Effect of Cold Storage Time (4°C) on Malondialdehyde (MDA) Level, Motility and Viability Spermatozoa of *Cyprinus carpio* L. Punten Strain

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Abstract

The aim of this research is to know the level of MDA, motility, and viability of spermatozoa of *Cyprinus carpio* L. in cold storage time at 4°C. This study used treatment extenders that were NaCl 0.9% as control, diluent of egg yolk with concentrate 0%, 5%, 10%, 15%, and storage in 0 h, 24 h, 48 h, 72 h, and 96 h at 4°C. The data were analyzed using ANOVA ($P<0.05$). The results showed that the quality (motility and viability) of spermatozoa *C. carpio* L. Punten strain in cold storage could be maintained at 96 h. The optimum storage for motility was found in a diluent of egg yolk concentrate 5% at 48 h. MDA levels of semen from *C. carpio* L. Punten strains after being stored at cold temperatures increased at 0 h to 24 h, then decreased after 48 h of storage and increase at 72 h - 96 h stored. Egg yolk concentration and storage time had a significant effect on MDA levels. There is a negative correlation between MDA levels and motility. There was no correlation between MDA levels and the viability of spermatozoa *C. carpio* L. Punten strain.

Keywords: *Cyprinus carpio* L**,** Egg yolk**,** Malondialdehyde, Motiliy, Viability.

INTRODUCTION

Cyprinus carpio L is classified as a type of fish that has high economic value [1]. One of the main factors needed to increase the cultivation and production of *C. carpio* L is created as the best quality of parental fish to produce good quality seeds. The process of producing the best quality of parental fish required a long time and high cost. Therefore, it must be utilized optimally. During the reproductive season, parental fish sperm can be stored and can be used when required.

The advantage of sperm storage is can be stored for a long time and can be used when necessary [2]. Sperm storage at low temperatures can cause cold shock [3], which can produce ROS (Reactive Oxygen Species) [4]. ROS is highly reactive, resulting in lipid peroxidation. Malondyladehid (MDA) is the final result of the lipid peroxidation process, which is toxic to cells. MDA can cause damage and decrease the integrity of the spermatozoa membrane, resulting in decreased sperm quality [5]. Sperm storage required diluent which protects sperm from low temperatures and provides an energy source during the storage process, and also to keep sperm from damaged and die during storage. The quality of sperm during storage is maintained using a good medium. The use of storage media can inhibit sperm movement, stabilize the physiology of sperm, and maintain the viability of sperm [6].

The diluent of sperm was supply nutrients and maintained the osmotic pressure and electrolyte balance to protected viability sperm. One of the diluents that can be used is egg yolk. Since 60 years ago, egg yolk has been shown to be an effective medium for storing sperm in various species, for example, the storage of salmon (*Salmon salar*) and rainbow trout (*Oncorhynchus mykiss*) [7].

Egg yolk contains LDL (Low-Density Lipoprotein), which can maintain sperm at low temperature, increase motility during storage, and binding to the sperm membrane to preserving the membrane from protein damage [8]. The content of egg yolks such as cholesterol, fatty acids, phospholipids, progesterone, and antioxidant compounds have the quality as a protective agent [9,10,11]. Therefore, it is necessary to choose a suitable and efficient medium storage of egg yolk. The research aimed to determine the quality and spermatozoa MDA levels of *C. carpio* L. Punten strain after cold stored at temperature 4°C with diluent of egg yolk, and evaluate the interaction between MDA levels with motility and viability of spermatozoa.

MATERIAL AND METHOD

Semen Collection

Semen was collected from six male *C. carpio* L. Punten strain aged one year and the weight ± 700 g by manual abdominal stripping.

