THE EFFECT OF ENDOSULFAN (INSECTICIDE) ON EXPRESSION OF VITELOGENIN GENE IN FEMALE SILVER SHARKMINNOW (Osteochilus hasseltii C. V.)

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Abstract. Endosulfan is an organochlorine insecticide that has high toxicity, persistent and bioaccumulative. Endosulfan entering the waters can cause sublethal toxicity to fish. Endosulfan residues that enter the body of the fish will inhibit their reproduction, such as the formation of vitelogenin. This study aimed to determine the effect of concentration and length of time endosulfan exposure which could reduce vitelogenin gene expression in female silver sharkminnow (Osteochilus hasseltii C. V.). Complete randomized design was applied using four treatments of endosulfan concentration i.e. 0 ppb, 0.88 ppb, 1.76 ppb and 2.64 ppb, respectively. The research was conducted for four weeks with sampling frequency every two weeks. The molecular study was carried out four steps include organ preparation, RNA isolation and DNAse treatment, measurement of RNA concentration and vitelogenin gene expression and RT-PCR was used in this study. Gene expression data analyzed by One Way ANOVA to each of the response variables to know whether differences existed among treatments. The results showed that the concentration and exposure time of endosulfan had not given different result on each treatment. This was presumably because the concentration of endosulfan given had not been able to influence changes in gene expression of vitelogenin in female silver sharkminnow.

Keywords: Endosulfan, Sublethal Toxicity, Vitelogenin Gene Expression
INTRODUCTION

Endosulfan is one of the cyclodien organochlorine insecticides whose use continues to increase. Since 1996, the use of endosulfan has been banned in Indonesia through Ministerial Decree. Agriculture No. 473 / KPTS / TP207 / 6/96. The use of endosulfan is restricted and prohibited due to its high toxicity, persistence, bioaccumulative nature and potential biomagnification. Biomagnification process occurs through the trophic level in living things [1], whereas bioaccumulative is characterized by absorption of endosulfan residues so that it accumulates in body tissues because it has high lipophilia properties, which are easily bound in fat tissue [2].

Endosulfan that enters the waters can cause sub lethal toxicity to aquatic biota (such as fish), one of which is that it interferes with the reproductive system [3]. Endosulfan residues that enter the body of the fish will give a signal to the brain and passed on to the hypothalamus. The hypothalamus will respond to these signals by releasing GnRH which works in the pituitary gland to regulate gonadotrophic synthesis and secretion (GtHs). Endosulfan in chronic concentrations and long exposure times cause GtHR-Iα mutations. Fish that lose the function of GtHR-Iα will cause small and infertile ovaries due to inhibited vitellogenesis [4]. Endosulfan contamination also causes changes in the distribution of primordial germ cells that can affect the structure and function of the gonads. Vitelogenin is synthesized in hepatocytes in response to the activation of estrogen receptors by estrogenic compounds such as endosulfan [5]. Endosulfan is competitive against androgens and estrogens in binding to receptors, has estrogenic effects, stimulates estrogen receptor production and inhibits aromatase activity [6].

Female silver sharkminnow (Osteochilus hasseltii C. V.) is one of Indonesia's native species that is widely cultivated and found along the watershed in the Banyumas region and also widely cultivated by the community. The existence of agricultural activities around the river by using endosulfan will cause endosulfan residues into the waters to be used in the maintenance of cultured fish. This residue can cause fish reproduction to be disrupted. The disruption of reproduction in fish is also greatly influenced by the concentration and time of exposure of endosulfan that enters the waters, so it is necessary to conduct research on the effect of endosulfan on the expression of the vitellogenin gene. This study aims to determine the concentration and duration of endosulfan exposure which can reduce the expression of the vitellogenin gene in female hard-lipped.

MATERIALS AND METHODS

Completely randomized design research (CRD) was used in this experiment. First, this study carried out a preliminary test to get the LC value of 50-96 hours. LC value of 50-96 hours was obtained at 5.87 ppb. The study was conducted with 4 treatment series, ie 0 ppb endosulfan concentration (0% LC concentration 50-96 hours), 0.88 ppb (15% LC concentration 50-96 hour), 1.76 ppb (30% LC concentration 50-96 hours) and 2.64 ppb (45% LC concentration of 50-96 hours) with three replications of individual silver sharkminnow in each treatment each sample. The treatment is given for 4 weeks with sampling intervals every two weeks.

