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Comparison of Neurotoxic Effects of Ethanol and Endosulfan on Biochemical Changes of Brain Tissues in Javanese Medaka (*Oryzias javanicus*) and Zebrafish (*Danio rerio*)

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ABSTRACT

An ideal model organism for neurotoxicology research should meet several characteristics, such as low cost and amenable for high throughput testing. Javanese medaka (JM) has been widely used in the ecotoxicological studies related to the marine and freshwater environment, but rarely utilized for biomedical research. Therefore, in this study, the applicability of using JM in the neurotoxicology research was assessed using biochemical comparison with an established model organism, the zebrafish. Identification of biochemical changes due to the neurotoxic effects of ethanol and endosulfan was assessed using Fourier Transform Infrared (FTIR) analysis. Treatment with ethanol affected the level of lipids, proteins, glycogens and nucleic acids in the brain of JM. Meanwhile, treatment with endosulfan showed alteration in the level of lipids and nucleic acids. For the zebrafish, exposure to ethanol affected the level of protein, fatty acid and amino acid, and exposure to endosulfan induced alteration in the fatty acids, amino acids,

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nucleic acids and protein in the brain of zebrafish. The sensitive response of the JM toward chemicals exposure proved that it was a valuable model for neurotoxicology research. More studies need to be conducted to further develop JM as an ideal model organism for neurotoxicology research.

Keywords: Biochemical changes, Fourier Transform Infrared, Javanese medaka, model organism, zebrafish

INTRODUCTION

Animal models play a fundamental role in drug discovery, biomedical, and ecotoxicology researches. The main goal of developing animal models is to understand biological phenomena in humans or a species other than the one investigated, depending on the questions asked by the scientist (Andersen & Winter, 2017). Traditionally, mammals have been used as model organisms in neurotoxicology research. However, since the use of these model organisms is time consuming, laborious, and expensive, they are not suitable for high throughput screening in which thousands of chemicals are tested in a short period of time. Therefore, using fish as a model organism is becoming increasingly popular among toxicologist as an alternative model organism, since they are relatively simple, easy to culture and can be maintained in the laboratory continuously (Schartl, 2014). Therefore, they offer a distinct cost benefit as compared to rodents, especially when dealing with high throughput testing. Fish have been used as models for various research disciplines such as engineering (Romano et al., 2017), environmental research (Cossins & Crawford, 2005), genetic research (Gerlai et al., 2000), toxicology (Peterson et al., 2015), pharmacology (Maximino et al., 2011) and diseases (Amal et al., 2019a; Amal et al., 2019b).

Zebrafish (*Danio rerio*) originated from South and Southeast Asia, Northeastern India, Bangladesh, and Myanmar. They are gaining popularity in neurotoxicology research as they have been proven to share approximately 70% of the human orthologous genes that are highly conserved and similarly regulated in humans (Howe et al., 2013). Another small fish that are resilient, with mapped and malleable genomes, namely Japanese medaka (*Oryzias latipes*) are used by a small, but gradually growing community of researchers for various types of research (Wittbrodt et al., 2002). Japanese medaka originated from Asian countries such as Japan, Korea and China, whereas, a related species namely Javanese medaka (*Oryzias javanicus*) has been highlighted as a new experimental model for environmental research. This species is hardy and highly adaptable to a high range of salinity (Inoue & Takei, 2002), and are found abundant in Peninsular Malaysia, Singapore, Indonesia, Thailand, and Western Borneo (Yusof et al., 2012). Recently, Javanese medaka has been utilized as model organism to understand ecotoxicity effects of environmental pollutants in marine and freshwater environment (Ismail & Yusof, 2011; Yusof et al., 2014; Aziz et al., 2017). Also, this fish has been utilized for bacterial diseases study (Amal et al., 2018). However, their potential as an animal model in neurotoxicology research remains to be explored.

