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The effects of different concentrations of ccBA-GFP promoter with electroporation methods on the quality of koi sperm (*Cyprinus carpio* var. koi)

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Abstract. The effectiveness of the use of promoter concentration which will be inserted into the Koi sperm as the medium of gene transfer is important. The objective of this research is to find out the influence of the adding of different concentrations of the ccBA-GFP promoter with electroporation methods to the motility, viability and the fertilization rate of the Koi sperm . This study was conducted at Central Lab of Life Sciences Brawijaya University in April 2017. Electroporation methods were conducted by using 30-volt voltage, 4 times shocks with 0.5 seconds per shock. The treatment of different concentration was done through 3 types of ccBA-GFP promoter concentration, namely: 10 ng/µl, 30 ng/µl, and 50 ng/µl. The best motility percentage with the score of 4 is at the treatment A (10ng/µl concentration), the best viability percentage is 77.83 % at the treatment A (10 ng/µl concentration) and the best fertilization rate is 73.09 % at the treatment A (10 ng/µl concentration). The result shows that there is a relationship between the treatment given to the motility and viability of the Koi sperm, at which, the higher the shocks, the lower the percentage of the motility and viability of the Koi sperm.

1. Introduction

Koi fish (*Cyprinus carpio*) is one of the leading tropical fish commodities that has a big potential in Indonesia, especially in West Jakarta, Blitar, Cianjur, Sukabumi, and Makassar. Japan is known as the biggest Koi cultivator country in the world and it is facing an obstacle on the cultivation area, hence Indonesia has a potential to utilize this big opportunity [1]. For that reason, the genetic quality of the local Koi has to be improved to make them able to compete with the imported ones. One of the genetic engineering techniques the can be applied is transgenesis.

Electroporation is one of effective and efficient transfer methods to be applied. The basic principle of this method is creating reparable-holes in the cell membrane with the help of power supply and then the cell is suspended in the DNA solution that later on will come into the cell through the whole shaped [2]. Electroporation methods conducted to the sperm functions as the vector of foreign gene holder that is going to be introduced (Sperm Mediated Gene Transfer). Several previous studies have shown that sperm has a unique ability to be natural foreign gene holder vector [3].

The success of gene transfer with electroporation methods depends on the level of voltage and the DNA concentration used [4]. In addition to the effective electroporation methods, it is also important to know the use of promoter concentration or foreign DNA that will be inserted. For that matter, proper concentration of promoter is essential and so is the effect of the sperm quality on the

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success of transgenesis by using the sperm as the gene transfer medium. During the transgenesis, the promoter has an important role to decide if a character coded by the gene transferred meets the expectation. The quality of the sperm is the main factor in succeeding the transgenesis process during fertilization. The quality of koi sperm includes sperm motility and viability.

The objective of this research was to find out the effects of the use of different concentrations of ccBA-GFP promoter inserted in sperm through electroporation process on the quality of the Koi sperm, which includes sperm motility, sperm viability, and fertilization rate of the sperm to the Koi fish eggs (*Cyprinus carpio*).

2. Methodology

This study was conducted in April 2017 at Central Lab of Life Sciences, University of Brawijaya (LSIH UB). The materials needed to conduct this study were Koi sperm, saline, eosin-nigrosin, ccBA-GFP DNA promoter, aluminum foil, and distilled water. The equipments used were a set of Gene Pulser Xcell Tm of BIO-RAD consisted of electroporator machine, shock pod electroporator, and a 0.2 mm cuvette, Olympus BX 51 inverted microscope, Olympus CX 41 binocular microscope, micropipette, microtube, tip, digital camera, microscope slide, Petri dish, and hand tally counter.

The research followed a Completely Randomized Design (CRD) with three treatments and one control with each of them was repeated three times. The treatments applied are as follows:
Treatment A = 10 ng/µl ccBA-GFP promoter concentration.
Treatment B = 30 ng/µl ccBA-GFP promoter concentration.
Treatment C = 50 ng/µl ccBA-GFP promoter concentration .
Treatment K = no treatment

2.1. Sperm Collection

The sperm was collected from the male parent Koi gonad through hand-stripping. It was then put into a 1.5 ml syringe and added with saline to dilute it with the ratio of 1:1, the next step was moving it into a microtube and keeping it in a room temperature [5].

