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The effects of laserpuncture on gonadal maturity and sperm quality of male striped catfish (*Pangasianodon hypophthalmus*)

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ABSTRACT

Laserpuncture is one of the applicative technologies used mainly in animal and fish reproductions. Laserpuncture technology has been used to improve gonadal maturity and sperm quality in fish rapidly. This study aimed to determine the effects of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish. Males striped catfish (800–900 g/fish body weight) and I gonadal maturity stage were used. Semi-conductor soft laser was used with doses of 0.2-, 0.4-, and 0.5-J, while the negative control (without the laserpuncture and the ovaprim™) and only the ovaprim™ were used as a comparison treatment, respectively. The soft-laser was treated on reproductive acupoint every week for four weeks, while the ovaprim™ was administered by intramuscular injection at dose of 0.2 mL/kg fish in final rearing period. Fish was reared in hapa at the controlled pond. Fish was fed with a commercial feed containing 32% crude protein. Gonadal maturity, gonadosomatic index (GSI), hepatosomatic index (HSI), and sperm quality of male striped catfish were measured in the final rearing period. The results showed that the laserpuncture on the reproductive acupoint had a highly significant effect ($P < 0.01$) on the gonadal maturity, GSI, HSI, and sperm quality of male striped catfish. In terms of the gonadal maturity, laserpuncture doses treatment of 0.4 and 0.5-J gave the most mature IV stage. While the highest levels of GSI and HSI were found in 0.5-J of laserpuncture dose, which was 2.17% and 1.54%, respectively. In addition, the best sperm qualities were observed in 0.5-J of laserpuncture dose, which were 81.75% motility, 82.75% viability, and 7.0×10^9 cell/mL concentration. These results suggest that the laserpuncture can accelerate a gonadal maturity and improve sperm quality in male striped catfish.

1. Introduction

Striped catfish has been considered as one of the main cultured fish in Indonesia due to several advantages, such as fast growth, easy cultivation, and high tolerance to low dissolved oxygen content in the rearing water [1]. The production of striped catfish in Indonesia was 339,060 metric tonnes (MT) in 2015 and increased rapidly in 2016 become 447,110 MT [2]. The high market demand triggers farmers to increase the amount of striped catfish production. However, one of the main problems faced by farmers was the supply of striped catfish seed, which depends on the spawning season. Zairin [3] stated that the reproductive cycle of striped catfish occurs naturally during the rainy season from October to April every year.

The process of gonadal maturity in striped catfish needed a long time and depended on the increase in gonadotropin hormone (GtH) and gonadal steroid. The productive period of spawning in male striped catfish peaks at the age of two years or about 1.5–2.0 kg body weight [4]. Other constraints of a male fish are the decrease in sperm quality, such as motility and viability after spawning.

To increase production of striped catfish seed requires new technology to improve the quality of reproduction using laserpuncture technology. Laserpuncture technology is a stimulation technique on acupuncture points (acupoint) by a laser beam [5].

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The use of the laserpuncture could reduce production costs [6]. Susan [5] stated that the application of laserpuncture in the reproductive organs could stimulate several reproductive functions of male and female animals. Laserpuncture technology has been proven to accelerate gonadal maturity, spawning processes, and to shorten the reproductive cycles of several aquatic species, such as catfish, *Clarias gariepinus* [7,8] and mud crab, *Scylla serrata* [9]. Other author explained that the main advantages of laserpuncture technology as a stimulation method are requiring a short time takes only 5–10 s, does not cause tissue damage, and provides a maximum response [10], depending on the type of soft-laser used.

The low-power laser affects the biology system of humans [11] and animals [12], including aquatic organisms (fish). The low-power laser around 5–30 mW improves tissue activity, such as increased production of hormones and enzymes [13]. The low-power laser gives a biological stimulus, such as changing the cell membrane's potential and permeability. On the other hand, it improves the nerve regeneration ability located both in central and peripheral sides will be produced to increase the cellular activities, and the ability to produce hormones and enzymes [13,14].

Several studies have shown that the laserpuncture improves the vascular and endocrine systems, and various other body systems [15]. The low-power laser also improves the male fertility of humans [16], animals [17], and aquatic organisms, such as fish [17] and sea urchins [18]. Kusuma et al. [7,8] found that the use of the low-power laserpuncture technology on the reproductive acupoint precisely in 2/3 ventral parts of the body through induction once a week is optimal for the maturation of catfish gonads. Moreover, the low-power laser induction at the reproductive acupoint for 15 s increases the production of the GtH [10], which is the regulator for produce steroid, oogenesis, and oocyte maturation [19]. On the other hand, induction of laserpuncture also increases the testosterone level in blood serum and the gonadosomatic index (GSI) of male catfish [8].

