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The Effect of Different Electric Field Strength (V/Cm) on Post-Electroporated Quality of Koi Fish Sperms

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ABSTRACT

This research was to understand the effect of different electric field strength on sperm quality of Koi fish and to identify the best electric field. Various electric field strengths were treated at range of 10, 20, 30, 40 and 50 V/Cm in form of square wave using electroporator Gen Pulser of BIO RAD. The length of shock was 0.5 ms with shock frequency of 4 times. The result showed The highest and lowest parameter tests of treatment Koi fish could be explained as following. (1) The highest motility was 77 % and found at electric field strength 10 V/Cm, and whereas the lowest motility was 58 % obtained from electric field strength 50 V/Cm. (2) The highest viability of Koi fish sperms was 80 % obtained from treatment with electric field strength 10 V/Cm, while the lowest viability was 59 % found at electric field strength 50 V/Cm (3). The highest fertility was 70 % acquired from electric field strength 10 V/Cm whereas the lowest fertility was 51% derived from electric field strength 50 V/Cm. It was suggested from this research that to obtain better motility and viability rates, electric field strength 10 V/Cm should be considered.

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Electric Field, Sperm Quality, Square Wave, Motility, Viability, Fertility

INTRODUCTION

Fish sperm was potential to be developed into a vector in transgenic process. Besides being a relatively new process, genetic transfer process was also relatively natural [1]. Transgenic technology in animal was an attractive technology to develop [2]. In modern biotechnology, the characters of certain living creatures could be changed by moving genes from a species to other species. Transgenic technology had been suggested since 1980 by Gordon and colleagues. In recent development, establishing transgenic fish by transferring "DNA construct" was facilitated through several methods [3], such as Micro-Injection, Retroviral Injection (intentionally injected with virus), Sperm-Mediated Gene Transfer (sperm as gene carrier), Particle Bombardment (gun particle or biolistic), and Electroporation [4]. Of these methods, electroporation method was the most frequent method used for gene transfer because it was more efficient, faster, simpler, and relatively natural. Gene transfer on fish using electroporation method with fish sperm as gene transfer medium, however, was rarely found in Indonesia [1].

Electroporation was a mechanical method used to embed certain molecule into host cell through cell membrane. In this procedure, electric shock was used to open temporarily phospholipids layers which allowed alien molecule, such as DNA, to enter into the cell and be the part of the cell [5].

Transfer of DNA molecules into cells, a transfer of gene from cells that contain foreign DNA through electric shock. Two different types of electric shocks that are Square wave and Exponential wave have been used in transferring the gene into the fish sperm. The voltage of the Square wave is more directed to the required amplitude

which is to maintain the length of the shock time, then returned to zero. While the Exponential wave, the voltage is intended for a desired amplitude, then gives exponential or continuous damage. Amplitude is the furthest distance from the equilibrium point, but in this wave the amplitude depends on the applied voltage [6].

The entry of DNA molecule into the cell was facilitated by electric shock against the DNA-carrying cell. Two wave forms were used, respectively square wave and exponential wave. Both were useful in transferring gene into fish embryo and fish sperm [7]. Square wave method could produce great voltage which kept more efficient the internalization of DNA due to the relatively wide opening of the pores. Other benefit was very short wave (*pulse length*) which could prevent cell damage and help the cell to recover faster because the involved heat was relatively low. *Square Wave* could also conduct higher electric field strength at short wave, and therefore, it produced low heat which made possible that certain molecule was transferred without killing cell or embryo [8].

Sperm is spermatozoa cells that are in seminal solution and are produced by hydration testes, one part of the fish reproduction apparatus [9]. Spermatozoa are solid and very distinctive cells, do not grow or divide and have no physiological role to the animals that produce them, just to fertilize eggs of the same kind [10]. As a gene transfer medium, sperm is potentially developed in transgenic fish because of its relatively natural and more efficient procedure [11]. Sperm have advantages as gene transfer media, because in the process of transfer of genetic material, sperm as a natural vector. Sperm cells have been used as gene transfer vectors in Carp, Catfish and Tilapia [6].