2.2. Koi Fish Eggs Collection

Koi fish eggs were collected from the female parent gonad through hand-stripping. The female parent Koi was previously given no food for 24 hours to empty the stomach and intestine and then injured using Ovaprim with a dose of 0.5 ml/weight and the last step was conducting an artificial spawning.

2.3. Electroporation

The koi sperm, which had been prepared in a microtube, was then electroporated using 1 set of Gene Pulser Xcell TM Electroporation System Bio-Rad. The mechanism was started with putting 25 µl of sperm into an electroporator cuvette and adding 10 µl of ccBA-GFP promoter according to the designated treatment [6]. Next, the electroporator was then pulsed by 30-volt electric field strength with the period of shocks was 0.5 m/s, and the process was repeated four times [7, 8]. The sperm was then added with 275 saline (to make total liquid of 300 µl) and t the sperm motility and viability were observed. Electroporation methods used on the koi sperm referred to the previous study conducted on goldfish sperm (*Cyprinus carpio*) [9].

2.4. Sperm Motility

Post-electroporation sperm motility was observed by collecting 1 µl Koi sperm and dropping it on a microscope slide. It was then dripped on with water to activate the sperm. Next, the slide was covered by glass cover and the motility was observed for 7 minutes under the inverted microscope that had been connected to the computer. Table 1 shows the percentage of sperm assessed by scoring methods according to Dewi [10].

Table 1. Motility scoring.

Percentage	Criteria	Score
>70 %	Spermatozoa moves forward and quickly with the variation of tail movement	5.0
55 – 70 %	Spermatozoa moves forward and shows a quick movement	4.0
40 – 55 %	Spermatozoa moves forward and several of them show a quick movement	3.0
25 – 40 %	Spermatozoa moves forward	2.0
10 – 25 %	Spermatozoa moves	1.0
1 – 10 %	Mostly spermatozoa are not moving	0.5
<10 %	Spermatozoa are not changing	0.0

2.5. Sperm Viability

An observation of the sperm viability or sperm survival was performed by coloring them using eosin-negrosin staining method. After being electroporated, 5 µl sperm was taken and 1 µl was dripped on a microscope slide. Next it was added with eosin-negrosin and painted using a cover glass. After dry, it was then observed under the CX41 Olympus microscope. Living sperm was marked by clear color and red color indicated dead sperm. According to Susilowati [11], the formulation used to assess sperm viability is:

$$\text{Viability} = \frac{\sum \text{Viable Sperm}}{\sum \text{Non Viable sperm}} \times 100\ \% \quad (1)$$

2.6. Fertilization Rate

The number of Koi fish eggs that had been fertilized by the sperm of electroporation was counted after 3 hour. Fertilization rate was calculated following the following formula [12]:

$$\text{Fertilization rate} = \frac{\sum \text{Fertilized egg}}{\sum \text{Egg}} \times 100\ \% \quad (2)$$

2.7. Data Analysis

Data was analyzed using Analysis of Variance (ANOVA). If there was significant different, LSD test was performed. In order to find out the relationship between treatment and the result, regression analysis was applied to decide the character and function of regression, giving a decision for the best treatment.

3. Result and Discussion

3.1. Pre-Treatment Sperm Quality

The success of electroporation includes motility and viability of sperm and also the sperm characteristics like color, smell, and pH.

Table 2. Sperm quality.

Parameter	Result
Concentration	11.7 x 109 cell.mL-1
Volume	2.3 mL
pH	7
Colour	White Milk
Head Diameter	± 3 µm
Length of Tail	± 8 µm

Motility	Score of 5
Movement Intensity	Progressive
Viability	90 %

Table 2 shows that the quality of the Koi sperm pre-electroporation was good and could be used to conduct the research. The condition or the quality of koi sperm was essential since it would be a vector of the foreign genetic carrier to maximize the method of SMGT (Sperm Mediated Transfer Gen) and the process of foreign gene binding. It would affect the success of the sperm in the insemination process with the egg cell after electroporation process.