When fish model organisms absorb a toxicant, biochemical and physiological responses may occur due to the toxicity mechanism (Begum, 2004). Several scientific studies shown that zebrafish exposed to alcohol demonstrated consistent neurotoxic effects with the mammal model organism and also with human (Joya et al., 2014). In addition, zebrafish also showed neurotoxicity effects after exposure to endosulfan (Silva et al., 2015). However, the study of both neurotoxicants on Javanese medaka is still limited and not extensively done compared to zebrafish. Biochemical or genetic accidents caused by toxic insults may provoke neurodegenerative disease and neurodevelopmental abnormalities (Yuan & Yanker, 2000). Thereby, identification of biochemical changes due to the neurotoxic effects of chemicals in the biological sample is a valuable approach in determining the toxic effects of chemicals. FTIR spectroscopy provides qualitative biochemical information for the assessment of structural and functional changes of macromolecules in biological samples (Cakmak et al., 2006). The changes in peak positions and bandwidths exhibited alterations in the structural and functional groups caused by the toxicants. In addition, FTIR spectroscopy is an efficient and reliable tool that utilizing infrared (IR) absorption spectra to enable the assessment of biochemical fingerprint from a micro-volume sample from complex biological systems (Ami et al., 2014). In the present study, FTIR was used to evaluate biochemical alterations in the brain tissues of Javanese medaka after acute exposure to ethanol and endosulfan, while zebrafish was used as a reference model.

MATERIALS AND METHODS

Animals and Housing

Adult wildtype zebrafish were purchased from the local supplier in Kajang, Malaysia. Javanese medakas were collected from estuary area in Sepang River, Selangor (2.6213° N, 101.7122° E). They were identified by the occurrence of a pair of silvery stripes at the dorsal part of the body and the presence of yellow sub marginal bands on the dorsal and ventral portions of the caudal fin (Yusof et al., 2013). They were acclimatized for 14 days and were kept afterwards in an aquarium (22.3 cm length × 12.2 cm width × 13.5 cm height), with the ratio of 3 females: 2 males.

The fish were maintained in light cycle 14 h light: 10 h dark controlled photoperiod and were fed four times a day with brine shrimp (*Artemia salina*) (San Francisco Bay Brand, San Francisco, CA) and supplemented with commercial dry flake food (Sera Vipan, Germany). The aquarium water was prepared 24 h before used, by dechlorinating it with anti-chlorine (Nutrafin, Hagen, Canada), aerated to increase oxygen concentration in the water, and treated with ultraviolet light. In order to promote good health and stable water quality for the fish, the water were maintained at pH 6.8 - 7.0, and the level of ammonia nitrogen, nitrite and nitrate were at low reading (0-0.25 ppm). The water temperature was maintained at 28°C ± 1°C. The fish tanks were cleaned once a week and the fish

were monitored frequently to ensure that they were free from any sign of diseases and healthy enough for further experiments. All procedures were conducted according to the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (IACUC/AUP-R024/2014).

Neurotoxicants Exposure to Adult Javanese Medaka and Zebrafish

In each treatment group (exposed to ethanol and endosulfan) and control, 15 adults of each species were used (n = 45 for each species of fish). The selected fishes were almost same size (3 - 4 cm), weight (0.6 g - 0.8 g) and only the healthy fish with no morphological abnormalities were selected for experiment. Ethanol (1%) was freshly prepared from 95% ethanol by diluting it with aquarium water. Fishes were individually exposed to 1% ethanol in a 500 mL glass beaker containing 250 mL of 1% ethanol solution for 1 h prior to behavioural assessment. A 1 h ethanol exposure was chosen based on several previous studies on zebrafish (Kurta & Palestis, 2010; Tran et al., 2015; Tran et al., 2016). Endosulfan (analytical standard α and β isomers, Pestanal®, Sigma-Aldrich Laboratories, Seelze, Germany) was used in this study. Both fishes were exposed to 1.6 µg/L endosulfan (Jonsson and Toledo, 1993) for 96 h according to OECD 203 (OECD, 2013). All treatment groups containing five fishes and were exposed in 3 L aquarium tank in a semi-static exposure where the exposure solution was renewed daily due to the short half-life of endosulfan, approximately 24 hours (Jonsson & Toledo, 1993). Control group of 15 fishes received treated aquarium water in the same route of administration with the same volume as the treatment groups, respectively.