Koi fish belonged to a family of Cyprinadae, and represented ornamental fish favored by Indonesians or foreigners. There was a future possibility that genetic engineering might be done to produce certain color and pattern, and it was realized through transgenic process. This paper would be a review against the optimization of different electric field strength with Koi fish sperm as transgenic vector in electroporation method.

The objective of research was to understand the effect of different electric field strength on sperm quality of Koi fish and to identify the best electric field

MATERIAL AND METHODS

Materials in this research were (1) Male prime of Koi Fish that was dissected for obtaining sperm to examine its motility and viability; (2) Female prime of Koi Fish that was stripped and taken its egg to be used in fertilization process; (3) ovaprim that was injected to fish prime to accelerate gonad maturity; (4) Fructose solution as the diluter before and after electroporation; (5) HCl 0.2 M to clean cuvette; (6) Ethanol 70% used to soak cuvette after cleaning with HCl; (7) Ice rock used to cool cuvette before cuvette was applied in sperm electroporation; and (8) Eosin-negrosin dye to color the sperm to facilitate the observation of its viability.

Several instruments were used, such as no (1) rearing batch of Koi fish primes; (2) spuit as sperm container during stripping; (3) micro pipette to move sperm cell, fructose, and aquadest; (4) digital weight METER PE 22 to weight Koi fish primes; (5) bowl to contain eggs from stripping; (6) ruler to measure total length of Koi fish; (7) fiber batch as the container of Koi fish primes after injected with ovaprim; (8) a set of electroporator (BIO-Rad) to give electric shock at Koi fish sperm; (9) cuvette as sperm container during electroporation; (10) Microscope DIC to observe motility of Koi fish sperm; (11) Ependorf as sperm container after electroporation; (12) Petri dish as the container of fertilized eggs; (13) Digital camera to take picture of the motility and viability of sperms and the development of ovum; (14) Set of incubator tools to incubate the eggs that had been fertilized by sperms.

Research was conducted at the Laboratory of Fish Biology and Reproduction (Breeding Laboratory), Faculty of Fishery and Marine Science, and also at the Central Laboratory of Natural Science (LSIH), University of Brawijaya, Malang, East Java. Research begun from May 2011 to finished.

Research design

Various electric field strengths were treated at range of 10, 20, 30, 40 and 50 V/Cm in form of square wave using electroporator Gen Pulser of BIO RAD. The length of shock was 0.5 ms with shock frequency of 4 times (1).

(a) Research procedure

Before exposing Koi fish sperm to electric field strengths, electroporation instruments must be prepared and programmed based on research design. Cuvette must be also ready, and cuvette size should be 0.2 cm. The sample was prepared through following steps: (1) 25 µl sperms were poured into cuvette using micro pipette and the cuvette was sealed tight; (2) The lid of "Shock Pod" was opened; (3) Cuvette with sample was set into "shock pod"; (4) "Shock Pod" was closed; (5) PULSE button was pressed; (6) Data were displayed on screen and it must be recorded;

(7) Physiological solution of 275 µl was added into 25 µl post-electroporated sperms in cuvette; (8) One drop was taken for motility observation and one other drop was obtained for viability observation, and sperms were colored with dye; and (9) Result of observation was recorded and compared with the quality of control sperm.

(b) Test parameters

1) Spermatozoa motility; 2) Spermatozoa viability; 3) Spermatozoa fertility

Fertility, or also called as fertilization, was a process when ovum core fused into cytoplasm to produce zygote. Basically, fertilization was the unification or fusion of male and female gamete cells to produce a cell [12]. The fertilized and unfertilized eggs might be enormous, and therefore, fertility percentage was counted (%).

Ethical approval

The review board and ethics committee of Fisheres and Marine Science Faculty approved the study protocol.

RESULTS AND DISCUSSION

Data of motility, viability and fertility

Data of observation on percentage rate of test parameters show that the higher electric field strength (V/Cm) could produce lower percentage rate of test parameters of Koi fish sperms. Table 1 showed that percentage rate of sperm quality of control Koi fish was still higher than that of treatment Koi fish.

The highest and lowest parameter tests of treatment Koi fish could be explained as following:

(1) The highest motility was 77% and found at electric field strength 10 V/Cm, and whereas the lowest motility was 58% obtained from electric field strength 50 V/Cm (Figure 1).