According to Dewi [13], good quality sperm can fertilize egg cell well and changes occur to the sperm will affect its performance. The changes refer to motility and survival. According to Havez [14], the cement, which meets the requirement of fertilization, has to contain a living and progressive sperm and the percentage of motile sperm is not more than 75 %.

3.2. Post-Electroporation Sperm Motility

Post-electroporation, each treatment showed a different result on the percentage of Koi sperm motility. The percentages of motility are shown in table 3.

Table 3. Sperm motility (%).

Treatment	Repetition			Total	Average
	1	2	3		
A	79	79.5	75	233.5	77.83
B	74	77	75.5	226.5	75.50
C	75.5	73.5	75.5	224.5	74.83
Total				684.7	
Control	89	93	90	272	90.66

From table 3, it can be seen that the highest average score of Koi sperm motility percentage gained by treatment A (10/µl concentration) with the score of 4, which means 55-70 % spermatozoa moved forward and showed a quick movement, while the lowest score was gained by treatment C (50 ng/µl concentration) with the score of 3.33, which means 40-55 % spermatozoa moved forward and several of them showed a quick movement. The analysis of the variety of Koi sperm motility can be seen in table 4.

Table 4. Analysis of the variety of koi sperm motility after electroporation treatment.

Source Diversity	Db	JK	KT	F Test		
				F	5 %	1%
Total	2	0.666	0.333			
Random	6	3.334	0.555	0.6 (ns)	5.14	10.92
Treatment	8					

Table 4 shows that F-table 1 % > F-count (6.21) < F-table 5 %, which means different concentration of ccβA-GFP promoter with electroporation method did not give significant influence on Koi motility.

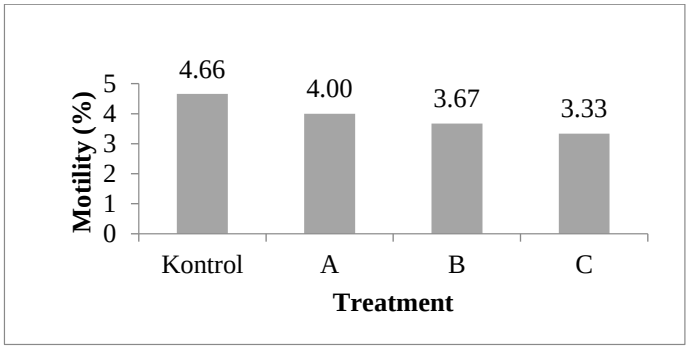


Figure 1. Sperm motility graph.

Figure 1 shows comparison between motility value of control (untreated) and treated sperm. The control sperm showed the highest result of motility that means the koi sperm used had a good quality. The graph indicates that the higher the concentration of the ccBA-GFP promoter given, the lower the percentage of the koi sperm motility.

The different concentration given had an influence on dilution ratio used in producing ccBA-GFP promoter. Different concentrations were collected from dilution using TE buffer according to the dilution formula. At lower concentration, more dilution solution was added compared to the amount added at higher concentration, causing differences in osmolarity value. Different dilution ratio where thicker and thinner solutions were separated by semipermeable membrane, made the diluted molecules flow from the thinner solution to the thicker one, similar to osmosis process. Therefore, the use of lower concentration caused easier diffusion into the sperm cell and resulted in a higher intensity of luminescence if compared to higher concentration with high numbers of DNA. In addition, higher concentration caused an incompatibility as a living medium for sperm; hence it decreased the rate of sperm motility and viability. There are at least two important parameters that have to be optimized in applying SMGT to reach an efficiency, namely cement quality and the entry process of sperm. Entry process of the sperm highly depends on the sperm viability and motility [15]. DNA concentration in the dilution also affects the success of gene transfer [16]. Spermatozoa membrane permeability tightly relates to the spermatozoa motility since membrane permeability is related to the nutrition transportation, which is important for cell metabolism. The sperm cell tends to be smaller after performing electroporation causing a decrease in sperm motility percentage [17, 18].

