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The role of laserpuncture exposure on gonad maturation mechanism of catfish (*Clarias* sp.) through Ca^{2+}, PKC and GABA neurotransmitter

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ABSTRACT

Laser puncture exposure at reproduction acupoint is proven to increase cellular activity like Ca^{2+} in the skin tissues. The aim of the study is to determine the role of laserpuncture exposure on gonad maturation by evaluating Ca^{2+} stimulation and PKC activity in skin tissue and the release of GABA from GABAergic neurons of the brain tissue of catfish (*Clarias* sp.). A total of 36 females and 36 males of 8–9-month old of F1 catfish broodstock *Sangkuriang* (female) and *Paiton* (male). This study used Completely Randomized Design (CDR) experimental method. Expression analysis was conducted using immunohistochemical staining with a streptavidinbiotin method with calcineurin kit, PKC kit, and GABA kit. The results showed that laserpuncture can stimulate calcineurin and PKC expression in skin tissue, and GABA expression in the brain tissue on the condition pre-spawn, spawn, and post-spawn ($P < .05$). It can be concluded that laserpuncture stimulates gonad maturation through Ca^{2+}, PKC, and GABA neurotransmitter.

Introduction

Laser is a short-wave light that can inhibit and stimulate biological tissues (Chaves, 2014). Low power laser can provide such biological stimulation improve cellular activity by affecting the membrane permeability to sodium, potassium, and calcium. This mechanism increases the activity of the enzyme (Hunt et al., 2009; Hashmi et al., 2011; Ross et al., 2015). Low-power laser with power 5 mW and 632.8 nm wavelength has been studied and applied to the Black varieties GIFT Tilapia (Tilapia Farmer Genetic Improvement) newly females spawned the first time. That laser inducted at the reproduction acupoint for 6 s on 2/6 ventral part of the body with the firing frequency of once a week can increase Gonado Somatic Index (GSI), proves that cellular activity occurs at the reproductive acupoint that is induced by laserpuncture (Pryor, 2011; Yao et al., 2014).

Cellular activity at the reproduction acupoint caused by laserpuncture exposure will affect to the release and storage process of Ca^{2+} from Endoplasmic Reticulum (ER). The laserpuncture plays role electromagnetic wave energy that can cause nerve cell membrane receptors such as G-protein subunit α would undergo phosphorylation to activate *phospholipase C* PLC (Kusuma and Mahendra, 2010). PLC will further hydrolyze phosphatidylinositol bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). In the ER Membrane, IP_3 bind to inositol triphosphate receptor (insP3R) to stimulate the release of RE Ca^{2+}. This pathway of cellular activities is known as a metabotropic path.

The release of Ca^{2+} from the ER will increase the levels of Ca^{2+} in the cytoplasm then Ca^{2+} will activate calcineurin. So calcineurin can be an indicator for Ca^{2+} stimulation. Ca^{2+} with DAG activates protein kinase C (PKC). PKC activity can increase the transcription of specific genes. Ca^{2+} and PKC play a role in physiological processes in the cell such as the synthesis of neurotransmitters. However, metabotropic path is not the main pathway. The induction could activate ionotropic pathway, starting from laserpuncture exposure on reproductive acupoint energy of the electromagnetic wave laser beam will be converted into an electrical signal. The electrical signal will cause nerve cell membrane depolarization. Membrane depolarization causes action potential of nerve cell membranes which responds with the opening of extracellular Ca^{2+} channels. Extracellular Ca^{2+} enters through the Calcium Sensing Receptor (CaSR) or via Voltage-Gated Calcium Channels (VGCC) (Clapham, 2007; Chow et al., 2011). Due to the influx of extracellular Ca^{2+}, Ca^{2+} meets with synaptic vesicles and membrane vesicles and opens to release neurotransmitters by exocytosis into the cleft at synapses. Neurotransmitter binds to

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specific receptors in postsynaptic can result in a positive or negative effect on the postsynaptic cell membrane. If the bond with neurotransmitter receptors in the postsynaptic matches, the impulse will continue towards the brain where causes a series of physiological reactions in activating the enzyme *Glutamic Acid Decarboxylase* (GAD-65) which will further stimulate the GABAergic neurons to synthesize GABA in brain tissue. The release of GABA can be seen from the expression of GABA-PKC and GABA-Calcineurin in brain tissue.