(2) The highest viability of Koi fish sperms was 80 % obtained from treatment with electric field strength 10 V/Cm, while the lowest viability was 59 % found at electric field strength 50 V/Cm (Figure 3).

(3) The highest fertility was 70% acquired from electric field strength 10 V/Cm whereas the lowest fertility was 51% derived from electric field strength 50 V/Cm.

Table 1. Percentage Rate of Test Parameters

Electric field strength (V/Cm)	Sperm quality of Koi fish		
	Motility (%)	Viability (%)	Fertility (%)
Control	82	85	72
10	77	80	70
20	72	75	65
30	67	73	60
40	62	65	55
50	58	59	51

a. Motility of Koi fish sperms

Figure 1 indicated that higher electric field strength would produce lower motility of Koi fish sperms. Spermatozoa plasma membrane was damaged with higher electric field strength, and it disturbed sperm metabolism. The consequence was that sperms lost their motility and even died. Higher electric field strength reduced the percentage of the living sperms. The disturbance against sperm's membrane or film permeability could reduce metabolism activity and damage sperm cells. According to Dewi et al. [13], sperm motility was reduced with higher electric field strength. Tsai [14], had said that sperm motility depended on the level of electric field strength (V/Cm) during electroporation.

According to Kalkianto [15], motility average rate of Koi fish sperms was 31.67% after treating sperms with electric shock at electric field strength 40 V/Cm and with shock length 0.5 ms using square wave method. The reduction of motility rate was due to the adaptation of sperms to electric field strength, and thus, it disturbed sperm's membrane or film permeability which led to metabolism activity decrease, cell damage and motility reduction.

As noted by Faqih [1], electric field strength 40 V/Cm brought relatively small negative impact effect on catfish, indigo fish and gold fish. The application of electric field strength 80 and 120 V/Cm had caused all sperms of gold fish and indigo fish to death. It could be said that sperms of gold fish and indigo fish could not survive normally in the medium exposed to electric field strength above 40 V/Cm, but catfish sperms were successfully survived until electric field strength 520 V/Cm although motility and viability of the sperms were relatively low.

Statistic measurement on the motility of Koi fish sperms indicated that F-table 5% < F-count < F-table 1 %, meaning that Ho was accepted. It also meant that treatment with electric field strength (V/Cm) in different forms of square wave was not influential obviously to the motility of Koi fish sperms.

The motility of Koi fish sperms was a measure of sperm feasibility. It declined with higher electric field strength (V/Cm) and with prolonged shock length. The survival rate of embryos that were fertilized by electroporated sperms and by control sperms (untreated) was not different because 30 millions sperm cells were fertilizing 500 eggs [16].

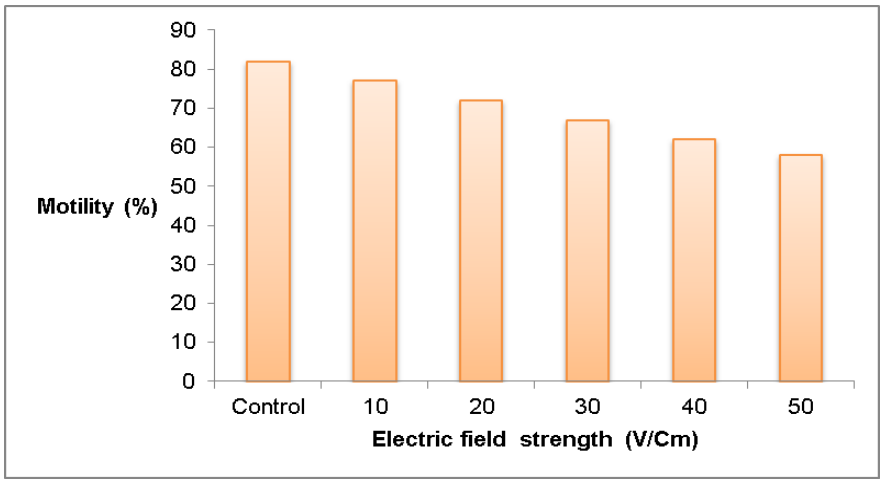

Figure 1. Bar Graphic of Motility of Koi Fish Sperms

b. Viability of Koi fish sperms

Result of observation with Inverted Microscope at 500x magnificence (Figure 2) had shown that dead sperms absorbed dyes because membrane permeability was increasing and facilitating the absorption of dyes. Only few living sperms absorbed the dyes, and therefore, the color was transparent. When sperms mixed with dyes, the living sperms did not absorb dyes or if any, only few of them could absorb it. Dead sperms would absorb great quantity of dyes because sperm's wall permeability was increasing after the death [17]. More understandings required examination on Figure 3. Graphic explained that higher exposure of electric field strength to the examined fish sperms might reduce viability of tested fish sperms. Higher electric field strength could also disturb the membrane of the treated sperms.

