Effect of free range chicken egg yolk concentration to spermatozoa viability of koi fish (Cyprinus carpio, Linnaeus 1758) post-cryopreservation

N G Zavitri and Abinawanto

Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok 16424, Indonesia

Corresponding author’s email: abinawanto.ms@sci.ui.ac.id

Abstract. Research on natural cryoprotectant such as chicken egg yolk, for sperm fish cryopreservation has already been done, however Using free range chicken egg yolk has yet to be done before in sperm fish, especially in koi fish. The aim of this research was to evaluate the effect of free range chicken egg yolk concentration on sperm viability of koi fish, Cyprinus carpio (Linnaeus, 1758) after freezing. Sperm was collected by hand stripping method, and diluted by a dilution solution (methanol 10 % and various concentrations of free range chicken egg yolk) with ratio 1:4. The given concentration of free range chicken egg yolk was 0 % (control), 5 %, 10 %, 15 %, 20 %, and 25 %. Freezing was done at -34 °C for 48 hours. The one factor ANOVA showed that various concentration of chicken egg yolk had effect (P < 0.05) on average value of viability of spermatozoa of koi fish 48 hours post-cryopreservation. Tukey’s multiple comparison test showed significant differences (P < 0.05) between controls with other chicken egg yolk treatment. Free range chicken egg yolk at concentration 15 % is the optimum concentration in this study for maintaining viability of koi fish sperm 48 hours post-cryopreservation, because it produced the highest average value of percentage viability 84.5 ± 7.32 %.

Keywords: Cryopreservation, free range chicken egg yolk, koi fish, spermatozoa, viability

1. Introduction
Koi fish (Cyprinus carpio, Linnaeus 1758) in Indonesia is an ornamental fish that has high economic value. The problem in cultivating koi fish is synchronization of mature fish for spawning and koi fish is susceptible to pathogens that can kill whole commodities [1]. Cryopreservation is an alternative solution for escaping such problems. Cryopreservation is a process of preserving genetic material (ovum, embryo, and sperm) at sub-zero temperatures by freezing for a long duration of time [2]. Sperm cryopreservation, reportedly, is more tolerable to extreme cold than ovum or embryo [3]. There are reports of fish cryopreservation for Cyprinus carpio [1], Osteochilus hasseltii [4], Osphronemus goramy [5, 6], Barbonymus gonionotus [7, 8], Salmon salar [9], and Salmo trutta macrostigma [1],

One of the factors affecting the cryopreservation process is the cryoprotectant. Adding a cryoprotectant solution can prevent spermatozoa from getting cryoinjuries during freezing and thawing [3]. Chicken egg yolk and its components reportedly can provide protection to spermatozoa against cold shock. One protective agent in chicken egg yolk that is low-density lipoprotein (LDL) [10].
Research on effect of chicken egg yolk has been reported in bull sperm [10], koi fish [1], and goat sperm [11]. Free-range chicken egg yolk when compared to commercial chicken egg yolk contains components such as chepalin, lecithin, and cholesterol that can provide more protection to cell during freezing [12]. The aim of this study is to evaluate the effect of various concentration of free-range chicken egg yolk (0 %, 5 %, 10 %, 15 %, 20 % and 25 %, respectively) on sperm viability after freezing for 48 hours in -34 °C.

2. Experimental method

2.1. Sperm collection
Sperm collection was done by hand stripping from four male koi fish (Cyprinus carpio).

2.2. Fresh sperm evaluation
Fresh sperm (milt) was evaluated macroscopically (volume, pH, milt colour) and microscopically (percentage viability of spermatozoa). The microscopic evaluation was done by making a eosin Y-0.5 % smear preparation.

2.3. Sperm dilution
The collected sperm were diluted by methanol 10 %, various concentration of free range chicken egg yolk, and glucose based extender with ratio 1:4, based on modified method of [7]. The given concentration of free range chicken egg yolk were: 0 % (control), 5 %, 10 %, 15 %, 20 %, and 25 %, respectively.

2.4. Sperm equilibration, freezing, and thawing
Equilibration of the diluted sperm was carried out at 4 °C for 10 min, and then sperm were frozen at -34 °C for 48 hours. Following equilibration, the sperm was thawed at 40 °C in water bath for 90 sec.