Fourier Transform Infrared (FTIR) Analysis

At the end of the exposure, the fish were euthanized with ice for 10 min. Then, the brains were dissected, washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) overnight at 4°C. Then, the brain were dried in a lyophilizer (VTIRTIS 6KBEL85) for 12 h at 50°C to remove water from the samples. The samples were then ground in an agate mortar and pestle to obtain brain powders. The brain powders were mixed with dried potassium bromide (100 mg) and subjected to a pressure of 5 tons for 5 min in an evacuated disc to produce a clear transparent KBr disc of 13 mm diameter and 1 mm thickness for use in the FTIR spectrometer (Palaniappan & Pramod, 2011). The measurements of the freeze dried samples were performed on a Thermo Nicolet Nexus, Smart Orbit spectrometer using the KBr disc method. The spectra were recorded over the themed infrared region of 500 - 4000 cm^{-1}. For each treatment group, the brains were harvested from 3 to 5 fish.

RESULTS AND DISCUSSION

Changes in the biochemical profile of the brain in both species were assessed using Fourier Transform Infrared (FTIR) spectroscopy. The range frequency for all functional groups and their peak assignment for the FTIR are presented in Table 1. The FTIR spectra in the 4000 - 500 cm^{-1} range were presented for both fishes. Comparison of FTIR spectra after exposure to ethanol or endosulfan in Javanese medaka is shown in Figure 1, while that of zebrafish is in Figure 2. The peak assignments are presented in Table 2, where the peak in the spectra corresponds to the functional groups of proteins, lipids, carbohydrates and nucleic acids (Senthamilselvan et al., 2012; Baker et al., 2014). Results showed that the intensities of the control *versus* exposed brain tissues for both fish species were different according to different neurotoxicant.

For Javanese medaka, treatment with either ethanol or endosulfan induced appearance of a new peak at 2937 cm^{-1} for ethanol and 2938 cm^{-1} for endosulfan, and these peaks were not observed in the control. These peaks represent the C–H stretch from alkanes of lipids. As for the zebrafish, the lipid molecules were detected at the peak 2939 cm^{-1} for the control, while treatment with either ethanol or endosulfan resulted in a reduction of these

Table 1
General band assignments of the FTIR spectra

Frequency (cm^{-1})	Functional group and peak assignment	Components
3700 -3200	Alcohol (O-H stretch)	Alcohol
2975 - 2850	Alkanes (C-H stretch)	Lipid
1730 -1720	Aldehydes (C=O stretch)	Lipid
1730 - 1690	Amide (C=O stretch)	Protein
1640 -1630	Alkene (C=C stretch)	Protein
1538 - 1529	Amide (N–H bending)	Protein
1452 – 1437	Alkanes (C-H stretch)	Lipid
1385 -1364	Alkanes ($C-H_3$)	Fatty acids, amino acids, lipid
1222 - 1203	Amines (C-N stretch) Alkyl halides (C-N stretch)	Glycogen
1131 - 1123	Alkyl halides (C-F stretch) Ethers (C-O stretch) Amines (C-N stretch)	Glycogen
1058 - 1051	Alkyl halides (C-F stretch) Alcohol (C-O stretch) Amines (C-N stretch)	Glycogen
1059 - 1026	Alkyl halides (C-F stretch)	Nucleic acid
968 - 953	Alkenes (C-H stretch)	Nucleic acid
888 - 784	Alkyl halides (C-Cl stretch) Aromatics (C-H stretch)	unknown

Note. The range frequency for all functional groups and their peak assignment for the FTIR spectra