The low-power laser has a significant effect on the reproductive performance of fish whose spawning cycle occurs throughout the season, however, does laserpuncture induction affect fish whose spawning cycle depends on the season? Therefore, this study was aimed to determine the effect of different laserpuncture doses on gonadal maturity and sperm quality of male striped catfish.

2. Materials and methods

This study was conducted at the Fish Breeding Research Centre, Subang, West Java, Indonesia. Histological analysis of fish gonads was held at the Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia. In this study, the experimental protocols were approved by the Scientific Committee, Institute of Research and Innovation, Universitas Airlangga (Protocol Number 886/UN3/2018).

2.1. Animal

Male striped catfish with the average body weight of 800–900 g/fish and the I gonadal maturity stage and never been spawned before was used in the present study. As a precaution, male striped catfish with the IV gonadal maturity stage were also prepared separately for positive treatment.

2.2. Laserpuncture

In this study, a semi-conductor soft-laser was used, which had a power specification of 20 mW. The preset doses and timer set were 0.2, 0.4, and 0.5 with 10, 20, and 25 s, respectively.

2.3. Experimental design

This study was used the completely randomized design structure consisting of five treatments with four replicates. Treatments used were doses of 0.2-, 0.4-, and 0.5-J, without laserpuncture and ovaprim™ treatments as a negative control, and treatment of the ovaprim™ dose of 0.2 mL/kg fish body weight in male striped catfish with the I gonadal maturity stage. The IV gonadal maturity stage of male striped catfish as positive treatment was treated the ovaprim™ dose of 0.5 mL/kg fish body weight, correctly was only used to observe sperm quality parameters.

Fish were adapted and reared in a hapa sized of $5.0 \times 3.0 \times 1.5$ m^3 at a controlled pond and fed commercial feed containing 32% crude protein. Laserpuncture treatment was performed on reproductive acupoint every week for four weeks. Reproductive acupoint located on the 2/3 ventral part of the body (governoer vessel) was measured from the anal to the pectoral fin. The determination of reproductive acupoint was also done using an electro-acupuncture device tool. On the other hand, the ovaprim™ was treated by using the intramuscular technique in the final rearing stage (week 4), 8–10 h before the end rearing of fish. Gonadal maturity (morphology and histology), gonadosomatic index (GSI) and hepatosomatic index (HSI), and sperm qualities, both macroscopic and microscopic, were measured.

2.4. Morphology and histology of gonadal maturity, GSI, and HSI

At the end of the rearing process, fish was anesthetized using MS222 (Argent Laboratories, Redmond, Washington, DC) of 100 ppm [20] for 10 min. Subsequently, the fish were dissected on the abdominal part from anal to ventral. Morphological and histological gonadal maturity was observed on the shape, the length, the weight, the color, and the gonadal development through histology preparation. Gonad sample was prepared according to Junqueira and Carneiro [21] to measure the GSI and HSI.

Next, the histological method was conducted by McCann [22] using Hematoxylin-Eosin (HE) staining method according to Genten et al. [23]. Scoring of testis cell development was conducted according to Çek and Yilmas [24].

2.5. Sperm qualities

The collection of sperms from the fish was conducted by using the stripping method. The sperm were stored in 1.5 mL microtubes in the refrigerator at a temperature of 4 °C before the analysis of microscopic qualities.

2.5.1. Motility of spermatozoa

Microscopic spermatozoa motility was observed under 100× and 400× magnifications using a BH2-RFCA Olympus binocular microscope (Olympus Optical Ltd. Shinjuku-ku, Tokyo, Japan), which was equipped with a camera. Motile and immotile of the spermatozoa were calculated using a modified method by Sohouka et al. [25], as well as a progressive or active movement forward and non-progressive movements (such as circular, backward or silent).

2.5.2. Viability of spermatozoa

The determination of spermatozoa viability was done by the staining protocol of 2% eosin yellow at sperm preparation. The viability of the spermatozoa was observed and counted under 400× and 1000× magnifications using the same microscope like the one used to observe motility of the spermatozoa. The spermatozoa viability was counted according to Sohouka et al. [25].

2.5.3. Concentration of sperm

The sperm concentration was calculated according to Stoss and Donaldson [26].

