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RESEARCH PAPER

A Histological Study on Mercury-Induced Gonadal Impairment in Javanese Medaka (*Oryzias javanicus*)

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Abstract

Mercury (Hg) is highly toxic and potent neurotoxin. Bioaccumulation of mercury in a new model fish species, Javanese medaka (*Oryzias javanicus*) under sublethal levels and effects of mercury exposure on gonads via histological studies were investigated in this study. A total of 150 adults of *O. javanicus* were exposed to mercury at five different concentrations; 0, 10, 20, 30, 40 ppb for 14 days. High accumulation of mercury were observed at the Mercury exposures of 30 ppb (3.17 mg/kg) and 40 ppb (2.92 mg/kg). Meanwhile, no significant difference was detected with 0 ppb (0.72 mg/kg) and 10 ppb (0.74 mg/kg) exposure. The accumulation increased by the increment of the concentrations of mercury exposure. Histological study revealed the increasing amount of oocyte atresia and decrease in vitellogenesis with high mercury exposure. Severity grading and degrading of ovaries were depicted at 30 and 40 ppb exposure, respectively. Testicular tissues showed severe effects, which resulted from exposure to various mercury concentrations. Complete disorganisation in cyst arrangement and decrease of progressive stages of spermatogenesis were observed in the morphology of exposed *O. javanicus* testis. The present study's finding suggested mercury potential for bioaccumulation and its effects on the morphology of gonad in *O. javanicus*.

Keywords: Mercury, *O. javanicus*, bioaccumulation, gonad, histology.

Introduction

Mercury (Hg) is one of the most toxic elements and threat to living organisms due to its high bioaccumulation and biomagnification that reach hazardous levels along the trophic chain (Lindberg *et al*., 2007). Human activities such as electronic industries, chloralkali industries and fossil burning into the atmosphere are the main contributors of both inorganic and organic forms of mercury. Mercury cannot be removed and it is rapidly transformed by microorganism into organic compounds that are generally more toxic than inorganic species (Azimi & Moghaddam, 2013). According to the US Environmental Protection Agency (EPA), mercury is considered as a highly dangerous element due to its accumulative and persistent character in the environment and biota (Beltran-Pedreros *et al*., 2011). Since mercury accumulates in the tissues and is biomagnified through the aquatic food chain, carnivorous fish exhibit higher mercury level compared to herbivorous and omnivorous fishes, and larger fish of the same species generally contain more mercury than smaller ones (Wang, Mao, Ma, & Yang, 2014).

Javanese medaka *(O. javanicus)* fish is a type of small bony fish that can be found in a diverse group distributed around Asia. They live in brackish water, freshwater and saltwater. *O. javanicus*could have equivalent capabilities as with Japanese medaka (*O. latipes*) which is one of the most established species that is widely used in experimental work on vertebrate biology for many years (Ismail & Yusof, 2011; Yusof, Ismail, Koito, Kinoshita, & Inoue, 2012). According to Imai, Koyama, and Fujii(2005), *O. javanicus* is commonly found in estuarine waters of southern to eastern Asia. Nevertheless, previous studies such as that carried out by Yusof *et al*. (2012) and Khodadoust, Ismail, and Zulkifli (2012) demonstrated that this fish is one of the most suitable species that can be easily reared in the laboratory. They have a short life cycle and life span with wide geographical range and availability. However, a suitable animal to use as a bioindicator for pollutants has to be abundant, sufficiently long-lived, reasonable in size and able to adapt well to laboratory surroundings. Moreover, *O. javanicus* is easily to be identified species, has a fast growth rate, and is easy

to culture (Khodadoust, Ismail, Zulkifli, & Tayefeh, 2013). *O. javanicus* also serves as an excellent candidate for fish model because it is sensitive to toxicants and endocrine disrupting chemicals at its early life stage (Lei *et al*., 2013). This study was carried out to determine the bioaccumulation of mercury in medaka fish under sublethal levels and to investigate its effects on *O. javanicus* gonad via histological studies.

