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RESEARCH PAPER

The Disruptive Effect Mercurychloride (HgCl) on Gene Expression of cGnRH-II, sGnRH, and Estradiol Level in Silver Sharkminnow (*Osteochillus hasseltii* C.V.)

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Abstract

Reproductive activities in fish are regulated by several environmental and physiological factors. In many aquatic ecosystems, metal concentrations are higher than natural levels, because of constant metallic releases from agricultural sources, industries, and mining operations. Mercury is a toxic and persistent pollutant, which bioaccumulates in the food chain. To investigate the effect of mercury chloride (HgCl) on fish reproduction, animals were keep with four aquaria containing increasing levels of HgCL (0 mg/L [control]; 0.025 mg/L [low]; 0.05 mg/L [medium]; 0.1 mg/L [high]) for 60 days. The effects of HgCl on reproduction performance on silver sharkminnow were evaluated by cGnRH-II and sGnRH gene expression, estradiol levels, GSI levels and proportion of oocyt. A significant decrease in by cGnRH-II and sGnRH gene expression, estradiol levels, GSI levels and proportion of oocyt were detected in fish receiving high mercury dose compared to controls on weeks 2,4,6, and 8 ($P<0.05$). On weeks 4, 6 and 8, all treatment groups had significantly lower cGnRH-II and sGnRH gene expression, estradiol levels, GSI levels and proportion of oocyt compared to the control group ($P<0.05$). These findings demonstrate a disruptive role of Mercury on the reproduction performance in Silver sharkminnow.

Keywords: HgCl, cGnRH-II, sGnRH, estradiols levels, GSI, Oocyt proportion.

Introduction

Several environmental and physiological factors will effect Reproductive activities in fish via HPG axis [Bromage *et al.,* 2001]. External environment exert it role on reproduction through hypothalamus regulating the synthesis and secretion of gonadotropins releasing hormone (GnRHs) (Piccione *et al.*,2015). GnRHs regulating the synthesis and secretion of gonadotropins (GtHs) (Mortehavizavizadeh *et al.,* 2010, Yaron and Levavi-sevan., 2011). The GtHs regulate the two main activities of the gonads i.e. hormone and gamete production [Rodriguez *et al.,* 2004]. Ovarian hormones especially estradiol and progesterone play an important role in maintaining and promoting gamete production (Miranda *et al.,* 2009). During the last years pollution by heavy metals in aquatic environment gained great importance because of the risk of toxicity and bioaccumulation in the food chain. Natural and anthropogenic sources including industrial and domestic sewage, harbor activities and atmospheric deposits, represent the sources of heavy metals in aquatic ecosystem. The concentrations of heavy metals accumulated in living organisms reflected the degree of environmental pollution. Among aquatic species, fish is a suitable biomarker of environmental pollution because of their position in the aquatic food chain. Naturally, the various forms of elemental, inorganic, and organic mercury are interconverted (Eisler 2004; Crespo- Lopez *et al.,* 2007), and it has been shown that HgCl is the most poisonous form of mercury compounds (Silva-Pereira *et al.,* 2005). Mercury poisoning lead to elevated death rate, weight loss, larvae deformation, depressed protein synthesis, hormonal imbalance, abnormal behavior, spawning reduction, sexual dysfunction, color changes, in fish (Oliviera Robeiro *et al.,* 2002; Devline 2006; Houck and Cech 2004; Dervnick and Sandheinrich 2003; Friedmann *et al.,* 1996).

Moreover, recent studies on human brain cell lines demonstrated genotoxic effects of HgCl such as inhibition of proliferation, increase in the metaphasic and micronucleation index and the index of nucleoplasmic bridges as well as decrease in the binucleation index (Crespo-Lo´pez *et al.,* 2007). So far, multiple mechanisms responsible for HgCl toxicity have been identified. Among these is high reactivity to thiols of bioactive proteins (Yasutake *et al.,* 1997; Zalups 2000), arachidonic acid (AA)

production (Shanker *et al.*, 2002), reactive oxygen species (ROS) occurrence (Limke and Atchison 2002), and intracellular calcium increase [Ca] (Castoldi *et al.*, 2001). However, despite extensive studies, the effect of HgCl on the fish reproduction has received little attention.

