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Exploration of natural cryoprotectants for cryopreservation of African catfish, *Clarias gariepinus*, Burchell 1822 (Pisces: Clariidae) spermatozoa

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ABSTRACT: Toxicity is a major limitation to successful spermatozoa cryopreservation of fish. Due to this problem, it is critical to find potential cryoprotectants which are more environmental-friendly, non-toxic, easily prepared, and available at affordable prices. Hence, the objective of the present study was to investigate several natural cryoprotectants for optimal cryopreservation of the African catfish, *Clarias gariepinus*, Burchell 1822 (Pisces: Clariidae) spermatozoa. Three natural cryoprotectants were tested – egg yolk, glucose, and honey, while DMSO was used as a control at different concentrations (5, 10, and 15%). Sperms were diluted with coconut water at a dilution level of 1 : 20 sperm to extender (v/v). Diluted sperms were kept at 4°C for 5 min, then at 0, –4, and –79°C for 5 min respectively, and stored in liquid nitrogen (–196°C) for 45 days. The cryopreserved sperms were thawed in a water bath (37°C) for 5 min and evaluated for fertilization and hatching rates. The data were subjected to analysis of variance (ANOVA), followed by comparison of means using Duncan's Multiple Range Test. The fertilization and hatching rates of African catfish in all cryoprotectants improved with concentration increasing from 5 to 10% but then decreased when concentration was increased to 15%. The ANOVA test showed that the differences in cryoprotectants used significantly affected fertilization and hatching rates of African catfish. Overall, the fertilization and hatching rates were higher in DMSO for all concentrations compared to other cryoprotectants. However, 10% egg yolk resulted in higher fertilization and hatching rates compared to other natural cryoprotectants. It was concluded that 10% egg yolk was the most suitable concentration for African catfish spermatozoa cryopreservation compared to other natural cryoprotectants tested.

Keywords: egg yolk; honey; glucose; coconut water; DMSO; fertilization and hatching rate

INTRODUCTION

The African catfish, *Clarias gariepinus* is one of the most important freshwater fish species currently being cultured both within and outside its natural range of tropical and subtropical environments (Adewolu et al. 2008). This species is known for its resistance to diseases, high growth rate, resistance to handling stress, and its ability to tolerate a wide range of environmental parameters and high stocking densities under culture conditions and high meat quality (Elnaggar et al. 2006;

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Rasowo et al. 2007; Wachirachaikarn et al. 2009), thus accounting for its commercial importance especially in southeast Asian countries.

In the wild, it has a discontinuous annual reproductive cycle (Van Oordt and Goos 1987) and the breeding season correlates with periods of maximal rainfall (Van Oordt et al. 1987). Outside this range of breeding seasons, the availability of high quality broodstocks is scarce, as in general the quality and quantity of spermatozoa decreased as the spawning season progressed (Moczarski and Koldras 1982; Fauvel et al. 1999; Suquet et al. 2000). Therefore, sperm cryopreservation is one way to overcome the problems associated with brood stock supply. For this purpose, sperm collection should be carried out during the spawning seasons because the quality and quantity of spermatozoa is the highest at this time (Muchlisin et al. 2004).

There are several reports on the investigations of the African catfish sperm cryopreservation (Steyn et al. 1985; Steyn and Van-Vuren 1987; Viveiros et al. 2000) where dimethyl sulfoxide (DMSO), ethanol, methanol, and glycerol have been used as cryoprotectants at various concentrations. Utilization of a suitable cryoprotectant is one of the factors important for the success of a cryopreservation protocol (Anil et al. 2011), in particular for long-term cryopreservation. Cryoprotectants are needed to protect the sperm cell from cold and hot shocks (Chao and Liao 2001). Moreover, they provide cryoprotection to labile enzymes (e.g. catalase) and stabilize proteins in unfrozen and aqueous solutions. However, two of their disadvantages are that they can induce protein denaturation at higher temperatures and cause cryoprotectant toxicity in cellular systems (Muchlisin and Siti-Azizah 2009). Therefore, toxicity is a major limitation to successful spermatozoa cryopreservation of fishes (Gwo and Arnold 1992; Chao et al. 1994). Toxicity of a cryoprotectant depends on the type, concentration, temperature, and exposure period (Tsai and Lin 2009). Due to these problems there is a strong need to search for other potential cryoprotectants for the African catfish spermatozoa which are more environmental-friendly, less or non-toxic, easily preparable, and available at affordable prices.