According to Partodihardjo [8], 100–2,000 sperms were involved to determine the percentage of sperm viability, and this could be seen in Figure 3. The figure 3 displayed that the highest viability rate was 80% obtained from electric field strength 10 V/Cm whereas the lowest was 59% from electric field strength 50 V/Cm. Sperm viability rate of sperms after electroporation treatment was lower than that of control sperms before electroporation, and the differential was 80%. The reduction of sperm viability rate was possible due to electric shock treatment at higher electric field strength that could damage plasma membrane, thus reducing sperm viability. It was consistent to Dewi et al. [13], sperm viability declined with the increase of electric field strength exposed to sperms. Stronger electric field could produce few number of sperms survived. It was evident because higher electric field strength could disturb sperm's membrane or film permeability, which would reduce metabolism activity and damage sperm cell [1].

Kalkianto [15], previously found that the percentage rate of viability of gold fish sperms was 50 % in average after it was given with electric shock at electric field strength 40 Volts at shock length 0.5 ms. This percentage rate was smaller compared to that of control sperms before treatment because the later was counted for 73.33%. Furizal [19], explained the result of observation on sperm dyeing by stating that viability rate after electroporation was 64.99%, and if compared to viability rate of control sperms (70.58%), this result remained in better category. The viability of post-eletroporated sperms was influenced by electric field strength and shock length during treatment. According to Rubinsky [20], electric current that produced heat could damage cells through electroporation where electric shock influenced cell membrane to transform into permanent pores which risked cells to death.

Statistic measurement on the viability of Koi fish sperms indicated that F-table 5% < F-count < F-table 1 %, meaning that Ho was accepted. It could be said that treatment with electric field strength (V/Cm) in different forms of square wave was not influential obviously to the viability of Koi fish sperms.

The reasons of this finding were some possibilities, such as:

1. Diluter material, respectively fructose 3%, increased the viability but declined the motility time to less significant portion. According to Hidayaturrahmah [21], treating sperms with fructose as diluter for fish sperms could provide energy and nutrient to fish sperms, and ATP energy might increase or elongate sperm motility time.
2. Replication was 3 times. More than 3 replications were assumed producing obviously different results, and therefore, more replications were more accurate the data would be.
3. This research was specified only to Koi fish, and therefore, the fish must be healthy. It was possible that other species could deliver obviously different results.
4. Sperms during stripping must be in good condition and good concentration. According to Adewumi et al. [22], sperm quality might vary depending on various external factors such as feeding schedule and feed quality. Physical characteristic of sperms was white-milk color and viscous.
5. The interval between one treatment and other was too high, respectively 10 V/Cm. If treatment interval was set on 5 V/Cm, the results might be obviously different. According to Cheng et al. [23], result of observation on sperm motility after electroporation at various shock strength and shock length had shown that sperm motility was reducing with the increase of shock voltage.