2.6. Data analysis

Gonadal maturity stage was statistically analyzed using Kruskal Wallis and Mann Whitney tests, while the morphology and histology of the organ were descriptively analyzed according to Genten et al. [23]. The other data were statistically analyzed using analysis of variant (ANOVA) with SPSS ver. 10 software and Duncan's multiple range test with a confidence level of 95%.

3. Results

3.1. Gonadal maturity stage, GSI, and HSI

This study showed that laserpuncture treatment affected the gonadal development of male striped catfish, as seen in Fig. 1. In general, gonad characteristics of fish with the I gonadal maturity stage are testicles like threads, smaller, shorter and limited, and visible ends in the body cavity. The II gonadal maturity stage (Fig. 1a and b) have more significant and more apparent the testis size and shape compared to the I gonadal maturity stage. The morphology characteristics of the III gonadal maturity stage are the surface of the testicles appear jagged, more prominent, and the whiter color (Fig. 1c). The IV gonadal maturity stage of male striped catfish has testis characteristics of more definite, denser, and milky white color (Fig. 1d and e).

The IV gonadal maturity stage achieved through laserpuncture treatment of 0.5-, 0.4-, and 0.2-J doses showed no significant difference between all the joules ($p > 0.05$). However, there was significant difference compared to the negative control and the ovaprim™ treatment ($p < 0.05$) as seen in Table 2. The results showed that ovaprim™ treatment does not have a significant effect on gonadal development; the gonadal maturity only reached the II stage (immature). It could not be stripped to collect sperms, then the laserpuncture-treated male fish compared with ovaprim™-treated male fish that has the IV gonadal maturity stage (as positive treatment) on sperm quality.

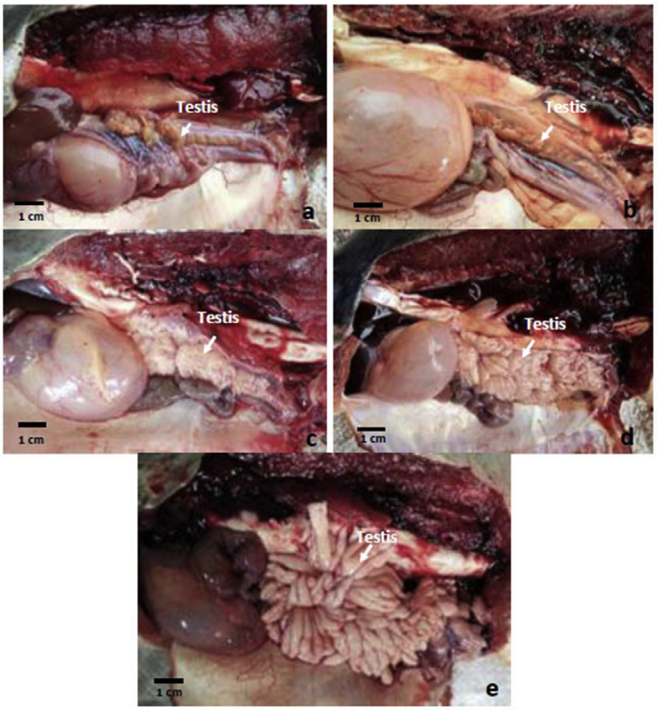

Fig. 1. The testicular morphology of male striped catfish in different gonadal maturity stages; the 2nd stage in the negative control (a) and the ovaprim™ treatments (b), the 3rd stage in the laserpuncture treatment of 0.2-J dose (c), and the 4th stage in the laserpuncture treatments of 0.4-J (d) and 0.5-J doses (e) (bar scale = 1 cm).

This study indicated that GSI and HSI had significant differences between treatments ($p < 0.05$). The laserpuncture treatment of 0.5-J had the highest GSI and HSI compared to other treatments, although the HSI is relatively not significantly different between treatments, except compared to the negative control (Table 1).

Similarity, the laserpuncture dose of 0.5-J had the highest effect on testicular development. This study also showed that the laserpuncture of 0.5-J dose has the highest scoring of gonadal or testicular histology in male striped catfish (Fig. 2) with testis containing spermatozoa of 75–100%, as seen in Table 2.

Based on the testicular histology of male striped catfish in Fig. 2 shows that the seminiferous tubules are still empty and the spermatozoa are not visible (Fig. 2a and b) as one of the II gonadal maturity stage characteristics in the fish. In the III gonadal maturity stage, seminiferous tubules already containing spermatozoa (Fig. 2c), while the IV gonadal maturity stage, seminiferous tubules containing more spermatozoa (Fig. 2d), even full spermatozoa (Fig. 2e).