Materials and Methods

Fish Sampling Method

Wild stocks of *O. javanicus* were collected from a recreational brackish lake in Sepang, Malaysia (2°37'15.2"N 101°42'38.4"E). Data on in situ water parameters were obtained using a Handheld Multiparameter Meter (YSI 556); temperature (28°C); dissolved oxygen (6.70 mg/L); pH 6.9; total dissolved solid (21.70 mg/L); salinity 18.50 ppt; and conductivity 0.45mS/cm. These data were used to setup an artificial environment for a toxicity testing system. Capturing process was conducted by using custom made hand nets. The fishes were kept in plastic container using the water from the fish's habitat. The fishes were transported to the laboratory in a well-padded container with aerating filter system to lessen transfer stress. Oxygen stone (Japan Pet Drugs Co. Ltd) was added into the water to provide oxygen to the water. A total amount of 150 adult *O. javanicus* (body weight [b.w], ± 0.30g) were acclimatized from the origin salinity level up until 0.00 ppt ± for freshwater condition in indoor glass aquaria (volume, ~8L per tank) and aerated with aerating filter system. The temperature was kept constant at 28 °C.

Treatment

Present exposure study has obtained an animal ethics clearance by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia. Since no data were available on mercury toxicity to *O. javanicus*, 96 hours- LC50 range finding was performed to determine the appropriate concentration ranges for the test chemical. The preliminary tested concentrations were 0, 10, 20 30, 40, 50, 60, 70, 80, 90, and 100 ppb (µg/L). Each experimental concentration consists of 30 individuals and replicates into three different glass aquaria. The number of dead specimens was recorded daily in order to calculate 14 days -LC50 (median lethal concentrations for 50% of sampled animals). Then, four concentrations were selected for sublethal exposure: 10, 20, 30, 40, ppb (µg/L) and an additional control treatment. In brief, there were 15 aquaria with the size of 38.1 cm x 17.8 cm x 17.8 cm each that had five different concentrations with three replicate of mercury (0, 10, 20, 30, 40 ppb). The size of samples was n=10 per tank. Every replicate receive fish at sex ratio of 3:1 (female: male) during the experiment due to common sex ratio that has been applied in fish breeding activity (Little & Hulata, 2000). The fish were placed at combination of three for male and seven for female. The water temperature was kept at 21-26 °C (nominal temperature ± 2 °C), pH: 6.0-8.5, total hardness: 10-250 mg $CaCO_3$/L, conductivity: ≤10 µS.cm^{-1}, dissolved oxygen: ≥60% of saturation level with photoperiod of 14 hours light and 10 hours dark. The chronic exposure was done in a static tank for 14 days. The fish were daily fed using freshly hatched brine shrimp (*Artemia salina*) nauplii. The fish were fed at three times (8:00 a.m., 12:00 p.m., 5:00 p.m.) during light period at interval between four hours. Nauplii of *A. salina* is highly suitable fish feeds for all ages of fish due to their high nutritional value (Zulkifli, Aziz, Ajis, & Ismail, 2014; Nguyen, Nguyen, Van Stappen & Sorgeloss, 2009), thus can promotes the development of *O. javanicus* and spawning of eggs by adult females.

Mercury Analysis

Homogenised amount of ± 0.001g dry sample was placed onto a sample boat. The sample was implanted into the instrument using an appropriate technique. The sample was covered with calcium hydroxide. To minimize the effect of interruptive materials, activated alumina and calcium hydroxide were used as described in the instruction manual of Mercury/MA-1s (Nippon Instrument Corp). The precision of the analysis was checked by performing the recovery test using the mercury standard solution (Wako Pure Chem, Japan) before every measurement. Heat vaporization method was conducted on the dried samples using a Mercury Analyzer Model MA-1s. The recovery for total mercury by spiking was measured between 98-102 %. Good recoveries of spiked samples demonstrate the accuracy of the methods used (Ertas & Tezel, 2004). The samples were then analyzed for total mercury concentration.

