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Preliminary study: the effect of cryopreservation on the gastrula-staged embryo of African catfish (*Clarias gariepinus*)

Preliminary study: the effect of cryopreservation on the gastrula-staged embryo of African catfish (*Clarias gariepinus*)

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Abstract. This study was aimed to observe the effect of cryopreservation on gastrula-staged embryo of African catfish. The gastrula-staged embryos were treated 5% (v/v) solutions concentration of dimethyl sulfoxide, propylene glycol, honey, and combined cryoprotectants, respectively and preserved at temperatures of -4 and -196ºC (in liquid nitrogen) for 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h, respectively. Thawing of embryos was conducted in freshwater at temperature of 28ºC. After thawing, the embryos were incubated in the aquaria at 28ºC temperature. The result showed that the cryopreservation of gastrula-staged embryo at temperatures of -4 and -196ºC affects damage and hatching percentages of African catfish embryos. The percentage of catfish embryo damage increases with the length of preservation at temperature of -4ºC for all treatments. A combination of DMSO+honey and PG+honey has the lowest damage percentage and the highest hatching rate of catfish embryo compared to other treatments ($p<0.05$). Meanwhile, total embryo damage occurs since the first 30 min of preservation at temperature of -196ºC for all treatments. Cryoprotectant toxicity and inability to protect the embryo are thought to be a cause of damage and death of catfish embryos on preservation, especially at temperature of -196ºC.

1. Introduction

The procedure of cryopreservation techniques of the fish embryo has been carried out over the decades [1, 2, 3, 4, 5]. The development of cryopreservation protocols for fish embryos has important potential for management of stocks in fisheries, creation of gene banks for fish species, facilitating the conservation of endangered populations of fish, and bioassay in ecotoxicological studies [6].

Cryopreservation by methods vitrification (-196ºC) is among the more effective cryopreservation techniques because cell and tissue injury are fewer. The vitrification of embryo, embryos are first immersed in a mild dehydrating solution to reduce intracellular water. The remaining water in the cell is then replaced with cryoprotectants so as not to cause crystals ice when cooled [7]. However, there are several factors that limit the success of embryo cryopreservation such as embryos have been large size, thick chorion, poorly permeable membranes, complex structure in development, high yolk content [8], and embryos were more sensitive to cryoprotectants [9].

So that to achieve successful cryopreservation of embryos, it is important to the knowledge of the cryoprotectant toxicity, suitable concentration and combination of cryoprotectants, along with

appropriate cooling protocols [6]. In addition, it also needs knowledge about the sensitivity of the embryo to cold which depends on the phase of embryonic development. Early embryonic stages are the most sensitive and the least stage the sensitive are post-gastrulation [10].

The study by Wang *et al.* [11] showed that fish embryo membrane permeability could be monitored in real-time after making use of cryoprotectant. The permeable cryoprotectants that commonly used, such as dimethyl sulfoxide (DMSO) and propylene glycol (PG). PG are functional fluids such as antifreeze, de-icing, and heat transfer, and unsaturated polyester resins [12], while DMSO is effectiveness to solubilize a wide range of polar and nonpolar compounds, inhibition of bacterial growth in aquatic test samples, low toxicity, and excellent ability to permeate biological membranes without inducing structural integrity changes [13, 14]. Non-permeable cryoprotectants, such as honey which contained of 38.4% fructose, 1.3% sucrose, and 30.3% glucose [15]. Honey has a potent role in providing antibacterial, energy, and antioxidant [16].

Several studies of embryos cryopreservation have been done, such as goldfish (*Carassius auratus auratus*) [17], snakehead (*Channa striata*) [18], zebrafish (*Danio rerio*) [8, 19], rohu (*Labeo rohita*) [20], common carp (*Cyprinus carpio*) [10], grouper (*Epinephelus septemfasciatus*) [9], seabream (*Pagrus major*) [21], and also *Steindachneridion parahybae* [22].