3.2. Sperm qualities

This study showed that laserpuncture treatment produces higher sperm quality, both macroscopic and microscopic, compared to negative and positive treatments, as seen in Tables 3 and 4. The laserpuncture treatment of 0.5-J power resulted in the production of the highest volume of sperms, i.e., 3.00–5.25 mL with a creamy color (Table 3). On the other hand, the treatments showed significant differences ($p < 0.05$) in microscopic sperm quality. The laserpuncture treatment of 0.5-J power had the highest microscopic sperm qualities compared to other treatments, as seen in Table 4.

4. Discussion

The induction of laserpuncture on the reproductive acupoint in fish has proven to accelerate the development and maturity of the gonad including male gonad of striped catfish from I stage to IV stage, while in the control treatments (negative control and ovaprim™ treatment), the maturity of the gonad developed from I stage to only II stage during one month. This study result was consistent with the study conducted by Matayborbir et al. [27] in the catfish that laserpuncture exposure accelerate gonadal maturity from II stage to IV stage of male rapidly. The condition of fish gonads at the beginning of the study was at the initial gonadal maturity stage

Table 1
Gonadal maturity stage, GSI, and HSI of male striped catfish in the different treatments.

Treatment	Gonadal maturity stage	GSI (%)	HSI (%)
Negative control	II^a	0.39 ± 0.15^a	1.10 ± 0.07^a
Ovaprim™	II^a	0.54 ± 0.29^{ab}	1.30 ± 0.14^{ab}
Ld of 0.2-J	III to IV^b	0.70 ± 0.38^{ab}	1.32 ± 0.15^{ab}
Ld of 0.4-J	IV^b	1.09 ± 0.19^b	1.39 ± 0.26^b
Ld of 0.5-J	IV^b	2.17 ± 0.68^c	1.54 ± 0.17^b

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p < 0.05$).

Table 2
Average testicular histology scoring of male striped catfish in the different treatments.

Treatment	Score	Description
Negative control	3[a]	Development of cells has reached spermatids
Ovaprim™	4[b]	Development of cells has reached spermatids
Ld of 0.2-J	5[c]	Already formed spermatozoa of 25.00–49.90%
Ld of 0.4-J	6[d]	Already formed spermatozoa of 50.00–74.50%
Ld of 0.5-J	7[e]	Already formed spermatozoa of 75.00–100.00%

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p < 0.05$).

Table 3
Macroscopically sperm quality of male striped catfish in the different treatments.

Treatment	Spermatozoa quality parameters			
	Volume (ml)	pH	Color	Consistency
Negative control	Negative	Negative	Negative	Negative
Ovaprim™	Negative	Negative	Negative	Negative
Positive	0.50 to 1.25	7 to 8	Clear White	Dilute
Ld of 0.2-J	0.50 to 1.50	7 to 8	White milk	Condensed
Ld of 0.4-J	0.50 to 2.00	7 to 9	White milk	Condensed
Ld of 0.5-J	3.00 to 5.25	7 to 9	Creamy	Condensed

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Positive = the male striped catfish with the IV gonadal maturity stage treated the ovaprim™ (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose.

with a small gonadal morphology and appeared clear. Then, after the laserpuncture treatment, the gonadal maturity reached IV stage (fully maturated).

GSI has been used as one of the indicators of the development and maturity of gonad [28] in both sexes. In general, GSI increases with the increasing gonadal maturity stage as well as a show in this study (Table 1). Increased GSI followed the bigger size of the gonad (testis), as shown in Fig. 1, and increase the number of spermatozoa produced by testis (Fig. 2). Kusuma [7] stated that laserpuncture stimulates cell activations in the area governoer vessel (reproductive acupoint) to produce energy. The formation of energy after laserpuncture exposure in the reproductive acupoint related to specific proteins in cells. As the results, the development of gonads gradually from spermatogonia, spermatocytes, spermatids, and spermatozoa, which are marked by the bigger testicles. The