Histological Analysis

Physical method was chosen for euthanizing the fish to provide an adequate fixation for the samples. Theywere immersed in 2-4 °C (36-39 °F) of water. The fish was humanely killed for 10-20 seconds and fixed for 24 hours in modified Davidson fixation (Johnson, Wolf, & Braunbeck, 2009). The samples were then transferred into 10% of buffered formalin and put through the dehydration steps with a series of alcohol. The samples were embedded in paraffin wax and undergone haematoxylin and eosin (H&E) staining after being sectioned for an interest cut of 8 µm. The slides were observed under the Leica DM3000 LED microscope and images captured using the Leica MC170 HD camera. This process was used to identify any abnormalities and deformations that

occurred in the male and female gonad of *O. javanicus*.

Data Analysis

The results of mercury accumulations and gonadal staging were expressed as means ± SE. Statistical differences (P<0.05) between the groups were determined by one way analysis of variance using StatPlus 2009 Professional 5.8.4. The comparison between the lesions of ovary performed by the nonparametric Kruskal–Wallis ANOVA. Statistical analyses were performed with the StatPlus 2009 Professional 5.8.4.

Results and Discussion

After 14 days of exposure, the mercury concentrations of *O. javanicus* were recorded. Preliminary exposure of *O. javanicus* in 90 ppb of mercury resulted in 100% mortality within 14 days (Figure 1). Meanwhile, the median lethal concentration (LC50) of *O. javanicus* with the exposure of mercury within 14 days was 40 ppb. Therefore, sublethal concentrations 0, 10, 20, 30 and 40 ppb were chosen to be used for the definitive testing. Figure 2 shows the accumulation of total mercury (mg/kg) in the whole body of *O. javanicus* when exposed to various mercury concentrations (0, 10, 20, 30 and 40 ppb) for 14 days, which from the range 0.72 mg/kg to 3.17 mg/kg. There were significant differences (P<0.05) observed in mercury accumulation in the whole fish with different concentrations of exposure. Bioaccumulation is the process where excess chemical concentration are present in the water, thus leading to the chemical uptake by aquatic organisms through all chemical exposure routes (dietary absorption, transportation across the respiratory surface, dermal absorption, and inhalation) (Gobas, 2001). Even though the data in this study were expressed as total mercury, it was estimated that 75-95% of total mercury accumulated in fish was in the form of methylmercury (MeHg) (Gochfeld, 2003). The highest level of mercury (3.17

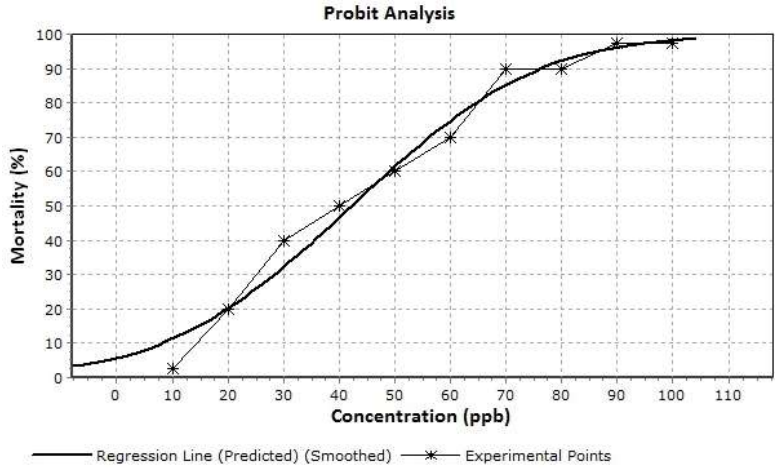

Figure 1. The mortality rate of *O. javanicus* at different concentrations of mercury in preliminary test (LC50 of 14days).