The present study therefore attempts to overcome this problem, particularly to find the optimum cryprotectants and their concentrations for long-term cryopreservation of the African catfish spermatozoa. Herein, we evaluated the efficacy of three different natural cryoprotectants (egg yolk, glucose, and honey) at different concentrations and compared their efficacies with the commonly used cryoprotectant, DMSO. Herein, a natural cryoprotectant is defined as a material of natural origin that does not contain any artificial chemical compounds.

Glucose and honey at concentrations of 5 and 0.5% have been reported suitable for cryopreservation of tilapia and black porgy sperm, respectively (Chao et al. 1987). In addition, egg yolk has been tested as a cryoprotectant in many cryopreservation studies, for example in yellow perch (Ciereszko et al. 1993) and rainbow trout (Lahnsteiner et al. 1996). However, these potential cryoprotectants have never been tested for African catfish spermatozoa.

MATERIAL AND METHODS

Extenders and cryoprotectants. The coconut water was obtained from mature green coconut collected from Balik Pulau, Penang, Malaysia, while the honey sample was collected from a local collector in Kedah, Malaysia. Three natural cryoprotectants (chicken egg yolk, glucose, and honey) at three concentrations (5, 10, and 15%) were tested in the study while DMSO was utilized as a control. Coconut water at a dilution ratio of 1 : 20 was used as an extender because a previous study revealed that this extender at this dilution ratio resulted in higher fertilization and hatching rate compared to sugarcane water and soybean milk (Muchlisin et al. 2010). The experiments were conducted in three replicates.

Sperm collection. Six male donors weighing 600–900 g were injected intra-peritoneally with 0.5 ml of ovaprim (Syndel Laboratories Ltd., Nanaimo, Canada) per kg body weight. After 24 h, the male fish donors were anesthetized with two drops of star anise oil extract dissolved in 10 l of tap water prior to sacrifice by spinal transaction. Testes were removed by dissection and perforated with a needle and semen were gently squeezed and polled into a glass tube which was placed on crushed ice (4°C) and mixed homogeneously.

Cryopreservation procedure. Fresh sperm suspension was diluted in coconut water at a dilution ratio of 1 : 20 in a 100 ml jar and kept at 4°C. A total of 1.9 ml diluted sperm suspension was filled

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into 12 tubes and then three tubes were added with 0.1 ml of each investigated cryoprotectant (i.e. triplicates of honey, chicken egg yolk, glucose, and the control DMSO) to give a final concentration of 5%. Another set of 12 tubes was filled with 1.8 ml of diluted sperm, and 0.2 ml of each cryoprotectant tested were added into the tubes to give a final concentration of 10%. The final set of 12 tubes was filled with 1.7 ml of diluted sperm suspension and added with 0.3 ml of each tested cryoprotectant in triplicates to give a final concentration of 15%. The tubes were kept at 4°C for 5 min and at +4 and –4°C for another 5 min respectively to allow time for the milt to be exposed to the cryoprotectant before freezing. Then, the tubes were placed into a container with liquid nitrogen where the tubes were first held at 6 cm above the liquid nitrogen surface (about –79°C) for 5 min, and then finally plunged into liquid nitrogen (–196°C) and stored for 45 days. Liquid nitrogen was refilled every 15 days to replenish the evaporated gas. After 45 days, the cryopreserved sperms were thawed in a water bath at 37°C for 5 min, and used for fertilization trials.