African catfish (*Clarias gariepinus*) is one of the most important species of freshwater fish. In Indonesia for example, African catfish production has been significantly increased significantly from 144,755 MT in 2009 to 543,461 MT in 2013 [23] and is expected to continuously rise in the future. However, development of African catfish culture is constrained by the limited supply of good quality fingerlings [24], demand for seeds is quite high and the catfish cultivation activity was growing rapidly [25]. Based on this statement, cryopreservation of the embryo was one approach to providing sustainable seed/larvae through embryo transport and allowing the storage of genetic material species for future applications. This study was aimed to observe the effect of cryopreservation on gastrula-staged embryo of African catfish (*Clarias gariepinus*).

2. Materials and methods

This study was conducted to Laboratory of Fish Reproduction, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, East Java, Indonesia and Wet Laboratory, Faculty of Fisheries and Marine and Teaching Farm, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia.

2.1. Artificial fertilization and eggs collection

Artificial fertilization was done by mixing 40,000 eggs and 3 mL sperm that have been diluted with physiological NaCl. Comparison dilution of sperm and physiological NaCl volumes were 1: 100. After sperm dilution was prepared, sperm are mixed with eggs and stirred evenly for 2 min, then eggs are rinsed with fresh water two times.

The fertilized egg is put into freshwater at temperature of 28ºC, serves as an incubation site for embryos. Eggs that have reached the embryonic stage of gastrula (6.5 h after fertilization) are fed into cryotube of 2 mL, each cryotube sample consists of 50 embryos. Respectively treatment group was repeated three times.

In this research, catfish eggs were obtained by artificial spawning. Catfish artificial spawning is carried out by injection of ovaprim^{TM} with a dose of 0.5 mL/kg for females and 0.3 mL/kg for male. Eggs collection done after 12 h injection of ovaprim^{TM} through striping method for a female to produced sperm, while the surgical method for mele produced eggs. After that, sperm and eggs are collected in containers for artificial fertilization.

2.2. Cryoprotectant solution preparation

In this research, the cryoprotectant used was propylene glycol ($C_3H_8O_2$) (Merck Millipore, Germany), dimethyl sulphoxide (Me_2SO), and honey solution (osmolarity 557.31 mOsm/L) (production by

Tawon Lawang Agro Tourism-Malang, Indonesia) and $C_3H_8O_2$ solution combined with honey solution and Me_2SO solution combined with honey solution.

The cryoprotectant dose used in this treatment was a 5% $C_3H_8O_2$ solution diluted into physiological NaCl (concentration 0.9%), 5% Me_2SO solution diluted into physiological NaCl, 5% solution honey diluted into physiological NaCl, 5% $C_3H_8O_2$ solution combined with 5% of honey diluted into physiological NaCl solution, and 5% Me_2SO combined with 5% of honey diluted into physiological NaCl solution, respectively dosage of cryoprotectant solution inserted into cryotube of 2 mL and each treatment is repeated three times.

2.3. Cryopreservation treatment

In this research, selected 50 embryos that have good quality at the stage of gastrula for each treatment. After 50 embryos were selected for each treatment, the embryos were rinsed with a physiological NaCl solution at a temperature of 28ºC for 5 min and the embryos were inserted into cryotubes of 2 mL. Then cryotube that has embryo, added cryoprotectant solution by using the equilibrium method at temperature of 28ºC for 30 min. The equilibrium method was modified according to Tian *et al.* [9] and Shaluei *et al.* [17].

After the treatment of the equilibrium method at 28ºC, each cryotube sample was prepared for storage at temperatures of -4 and -196ºC, respectively. Each storage temperature treatment is always controlled to maintain a temperature constant. At a storage temperature of -4 and -196ºC, 90 cryotube samples were added to the freezing refrigerator and liquid nitrogen container (Thermolyne Bio Cane 20, 20 L capacity), respectively with rapid cooling or vitrification methods. Each cooling treatment is stored for 30 min, 1, 2, 3, 4, 5, and 6 h, respectively and each treatment was repeated three times.