Test Media Preparation and Manufacturing

The maintenance container is in the form of 4 round tubs of water fiber as much as 380 L. The fiber tub is made aeration and recirculation system to maintain water quality. After that, the four-month-old female silver sharkminnow with a stocking density of 50 fish per tank was acclimatized in advance for three days. Preparation of a 10 ppm endosulfan stock solution was carried out by dissolving 2.86 mL of Akodan 35 EC in 10 mL aceton pro analyst (100%), then dissolving in 99,987.14 mL of water. The concentration of the treatment is obtained by the gradual dilution formula.
Female silver sharkminnow is maintained for 4 weeks. Every day the condition of the fish is checked, which is around 07.00, 12.00 and 18.00 WIB. Feed in the form of pellets (Prima Feed GP-2 brand with a protein content of 27-29%) is given as much as 3% of body weight, namely in the morning and evening.

**Tissue Preparation**

Female silver sharkminnow in each treatment were randomly taken with a total of 3 fish in each treatment tank. Fish brain and pituitary are taken by earpick, then the sample is weighed and put into a tube. Tube containing the sample is stored in a freezer of -80 °C until the organ sample is isolated.

**RNA isolation**

Brain and pituitary samples were taken with tweezers and weighed ± 25 mg for each fish sample. Isolate RNA samples using products from Geneaid, namely the Total RNA Mini-Kit.

**DNase Treatment**

DNase Treatment uses the DNA-free Thermo Scientific-Kit. DNase treatment components consist of RNA in RNase-free Water (1 μL), 10X reaction buffer with MgCl₂ (1 μL), DNase I, RNase-free (1 U), DEPC-treated Water (8 μL). All DNase components were put into a tube then incubated at 37 °C for 30 minutes. After that, 1 mL of 50 mM EDTA was added and incubated at 65 °C for 10 minutes.

**Measurement of Vitelogenin Gene Expression**

Vitelogenin encoding gene expression using KAPA ™ SYBR® FAST One-Step qRT-PCR Kit.

**Making the qPCR Mix Master**

Components in making a master mix consist of 2x Sensi FAST (5 µL), Forward Primer (0.4 µL), Reverse Primer (0.4 µL), RNA Template (2 µL) and Nuklease is free water (up to 10 µL).

**Quantitative real-time analysis**

The One Step qPCR setting starts from synthesizing cDNA at 42 °C for 5 minutes, then deactivating RT (Reverse Transcript) at 95 °C for 2-5 minutes. Furthermore the denaturation process at a temperature of 95 °C for 3 seconds and aneling at a temperature of 60 °C for ≥20 seconds for as many as 40 cycles. The last step is the extension process for 5 minutes. The primers used in this study used specific real time primers amplifying the vitelogenin gene in female silver sharkminnow. Whereas house keeping gene uses primary beta actin, namely FA and RA (Table 1).

The results of the amplification using Real Time PCR are then used to compare the number of DNA molecules from the amplification of the vitelogenin coding gene with the results of the amplification of the β-Actin gene. The comparison values obtained were then compared again with the fish groups in various endosulfan treatments according to Forlenza et al. (2012) with the formula.  

$$
\Delta\Delta CT = (CT\ vtg - CT\ actin)\ sampel - (CT\ vtg - CT\ actin)\ Kalibrator, \ R_{vtg} = 2^{-\Delta\Delta CT}
$$

Keterangan:

- ΔΔCT = Threshold Cycle
- Ct vtg sample = Vitogenogenin Ct value of the sample-i
- Ct β-actin sample = nilai Ct β-actin sampel ke-i
- Ct vtg calibrator = Vitogenogenin Ct values of samples with the lowest β-actin
- Ct β-actin calibrator = the lowest Ct β-actin value of the amplification results
- Rvtg = Vitelogenin gene expression level
Table 1. The primary design used in Real Time PCR [16,17]

| No | Nama/kode primer | Sekuens komplemen DNA (primer) | Tm  | Produk PCR |
|----|-------------------|--------------------------------|-----|------------|
| 1  | Forward Vitelogenin Real Time | CGTGGGATCHYTGMARTACGA GTT | 62,81 | 1100 bp |
| 2  | Reverse Vitelogenin RealTime | ATGGTGGCRCTATTGAT | 62,83 |
| 3  | Forward Beta Actin (FA) | GAG CTA TGA GCT CCC TGA CGG | 58,30 | 53 bp |
| 4  | Reverse Beta Actin (RA) | AAA CGC TCA TTG CCA ATG GT | 55,60 |

Data analysis

Quantitative data on the level of vitelogenin gene expression were analyzed by One Way ANOVA to determine the concentration and duration of endosulfan exposure that could interfere with the expression of the vitelogenin gene in female silver sharkminnow (*Osteochilus hasseltii* C.V.).

Results and Discussion

The results of measurement of vitelogenin gene expression in female silver sharkminnow (*Osteochilus hasseltii* C.V.) exposed to endosulfan for 4 weeks with different concentrations can be seen in Figure 1.