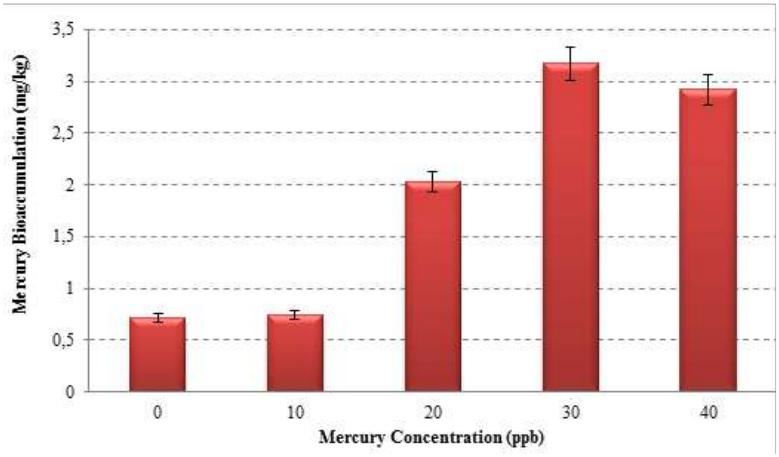

Figure 2. Readings of total average of mercury bioaccumulation (mg/kg) in *O. javanicus* after 14 days of exposure to different mercury concentrations (ppb).Statistically significant differences in Mercury (Hg) content in fish groups exposed to this metal were found between the control group and groups exposed to different concentrations of mercury (P<0.05).

mg/kg) was detected in the 30 ppb group exposure. The accumulation increased with the increase in concentration of mercury exposure. However, the accumulation of mercury was decreased at 40 ppb (2.92 mg/kg) on the trend of accumulation and showed a more similar reading to the 30 ppb exposed group. The decreased rate of accumulation might be due to cellular damage that counteracted the toxicity (Paulose, 1987). To demonstrate the toxic effects of mercury concentrations, histological observations were performed on the *O. javanicus* gonad. In this study, changes in the gonads were examined over the distinct exposure concentration. Johnson *et al*. (2009) introduced a consolidated set of diagnosed criteria, which are divided into two categories, known as primary and secondary criteria. Johnson *et al*. (2009) found that the atretic oocyte was considered to be primary interest to act as an indicator for identifying the reproductive performance. The deformation is the increment towards degradation and resorption of oocytes at any developmental point, which is characterised by the clumping of chorion, fragmentation of nucleus and disorientation of the ooplasm.

The gonadal reproductive stage determination (Table 1) of the testes and ovaries of fish from exposed group indicated developing and mature (final stages of gametogenesis predominant) gonads. The developmental stage of the exposure gonads was classified as mature except for testis in 10 and 20 ppb that showearly spermatogenic. This current study has demonstrated the estrogenic effect of mercury may influence the fertility of male fish. According to Kirubagaran and Joy (1992), increased of mercury concentrations in the freshwater teleost had inhibited testicular activity as shown by the arrest of streoidogenesis, degenerative changes in the Leydig cells and significant reduction in the GSI. This was confirmed by the histological assessment. The main histopathological findings in female ovaries from this study was retraction of the cytoplasm from the follicular cells, karyoplasmic clumping, which generate a space between cytoplasm and karyoplasms and follicular atresia where follicles had changes within their supportive cells (e.g., break down of yolk granules) characterized by broken zonaradiata and proliferation of follicular cells (Table 1) (Louiz, Ben-Attia, & Ben-Hassine, 2009). In fact, ovaries from control and 30 ppb concentrations showed a significantly different percentage of follicular atresia, 4.25% and 25.15% ($P<0.05$) respectively, compared with the females from the other concentration (Table 1).

The normal ovarian architecture in adult *O. javanicus* as shown Figure 3 (A) demonstrated the complete four general stages of small fish ovaries, which are perinuclear oocytes, cortical alveolar oocytes, vitellogenic oocytes and mature oocytes. Besides, it did not show any obvious abnormalities especially in the physical changes of oocytes compared to the exposed fish. Primary and secondary criteria such as the increase of oocytes atresia, decreased vitellogenesis, changes in gonadal staging, increased fibrosis and decreased post-ovulatory are clearly shown in ovaries of *O. javanicus* which had been exposed to different concentrations of mercury. Figures 3 (B) and 3 (C) present the exposure results of *O. javanicus* in 30 and 40 ppb mercury and clearly depicted increased oocytes atresia and decreased of vitellogenesis.