2.4. Thawing

Cryotubes that contained embryos were removed quickly from the refrigerator and liquid nitrogen container for the liquefaction treatment at a temperature of 28°C for 30 min. After the embryo is removed from cryotube and rinsed with freshwater. Embryos that have been rinsed with freshwater are transferred to incubation hatching with a temperature of 28ºC.

Next, evaluated the stages of embryo development, hatching rate, and embryo damage are evaluated. Evaluation of the stages of embryonic development was observed with an Olympus CX41 light microscope. Hatching rate was determined by counting the number of larvae in the number of eggs contained in incubation and evaluate cryoprotectant toxicity by counting the number of embryos damaged after cryopreservation.

2.5. Data analysis

The results of data analysis used analysis of variance (ANOVA). The treatment differences were determined using the Tukey test. The statistics are analyzed using IBM SPPS 20 (statistical software). The significant level was determined at $p<0.05$.

3. Results and discussion

3.1. Results

Based on this research showed that the gastrula-staged embryos added cryoprotectants of $C_3H_8O_2$, Me_2SO, honey, and combinations of $C_3H_8O_2$+honey and Me_2SO+honey during a length of storage of 6 h at a temperature of -4 and -196ºC have significantly affected the damage percentage and hatching rate of catfish embryos ($p<0.05$). The results of embryo damage in Table 1 show each treatment and cryopreservation over a 2 h of embryo damage was 100%, especially embryo storages at temperature of -196ºC. Embryo damage of 100% is directly proportional to the hatching of catfish embryos in Table 2 shows 100% there is not hatching of catfish embryos at temperature of -196ºC.

This indicates that the results of the study did not differ significantly between different cryoprotectant treatments and over the 3-hour embryo storage period to the percentage of embryo

damage and the percentage of embryo hatching. Catfish embryo damage in this study showed changes in embryonic shape after the storage and thawing process in the embryonic storage stage of gastrula.

Table 1. Damage percentage of catfish embryos in different cryoprotectants and temperatures

Treatment	Temp. (ºC)	Long time of preservation						
		30 min	1 h	2 h	3 h	4 h	5 h	6 h
Honey	-4	66.7±1.2^{c}	68.7±1.2^{b}	100.0±0.0^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
	-196	100.0±0.0^{d}	100.0±0.0^{c}	100.0±0.0 ^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
DMSO	-4	65.3±1.2^{bc}	66.7±1.2^{b}	100.0±0.0^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
	-196	100.0±0.0^{d}	100.0±0.0^{c}	100.0±0.0^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
PG	-4	63.3±1.2^{b}	66.0±2.0^{ab}	100.0±0.0^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
	-196	100.0±0.0^{d}	100.0±0.0^{c}	100.0±0.0^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
DMSO +	-4	60.7±1.2^{a}	63.3±1.2^{a}	86.0±0.0^{a}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Honey	-196	100.0±0.0^{d}	100.0±0.0^{c}	100.0±0.0^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
PG +	-4	59.3±1.2^{a}	62.0±2.0^{a}	84.0±2.0^{a}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Honey	-196	100.0±0.0^{d}	100.0±0.0^{c}	100.0±0.0^{b}	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0

Note: Temp. = temperature of preservation. Different superscripts in the same column show no significant differences ($p<0.05$).

Table 2. Hatching rate of catfish embryos in different cryoprotectants and temperatures

Treatment	Temp. (ºC)	Long time of preservation						
		30 min	1 h	2 h	3 h	4 h	5 h	6 h
Honey	-4	33.3±1.2^{bc}	31.3±1.2^{ab}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	-196	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
DMSO	-4	34.7±1.2^{b}	33.3±1.2^{ab}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	-196	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
PG	-4	36.7±1.2^{b}	34.0±2.0^{a}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	-196	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
DMSO +	-4	39.3±1.2^{a}	36.7±1.2^{a}	14.0±0.0^{a}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Honey	-196	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
PG +	-4	40.7±1.2^{a}	38.0±2.0^{a}	16.0±2.0^{a}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Honey	-196	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Note: Temp. = temperature of preservation. Different superscripts in the same column show no significant differences ($p<0.05$).