![Figure 1. Vitelogenin Gene Expression Values of Endosulfan Fish Exposed for 4 Weeks](image-url)
Based on Figure 1, it can be seen that the value of female nilema vitellogenin gene expression values at week 0 in each treatment concentration showed the same value, which was 41.8259. This is because at week 0 the female silver sharkminnow has not been given treatment so that there is no fluctuation in each concentration treatment.

At the second week the value of vitellogenin gene expression ranged from 0.9419 - 31.8288. The results of measurement of the silver sharkminnow vitellogenin gene expression at week 2 showed results that were not different in each treatment. This can be caused because the concentration of endosulfan given has not been able to affect changes in the expression of the vitellogenin gene in female nilem.

At the 4th week the expression value of the vitellogenin gene ranged from 0.9062 to 1.0667. The results of the measurement of vitellogenin gene expression at the 4th week showed that the inter-treatment did not differ. Apart from giving too low endosulfan concentrations, this can also be caused by the endosulfan concentration at the 4th week the level of toxicity has been reduced so that it has not so affected the expression of the silver sharkminnow vitellogenin gene. According to Reyes et al. [7], the half-life of endosulfan-α is 19 - 33 days, while endosulfan-β is 45 - 58 days.

The results of the measurement of vitellogenin gene expression at the 2nd and 4th week also showed no different results. This is allegedly due to exposure time that is too short. So that the exposure time of endosulfan which was carried out for 4 weeks has not been able to reduce the expression of the vitellogenin gene.

Measurement of vitellogenin gene expression in female silver sharkminnow that showed results that were not significantly different were estimated to be influenced by several factors, namely endosulfan concentrations given were still low and shorter exposure times. In the sublethal toxicity test, the concentration of treatment that is usually used is 0%, 20%, 40% and 60% of the LC-96 hour value [8,9,10,11,12,13]. Thus, endosulfan concentration and exposure time given have not been able to reduce the expression of the vitellogenin gene. Based on the results of research conducted by Hemmer et al. [14], endosulfan concentrations of 1.5 ppb which were exposed for 42 days on Cyprinodon variegatus have not been significantly different. Although endosulfan can bind to estrogen receptors at very high concentrations, pharmacokinetic considerations such as absorption, metabolism and distribution and concentration of end tissues can negate the potential estrogenic endosulfan so that it becomes weak[18]. In addition, fish used as test animals will also affect the level of endosulfan toxicity concentration.

The results of the analysis of the expression value of the vitellogenin gene in female silver sharkminnow exposed to endosulfan for 1 month with different concentrations can be supported by the results of the analysis of the value of the Somatic Gonado Index (IGS) because vitellogenin activity is closely related to the IGS value. The results of the analysis of IGS values showed no significant difference so that it showed that different endosulfan concentrations and exposure times did not have a significantly different effect on IGS values in female nilem.

The value of the Somatic Gonad Index (IGS) in silver sharkminnow (Osteochilus hasseltii C.V.) female used as a supporting factor in measuring the expression value of the vitellogenin gene can be seen in Figure 2.

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Based on Figure 2, the value of silver sharkminnow IGS percentage in each endosulfan treatment which was exposed for 4 weeks showed fluctuations. The IKG value of week 0 for each treatment is the same, that is 0.250%. While the second week the IKG values ranged from 0.077 to 0.235% and the fourth week ranged from 0.080 to 0.150%. IKG values in each treatment have a gonad maturity stage <4% which means the fish are not ready to spawn [15]. In the condition of fish that are not ready for spawning, gonad damage can be seen by observing gonad histology.

CONCLUSION

Based on the results of the study, it can be concluded that the level of endosulfan concentration with various concentrations given (<2.64 ppb) and the length of time of endosulfan exposure for 4 weeks did not give different results on the decrease in the value of vitellogenin gene expression in silver sharkminnow (*Osteochilus hasseltii* C.V.).

REFERENCES

[1] Kostoer, Y. 2006. Kimia dan Ekotoksikologi Pencemaran. UI-Press, Jakarta.

[2] Taufik, I. 2005. *Pengaruh Lanjut Bioakumulasi Insektisida Endosulfan Terhadap Pertumbuhan dan Kondisi Hematologis Ikan Mas (Cyprinus carpio)*. Tesis. Institut Pertanian Bogor, Bogor.

[3] Putri, A. C., Razak, A., Sumarmin, R. 2015. *Pengaruh Insektisida Organoklorin Endosulfan Terhadap Daya Tetas Telur Ikan Nila (Oreochromis niloticus)*. Universitas Negeri Padang, Padang.

[4] Murozumi, N., Nakashima, R., Hirai, T., Kamei, Y., Ishikawa-Fujiwara, T., Todo, T., Kitano, T. 2014. Loss of Follicle-Stimulating Hormone Receptor Function Causes Masculinization and Suppression of Ovarian Development in Genetically Female Medaka. *Endocrinology, 155*(8): 3136–3145.