We investigated the specific effects of HgCl concentration, on gene expression of cGnRH-II, sGnRH, estradiol, testosterone, GSI and oocyt proportion in silver sharkminnow (Osteochilus hasseltii, C.V 1842). Silver sharkminnow were economically fish in Indonesia and had a highly market. Biology, physiology, and nutrition of this species have been profoundly studied, and there have been several global efforts to conserve this species. Thus, Silver sharkminnow can be a suitable freshwater model species that provides insight into the toxic effect of HgCl in other cyprinids species. Hard-lipped barb is a synchronous batch spawner fish capable of spawning several time during the peak of the spawning period.

Materials and Methods

Experimental Fish

144 females hard-lipped barb weighing of 100 g in average were purchased from local farmer in Banyumas regency and maintained at Laboratory of Fisheries and Marine, Jenderal Soedirman University. The females were divided into 4 groups. Each group consisted of 4 aquaria with 9 fish/50 L water. In this study, three types of HgCl concentration namely 0.025 mg/L (P1) low concentration, 0.05 mg/L (P2) medium concentration and 0.1 mg/L (P3) high concentration and control have been tested toward concentration of estradiol, GSI and oocyt proportion. The aquaria were design with recirculation for oxygen supply. Then, the fish were reared for two months at the laboratory of Aquaculture Department of Marine and Fisheries, University of Jenderal Soedirman. During the research, fish were fed on commercial pellet (protein 37% and fat 10%) as much as 3% of total body weight daily. The water was siphoned regularly to maintain water quality. The water temperature, disolved oxygen, pH and carbondioxide were monitored every 2 weeks. Every sampling time, 9 fish captured for the samples. All fish were anesthetized with tricaine methane sulfonate (Sigma) and decapitated prior to tissue collection. Gonads samples from the fish were removed, and after being dissected and weighed, the gonads were fixed in Bouin's solution and subjected to histological observation. The GSI was calculated for each fish. The serum samples were separated by centrifugation (4°C, 10.000 g, 5 min) and stored at −80°C until analysis.

RNA Isolation and RT-PCR

Total mRNA was extracted from whole brain using blue Sepasol R- RNA super 1 reagent (nacalai tesque), based on Etanol-phenol-chloroform extraction method. The sample were treatment with DNAse free RNAse (Takara). The quality and concentrations of total RNA were assayed by agarose gel electrophoresis and optical density reading at 260 and 280 nm, the RNA were loaded in batches and frozen at -70°C.

RT-PCR

Total mRNA samples (1µl) were reverse transcripted using cDNA synthezis kit (PrimeScript™ Reverse Transcriptase) from Takara using Random 6 mers (50µM) primers and Prime script R-tase with manufacture instruction.

Quantitative Real Time Analysis

The primers were designed based on cGnRH-II (accession numbers JN867720) and sGnRH (accession numbers JN867721) using the Primer 3.0 software. The generated primer used in this study were as follows: sGnRH forward 5'-TGGTGTGTGTGTTGGAGG TT -3', sGnRH reverse 5□'-AATGTTGCCTCCACTTCACC-3'; cGnRH-II forward 5'-CATCTGCAGGCTGTTTGTGG-3', cGnRH-II reverse 5'-TGCTGAGAGCTGGCAAACTG -3' (Prayogo *et al.*, 2011). Silversharkminnow □actin used as endogenous control (housekeeping gene), was amplified by the following primers—actin forward 5'-GAGCTATGAGCTCCCTGACGG-3', and actin reverse 5'- AAACGCTCATTGCCAATGGT-3' were used to normalize variations in RNA (Table.1). After optimization, PCR reactions were performed in a 10 µl volume containing 2 µl cDNA, 5 µl SYBR mix (Applied Biosystem), 0.3 µl forward primer, 0.3 µl reverse primer, and 2.4 µl DDW using the following

Table 1. The primer used to amplify the GnRH genes and their PCR product

No	Primer code	DNA komplement sequence (primer)	Tm	PCR Product
1.	Forward sGnRH Real Time (F2)	TGGTGTGTGTGTTGGAGG TT	62,81	75 bp
2.	Reverse sGnRH Real Time (R2)	AATGTTGCCTCCACTTCACC	62,83	
3.	Forward cGnRH-II Real Time (F3)	CATCTGCAGGCTGTTTGTGG	59,89	80 bp
4.	Reverse cGnRH-II Real Time (R3)	TGCTGAGAGCTGGCAAACTG	59,97	
5.	Forward Actin (FA)	GAGCTATGAGCTCCCTGACGG	58,3	53 bp
6.	Reverse Actin (RA)	AAACGCTCATTGCCAATGGT	55,6	

condition: 95°C for 45 s, (45 cycles of 95°C for 15 s and 60°C for 1 min), then 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Relative fold change of gene expression was calculated using the ΔΔCt method. Elongation actin gene, a stable reference gene in Silversharkminnow (Prayogo *et al.*, 2012), was used to normalised the Ct values of the target genes. Normalized qPCR data were LOG transformed prior to statistical testing.

