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The Effectivity of Arowana Pinoh (*Scleropages macrocephalus*) Vitellogenin Production using Estradiol Stimulation by Injection and Oral.

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Abstract. Arowana pinoh (*Scleropages macrocephalus*) is an Indonesian native commercial ornamental fish which it's spreading are was in Sumatra, Kalimantan and Papua. Vitellogenin is an egg yolk protein precursor which can be utilized for the detection of female fish sex and gonadal maturity. This study was aimed to stimulate the synthesis of Vitellogenin on *arowana pinoh* through Estradiol stimulation either by induction or oral. Three arowana fishes was given Estradiol stimulation 50 mg/kg body weight, two of them through intraperitoneal induction and one through orally by accumulation in feed. Plasma samples were taken before (control) and seven days after hormonal stimulation. Investigation using SDS-PAGE on a 7.5% Acrylamide gel showed that there was band difference on wells that contain plasma control and after treatment both with injection and oral. Wells containing plasma after oral and induction showed a thick band that is different from the control on 180 kiloDalton of the molecular weight. These results indicated that orally Estradiol stimulation as efficient as injection for the Vitelogenin production of Arowana pinoh. Eventhough both have the same effect, the process of orally estradiol stimulation to produce Vitelogenin was a new better method that can be used rather than injection regarding to animal welfare.

Keywords : Arowana pinoh, Estradiol, oral, Scleropages macrocephalus, Vitellogenin,

INTRODUCTION

Arowana fish (*Scleropages sp*) is a monomorphic species, that is animals that are physically incapable or indistinguishable between males and females. Indonesia has five species of arowana fish. They are arowana green (*S. formosus*), arowana super red (*S. legendrei*), arwana pinoh (*S macrocephalus*), arowana golden (*S. aureus*) and arowana papua (*S. jardinii*).

To solve the problem of monomorphism in arowana, it needs identification process of Vitelogenin. One of the phases of gonadal maturation in female fish is the occurrence of vitelogenesis. Vitelogenesis is the synthesis of vitelogenin (Vtg) or yolk precursors that occur in the liver. Vitelogenesis is triggered by the influence of estrogen-estradiol [5] hormone estrogen by phosphorylation, glycosylation and lipidation in the liver which results in Vtg [13]. Vtg is a glycophosphoprotein consisting of about 20% fat, especially phospholipids, triglycerides and cholesterol [17]. This protein is transported to the ovaries by the blood and fed into expanding oocytes and then degraded to lipovitelin in the yolk [13].

Vtg which is an egg yolk precursor is a specific characteristic of the female mother until it is detected in a fish, it is confirmed that the fish is female. Vtg as a parameter of gonadal maturity has been observed in Arapaima gigaz fish and successfully found differences in males and females based on these Vtg levels [6].

The initial process for identifying Vtg is isolating Vtg derived from arwana pinoh fish, where for this isolation hormone stimulation is needed to stimulate the production of Vtg in fish. During this process of

stimulating the hormone Estradiol to produce Vtg in fish is used by injection or injection on the intraperitoneal (abdominal cavity). Since this method causes injury and possibly results in fish mortality, other methods of hormonal stimulation should be tried. One alternative that will be tested in this research is through oral method that is given through the given feed. The purpose of this study was to test the results of hormonal stimulation through injection or oral.

EXPERIMENTAL PROCEDURES

Materials

The material used for hormone stimulation is the hormone Estradiol (Sigma), Ethanol absolute and cocoa butter. The ingredients for blood sampling are Heparin as anti coagulant and Phenoxy ethanol as anesthesia. While for electrophoresis process the materials used are Bio-Rads 30% Acrylamide / Bis solutions 37.5: 1 mixture, Tris-HCl, pH 8.8 1.5 M, Tris-HCl, pH 6.8 0.5 M, SDS 10%, Sample buffer (3.8 mL H2O , 1.0 mL 0.5 M Tris-HCl, pH 6.8, 0.8 mL Glycerol, 1.6 mL 10% (w / v) SDS, 0.4 mL β mercapto ethanol, and 0.4 mL 1% (w / v) bromophenol blue). 5x electrode (running) buffer, pH 8.3 (Tris base 15 g / L, Glycine 72 g / L, SDS 5 g / L), Ammonium persulphate 10%, Staining solution (0.1% coomassie blue in Methanol 40% and acetic acid 10 %), fixative solution (Methanol 40% and 10% acetic acid) and Standard molecular weight protein (Bio-Rad). Stacking gel (Aquadest 3.05 mL, tris-HCl <pH 6.8 0.5 M 1.25 mL; SDS 10% (w / v) 50 μL; Acrylamide / bis (30% stock) 0.665 mL; ammonium persulphate 10% 25 μL and TEMED 5 μL). Separating gel (Iades 4.85 mL, tris-HCl <pH 8.8 1.5 M 2.5 mL; SDS 10% (w / v) 100 μL; Acrylamide / bis (30% stock) 2.5 mL; ammonium persulphate 10% 50 μL and TEMED 5 μL).