3.3. Post-Electroporation Sperm Viability

Sperm viability is the percent comparison of living and dead sperm. The average values of viability percentage can be seen in table 5.

Table 5. Sperm viability (0%).

Treatment	Repetition			Total	Average
	1	2	3		
A	79	79.5	75	233.5	77.83
B	74	77	75.5	226.5	75.50
C	75.5	73.5	75.5	224.5	74.83
Total				684.7	
Control	89	93	90	272	90.66

According to table 5, the highest result was given by treatment A (10 ng/µl concentration), which was 77.83 %, while the lowest result was shown by treatment C (50 ng/µl concentration) with 74.83

%. The control (untreated sample) was higher than treatment A by 90.66 %, which showed the higher the ccBA-GFP promoter concentration given, the lower the viability percentage resulted. Improper living medium caused sperm viability decreased.

According to Table 6, F table 1 % > F count (2.279) < F table 5 %, which showed different concentration of ccβA-GFP promoter given with electroporation methods did not give significant influence over the Koi viability. The analysis of the variety of Post-electroporation Koi Sperm Viability is shown in table 6.

Table 6. Analysis of the variety of post-electroporation koi sperm viability.

Source Diversity	Db	JK	KT	F Test		
				F	5%	1%
Total	2	14.68	7.34			
Random	6	19.36	3.22	2.279	5.14	10.92
Treatment	8					

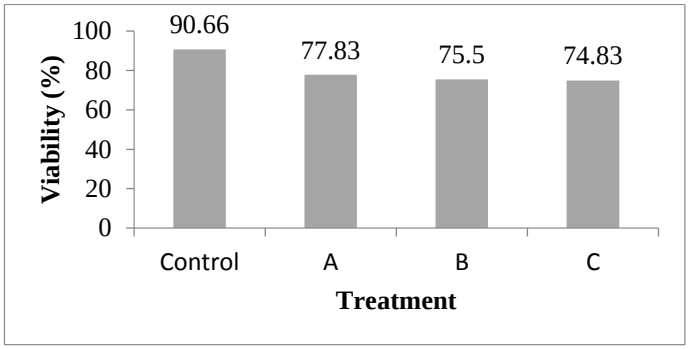


Figure 2. Viability of control and treated sperm.

Sperm viability normally ranges from 1-2 minutes after coming out of the testes. Change of plasma membrane structure and the loss or decrease of some of the mitochondrial matrix can result in loss of spermatozoa viability [19].

Electroporation treatment with high electric field can stretch the sperm cell and temporarily destabilize the membrane cell. During that period, the membrane is highly permeable to exogen molecules (DNA or RNA) around the cell medium. DNA can then move into the cell (internalization process). When the electric field is turned off, the membrane's deep hole closes and the exogen DNA comes into the cell [20, 21].

Therefore, the best result was gained when low concentration of promoter was used to produce transgenic Koi fish. During the transgenesis using sperm as the genetic holder vector, the motility and viability of sperm were important.

3.4. Sperm Fertilization Rate at the Cell Eggs

Fertilization rate is the number of eggs cell that can be fertilized, i.e., the percentage of ratio between number of eggs cell spread and fertilized by sperm. A total of 0.25 gram of eggs (±114 eggs) was fertilized The egg cell was fertilized with the electroporated sperm . Fertilized eggs cells were then observed after the fertilization process and calculated according to the fertilization rate formulation. The percentage of successfully fertilized eggs is presented in table 7.

Table 7. Fertilization rate (%).