Measurement of Estradiol Levels by Enzyme Linked Immunoasorbent Assays

At the sampling points, 1mL blood was taken from caudal vein of each female. The blood was allowed to clot at room temperature for 15 minutes. The serum was aspirated by centrifugation at 3.000 g for 10 min then was kept at -20 °C until further analysis. Serum estradiol (E2) levels were determined DRG Estradiol ELISA (EIA- 2693) according to manufacturer's instruction. Duplicate were provided for each measurement.

Histological Analysis

The gonadal tissues of each HgCl treatments group, were fixed in Bouin's solution (formaline, picrat acid, and Glacial acetat acid) to analyze the gonads during sexual maturation. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin. Sections (6 μm thick) were selected and stained with hematoxylin-eosin for observation under a light microscope (DM 100; Leica, Wetzlar, Germany). The images were captured using a digital camera (DFC 290; Leica).

Data Analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). A one-way analysis of variance followed by Tukey's post hoc test was used to test for significant differences in the data ($P \leq 0.05$). The values are expressed as the means ± standard error (SE).

Results

Expression of cGnRH-II mRNA and, sGnRH mRNA

In silver sharkminnow females, relative cGnRH-II mRNA expression level in four months were 0.52-2.35 (Figure. 1A). The highest cGnRH-II mRNA expression (2.35) was observed in control group (PK) at eight weeks' treatments ($P<0.05$). mRNA expression for control group increased with treatments periods ($P<0.05$). The cGnRH-II mRNA expression for other HgCl treatment at second weeks and fourth weeks had non significantly different ($P>0.05$), but in the six and eight week, control treatment had higher gene cGnRH-II than HgCl treatment. ($P<0.05$).

The relative level of sGnRH mRNA expression throughout the study were 0.009-4.20 (Figure.1B). The highest sGnRH gene expression were (4.20) were found in control group at the six weeks ($P<0.05$). The sGnRH mRNA expression for HgCl treatment in second weeks untill fourth weeks had non significantly different ($P>0.05$), but in sixted weeks and eighted weeks sGnRH gene expression in control group were higher trends than HgCl treatments ($P<0.05$). This result showed a high correlation between the bioaccumulation rates of mercury and the GnRH gene expression levels in silver sharkminnow.

Serum Estradiol 17β Levels

The concentrations of estradiol in serum silver sharkminnow on weeks 2, ,4 6, and 8 treatments by HgCl are illustrated in Figure 2. On weeks 2, 4, 6, 8, estradiol level in serum silver sharkminnow among the treatment and control groups were statistically different ($P<0.05$). All treatment groups showed a significant lower estradiol level compared to control group ($P<0.05$). This result showed a high correlation between the bioaccumulation rates of mercury and the serum estradiol levels in silver sharkminnow.

Gonada Somatic Index (GSI)

The precentage of GSI in silver sharkminnow on weeks 2, ,4 6, and 8 treatments by HgCl are illustrated in Figure 3. On weeks 4, and 8, GSI level in silver sharkminnow among the treatment and control groups were statistically different ($P<0.05$). treatment groups showed a significant lower GSI level compared to control group ($P<0.05$). This result showed a high correlation between the bioaccumulation rates of mercury and the GSI levels in silver sharkminnow. Higher mercury concentration will decreasing GSI level. GSI level were representation of gonad activity in silver sharkminnow.