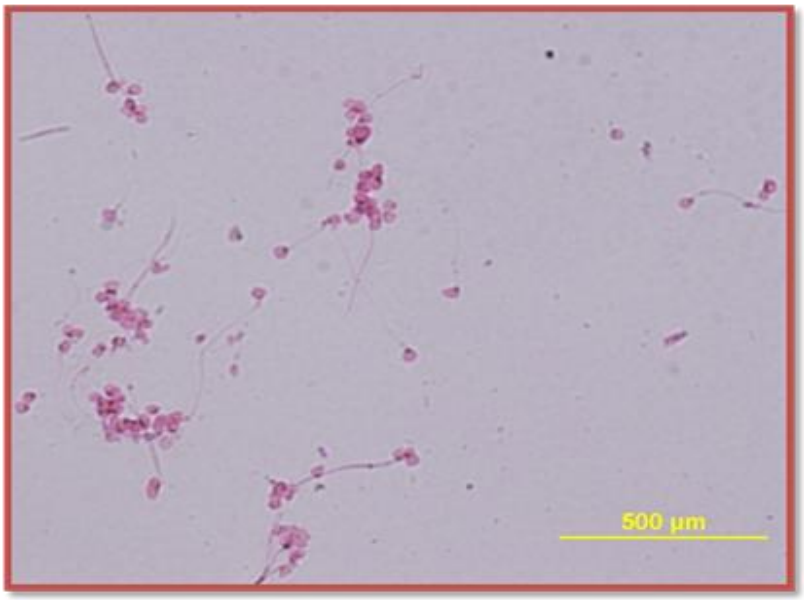

Figure 2. Viability of Koi fish sperms after dyeing with *hematoxin eosin*;
A) Red-colored dead sperms were due to absorbing many dyes; B) Pink-colored living sperms were due to absorbing few dyes.

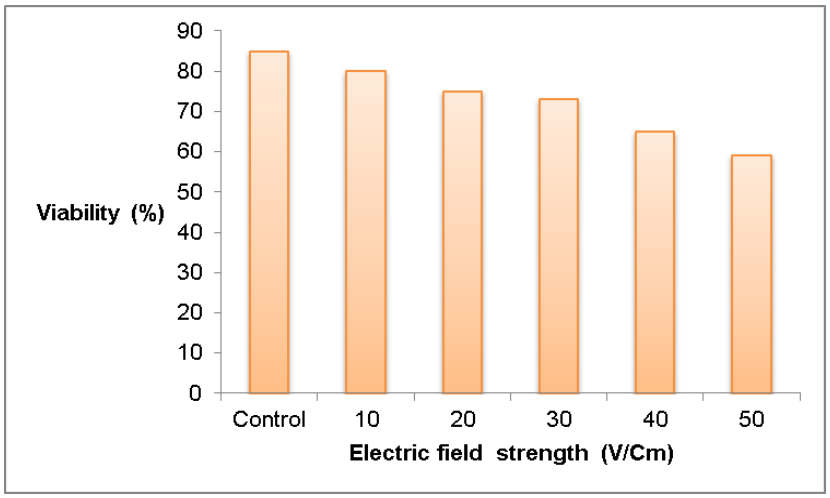

Figure 3. Bar graphic of viability of Koi fish sperms

c. Fertility

To understand the ability of sperms in fertilizing eggs, it was measured through sperm fertility [1]. This research was aimed to acknowledge how many eggs could be fertilized by sperms after exposing sperms with electroporation at electric field strength 50 V/Cm. It was assumed that if highest electric field strength at 50 V/Cm had been useful for egg fertilization, then it was possible that the electroporated-sperms with lower electric field strength 10 V/Cm was also capable to fertilize the eggs.

Graphic above showed that treating sperms with electric field strength 50 V/Cm was producing average rate of fertility counted for 50%. Electric field strength of electroporation at 50 V/Cm had produced higher motility rate and thus, it stimulated higher fertility. Successful egg fertilization by sperms was influenced greatly by sperm motility because higher viability could produce higher fertility. In this condition, sperms needed great energies to fertilize the eggs [21].

High sperm concentration could increase the possibility of fertilization. Fishes that produced hundreds thousands eggs could have high egg concentration, and thus, might need high sperm volume [24]. According to Dacie and Lewis [25], the concentration of fish sperms was ranged about 3.7–11.9 x 10^9 sperms/ml liquid. Sperm density was observed about 40.294 x 10^9 cells/ml liquid.

The dimension size of observational result of trialed fish sperms was displayed in Figure 5. According to Anonymous [26], sperm cells had total length of 50-60 µm. Sperm comprised of two parts, respectively head and tail. Head dimension was 4-5 µm length and 2.5-3.5 µm width. The ratio of length to width was 1.5-1.75 µm.