[5] Chow, W. S., Chan, W. K. L., Chan, K. M. 2013. Toxicity Assessment and Vitellogenin Expression in Zebrafish (*Danio rerio*) Embryos and Larvae Acutely Exposed to Bisphenol A, Endosulfan, Heptachlor, Methoxychlor and Tetrabromobisphenol A. *Journal of Applied Toxicology, 33*: 670–678

[6] Mnif, W., Hassine, A. I. H., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B. 2011. Effect of Endocrine Disruptor Pesticides : A Review. *Int. J. Environ. Res. Public Health, 8*: 2265-2303.
[7] Reyes, J. G. G., Rodriguez, G. R. G., Osuna, M. D. C. C., Jaward, F. M. 2014. Bioaccumulation and Evidence of Hormonal Disruptions in Tilapia Fish (Oreochromis ssp.) Exposed to Sub-Lethal Concentrations of Pesticides in Sinaloa, Mexico. *International Journal of Biochemistry Research*, 4(4) : 333-343.

[8] Rudiyanti, S., Astri, D. E. 2009. Pertumbuhan dan Survival Rate Ikan Mas (Cyprinus carpio Linn) pada Berbagai Konsentrasi Pestisida Reagent 0,3 G. *Jurnal Saintek Perikanan*, 5(1) : 39-47.

[9] Prayogo, N. A., Hidayati, A., Siregar, A. S., Yunasfi. 2016. Uji Toksisitas Letal dan Subletal Logam Berat Merkuri Terhadap Ikan Nilem (Osteochilus hasseltii). *Omni Akuatika*, 12(1) : 86-94.

[10] Prayogo NA, Wijayanti GE, Murwantoko, Kawaichi M, Astuti P. 2012. Effect of photoperiods on melatonin levels, estradiols level and the expression of cGnRH-II and sGnRH genes, in hard-lipped barb (osteochilus hasselti c.v.). Journal of global veterinaria, 8(6) : 591-597.

[11] Prayogo NA, Wijayanti GE, Sulistyo I, Sukardi P. 2016a. Cloning and expression cgnrh-ii and sgnrh genes in hard-lipped barb (osteochilus hasselti c.v.). Biodiversitas, 17(29) : 523-530

[12] Prayogo NA, Siregar AS, Sukardi P. 2016b. The disruptive effect mercurychloride (hgcl) on gene expression of cgnrh-ii sgnrh, and estradiol level in silver sharkminnow (osteochillus hasseltii c.v.). Turkish journal fishery and aquatic science. 16(2) : 1003-1009

[13] Prayogo NA, Siregar AS, Sukardi P, Bessho Y. 2018. Molecular Cloning Of The Vitellogenin Gene In The Hard-Lipped Barb (Osteochilus Hasseltii C.V) And Photoperiod’s Effects On Gene Expression. Biotropia journal. 25(3) : 211-233.

[14] Hemmer, M. J., Hemmer, B. L., Bowman, C. J., Kroll, K. J., Folmar, L. C., Marcovich, D., Hoglund, M. D., Denslow, N. D. 2001. Effects Of P-Nonylphenol, Methoxychlor and Endosulfan on Vitellogenin Induction and Expression in Sheepshead Minnow (Cyprinodon variegatus). *Environmental Toxicology and Chemistry*, 20(2) : 336–343.

[15] Sumantadinata, K. 1983. *Pengembangan Ikan-Ikan Peliharaan di Indonesia*. Satra Hudaya, Jakarta.

[16] Siregar, A. S., Prayogo, N. A. 2017. The Disruptive Effect of Mercury Chloride (HgCl) on Gene Expression of Gonadotrophin Hormones and Testosterone Level in Male Silver Sharkminnow (Osteochilus hasseltii C.V.) (Teleostei: Cyprinidae). *The European Zoological Journal*, 84(1), pp. 436–443.

[17] Siregar, A.S., Prayogo, N.A. 2018. The disruptive effect of mercury chloride (HgCl) on gene expression of gonadotrophin hormones and testosterone level in male silver sharkminnow (Osteochilus hasseltii C.V.) (Teleostei: Cyprinidae). European Zoological Journal 84(1), pp. 436-443

[18] Sukardi P, Hana H, Prayogo NA, Sulistyo I, Soedibya PHT, Harisam T, Wlnanto T. 2018. A lipid-walled microcapsule diet as co-feed for early feeding the Osphronemus gourami (Lacepede) larvae | [Dieta micro encapsulada com lipídeos (Lipid-walled) como co- alimentação na alimentação precoce da larva de Osphronemus gourami Lacepede]. Acta Scientiarum. Animal Sciences, v. 40, e38335. Doi: 10.4025/actascianimsci.v40i1.38335.