Oocyt Proportion

The oocyt proportion of GSI in silver sharkminnow on weeks 2, 4 6, and 8 treatments by HgCl are illustrated in Figure 4. On weeks 2 and 4, no post vitelogenic phase were found in oocyt and on weeks 6 and 8 had phase of post vitelogenic in oocyt. Oocyt proportion post vitelogenic post in silver sharkminnow among the treatment and control groups at weeks 6 and 8 were statistically different ($P<0.05$). treatment groups showed a significant lower post vitelogenic phase compared to control group ($P<0.05$). This result showed a high correlation between the bioaccumulation rates of mercury and the development of oocyt in silver sharkminnow. Higher mercury concentration will decreasing proportion post

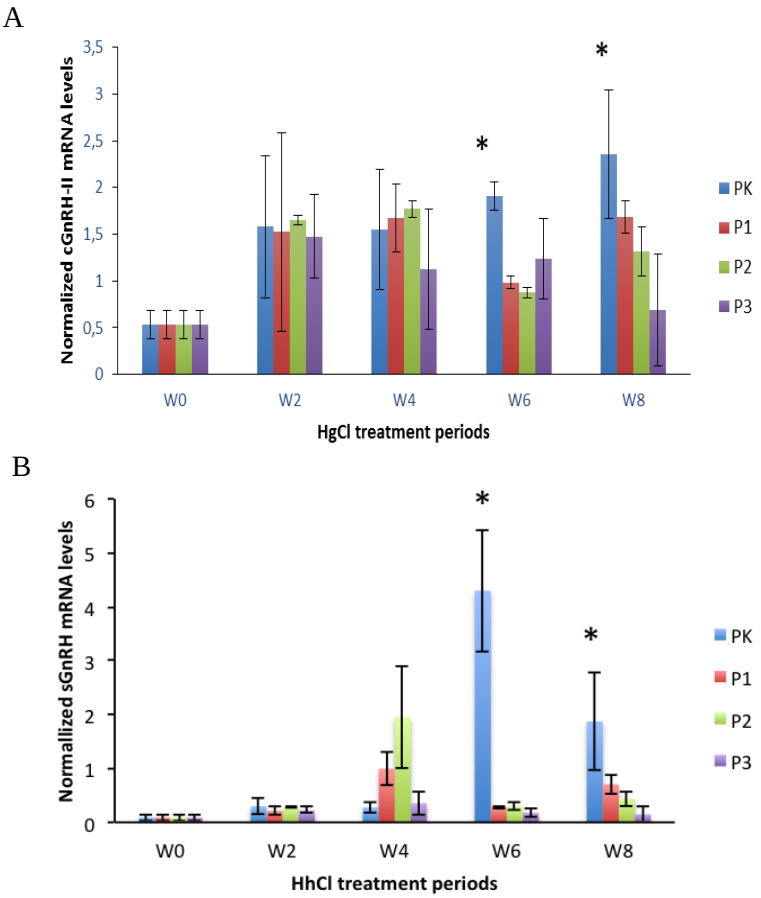

Figure 1. mRNA levels of cGnRH-II (A) and sGnRH (B) of hard-lipped barb kept under HgCl treatment for 8 weeks. (PK=control, P1=14L:10D, P2=16L:8D, P3=18L:6D, M=Month). (* : Significantly different).

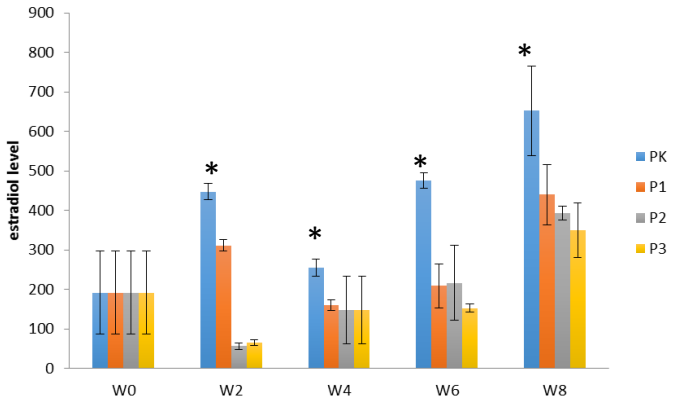

Figure 2. Serum Estradiol Levels of hard-lipped barb kept under different mercury concentration for 8 weeks. PK=control, P1=0.025 mg/L, P2=0.05 mg/L, P3=0.1 mg/L, W=Weeks). (*: Significantly different).

vitelogenic phase.

