from this species.

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