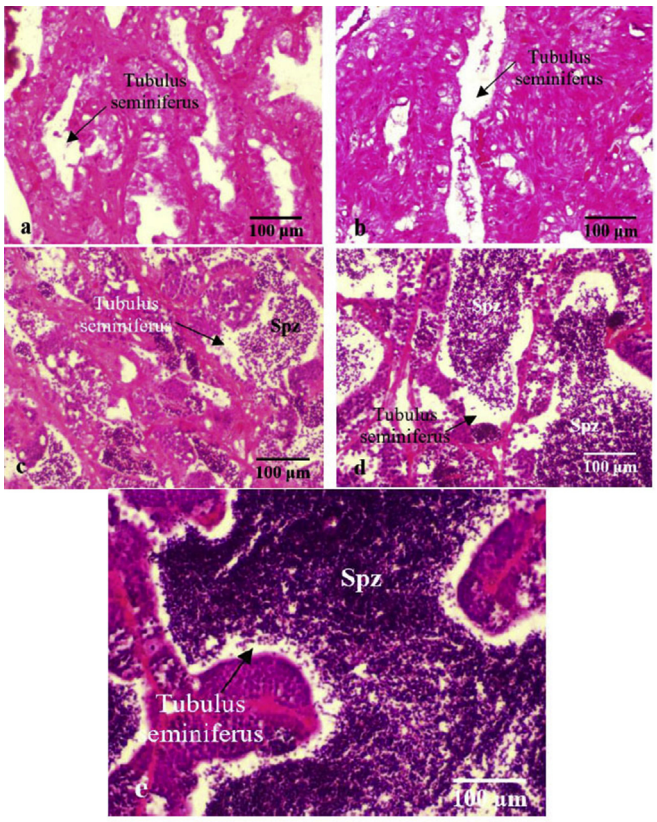

Fig. 2. The testicular histology of male striped catfish in different gonadal maturity stages and treatments observed under a microscope at 400× magnification; the 2nd stage in the negative control (a) and the ovaprim™ treatments (b), the 3rd stage in the laserpuncture treatment of 0.2-J dose (c), and the 4th stage in the laserpuncture treatments of 0.4-J (d) and 0.5-J doses (e) (Spz = spermatozoa, bar scale = 100 µm).

laserpuncture exposure at reproductive acupoints in male striped catfish increased GSI and HSI. GSI will continue to increase along with the maturation of the fish gonads and will reach the maximum value when the peak period of gonad maturity [29].

The exposure of laserpuncture on the reproductive acupoint stimulate FSH and LH from the pituitary, which has a vital role in the development and maturity of the gonad. Although FSH and LH levels were not measured in this study, a previous study [7] have reported that laserpuncture exposure to reproductive acupoint increased FSH and LH levels and also accelerated LH formation which play a role in the final stage of spermatogenesis or spermatozoa production, testicular maturation, ovulation, and spawning stimulation in catfish. It indicated that the induction of laserpuncture could increase the performances of the activity of the hormone, which takes part in a reproduction control system to accelerate the provision of growth, development, and gonad maturation of fish.

Furthermore, the induction of laserpuncture on the tissue increased gonadotropin-releasing hormone (GnRH) released by the hypothalamus and it stimulated the anterior pituitary to secrete FSH and LH which act on theca cells in the male gonad (testis) to produce testosterone hormone [30]. Similarly, a study by Chang et al. [31] showed that laserpuncture stimulated the release of neurotransmitters. The laser beam transduced into chemical signals band being received by various ion channels, such as G-proteins (GTP-binding protein)-coupled receptors subunit α and VGCC (voltage-gated Ca2+ channels). Other possible mechanisms is through calcium receptors, such as calcium-sensing receptor (CaSR), located in the nervous membrane cells. Then, ligands binding on the specific receptors triggers the release of second messengers causing a chain reaction and bringing changes in the cell. Electrical signals caused by depolarization of the nerve cell membrane were propagated from cell to cell along the axon. Next, the electrical signals were inserted from the pre-synapse membrane into the post-synapse membrane and trigger the release of neurotransmitter molecules in the synapse. The same mechanism, the electrical signals were transmitted to the brain. intracellular and extracellular Ca2+ ions mediated the electrical signals through changes in spontaneous membrane potential play an essential role in stimulating the release of GnRH from the hypothalamus and it stimulates the pituitary to release FSH and LH. Next, FSH and LH are then channeled into the bloodstream towards the gonads (testis), which enable various activities. This process repeats when the nerve cell membrane is depolarized [32].

Other study by Anglade et al. [32] explained that the release of neurotransmitters such as gamma-aminobutyric acid (GABA) from GABAergic neurons depends on nerve cell membrane depolarization, action potential, calcium ions, decarboxylation of glutamate, and glutamic acid decarboxylase (GAD). The anterior pituitary directly innervates GABAergic neurons, so it has a stimulatory effect on the release of LH [33].