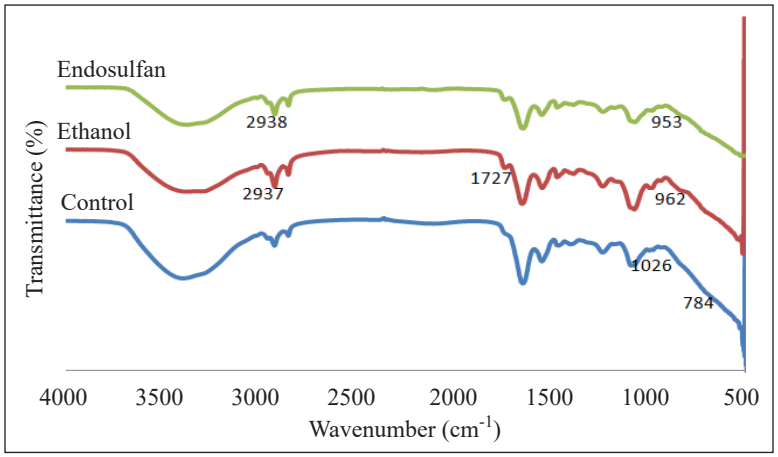

Figure 1. FTIR spectra of the brain tissue in Javanese medaka in the region 4000 - 500 cm^{-1}

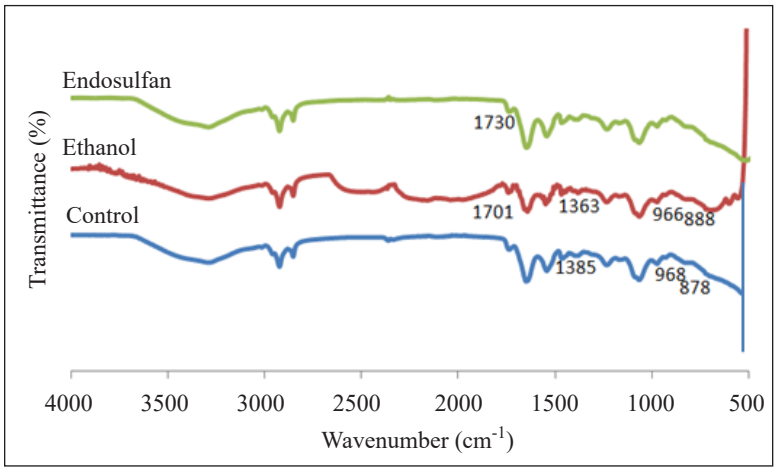

Figure 2. FTIR spectra of the brain tissue in zebrafish in the region 4000 - 500 cm^{-1}

peaks. Thereafter, a new peak appeared at 1727 cm^{-1} in the Javanese medaka brain after treatment with ethanol, but not in the endosulfan and control group. This peak is associated to the stretching of the C=O group in aldehydes of lipids. This peak was not observed in the zebrafish in any treatment group. In the Javanese medaka, another peak of the lipid component appeared at 1437 cm^{-1} in control, ethanol and endosulfan groups. However, for the zebrafish this peak was observed at higher frequency, 1442 cm^{-1}. Treatment with ethanol has exhibited an increment to approximately 1452 cm^{-1} and treatment with endosulfan exhibited decrement approximately to 1437 cm^{-1} in the zebrafish. The appearance of the new peaks, disappearance of peaks, increment and decrement of the peaks in the Javanese medaka and zebrafish indicated disruption of the lipid molecules after treatment with ethanol and endosulfan.

Table 2
The band area of brain tissue in Javanese medaka and zebrafish exposed to ethanol and endosulfan

Javanese medaka frequency (cm^{-1})			Zebrafish frequency (cm^{-1})			Functional groups and peak assignment	Components
Control	Ethanol	Endosulfan	Control	Ethanol	Endosulfan		
3307	3216	3302	3371	3341	3344	Alcohol (O-H stretch)	Alcohol
Not observed	2937	2938	2939	2937	2927	Alkanes (C-H stretch)	Lipid
2917	2917	2918	2919	2917	2921	Alkanes (C-H stretch)	Lipid
2847	2847	2848	2847	2845	2849	Alkanes (C-H stretch)	Lipid
Not observed	1727	Not observed	Not observed	Not observed	Not observed	Aldehydes (C=O stretch)	Lipid
1694	Not observed	1701	Not observed	1701	1730	Amide (C=O stretch)	Protein
1635	1631	1632	1635	1639	1637	Alkene (C=C stretch)	Protein
1528	1529	1531	1538	1532	1528	Amide (N–H bending)	Protein
1437	1437	1437	1442	1452	1437	Alkanes (C-H stretch)	Lipid
1363	1363	1363	1385	1363	Not observed	Alkanes ($C-H_3$)	Fatty acid, amino acid, lipid
1214	1209	1208	1212	1222	1203	Amines (C-N stretch) Alkyl halides (C-N stretch)	Glycogen
1123	Not observed	1131	Not observed	Not observed	Not observed	Alkyl halides (C-F stretch) Ethers (C-O stretch) Amines (C-N stretch)	Glycogen
1051	1051	1058	Not observed	Not observed	Not observed	Alkyl halides (C-F stretch) Alcohol (C-O stretch) Amines (C-N stretch)	Glycogen
1026	Not observed	Not observed	1059	1056	1037	Alkyl halides (C-F stretch)	Nucleic acid
Not observed	962	953	968	966	Not observed	Alkenes (C-H stretch)	Nucleic acid
784	Not observed	Not observed	878	888	Not observed	Alkyl halides (C-Cl stretch) Aromatics (C-H stretch)	unknown