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Analysis of Spermatozoa Quality

Analysis of sperm quality performed by dripping 10 µL of semen, then added 10 µL of distilled water on the preparation object glass and then observed using a microscope. Sperm used in this research had a motility of more than 50%. Treatment extender that was NaCl 0.9% as control, Egg yolk with concentrate 0%, 5%, 10%, 15%. Sperm and extender ratio were 1:9 (100 µL : 900 µL) [12,13], and then antibiotic penicillin-streptomycin was added. The sample was applied in five different cold storage times i.e., 0 h, 24 h, 48 h, 72 h, and 96 h at 4°C. Motility, Viability, and MDA level of sperm were observed.

Motility evaluation was performed by dripping 10 µL semen, and 10 µL distilled water semen on the preparation object glass and then observed using a microscope. Viability evaluation was carried out by dripping 10 µL semen and 10 µL eosin-nigrosin on the preparation object-glass, and then it was mixed, swabbed, and then observed using a microscope. Spermatozoa that absorbed the color was dead, whereas the sperm that did not absorb color was alive. the observation was performed by a light microscope (Olympus BX51) with magnification at 400x.

MDA Level Anaylsis

A solution of each treatment was taken 400 µl and added with 400 µL of Trichloroacetic acid (TCA) 20% into the TBA 0.5% then were mixed. 200 µL HCl 1N was added and 1000 µL distilled water and mixed, incubated at 95°C for 15 minutes, and centrifuged at 10000 rpm at 4°C for 10 minutes. The supernatant was read in absorbance 532 nm wavelength [14].

Data Analysis

Data were analyzed by one-way ANOVA. Kolmogorov-Smirnoff and homogeneity Levene's test were used for data normality. If there was significant differentiation, it was continued by Duncan.

RESULT AND DISCUSSION

The quality of Spermatozoa (Motility and Viability)

The quality of Spermatozoa has two parameters analysis, such as motility and viability. Motility spermatozoa have variated based on strength and duration [15]. The result of motility spermatozoa *C. carpio* L. Punten strain has shown in Table 1. Diluent of egg yolk concentrate 0%, 5%, 10%, and 15% have decreased after storage at 4°C. The time of storage 0 h and 24 h showed that the percentage of motility has more than 70% and decreased to 50% at 48 h – 72 h.

The time of cold treatment storage is significantly different from the percentage of motility spermatozoa with *a P-value* = 0.000. EYC 0% at time 0 h – 24 h has high percentage of motility 95.14% ± 4.84% and 82.74% ± 6.55%. Furthermore, motility at time 96 h has the lowest percentage 19.82% ± 8.34%. It was shown that cold storage time at 4°C would decrease the motility of spermatozoa. Motility spermatozoa of sturgeon fish *A. gueldenstaedtii* and *A. baerii* were decreased significantly after 72 h of storage at a cold temperature [16].

Viability spermatozoa can be observed by an eosin-negrosin dye. Living sperm did not absorb color caused it still has membrane function, whereas sperm absorbed color was died [17]. The result of viability spermatozoa *C. carpio* L. Punten strain has shown in Table 2. Percentage of viability shown that sperm has decreased by storage at 0 h – 96 h (Table 2). EYC 15% has a high percentage of viability spermatozoa and a significant difference with EYC 0% and 5%, whereas there was no significant difference between EYC 10% and EYC 15%. It was shown that EYC 10% and 15% have the ability to maintan viability of spermatozoa at 4°C compared to other concentrations.

The percentage of viability spermatozoa was significantly different at 0 h and 24 h with percentage viability at storage time 72 h and 96 h. However, the average percentage optimum of viability spermatozoa stored through 96 h ≥ 76.07% ± 7.50%.

The change in storage temperature influences the quality of spermatozoa. The level of ROS production in the population of sperm has a negative correlation with the quality of the sperm. Leukocytes can increase nitric oxide as free radical compounds in seminal plasma sperm of abnormal and dead [17].

The previous study has demonstrated that a concentration of 10% egg yolk medium has the ability to maintained spermatozoa compared with honey and glucose at the same concentration [7]. The current study was also shown that the percentage of viability spermatozoa, which stores in a diluent of egg yolk, keeps maintained.