Evaluation of fertilization and hatching rates. Three females weighing 800 g and 950 g were injected with 0.5 ml of ovaprim (Syndel Laboratories Ltd.) per kg body weight. After 24 h, the ovulated females were anaesthetized by using five drops of star anise oil extract dissolved in 10 l tap water and the eggs from two females were gently squeezed out into a jar in ice box (4°C) and mixed homogeneously. Aliquots of 1 ml of egg batches (about 500 eggs) were randomly taken from the jar and mixed with 0.5 ml volume of thawed sperm suspension (egg : sperm ratio was 1000 eggs/ml of sperm), then three drops of tap water were added to activate the sperm, stirred with a feather, and then left for 5 min to allow the eggs to make sufficient contact with the sperms. Approximately 5 min after fertilization, 100 eggs were randomly taken and incubated in an aerated plastic container with 5 l tap water. Each trial was repeated three times. Successful fertilization was recorded 2 h after fertilization. Unfertilized eggs, identified by their opacity, were removed from the container, while hatching rate was monitored at two-hour intervals.

Statistical analysis. All data were subjected to analysis of variance (ANOVA), followed by comparison of means using Duncan's Multiple Range Test (Zar 1984). Percentage data were arcsine transformed prior to analysis. All statistical analyses were performed using SPSS software (Version 14.0, 2005).

RESULTS AND DISCUSSION

The ANOVA test showed that the effect of different cryoprotectants on fertilization and hatching rates of African catfish was significant ($P < 0.05$). The thawed sperm cryopreserved in 10% DMSO

Table 1. Fertilization and hatching rates of eggs treated with cryopreserved sperm of African catfish (*Clarias gariepinus*) tested on four different types and concentrations of cryoprotectants after a 45-day storage in liquid nitrogen

Cryoprotectant	Concentration (%)	Fertilization rate (%)	Hatching rate (%)
DMSO	5	79.67 ± 6.66^{d}	30.00 ± 1.00^{fg}
	10	91.33 ± 3.79^{e}	31.67 ± 4.04^{g}
	15	66.67 ± 3.51^{c}	27.00 ± 4.58^{ef}
Chicken egg yolk	5	68.67 ± 7.23^{c}	18.67 ± 0.58^{d}
	10	80.67 ± 5.69^{d}	24.33 ± 1.53^{e}
	15	60.67 ± 4.73^{bc}	18.67 ± 2.52^{d}
Glucose	5	59.00 ± 12.12^{abc}	16.00 ± 2.65^{bcd}
	10	65.33 ± 4.04^{c}	17.00 ± 1.73^{cd}
	15	49.33 ± 2.52^{ab}	11.33 ± 1.53^{a}
Honey	5	47.67 ± 7.51^{a}	12.00 ± 2.00^{ab}
	10	53.33 ± 3.79^{ab}	13.33 ± 0.58^{abc}
	15	47.67 ± 9.29^{a}	9.33 ± 1.53^{a}
Fresh sperm (non-cryopreserved sperm)	–	95.67 ± 2.67^{e}	68.63 ± 4.28^{h}

$^{a–h}$mean values in the same column followed by a different superscript indicate significant difference ($P < 0.05$)

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showed the highest fertilization rate (91.33%), which was significantly different from the other groups ($P < 0.05$). There was also statistically significant difference in fertilization rate between 10% and 5% DMSO. But there was no statistical difference in hatching rate between 10% and 5% DMSO ($P > 0.05$). Furthermore, there was also no statistical difference in fertilization rate when 5% DMSO and 10% egg yolk were used ($P > 0.05$), although they showed differences in hatching rate ($P < 0.05$) (Table 1).

Among the tested natural cryoprotectants (chicken egg yolk, honey, and glucose), the fertilization and hatching rates were the highest for the spermatozoa cryopreserved in the 10% egg yolk (80.67 and 24.33%, respectively), being significantly different from honey and glucose at all concentrations ($P < 0.05$), but not significantly different with 5% DMSO ($P > 0.05$). In addition, cryopreserved sperm in honey resulted in a lower percentage of fertilization and hatching rates compared to egg yolk and glucose at the same concentrations.