Table 4
Microscopically sperm quality of male striped catfish in the different treatments.

Treatment	Spermatozoa quality parameters		
	Motility (%)	Viability (%)	Concentration ($\times 10^9$ cells/mL)
Negative control	Negative	Negative	Negative
Ovaprim™	Negative	Negative	Negative
Positive	58.88 ± 1.93^a	58.00 ± 1.58^a	4.31 ± 4.26^a
Ld of 0.2-J	65.75 ± 2.32^b	66.25 ± 1.75^b	5.25 ± 4.56^b
Ld of 0.4-J	73.00 ± 2.73^c	73.00 ± 2.27^c	6.06 ± 6.25^c
Ld of 0.5-J	81.75 ± 1.19^d	82.75 ± 1.84^d	7.00 ± 5.40^d

Note: Negative control = without the laserpuncture and the ovaprim™ treatments, Positive = the male striped catfish with the IV gonadal maturity stage treated the ovaprim™ (0.5 mL/kg of fish body weight), Ld = Laserpuncture dose. Different superscripts the same column show significant differences ($p < 0.05$).

Factors which determined the volume of sperm production include environmental conditions, such as temperature, pH, and dissolved oxygen levels. Fish which are in stress condition due to environmental condition cannot produce large volumes of sperm. The same factors have been confirmed by a study of Salisbury and VanDenmark [34] where the volume of sperm production was influenced by environmental factors, age, body size, feeding management, and sperm release frequency.

The low-power laser has significantly improved sperm quality, such as volume [35], concentration [36], motility, movement, and viability of spermatozoa [37]. The highest motility obtained in 0.5-J treatment because this dose can provide a stimulatory effect large enough to stimulate the hypothalamus neuron to release the GnRH. GnRH stimulates pituitary neurons to release FSH and LH. FSH produces testosterone hormone, and LH produces an androgen-binding protein (ABP). Testosterone hormone and ABP control spermatogenesis and initiate spermatogenic development into motile spermatozoa [38]. Mantayborbir et al. [27] also found that laserpuncture induction has spontaneous stimulation power and rapidly influences the increase in the number of Leydig cells produced. The function of Leydig cells is to produce testosterone hormone, which binds to androgen receptors in Sertoli cells, which secretes ABP and helps form spermatozoa.

Several studies indicate the influence of laserpuncture on improving the sperm quality of animals including motility [35,37] and fertility [35] of spermatozoa, the adenosine triphosphate (ATP) content [39], Ca^{2+} concentration [40], and cell life [41]. Ca^{2+} stimulates the work of the mitochondria and the ATP synthesis in the cell [42], while mitochondria and ATP play an important role in supporting spermatozoa motility [43]. Furthermore, laserpuncture improved protein synthesis, cell growth, differentiation and motility, membrane potential, binding affinities, neurotransmitter release, and ATP synthesis [44].

The best spermatozoa viability obtained in the present study after laserpuncture treatment was 82.75% at dose of 0.5-J. The result was considered to be good according to Rahardhianto et al. [45], who stated that the quality of spermatozoa is good based on spermatozoa viability of 80%. Spermatozoa viability also determines successfullness of fertility including in the fish.

The FSH will stimulate Leydig cells to produce testosterone hormone. Testosterone hormone as a part of an androgen steroid hormone plays a vital role in the reproductive tissue development and the secondary sexual characteristics expression of male. GnRH promotes the secretion of LH by stimulating the pituitary gland. Then, LH promotes the synthesis of testosterone hormone by stimulating the Leydig cells of the testis [46]. Alves et al. [47] also have proven that the induction of low-level laser improves the testosterone hormone level in males by increasing 11-ketotestosterone hormone level in spermatogenesis.

The result was consistent with a study by Schulz et al. [48] in which 11-ketotestosterone hormone influenced more in the spermatogenesis than testosterone hormone. The concurrent elevation of 11-ketotestosterone and testosterone hormones level is another typical pattern in male fish [49]. Therefore, the measurement of 11-ketotestosterone and testosterone hormones will be investigated for further laserpuncture studies of fish.

5. Conclusion

Laserpuncture induction of 0.5-J dose at the reproductive acupoint increase gonadal maturity and sperm quality of male striped catfish. Further studies on higher doses of laserpuncture and its effects on hormonal mechanisms are critical, especially in the fish.

Declaration of competing interest

No conflict of interest.

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