Table 1. The average percentage of motility spermatozoa *C. carpio* L. Punten strain at cold storage 4°C used diluent of egg yolk

Storage Time (h)	Percentage of motility Spermatozoa (%)			
	EYC 0%	EYC 5%	EYC 10%	EYC 15%
0	91.83 ± 4.98	95.14 ± 4.84	88.82 ± 6.03	89.63 ± 5.83
24	88.24 ± 8.06	88.77 ± 8.03	84.40 ± 5.91	82.74 ± 6.55
48	65.82 ± 9.73	78.51 ± 4.60	73.25 ± 8.81	67.03 ± 10.64
72	52.69 ± 8.53	69.03 ± 5.53	65.88 ± 8.20	60.98 ± 14.74
96	19.82 ± 8.34	38.89 ± 7.91	41.80 ± 7.59	39.37 ± 7.21

Notes: EYC= Egg Yolk Concentration

Table 2. The average percentage of viability spermatozoa *C. carpio* L. Punten strain at cold storage 4°C used diluent of egg yolk

Storage Time (h)	Percentage of viability Spermatozoa (%)			
	EYC 0%	EYC 5%	EYC 10%	EYC 15%
0	92.68 ± 1.39	96.14 ± 2.04	96.64 ± 2.35	98.17 ± 0.79
24	92.17 ± 3.31	94.84 ± 3.92	96.09 ± 2.76	96.92 ± 2.30
48	84.63 ± 6.78	86.22 ± 2.98	95.08 ± 3.36	94.31 ± 5.34
72	76.35 ± 7.00	83.48 ± 5.22	85.87 ± 8.06	88.83 ± 3.58
96	76.07 ± 7.50	80.61 ± 8.73	82.55 ± 7.95	87.19 ± 5.15

Notes : EYC= Egg Yolk Concentration

Level of Malondialdehyde (MDA)

Level of malondialdehyde was observed by thiobarturic acid reactive subtances (TBARS) method using spectrophotometer with absorbance wavelenght of 532 nm. Based on table 3 the result shown that the effect diluent of spermatozoa *C. carpio* L. punten strain EYC 0% was significantly different compared to MDA level in EYC 15%, while EYC 5%, 10% and 15% was not significantly different. EYC 15% had highest MDA level, while EYC 10%, 5% and 0% was in lowest position.

Egg yolk has low density lipoprotein to protect the membrane of sperm from heat shock [8,18,19]. The treatment of storage time in this study had a significant effect on percentage MDA levels. The storage time of 48 hours had the lowest levels 119.88 ± 36.11 ng/ml - 137.82 ± 39.49 ng/ml, while the storage time of 96 hours had the highest levels 137.24 ± 25.07 ng/ml - 164.44 ± 29.36 ng/ml). The effect of storage time was increased MDA levels of spermatozoa C. *carpio* at 0 h to 24 h. Furthermore, there was decreased in MDA levels at 24 h to 48 h. Then the MDA levels increase at 72 h to 96 h (Table 3).

Table 3. The average percentage of MDA level *C. carpio* L. punten strain at cold storage 4°C used diluent of egg yolk

Storage Time (h)	Percentage of MDA Level (ng/ml)			
	EYC 0%	EYC 5%	EYC 10%	EYC 15%
0	139.74 ± 31.03	138.71 ± 26.07	142.68 ± 25.29	156.06 ± 36.09
24	150.91 ± 56.38	162.38 ± 49.12	165.03 ± 38.09	171. 50 ± 50.55
48	119.88 ± 36.11	120.03 ± 23.47	122.68 ± 27.78	137.82 ± 39.49
72	133.71 ± 32.49	139.44 ± 25.70	140.62 ± 34.17	140.47 ± 30.65
96	137.24 ± 25.07	141.21 ± 22.50	149.88 ± 29.10	164.44 ± 29.36