Besides that, these exposed groups also showed other additional criteria of severity grading such as the folding of retraction of cytoplasm in the 30 ppb exposed group. This indicated a compound-associated, presumably degenerative (atretic) process characterised by abrupt, usually multiple invigations of the chorion. There are two secondary criteria ovary severe degradation diagnosed in the 40 ppb exposed fish as shown in Figure 3 (C), which are disrupted cortical alveoli and interstitial fibrosis.

Untreated testicular tissues of *O. javanicus* presented a characteristic organisation with many spermatogenic cysts surrounded by interstitial tissues as shown in Figure 4 (A). From observation in Figure 4, evaluation in male gonad of *O. javanicus* was characterized as the spawning class which compacted

Table 1. Gonadal staging in Oryzias javanicus after exposure of Hg and lesions in ovary

Treatment (µg/L)	Males		Female				
	n	Median Stage[1]	n	Median Stage[2]	Lesions (%)		
					Atretic oocyte	Cytoplasmic retraction	Karyoplasmic Clumping
Control	8	4*	8	2	4.25*	0.25	6.67
10	8	1*	8	3*	20	22.54	19.64
20	8	1*	8	3	11.8	6.07	10.20
30	8	3	8	3	25.15*	22.83	17.93
40	8	2	8	2*	17.5	11.96	16.16

N: sample size; Asterisk denotes significant differences between concentration of Hg (*P< 0.05). %: percentage of altered oocytes per section.

[1]The guideline recommends the following gonadal staging scale for male *O. javanicus*: 0=undeveloped, 1=early spermatogenic, 2=mid-spermatogenic, 3=late spermatogenic, 4=spent.

[2]The guideline recommends the following gonadal staging scale for female *O. javanicus*: 0=undeveloped, 1=early development, 2=mid-development, 3=late development, 4=late development/hydrated, 5=post-ovulatory.

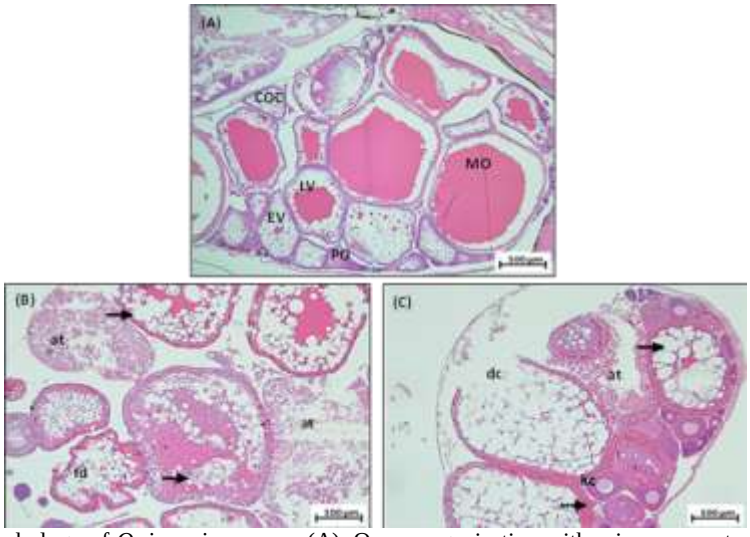

Figure 3. Normal morphology of *O. javanicus* ovary (A). Ovary organisation with primary oocytes (PO), cortical oocyte, (COC) early vitellogenic oocytes (EV), late vitellogenic oocytes (LV), and mature oocytes (MO). Hence, (B) and (C) are the Hg exposed fishes for 30 ppb and 40 ppb. After Hg treatment, the fishes showed destruction evident in oocyte arrangement such as atretic oocytes (at), cytoplasmic retraction (fd), disrupted cortical alveoli (dc) and karyoplasmic clumping (kc). Complete arrow showed the minimal decreased yolk formation. Besides that, in exposed ovary, the number of oocytes was reduced at every stage. Meanwhile, the dashed arrow showed the interstitial fibrosis in the ovary of exposed fishes.