Note. The FTIR spectra in the 4000 - 500 cm^{-1} range were presented for both fishes after exposure to ethanol or endosulfan where the peak in the spectra corresponds to the functional groups of proteins, lipids, carbohydrates and nucleic acids.

The band observed at 1694 cm^{-1} in the control Javanese medaka corresponds to C=O stretching of amide functional groups in proteins. Treatment with ethanol in Javanese medaka has caused a disappearance of this peak. Exposure to endosulfan (1701 cm^{-1}) had exhibited increment in the treated Javanese medaka. In the normal zebrafish, there was no peak occurrence at this band. However, these peaks could be observed when treated with ethanol (1701 cm^{-1}) and endosulfan (1730 cm^{-1}). From this, the aspect to be taken is that any alteration at these peaks showed that treatment with ethanol and endosulfan induced disruption in the protein molecules for both fishes.

Furthermore, functional group of alkyl halides, ethers, amines and alcohol for glycogen due to the stretching of C-F, C-O and C-N was only detected in the control of Javanese medaka from 1051 cm^{-1} to 1123 cm^{-1}, and not available in the zebrafish for all groups. Treatment with ethanol causes these peaks to disappear. Treatment with endosulfan (1131 cm^{-1}) caused an increment of these peaks in the Javanese medaka, as compared to the control. The disappearance of this peak as a result of ethanol exposure and endosulfan exposure caused a large shifted peak. This showed that both toxicants severely disrupted glycogen in the Javanese medaka.

Additionally, the peak observed at 1026 cm^{-1} in the control Javanese medaka and 1059 cm^{-1} in the control zebrafish corresponds to C-F stretching of alkyl halides in nucleic acids. In Javanese medaka, treatment with ethanol or endosulfan caused this peak to disappear. For zebrafish, treatment with ethanol (1056 cm^{-1}) and endosulfan (1037 cm^{-1}) also exhibited a reduction as compared to the control (1059 cm^{-1}). Whereas, treatment with ethanol (962 cm^{-1}) or endosulfan (953 cm^{-1}), showed a new appearance of these peaks in the Javanese medaka which also corresponded to nucleic acid. In zebrafish, this peak disappeared after treatment with endosulfan.

This study revealed that untreated Javanese medaka had different macromolecules composition where they had lower lipids and nucleic acid in comparison to zebrafish. In addition, Javanese medaka also had higher protein and glycogen component in their brain as compared to the zebrafish. The dissimilarity may be due to the differences in rearing conditions in which that zebrafish may be undergone domestication, while Javanese medaka is from the wild habitat. Although the Javanese medaka used in this study was already being acclimatized in the laboratory settings condition similarly with the zebrafish for two months, this length of period may be not long enough for the Javanese medaka. Domestication is a process of adaptation to a captive environment (Price, 1999). The process of adaptation for wild population of animals to preadaptation for domestication may be differed among species depending on the ability of species members to adapt through developmental and evolutionary processes to a variety of husbandry conditions and the species able to exhibit the behavioural patterns compatible with husbandry techniques (Price, 1999). Furthermore, a distinct biochemical comparison observed in Javanese medaka could be explained by

obvious morphological characteristics in both fishes. Javanese medaka has transparent body, while zebrafish has black striped body. In agreement, a previous study showed that seven different bivalve species which had different morphological characteristics also showed divergent biochemical composition (Bouhlel et al., 2017). Moreover, the variance of biochemical composition could be determined by the natural habitat of the fishes. Javanese medaka is a fish that originated from marine or brackish water, while zebrafish from freshwater.