Notes : EYC : Egg Yolk Concentration

Table 4. Pearson Correlation Coefficient Analysis-SPSS

		MDA	Motility	Viability
MDA	Pearson Correlation	1	-.263**	-.015
	Sig. (2-tailed)		.004	.870
	N	120	120	120
Motility	Pearson Correlation	-.263**	1	.567**
	Sig. (2-tailed)	.004		.000
	N	120	120	120
Viability	Pearson Correlation	-.015	.567**	1
	Sig. (2-tailed)	.870	.000	
	N	120	120	120

**. Correlation is significant at the 0.01 level (2-tailed).

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In this research, the high concentration of egg yolk indicated that the level of MDA was high (15% egg yolk). Egg yolk contains lipid macromolecules and proteins, which are the target of lipid peroxidation by free radical compounds. Free radical compounds are reactive and can modify the structure of several biomolecules such as lipids, proteins, and nucleic acids. Lipid peroxidation products may cause an oxidation reaction cycle, which affects the pH. Furthermore, the amount of egg yolk is assumed to cause the diluent medium to become more concentrated and decrease the pH. Production of MDA was increased at low pH by lipid peroxidation chain reactions [20].

Interaction of Malondialdehyde (MDA) with Motility and Viability Spermatozoa

This research was found that MDA levels have a negative correlation with spermatozoa motility and viability percentage of *C. carpio* L. Punten strain. A significant correlation occurs in MDA levels with the percentage of motility (correlation coefficient = -0.263) (Table 4).

There is a positive correlation of motility with significant viability (correlation coefficient = 0.567), and it is classified as a strong correlation. Meanwhile, there was no significant correlation between MDA levels and viability (p = 0.870> 0.05). It indicates that when the MDA level increases, the motility of the sperm would be decreased. Furthermore, there is no correlation between MDA levels and viability spermatozoa. It indicated that MDA levels with a storage at 96 h have not provided a significant correlation. In addition, the interaction between motility and viability shows that when the motility of the sperm decreases, the viability would be also decreased.

Malondialdehyde is a marker of oxidative stress caused by ROS [21] and a marker of lipid peroxidation [16]. The results of this study were related to a previous study [22], which show that increased levels of ROS were negatively correlated with sperm concentration, motility, morphology, and parameters of semen. ROS may cause oxidative damage to the membrane, midpiece, axonema, and DNA of sperm, which leads to apoptosis [23,24]. Polyunsaturated Fatty Acid (PUFA) is able to cause lipid peroxidation of membrane spermatozoa [25]. Lipid peroxidation increases the membrane integrity of sperm, so it can increase cell permeability and enzyme inactivation [16]. ROS will increase the expression of lipid peroxidation were affect the MDA that causes the death of the sperm cell. It caused a decrease in motility, so that level of MDA was high.

This research has shown that egg yolk has the ability to preserve sperm in cold storage time at 0 h – 96 h. Egg yolk diluent is appropriate to use as an applicative diluent caused it was easy and cheap. Also, the quality (motility and viability) of the sperm of *C. carpio* L. Punten strain was maintained by the addition of egg yolk during cold storage at 0 h - 48 h and also suitable to management and breeding of *C. carpio* L. Punten strains by providing transfer of male gametes to reducing the risk of death or stress of male parental *C. carpio* L. Punten strain fish species.

CONCLUSION

The quality (motility and viability) of spermatozoa fish *C. carpio* L. Punten strain was maintained at 96 h during cold storage. The optimum storage for motility was found in egg yolk diluent 5% at 48 hours. The MDA level of the semen of *C. carpio* L. Punten strain stored at cold temperatures increased at 0 h - 24 h, then decreased at 48 h of storage and increased at stored 72 h - 96 h. There is a negative correlation between MDA levels and motility. There was no correlation between MDA levels and the viability of spermatozoa *C. carpio* L. Punten strain.

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