2.5. Sperm evaluation
After the sperm was thawed, the sperm was evaluated using a light microscope that integrated with computer and supported by image driving software: Leica. The following parameter evaluated was sperm viability.

3. Results and discussion

3.1. Macroscopically and microscopically fresh sperm evaluation
The average value of fresh sperm volume collected in the research was 0.72 ± 0.05 mL with individual volume ranging between 0.69–0.8 mL. The average value of fresh sperm pH was 7.9 ± 0.14, while the colour of milt was milky-white. In a previous study, some comparable results were found in which the fresh sperm volume was 0.5 mL [4], the colour of milt was milky-white [7], and the pH of fresh sperm were in range 7.7 ± 0.26–8 ± 0.26 [13]. Overall result for macroscopic evaluation of fresh sperm can be seen in table 1.

Results for microscopic evaluation of fresh sperm can be seen in the percentage of fresh viable sperm. The average value of fresh viable sperm was 81.5 ± 1.29 %, with range between 80 % to 83 % (table 1). The difference between viable sperm and non-viable was in the colouring of the sperm head; has viable sperm has green coloured or transparent sperm head, while non-viable sperm has a pink or red colour sperm head (data not shown). This present result was similar to another similar study [7] which the average value of sperm viability is 82.57 ± 5.38 %. Samples obtained was above 80 %, which could be used for cryopreservation experiments [14].
3.2. Sperm evaluation after freezing

The percentage of sperm viability after freezing with various concentrations of free-range chicken egg yolk can be seen in table 2. Based on a one factor ANOVA test, various concentration of free-range chicken egg yolk had an effect (P < 0.05) on the average value of spermatozoa viability of koi fish 48 hours post-cryopreservation. Tukey’s multiple comparison test showed significant difference (P < 0.05) between control and experimental group. Treatment with 15 % of free range chicken egg yolk concentration showed the highest percentage of sperm viability after freezing.

The average value of sperm viability obtained after freezing showed a higher value than fresh sperm viability, except for the control (0 %). A previous study with Cyprinus carpio showed average value of sperm viability 75.8 % when preserved with DMSO 10 % [15]. The results from the previous study were lower than the data obtained in this research. This difference in data could be caused by the fact that the previous study used just one cryoprotectant, while this research used two cryoprotectants: methanol 10 % and free range chicken egg yolk 15 %. It is possible that using one cryoprotectant wasn’t enough to protect the integrity of the cell membrane of the sperm while freezing, compared with using two cryoprotectants.

A previous study using two cryoprotectants were carried out in Osphronemus goramy [5] and Barbonymus gonionotus [7] sperm using a combination of methanol 10 % with sucrose 0.5 % and chicken egg yolk 15 %, respectively. The average value of this previous study were 82.17 ± 2.56 % [5] and 85.50 ± 3.11 % [7], respectively. The data obtained in this research were similar with these studies because of two cryoprotectants in combination. The combination of two cryoprotectants working together provided an optimum condition to protect cell from cryoinjury while freezing.

The low-density lipoprotein (LDL) in free-range chicken egg yolk had an ability to coat the cell membrane of sperm while cryopreservation, especially in freezing at -34 ºC and thawing at 40 ºC. Extreme temperature change in cryopreservation could be a factor of cryoinjury [16]. The LDL is thought to protect the sperm while freezing by using phospholipid fraction in LDL to form a membrane coating throughout the cell membrane, and phospholipid fraction could substitute a loss or damage in the phospholipid bilayer in the cell membrane of sperm while sperm goes through cold shock [10].

Cryoinjury, (such as cold shock) happens because of contraction of lipoprotein in the sperm cell membrane is more powerful than contraction the intracellular space of the cell, and the intracellular substance in the cell could be damaged [16]. Cryoinjury could also happen because of the sperm plasma binding with LDL and lechitin on the surface of the sperm cell membrane. The binding could initiate an efflux of cholesterol and phospholipid in the cell membrane cellular, and it could cause a great loss of phospholipid in the sperm cell membrane. The risk caused by efflux of cholesterol and phospholipid loss could be reduced by adding chicken egg yolk as cryoprotectant, so LDL and lechitin in chicken egg yolk could bind with sperm plasma while freezing, instead of in the sperm cell membrane [17].