In our laboratory, zebrafish has been constantly fed with artemia. Meanwhile, Javanese medakas were collected from the wild and were fed with brine shrimps for only two months during laboratory acclimatization. Owing to their differences in the composition of feeding materials between wild Javanese medaka and domestic zebrafish (Tasbozan & Gökce, 2017), we found that Javanese medaka had lower lipids component in the brain as compared to the zebrafish brain. In this study, as Javanese medakas were collected from the wild, their dietary intakes were influenced by the particular microenvironment and food availability. While, zebrafish were maintained in the laboratory condition and properly fed with brine shrimps on regular basis. This could explain the differences in their lipids component between the fishes, as lipid composition is dependent on the fatty acid composition of their feed and dietary intakes (Cahu et al., 2004).

The present study evaluated the neurotoxic effect of acute exposure to ethanol and endosulfan on the biochemical contents in the brain tissues of Javanese medaka and zebrafish. We found that ethanol and endosulfan exposure changed transmission intensity, shifted peak positions, and caused disappearing or addition of new peaks in FTIR wavelength. This prove that ethanol and endosulfan impaired biochemical structures of proteins, lipids and nucleic acids in the brain. The destructive effects of ethanol and endosulfan on the brain are more prominent in the Javanese medaka, in comparison to the zebrafish. This is due to more macromolecules were affected in the Javanese medaka as compared to the zebrafish. The alteration of the proteins, lipids and nucleic acids structure in the brain will lead to neurotoxicity or neurobehavioural deficits (Zahir et al., 2006).

Exposure to ethanol has been proven to induce oxidative stress, alteration in lipid components and dysfunctional membranes which lead to neurotoxicity and neurodegeneration (Hernández et al., 2016). Previous study also showed that exposure to alcohol altered protein expression and generated more reactive oxygen species (ROS) in the Purkinje's cells of the brain (Oyinbo et al., 2016). Jang et al. (2016) discovered that Sprague-Dawley rats which were exposed to endosulfan demonstrated elevation of ROS and oxidative damages leading to the reduction in glutathione, lipid peroxidation and protein carbonylation. Additionally, the brain contains high lipid content with sufficient macromolecules, a prerequisite for proper central nervous system function (Carlson, 2009). The integrity of the cell membrane is highly dependable on the sufficiency and balance

amount between the lipids and proteins molecules. Any disruption of the macromolecules in the brain will affect the proper biological functions and mechanisms, which subsequently contribute to the induction of adverse toxicity effects in the fishes.

Zebrafish has been commonly used as a model organism for alcohol researches (Sylvain et al., 2010; Joya et al., 2014; Tran et al., 2016). However, in this study, we found that Javanese medaka had higher sensitivity towards ethanol exposure, as compared to the zebrafish. This finding leading to a suggestion that Javanese medaka is more suitable for alcohol research and also can be a valuable model organism for neurotoxicology research. Important to note, biochemical endpoints evaluation by using FTIR alone is not sufficient to draw a concrete conclusion about the suitability of Javanese medaka as model organism for neurotoxicology research. Thus, utilization of Javanese medaka for neurotoxicology research requires concerted effort by scientists from various research fields to generate the fundamental knowledge about their genetic variations, biology and physiology. Based on the history, development of model organism for research took decades of continuous efforts by the research community. Therefore, more studies need to be conducted to further develop Javanese medaka as an ideal model organism for biomedical research. These data can be referred as a fundamental knowledge for the adverse neurotoxic effects of neurotoxicants for both fishes.

CONCLUSION

As a conclusion, we found that Javanese medaka is a valuable aquatic model organism for neurotoxicology research, as this fish is sensitive to toxicant exposure. To fully utilize this fish as a model organism for neurotoxicology research, it has to be further developed by using different sophisticated platforms such as their genome has to be fully sequenced to allow further studies for genetic modifications.

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