3.2. Discussion

In this study, cryopreservation of gastrula stage embryos treated with a combination of permeable cryoprotectants and non-permeable cryoprotectants over a period of 3 h at a storage temperature of -196ºC could not protect the embryos and affect damage to the embryos by 100% and cannot maintain hatching rates catfish eggs by 100%. This is thought to be due to osmotic differences and sensitivity of catfish embryos that occur during the cryopreservation process at a temperature of -196ºC. Bhattacharya dan Prajapati [26] states that rapid cooling can cause serious intracellular cell damage, where the cytosol, nucleus, parenchyma, and almost all cellular component rapidly freeze which causes intracellular formation. All integral parts of the cell can get out, and cell can die due to shrinking. Embryo shrinkage is related to membrane permeability and cold sensitivity, which is one of the obstacles to the success of egg hatching rates embryo damage. This statement is reinforced by Hagedorn *et al.* [27] states that the permeability barrier in zebrafish embryos is the yolk synchronization layer (YSL). The syncytial layer of the eggshell physiologically prevents the entry of some cryoprotectants and the release of water, but cryoprotectants can penetrate the egg yolk syncytial layer to enter the yolk, resulting in the process of removing water from the yolk.

In addition, one of the factors causing the failure of cryopreservation of catfish embryos is due to the use of physiological NaCl as a solvent for each cryoprotectant treatment. Cryoprotectants used in this study, such as $C_3H_8O_2$, Me_2SO, honey, and combinations of $C_3H_8O_2$+honey and Me_2SO+honey,

respectively was dissolved with physiological NaCl. The use of DMSO cryoprotectant acts as a universal aprotic solvent, is able to be absorbed by biological membranes, and has low toxicity to biological materials. DMSO does not cause significant mortality but can cause significant biological and material abnormalities in sublethal concentrations in all species [28], propylene glycol is used to prevent cytotoxicity because it has a lower concentration that will diffuse from embryo to the cryoprotectant, thereby reducing freezing tolerance [29]. Sucrose as a non-permeable cryoprotectant is known to be able to provide physical and chemical energy requirements for the process of egg or embryo metabolism during the storage process. Besides that sucrose also acts as cryoprotectant which is not penetrating into fish egg cells [30]. So from this statement, honey containing ingredients such as fructose, sucrose, and glucose [15] have the potential to minimize toxicity and increase osmotic pressure of the embryo.

However, the giving a different cryoprotectant treatment by adding NaCl as a solvent is less effective in protecting the embryo during the vitrification cryopreservation (-190ºC) at the gastrula stage. Shaluei *et al.* [17] observes that the solvent used in cryoprotectant (DMSO, MeOH, PG, Gly, EG, PVP, and sucrose) combinations for cryopreservation of goldfish embryos is 8.00 g/L NaCl + 0.40 g/L KCl + 0.14 g/L $CaCl_2$ + 0.10 g/L $MgSO_4$ -$7H_2O$ + 0.10 g/L $MgCl_2$-6H2O + 0.06 g/L $NaHPO_4$-$12H_2O$ + 1.00 g/L glucose, and 0.35 g/L $NaHCO_3$. In cryopreservation of Persian sturgeon embryos (*Acipenser persicus*) the solvent used is Ringer solution (2.99 g/L KCl, 6.49 g/L NaCl, 0.29 g/L $CaCl_2$, and 0.20 g/L $NaHCO_3$) [31] and cryopreservation of embryo grouper (*Epinephelus septemfasciatus*) the solvent used is the diluent BS2 (24.72 g/L NaCl, 1.46 g/L $CaCl_2$-$2H_2O$, 0.865 g/L KCl, 4.86 g/L $MgCl_2$-$6H_2O$, and 0.19 g/L $NaHCO_3$) [9].

4. Conclusion

Type and combination of cryoprotectants and preservation temperature strongly determined successfulness of catfish embryo cryopreservation.

5. References

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