Discussions

In this study, changes in cGnRH-II and sGnRH gene expression, estradiol level, GSI levels and oocyt proportion were analyzed to characterize the role of endocrine disruption of reproduction under mercury treatment. This study confirms previous results from fisheries and marine laboratory showing decrease in cGnRH-II and sGnRH gene expression, serum estradiol levels during mercury treatment in hard-lipped barb at weeks 2, 4, 6, and 8. In this study showed that cGnRH-II and sGnRH gene expression

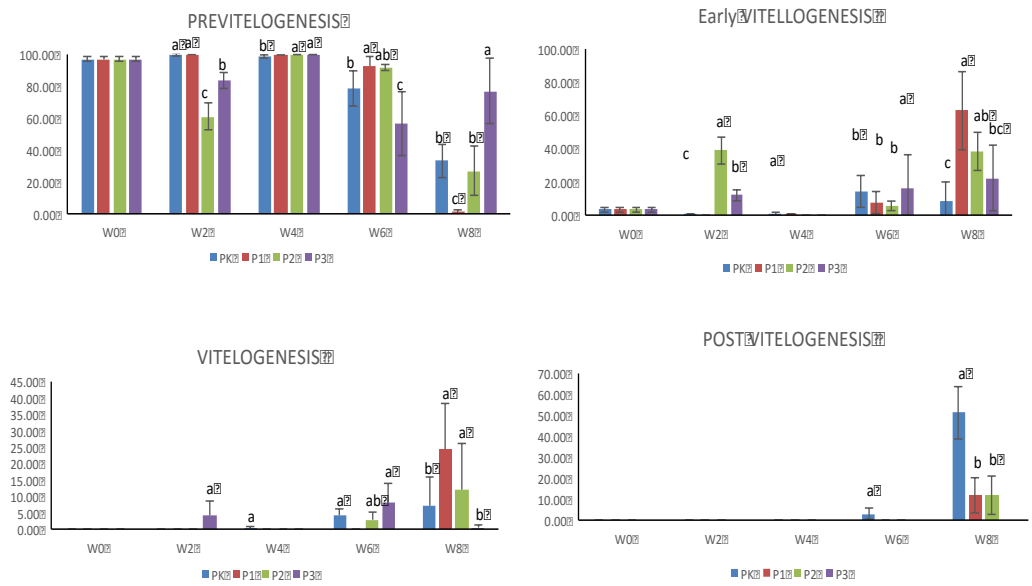

Figure 4. Oocyt Proportion vitelogeneis phase of hard-lipped barb kept under different mercury concentration for 8 weeks. PK=control, P1=0.025 mg/L, P2=0.05 mg/L, P3=0.1 mg/L, W=Weeks). (different letter: Significantly different).

and estradiol level decreased equivalent with the mercury concentration increased. This is proved that mercury act to inhibit protein GnRH and GtH synthesis and finnaly will inhibit secretion of estradiol. Mercury poisoning lead to elevated death rate, weight loss, larvae deformation, depressed protein synthesis, hormonal imbalance, abnormal behavior, spawning reduction, sexual dysfunction, color changes, in fish (Oliviera Robeiro *et al.*, 2002; Devline 2006; Houck and Cech 2004; Dervnick and Sandheinrich 2003; Friedmann *et al.*, 1996). Mercury depressed protein synthesis via inhibit transcription factors for gene expression. Mercury also act mimicking the effects of endogenous hormones, such as the estrogens and androgens, antagonizing the effects of endogenous hormones, altering the pattern of synthesis and metabolism of normal hormones; and modifying hormone receptor levels (Soto *et al.*, 1995).

The results presented here, furthermore indicates that the hypothalamic–pituitary–reproductive axis is a potential target of HgCl even before sexual maturity and that HgCl exerts its toxicity by altering the transcription of key genes involved in reproductive physiology. Estradiol production were decreased when exposure in high mercury doses at weeks 2, 4, 6, and 8. The major estrogen in female fish 17β estradiol (E2) is produced primarily in the ovary by the follicular cells. Mercury can also modifying GtH receptor level in follicular cells, decreasing production of estradiol (Friedman *et al.*, 1996).