The fertilization and hatching rates of African catfish in all cryoprotectants improved with increasing concentration from 5 to 10% but then decreased when concentration was increased to 15%. In general, the hatching rate in the control (non-cryopreserved fresh sperm) was significantly higher than in all cryopreserved sperm, but fertilization rate of sperm cryopreserved in 10% DMSO did not differ from that of control.

The present study showed that DMSO could be considered as an effective cryoprotectant for cryopreservation of African catfish sperm, resulting in higher fertilization and hatching rates. However, the values were lower compared to fresh sperm (non-cryopreserved sperm). Regarding to cryoprotectant activities, the tested natural cryoptectants (chicken egg yolk, honey, and glucose) are considered as non-permeating, while DMSO is a permeating cryoprotectant. Most permeating cryoprotectants are composed of small molecules compared to non-permeating which are typically in the form of polymers. Hence, DMSO can readily penetrate the sperm cell membrane and enter the cytosol through its interaction with the phospholipids of the sperm membrane (Ogier de Baulny et al. 1996) and act both intracellularly and extracellularly, while egg yolk, honey, and glucose act only extracellularly. Moreover, Thapliyal et al. (2011) stated that permeating cryoprotectants can reduce the rate of diffusion of water from cell to extra-cellular ice crystal. This reduces the cell volume change or salt concentration colligatively, reducing the homogeneous nucleation temperature, and demoting the rate of ice crystal growth. Therefore, it also functions efficiently in sperm protection from cold and heat shock during freezing and thawing.

It is an interesting finding that the fertilization and hatching rates were recorded in sperm cryopreserved without intra-cellular cryoprotective agents, indicating that African catfish sperm is cryopreservable with a natural non-permeating cryoprotectant as recorded in this study, however, it is less effective in comparison with DMSO as an intra-cellular cryoprotectant. With respect to a natural cryoprotectant agent, the present study revealed that 10% egg yolk was the optimal treatment compared to other natural cryoprotectants investigated. The egg yolk has become a popular cryoprotectant for cryopreservation of sperm in various species during the past 60 years (Witte et al. 2009), for example for Atlantic salmon, *Salmo salar* (Jodun et al. 2006) and rainbow trout, *Oncorhynchus mykiss* (Perez-Cerezales et al. 2010).

It has been well documented that egg-yolk prevents sperm cell damage during freezing and thawing. In addition, it has protective effects against harmful environmental conditions such as changes in the temperature, pH, and osmotic pressure or accumulation of harmful substances as e.g. reactive oxygen and toxicity of the diluents and cryoprotectants (Manjunath et al. 2002; Aboagla and Terada 2004). However, the mechanism of membrane protective capacity by egg yolk component has remained unclear. Polge (1980) suggested that the low-density lipoprotein fractions (LDL) in egg yolk are the main cryoprotective agent and therefore one possible explanation may be a specific interaction effect between the LDS fractions with some major proteins of seminal plasma. However, Babiak (1999) reported that addition of LDL to sperm extender did not improve the hatching rate of northern pike fish (*Esox lucius*) eggs. It was presumed that the egg yolk contains gelatin and certain gums which increase the viscosity of diluted semen and reduce the motility during storage thus maintaining their energy and therefore resulting in higher fertility after thawing. Furthermore, egg yolk contains cholesterol, fatty acids, and phospholipids. These compounds have been identified as protective agents (Watson 1976 cited by Bozkurt et al. 2014).

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Another hypothesis is that the egg yolk also contains considerable concentrations of progesterone (Mostl et al. 2001) and natural antioxidant (Sakanaka et al. 2004), but their role in protecting fish sperm cell during cryopreservation has not been investigated. However, Mayer and Lesley (1945) found that egg yolk contained a harmful acetone-insoluble, alcohol-soluble fraction and a fraction insoluble in alcohol, acetone or ether, which was beneficial in minute amounts and these authors suggested that the protective action of egg yolk might be obtained at concentrations lower than 50%. Thus in conclusion, among natural cryoprotectants tested, 10% chicken egg yolk was the most suitable concentration for African catfish spermatozoa cryopreservation.

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