γ-Amino butyric acid (GABA) is an immunoreactive group of amino acids that are found in the main neuroendocrine area (Mueller et al., 2008; Lado et al., 2014; Watanabe et al., 2014). Telencephalon and hypothalamus have the most prominent stimulatory acts to increase the presence of GnRH release and inhibit dopamine (DA) in the hypothalamus-pituitary complex (Popesku et al., 2008). In principle, DA may reduce the influx of extracellular Ca^{2+} and inhibit the release mechanism of the GnRH release. Thus DA is the control center in the release of GtH-I and GtH-II of the pituitary (Chang et al., 2009; Popesku et al., 2010; Kim et al., 2011). GtHI plays a role in growth, steroidogenesis and vitellogenesis process while GtH-II has a role in end of gonad maturation, ovulation and spawning (Vijayan et al., 2007; Pham et al., 2010; Yousefian and Mousavi, 2011; Pandey, 2013; Swanson, 2014; Shi et al., 2015). Gonad development and spawning regulated by the hypothalamic-pituitary axis regulates the gonads and liver (Kah and Dufour, 2011; Urbatzka et al., 2011).

However, basic information about laserpuncture effect on Ca^{2+} and PKC expression in skin tissue and the release of GABA is important. Therefore, we examined the role of laserpuncture exposure on gonad maturation by evaluating Ca^{2+} stimulation and PKC activity in skin tissue and the GABA release in the brain tissue of catfish (*Clarias* sp.)

Materials and methods

This research was conducted in Freshwater Aquaculture Management Unit (FAMU) Kepanjen, Indonesia. Samples used prospective parent catfish (*Clarias* sp.) gonads that have not been spawned. F1 catfish aged 9 months old were collected from broodstock *Sangkuriang* (female) and *Paiton* (male). A total of 36 females and males with weight of 1010–1690 g and 1140–1750 g were used as samples. The female catfish were treated for 15 s with laserpuncture (soft laser Helium-Neon (He-Ne); 5 mW and wavelength 632.8 nm) at the reproductive acupoint (2/3 of the ventral body) until gonads matured (Hashmi et al., 2011; Yao et al., 2014). Completely randomized design was used as an experimental method. The treatments consisted of 6 levels with six repetitions. The treatments were pre-spawn, spawn and post-spawn.

Calcineurin, PKC and GABA expression analysis

The experiment was performed to determine the histology scores calcineurin (expression (Ca^{2+} indicator) and PKC in skin tissue, and GABA expression in the brain tissue by isolating the skin and brain tissues after exposure to by laserpuncture. Five micrograms of skin thickness and brain tissue was prepared and stained by immunohistochemical staining. The activity of calcineurin, PKC and GABA detected by cell presentation and colour intensity that arise as a result of enzymatic reactions that occur between the HRP enzyme with its substrate. Cell expression indicated calcineurin was red, PKC indicated brown and GABA expression showed dark blue colour under a light microscope with 1000x magnification. The results of calculations performed 10 fields of view to obtain an average of 1 time the field of view. The obtained data were compared between control and treated.

Statistical analysis

Data was analyzed using SPSS software (one-way ANOVA). Significant different used $p < .05$ and LSD test at 95% used for the confidence level.

Result

Expression of calcineurin-PKC in the skin tissue of *Clarias* sp. on pre-spawn, spawn and post-spawn after treatment showed high expression respectively by 117%, 43.8%, and 33.3% compared with the control group. The expression of calcineurin-GABA after laserpuncture exposure on the pre-spawn, spawn and post-spawn were higher and precisely at 300%, 46.4% and 97% respectively than the control group. The expression of PKC-GABA after laserpuncture exposure on the pre-spawn, spawn and post-spawn were also higher and recorded as 188.3%, 83.5% and 86.7% respectively than the control. The statistical analysis showed calcineurin, PKC and GABA expression on the pre-spawn, spawn and post-spawn were significantly different ($P < .05$) than the control (Table 1).