Treatment	Fertilization Rate			Total	Average
	1	2	3		
A (10 ng/µl)	76.3	74.5	68.4	219.2	73.09
B (30 ng/µl)	73.6	52.6	66.6	192.9	64.32
C (50 ng/µl)	39.4	58.7	68.4	166.6	55.55
Total				578.92	
Control	85,09	83,33	71,92	240,34	80,11

The highest average of fertilization rate was 73.09 % given by treatment A, followed by treatment B with 64.32% and the lowest was treatment C with 55.55 % (table 7). The control sample had higher fertilization rate than treated samples. Fertilization rate was equal to sperm quality used in fertilization including sperm motility and viability. The best quality sperm came from treatment A, thus the best fertilization occurred at treatment A since sperm quality of highly affects fertilization rate. Sperm with good motility can move maximally during fertilization.

Table 8 shows, F table 1 % > F count (1,982428) < F table 5 %, which showed different concentration of ccβA-GFP promoter given with electroporation methods did not give significant influence over the Koi Fertlization Rate. The analysis of the variety of Post-electroporation Koi Fertlization Rate is shown in table 8.

Table 8. Analysis of the varians of post-electroporation koi fertilization rate.

Source Diversity	db	JK	KT	F.Hit		
				F	F 5%	F 1%
Total	2	461.6528	230.8264	1.982428	5.14	10.92
Random	6	698.6172	116.4362	ns		
Treatment	8					

Figure 3 shows the fertilizaton rate control showed the highest result of fertilizaton rate that means the koi sperm used had a good quality. The graph indicates that the higher the concentration of the ccBA-GFP promoter given, the lower the percentage of the koi fertilizaton rate.

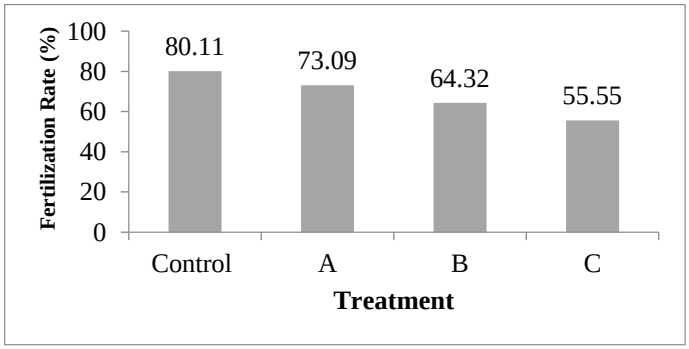


Figure 3. Fertilization Rate of control and treated sperm.

The difference between control sample and treated samples was caused by the difference in sperm quality was used to fertilization. The control sperm had better quality since it was not given electroporation so it did not experience environment stress and it still had energy reserve coming from the medium used to protect the sperm immotility. In addition, the control sperm was able to

maintain the sperm quality so that during the artificial fertilization, the sperm went into cell micropyle and had adequate energy to go through embryogenesis process. Moreover, unlike the electroporated sperms, the control sperm did not cause the plasma membrane to get broken and it did not lose the mitochondria contained in its tail. The loss of mitochondrial matrix can decrease viability and percentage of the sperm can influence the rate of the hatching eggs.

The electroporated sperm decreased the viability and the fertilization rate due to its broken tail [22]. The low rate of spermatozoa fertilization is also influenced by the short time of motility and the sperm viability, while the success of egg fertilization is highly influenced by the sperm motility [23, 24]. Long viability does not guarantee high fertilization rate, thus spermatozoa needs high energy to fertilize cell. The success of post-electroporation sperm fertilization is highly influenced by the sperm motility. There is a positive correlation between the capability of foreign DNA to come and integrate into the main sperm with the quality of cement and sperm motility rate. Therefore, it needs the sperm mobility of at least 80% and not lowers than 65%. In addition, a motile sperm is able to tie foreign DNA transferred up to 30% from the total [25].

4. Conclusion

Different concentration of ccBA-GFP promoter given with electroporation method had effect on sperm motility and viability. The higher the concentration promoter given, the lower the quality of Koi sperm. The best result was shown by treatment A that had the lowest concentration of promoter (10 ng/µl).

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