In addition to these materials are also used technical ethanol and akuades. Fish test used was three Arowana pinoh A, B and C with the weight of each 397; 381.2 and 440 grams. Percil (small frog) was used as feed about two individues per day.

Equipments

Common equipment used is the centrifugation, scales, magnetic stirrer, water bath, and bottle falcon. Equipment for injecting hormone and blood taking is 1 mL syringe and 1.5 mL micro tube. Equipment used for SDS-PAGE process is Bio-Rad Mini-protean 3. Other equipment used is bottle of solution, gel immersion container and camera for documentation. The micropipette used is 0.5-10 μL; 2 - 20 μL; 10 - 100 μL; 20 - 200 μL; 100 - 1000 μL; 0.5 - 5 mL and 1 - 10 mL. The micro-tip used is 20 μL, 200 μL, 1000 μL, 5 mL and 10 mL. In addition to the above equipment is also used Cup glasses 100, 250, 500 and 1000 mL; measuring cups 100, 500 and 1000 mL. Tissue smooth and rough cloth as a complementary tool.

Procedure

Embellishment of fish

Stuttering of test fish is done before measurement of length and body weight, blood taking and hormone injections. Stunning is done by using Phenoxyethanol 0.4 mL / L of water. Fish conscious a few minutes after being put into a container filled with aerated water.

Collection and preservation of blood samples

Blood is taken by injection at the base of the tail (Figure 1) and then inserted into centrifuge type plastic tubes that have been given heparin as anti-coagulant.

Fig 1. Fish Bleeding at the Base of the Tail

Fish bleeding was conducted before hormone stimulation (0) and seventh day. The blood that has been taken then centrifuged at a rate of 10000 rotation per minute (rpm) for five minutes at 4 ° C. Plasma is then made in aliquots and stored at -20 ° C.

Preparation of the Hormone Estradiol

250 mg of Estradiol hormone dissolved in 5 mL of absolute ethanol on a falcon bottle, the solution is warmed to a maximum temperature of 62.5 oC and divortex to a clear color solution. The solution is allowed to evaporate until saturated, then added with warm cocoa butter liquid to a volume of 8 mL and then divortex. After evenly the suspension is silenced to form solids. Before use, the suspension is warmed to melt and then injected immediately to either the fish stomach directly or to the spill.

Hormonal Stimulation

Stimulation of hormones is given as much as 50 mg / kg of first fish weight by direct injection to the intraperitoneal (abdominal cavity). The second is orally by injecting the hormone into the stomach of the spill which is then given to the fish as much as 2 feed per day.

Characterization of Protein Expression

Vtg characterization was performed by comparing blood plasma protein content before and after hormone stimulation using Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) [7] method by migrating samples through stacking gel and separating gel.

The 5 μL sample was dissolved in a 100 μL sample buffer and then fed into a gel well of 10 μL per well. The protein migration is done for 45-60 minutes or until the sample reaches the bottom of the gel. The gel was then soaked using a staining solution for 2 hours then rinsed with fixative solution. The bands seen in hormone-stimulated samples were then compared with the control sample bands. Different bands can be considered as Vtg. The determination of the molecular weight of Vtg is done by comparison with the standard protein used.

RESULT AND DISCUSSION

Embellishment of fish

It fainted on average after 3 minutes of immersion of water that had been given phenoxy ethanol. Characteristics of fainting will be seen when the fish's body has been tilted and can not be erect, tail and tail slippage faltered and the movement of the gill decreased. After taking blood or weight measurements, the fish are transferred to aerated water to recover and regain consciousness. Other fishes were anaesthetized using Fish were anaesthetized in 0.05% tricaine methane sulfonate (TMS) [14] [15] [16] while other using 4-ethyl-aminobenzoate [2].

Collection and preservation of blood samples

The blood taken from each test fish ranged from 1 - 1.5 mL. After centrifugation, the plasma produced about 0.5 - 1 mL (Figure. 2).

Fig 2. Plasma after centrifugation

Storage at -20 °C (Chu-koo et al, 2008) can maintain the freshness of the plasma until the analyzes of vitelogenin are undamaged and preserved. Storage in aliquots facilitates retrieval to avoid repeated warming of any plasma samples. Vtg aliquot also better to storage at -80 ^{o}C [1].

Preparation of the hormone estradiol

The hormone estradiol will dissolve in ethanol when accompanied by heating solution. The dissolution of estradiol is characterized by the change of the solution from milky white to clear. Evaporation is done so that less ethanol and higher concentrations of estradiol in solution. The higher the concentration of estradiol, the less solvent (ethanol) will be injected into the fish and consumed. Mixing with cocoa butter forms an easier emulsion inserted into the fish's stomach as well as the percussion [6]. Cocoa butter is a fat will facilitate the absorption of estradiol into the fish body.