Table 1. Macroscopic and microscopic fresh sperm evaluation. Values are means ± SD of four replicates.

| Volume (mL) | pH     | Color of milt | Viability (%) |
|------------|--------|---------------|---------------|
| 0.72 ± 0.05 | 7.9 ± 0.14 | Milky-white   | 81.5 ± 1.29%  |

Table 2. Evaluation of sperm evaluation after freezing process. The values are means ± SD from four replicates. The different superscript in the same row denotes significant difference (P < 0.05).

| Sperm | Free range chicken concentration |
|-------|---------------------------------|
|       | 0 %    | 5 %     | 10 %    | 15 %    | 20 %    | 25 %    |
| Viability (%) | 68 ± 3.26a | 79.5 ± 5.90b | 75.5 ± 5.06a | 84.5 ± 7.32b | 81.5 ± 4.79b | 81.5 ± 3.10b |
Adding methanol 10 % also could avoid formation intracellular crystal ice. Methanol could replace intracellular substance in cell membrane while freezing [18]. Using two cryoprotectants (intracellular and extracellular) can optimize the protection of cell membrane against cold shock during freezing.

4. Conclusion
This study demonstrated that free-range chicken egg yolk in combination with 5 % methanol can be use as cryoprotectant for sperm of Koi Fish, *Cyprinus carpio* for short term storage. Optimum concentration of free range chicken egg yolk to preserve sperm viability is fifteen percent with average percentage value of 84.5 ± 7.32 %. However, fertilization studies have to be carried out for further clarification of the cryoprotectant effects.

Acknowledgments
This work was facilitated by Department of Biology, FMIPA Universitas Indonesia. The author would like to thank to Dr. Drs. Abinawanto, M.Si. as a supervisor during the research.

References
[1] Bozkurt Y, Yavas I and Karaca F 2012 Cryopreservation of brown trout (*Salmo trutta macrostigma*) and ornamental koi carp (*Cyprinus carpio*) sperm Current Fronties in Cryopreservation ed. I Katkov (London: IntechOpen Publisher) chapter 4 pp. 293-304
[2] Baust J G, Gao D and Baust J M 2009 Organogenesis 5 90-6
[3] Kopeika E, Kopeika J and Zhang T 2007 Cryopreservation of fish sperm Cryopreservation and Freeze-drying Protocols 2nd edition ed. J G Day et al. (Totowa: Humana Press Inc.)
[4] Sunarma A, Hastuti D W B, Saleh D M and Sistina Y 2007 Journal Perikanan 10 76-64
[5] Abinawanto, Nurman K and Lestari R 2012 Int. J. of Aquatic Science 3 23-8
[6] Abinawanto, Pratiwi I A and Lestari R 2017 AACL Bioflux 10 156-63
[7] Abinawanto, Rahayu S and Lestari R 2013 Glob. Vet. 10 318-21
[8] Abinawanto, Zuraida and Lestari R 2016 AACL Bioflux 9 326-33
[9] Jodun W A, King K and Farrel P 2006 N. Am. J. Aquacult. 69 36-40
[10] Manjunath P 2012 Anim. Reprod. 9 809-15
[11] Janice C W K, Kanwai K D S, Wan K W E and Abdullah R B 2013 Malaysian Journal of Science 32 38-42
[12] Adeyeye E I 2012 Agric. Biol. J. N. Am. 3 374-84
[13] Aliniya M, Hossein K, Shahrouz B N and Hadiseh D 2013 Turk. J. Fish Aquat. Sc. 12 19-25
[14] Carbita E, Anel L and Herraez M P 2001 Theriogenology 56 623-35
[15] Rani K U and Munuswamy N 2014 J. Coast. Life Med. 2 181-6
[16] Anand M, Yadav S and Shukla P 2014 Livest. Res. Int. 2 48-53
[17] Bergeron A, Crete M H, Brindle Y and Manjunath P 2004 Biol. Reprod. 70 708-17
[18] Best B P 2015 Rejuvenation Res. 18 422-36