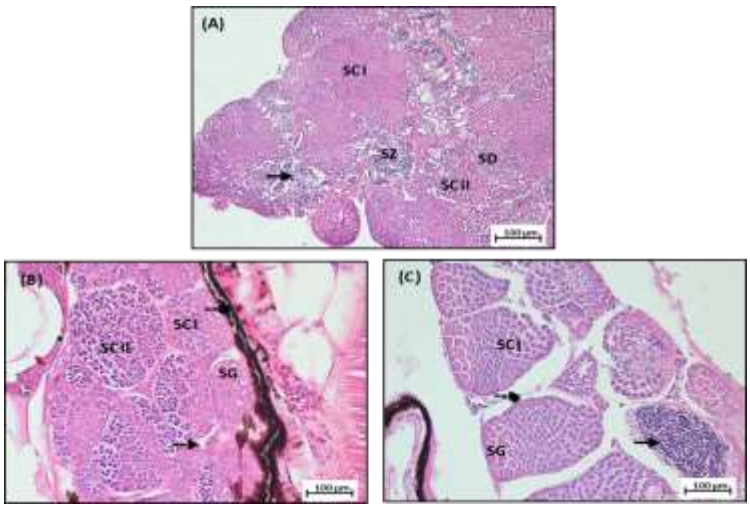

Figure 4. Normal morphology of *O. javanicus* testis (A) showed testicular organisation with seminiferous tubules and interstitial tissues. Seminiferous lobes containing germ cells: spermatogonia (SG), primayspermatocysts (SCI), secondary spermatocyts (SCII), spermatids (SD), spermatozoa (SZ). Exposed fishes (B) for 10 ppb and (C) 20 ppb Hg showed the disorganisation of testicular arrangement (incomplete stages). Complete arrow in figure (A) and (B) indicate a spent or unspent spermatozoa, normal testis in (A) displayed a spent spermatozoa. However, (B) showed packed and unspent spermatozoa. The dashed arrow in figures (B) and (C) indicated the reduction of germ cells interstitial and lobular disintegration in testis of exposed *O. javanicus*.

seminiferous tubules with spermatids and spermatozoids. These tubules usually appear engorged and huge (Amal, Ismail, Nasarruddin, & Rahman, 2015). In contrast, a spent gonad was flaccid which most of the spermatids had been releases resulting semi empty seminiferous tubules with noticeable spermatogenesis cells like in control treatment (Figure 4 (A)). Mercury caused changes in the treated fishes at all the concentrations administrated and the effects became more severe with the increase of dose. Mercury treatment induced complete disorganisation in cyst arrangement and showed a decrease in the progressed stages of spermatogenesisand caused a reduction of spermatozoa. Spermatozoa reduction will cause the negative effect on reproductive performance in term of fertilization success (Hinton & Lauren, 1990). It is clear that the response towards sublethal level of mercury would lead to sperm reduction displayed in Figures 4 (B) and (C).

Besides, testis exposed to mercury also showed the increase of an early stage spermatogonia. Severe damages were observed in 20 ppb (Figure 4 (B)) as the interstitial germ cells, lobular disintegration, and sperm aggregation were reduced (Vergilio, Carvalho, & Melo, 2012). The testis suffered progressive morphological alterations, which reflected the elevated mercury concentrations. This result is crucial as such damages could induce male reproductive dysfunction and impairment of both gonadal development and growth, thereby affecting fertilisation success and offspring survival (Crump & Truden, 2009; Friedmann, Watzin, & Brink-Johsen, 1996). Histological sections revealed degeneration of the germ cells in the testicular lobules, reduced spermatogenic activity, rupture of the testicular lobules and the appearance of inter-follicular spaces due to the shrinkage of the oocytes.

Conclusion

Histopathological analysisshowed different morphological alterations in types and severity of lesions in *O. javanicus* gonads afterexposed to mercury. Consequently,it helps to characterize the mechanism of mercury induced pathogenesis. Besides, we have revealed herein that the *O. javanicus* could be considered as a good bioindicator for ecotoxicology purpose.

Acknowledgement

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