Estradiol will lead production of protein vitelogenin in hepatocyte and act to vitelogenesis in oocyt. Oocyte development resulted in an increase in ovarian size and weight as well as the production of reproduction hormone by the ovarium (Figure 4). Protein vitellogenin will enter the oocyt and role in oocyt development. Mercury may also interfere with the binding protein that act to transport hormone to their desnitation. The high level of mercury will able to bind to the estrogen receptor in the cell and interfere the regulation of estrogen, estrogen receptor and heat shock protein 50, 70 and 90 as the protein binding. The interfere of mercury might very well result in transcription response for vitelogenin production as genomic action of estrogen. (Bjornstorm and Sjoberg., 2005)

According to the histological observations of oocyt, formation of vitelogenic phase in silver sharkminnow will slower in mercury treatment than control treatment. The oocytes had developed and were largest in size at control treatment at weeks 6 and 8 compared the all treatment (Figure 5). Estradiol will stimulate the formation of the yolk and affects final maturation. This result was supported by histological observations: post vitelogenic oocyte cells were observed in many control female gonad tissues. For the highest levels of mercury treatment, ovary not yet reach post vitelogenic phase at weeks 8 contrally with the control treatment, in which we confirmed that sexual maturation under high level of mercury was decreased

Sex steroids are known to be involved in the progression of oogenesis. In general, levels of estrogen, particularly 17-estradiols, are shown to increase gradually as oogenesis progresses (Prayogo, *et al.*, 2012, Schulz *et al.*, 2010). Thomas *et al.* (2005) reported that circulating levels of estradiol in fish increased in correlation with an increase in the gonadosomatic index (GSI) and progression of

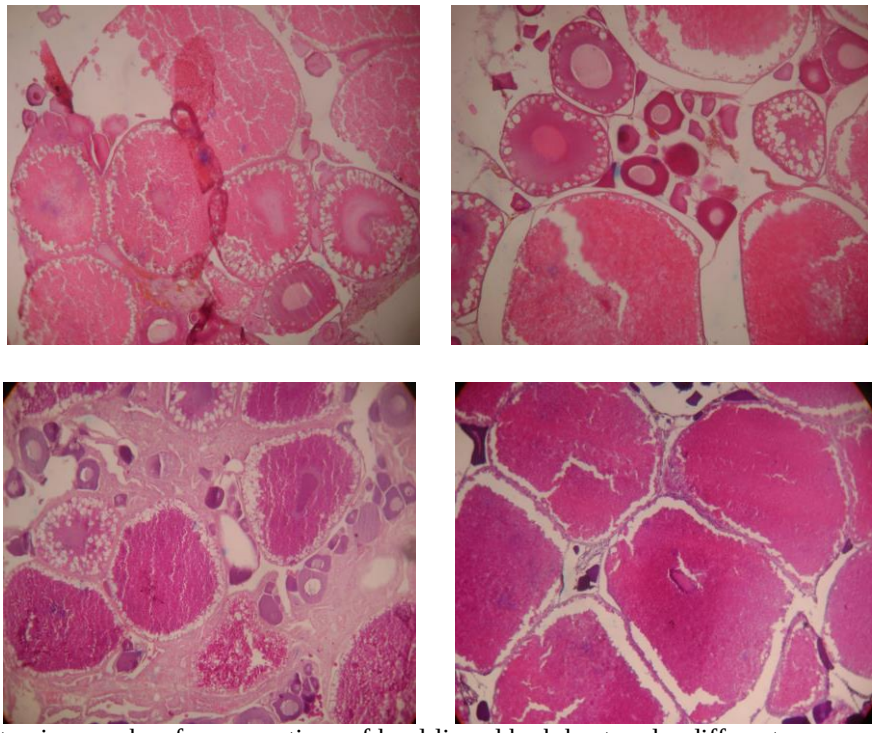

Figure 5. Photomicrographs of cross sections of hard-lipped barb kept under different mercury concentration for 8 weeks. A. oocyt from high concentration mercury (0.1 mg/L), B. oocyt from medium concentration mercury (0.05 mg/L), C. oocyt from low concentration mercury (0. mg/L), and D. Control. Scale bar = 6 µm (400x).

oogenesis, suggesting their involvement in the acceleration of oogenesis in fish. Inhibiting the action of estradiol will decreasing GSI level and oogenesis progression. After mercury treatment, rare mature oogenesis were observed in the gonadal tissue of female silver sharkminnow at 6 and 8weeks (Figure 4). Therefore, mercury act as endocrin disruption of oocyt development in Silver sharkminnow.

Conclusion

In summary, the results obtained in the present study highlighted the toxic effects of mercury on reproductive performance of silver sharkminnow. In particular, female silver sharkminnow exposed to HgCl showed an inhibited cGnRH-II and sGnRH gene expression and a decreased the secretion of estradiol. Mercury played an endocrine disruption action on gonad activity and it disrupted the final maturation of the oocyte, as well.

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