Discussion

The calcineurin and PKC expression in the skin tissue of *Clarias* sp. on pre-spawn, spawn and post-spawn were higher after exposure to laserpuncture. The laserpuncture properties was adopted from Kusuma and Mahendra (2010) that has lowpower *(soft laser)* Helium-Neon (He-Ne) with a wavelength of 632.8 nm, the power of 5 mW/cm^2 and 0.2 cm^2 wide light outputs which 15 s are equivalent to an energy of 0.375 Joules/cm^2/reproduction acupoint. This energy can induce ER for releasing Ca^{2+}. It happens because when laserpuncture expose the reproductive acupoint, the energy of the electromagnetic wave laser can penetrate the skin tissue to the dermis and epidermis which is suspected to have on peripheral nerve endings. Also, IP_3 headed into the cytosol and binds to specific receptors on the ER and associated with Ca^{2+} channels trigger the release of Ca^{2+} from ER to the cytosol, thereby it may increase the intracellular levels of Ca^{2+}. The receptor activation of phospholipase pathway gains some second messengers such: DAG, IP_3, and Ca^{2+}. DAG and Ca^{2+} plays a role in activating PKC. PKC activity depends on Ca^{2+}. PKC plays a role in the synthesis of specific genes. Ca^{2+} activity intracellular can be seen by calcineurin expression.

Table 1
Calcineurin and PKC expression in the skin tissue compare with calcineurin and GABA, PKC-GABA expression in the brain tissue of catfish (*Clarias* sp.).

Group		N	Calcineurin-PKC	Calcineurin-GABA	PKC-GABA
Control	Pre-spawn	6	4.7 ± 1.9^{a}	3.2 ± 2.4^{a}	6.0 ± 3.6^{a}
	Spawn	6	13.0 ± 4.0^{c}	13.8 ± 3.7^{d}	15.8 ± 2.9^{c}
	Post-spawn	6	9.0 ± 3.0^{b}	6.5 ± 2.9^{b}	9.8 ± 2.3^{b}
Laserpuncture exposure	Pre-spawn	6	10.2 ± 1.5^{d}	12.8 ± 3.3^{c}	17.3 ± 3.4^{cd}
	Spawn	6	18.7 ± 1.8^{f}	20.2 ± 2.0^{e}	29.0 ± 1.4^{e}
	Post-spawn	6	12.0 ± 4.0^{e}	12.8 ± 3.5^{c}	18.3 ± 4.9^{d}

Notes: different letter means significant different.

Ca^{2+} along with PKC play a part in the physiological activity of neurotransmitter release. Electromagnetic wave energy of laserpuncture exposure may be converted to electrical signals. This depolarization effect will cause an action potential in nerve cell membranes and the membranes of nerve cells will respond by opening Ca^{2+} channel allowing extracellular Ca^{2+} entry through the Calcium Sensing Receptor (CaSR) or via VoltageGated Calcium Channels (VGCC) (Clapham, 2007; Chow et al., 2011). Due to the influx of extracellular Ca^{2+}, intracellular Ca^{2+} will increase. Increased intracellular Ca^{2+} will stimulate the release of neurotransmitters, which will activate a GAD-65 that is responsible for GABA synthesis (Deidda et al., 2014).

GABA stimulates neurons in the hypothalamus and then released GnRH (Maffucci and Gore, 2010; Shi et al., 2015). So GABA can trigger gonadotropinhormone release (GtH-I and GtH-II) (Popesku et al., 2008; Dedden, 2011; Bencic et al., 2013; Karigo and Oka, 2013; Ogawa and Parhar, 2014). GtH-I has a role in stimulating growth, gametogenesis and gonad steroidogenesis fish. While GtH-II has a role in spawning and gamete maturation (Pandolfi et al., 2009; Martyniuk et al., 2009).

This study demonstrates that exposuring laserpuncture at reproductive acupoint of *Clarias* sp. could activate enzyme GAD-65 in the brain tissue which will further stimulate the GABAergic neurons to synthesize GABA. In the control group, there was also an increase in GABA expression in the brain tissue on pre-spawn, spawn, and post-spawn. But GABA expression in the brain tissue was still low when compared with the group exposed to laserpuncture. Improvement can occur due to external stimuli such as chemicals (pheromones) released by the opposite sex in a pool. The previous study reported other factors that can affect the spawning as photoperiod, a substrate for spawning, the addition of new water and pheromones that come from the opposite sex in a pool (Reed and Jennings, 2011).

Conclusion

Laserpuncture stimulates gonad maturation through Ca^{2+}, PKC, and GABA neurotransmitter. Its exposure at skin tissue of brain activates enzyme GAD-65 in the brain tissue for stimulating GABAergic neurons to synthesize GABA.

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