In addition, the cocoa butter properties that will harden at room temperature help to keep the estradiol solution persist in the fish's abdominal cavity and percill before being absorbed. If estradiol is still a solution in ethanol it will be great risk of the solution out of the fish body and percil. Other side effects if given directly to ethanol will be at risk for fish and may cause fish death. Parts of the body in fish exposed to ethanol usually show symptoms of drying and hardening such as being exposed to burns. Cocoa butter also could be use for emulsifying LHRH and cholesterol in ethanol mixture [8].

Hormonal Stimulation

Fish that are given hormone stimulation through direct injection show less healthy clinical symptoms. Slow movement and response to less feed. On the abdomen looks slightly swollen and soft. Fish given orally indicate healthy conditions in which the response to aggressive and agile feed moves. After the last blood-taking, the fish given hormone stimulation by injection suffered death while the orally survived and could be preserved for further research. This indicated that the oral induction is better than injection regarding the animal welfare eventhough the fish welfare still debatable [4].

Characterization of protein expression

The results showed that there were differences in protein characteristics between plasma before and after hormone induction (Figure 3). The images show that there are differences in the protein bands appearing on the plasma of test fish before (0) and after hormone induction. This occurs in all test fish (A, B and C). On this seventh day there has been a specific protein-produced response to the hormone estradiol. There is no visible difference in the thickness of the tape as a characteristic of the quantity of Vtg produced on each test fish. The response of the three test fish to estradiol was still relativally the same on the seventh day.

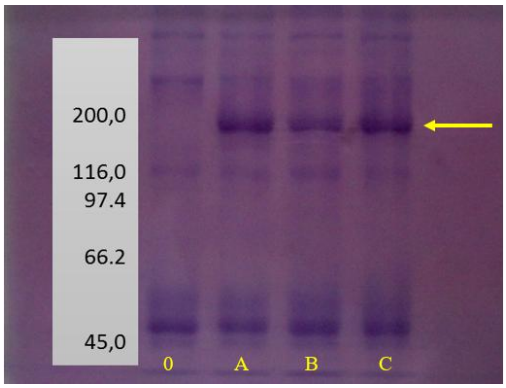

Fig 3. Expression of Protein in 7.5% Acrylamide Gel. Caption: 0 is The Control, A, B And C Refers to the Test Fish, the Yellow Arrow Refers to the Specific Protein Produced. Figures 45 - 200 Refer to the Molecular Weight (Kda)

The test results show that Vtg production process successfully done. Estradiol is effective for stimulating Vitelogenesis and producing many of the vitaminsin produced in fish liver [5] [9]. The types of proteins that appear in samples before (0) and after (A, B and C) hormone induction are shown in Table 1.

Table 1**.** Molecular Weight (Dalton), Alleged protein based on molecular standards and protein types found in control samples (0) and after all three test fish (A, B and C). Description of the table: the sign Ö indicates the presence of proteins and signs - indicates no

Molecule Weight (dalton)	**Alleged Protein**	**0**	**A**	**B**	**C**
>200.000	-	Ö	Ö	Ö	Ö
200.000	Myosin	-	-	-	-
116.250-200.000	**-**	**-**	Ö	Ö	Ö
116.250	B-galactosidase	Ö	Ö	Ö	Ö
97.400-116.250	-	-	-	-	-
97.400	Phosphorylase b	-	-	-	-
66.200	Serum albumin	-	-	-	-
45.000-66.200	-	Ö	Ö	Ö	Ö
45.000	Ovalbumin	Ö	Ö	Ö	Ö

The difference in the band looks striking at 200 kiloDalton (kDa) and 116 kDa molecular weight. There were bands in A, B and C but not in control. Measurement results show that the protein is at 180 kDa molecular weight which can be determined as Vtg. The single Vtg of 180 kDa is equal to Vtg of albino patin (*Pangasianodon hypophthalmus*) and gurame padang (*Osphronemus goramy*) also weighs 180 kDa [10] [11]. In contrast to the species Arapaima gigas which has 2 Vtg Vtg 1 and Vtg 2 [6] with suspected molecular weight of 180 kDa and 110 kDa respectively. Two species of Vtg were also found in Rainbow Trout (*Oncorhynchus mykiss*) fish at 390 and 176 kDa [3]. Ringau fish (*Datnioides microlepis*) has two types of Vtg with molecular weight of 180 and 110 kDa [10] [11].

Vtg obtained can be utilized for the immunoassay process which is very useful for Vtg detection process both qualitatively and quantitatively [6] [12]. The problem with monomorphic pinnacle arises with the acquisition of Vtg could be solved. If fish sexually could be identified then it will facilitate the cultivation process effectively. Pairing of male and female parent with a certain ratio could be decided to facilitate the spawning process.

CONCLUSION

Oral hormonal stimulation also efficient as injection for the production process of Vitelogenin Arowana and better than injection regarding to animal welfare. The process of oral hormone estradiol stimulation for the

production of Vitelogenin is a new method that can be used in rather than injection. Oral hormone stimulation more recommended in order to avoid the risk of fish mortality.

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