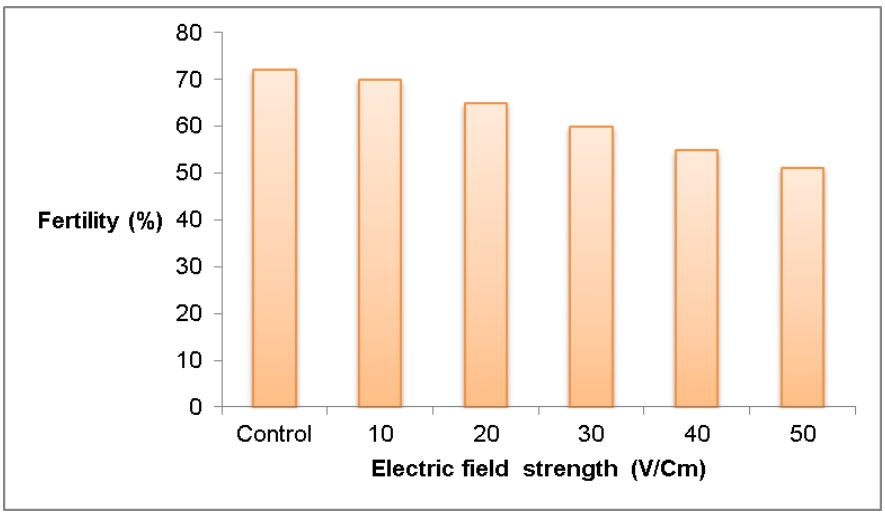

Figure 4. Graphic of Fertility of Koi Fish Sperms

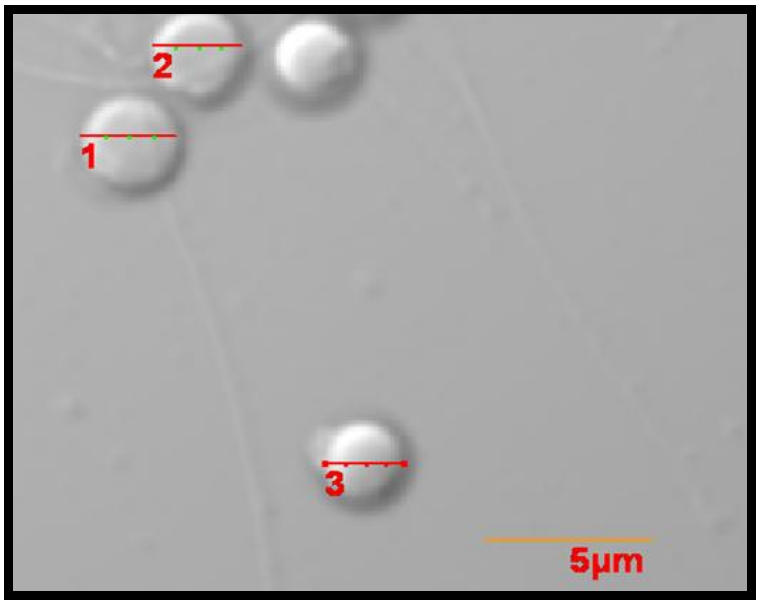

Figure 5. Total length of Koi fish sperms was 15 µm with head width 2.75 µm and tail length 12.5 µm.

CONCLUSION AND RECOMMENDATION

Some conclusions were made from this research, such as:

1. The density of sperm cells was 8.4 x 10^9 cells/ml.
2. The highest motility was 77 % and it was obtained at electric field strength 10 V/Cm whereas the lowest was 58 % attained at electric field strength 50 V/Cm.
3. The highest viability was 80 % and it was obtained from electric field strength 10 V/Cm whereas the lowest was 59 % attained from electric field strength 50 V/Cm.
4. The higher electric field strength was the fewer surviving sperms.
5. Treating sperms with different levels of electric field strength (V/Cm) with different form of square wave was not producing obvious difference on motility and viability of Koi fish sperms.
6. The average rate of fertility of control sperms was 72 %, while that of sperms treated with electric field strength 50 (V/Cm) was 51 %.

It was suggested from this research that:

1. To obtain better motility and viability rates, electric field strength 10 V/Cm should be considered.
2. Further research could be focused on electroporation with square wave method against other fish species.

DECLARATIONS

Acknowledgements

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Authors' Contributions

All authors contributed equally to this work.

Competing interests

The authors declare that they have no competing interests.

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