CHRONIC EMBRYONIC ARSENIC AND MERCURY EXPOSURE-INDUCED DEVELOPMENTAL NEUROTOXICITY, NEUROBEHAVIORAL DYSFUNCTION AND METABOLIC ALTERATION IN ZEBRAFISH (*Danio rerio* F.Hamilton, 1822)

By

NORAINI BINTI ABU BAKAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

CHRONIC EMBRYONIC ARSENIC AND MERCURY EXPOSURE-INDUCED DEVELOPMENTAL NEUROTOXICITY, NEUROBEHAVIORAL DYSFUNCTION AND METABOLIC ALTERATION IN ZEBRAFISH (*Danio rerio* F.Hamilton, 1822)

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Arsenic and mercury are ubiquitous in the environment. Exposure to these toxicants at low concentrations is unremarkable in developing organism. Nevertheless, their long-term adverse effects are underestimated and their association with cognitive deficits, one of the major criteria for neurodevelopmental disorders (NDD) remains a challenge to understand. This study investigated the long-term effects of embryonic exposure to arsenic and mercury at low environmentally relevant concentrations on developmental neurotoxicity, neurobehavioral dysfunction, and metabolic alteration in the developing zebrafish. Chronic embryonic exposure to arsenic trioxide (As_2O_3) or mercury chloride ($HgCl_2$) from gastrulation to hatching resulted in developmental neurotoxicity, neurobehavioral dysfunction, and metabolic alteration in the treated larvae. Embryonic exposure to As_2O_3 or $HgCl_2$ induced hypo-/hyperlocomotion, affecting common metabolic pathways, particularly arachidonic and linoleic acid metabolism. Downregulation of docosahexaenoic acid (DHA) metabolites results in reduction of eyes, lens, and retinas diameter, and impaired microridges for both As_2O_3 and $HgCl_2$ exposure. Long-term impairment of learning by As_2O_3 exposure was evident in a lack of directional and color preference responses in adult zebrafish correspond with depletion of both arachidonic acid and DHA metabolites. Lower survivability in $HgCl_2$ exposed larvae compared to As_2O_3 could be related to the inflammatory response and abnormal inflation of swim bladder. Transcriptional evaluation of *adsl, shank3a, tsc1b* and *nrxn* revealed association between embryonic exposure to As_2O_3 and $HgCl_2$ with autism spectrum disorder (ASD). In this study, zebrafish was successfully developed as an animal model for evaluation of the long-term neurotoxicity effects of toxicants by integrating toxicity, behavior, metabolomics, and gene expression to understand the underlying behavioural disorders associated with NDDs. Thus, highlighting the risk of

exposure to low concentrations of As/Hg for long-term duration. Considering the ubiquity of As_2O_3 and $HgCl_2$ in the environment, this study proves that arsenic and mercury as one of the risk factors for NDDs, especially ASD in the human population

Keywords: zebrafish; mercury; arsenic; behavior; metabolomics

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

DEDAHAN KRONIK EMBRIONIK ARSENIK DAN MERKURI MENYEBABKAN PERKEMBANGAN KENEUROTOKSIKAN, DISFUNGSI TINGKAHLAKU-NEURO DAN PERUBAHAN METABOLIT TERHADAP ZEBRAFISH (*Danio rerio* F.Hamilton, 1822)

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Arsenik dan merkuri terdapat di mana-mana di alam sekitar. Pendedahan kepada bahan toksik ini pada kepekatan rendah adalah tidak ketara dalam perkembangan organisma. Walau bagaimanapun, kesan buruk jangka panjang mereka dipandang remeh dan kaitannya dengan defisit kognitif, salah satu kriteria utama untuk gangguan perkembangan saraf (NDD) kekal sebagai cabaran untuk difahami. Kajian ini menyiasat kesan jangka panjang pendedahan embrio kepada arsenik dan merkuri terhadap perkembangan keneurotoksikan, disfungsi tingkahlaku-neuro dan perubahan metabolit yang dikaitkan dengan NDD dalam ikan zebra yang sedang berkembang pada kepekatan yang rendah dan berkaitan dengan alam sekitar. Pendedahan embrionik kepada arsenik trioksida (As_2O_3) atau merkuri klorida ($HgCl_2$) secara kronik daripada gastrulasi hingga penetasan mengakibatkan perkembangan keneurotoksikan, disfungsi tingkahlaku-neuro dan perubahan metabolit dalam larva ikan yang dirawat. Pendedahan embrio kepada As_2O_3 atau $HgCl_2$ menyebabkan hypo-/hyperlocomotion, menjejaskan laluan metabolik sepunya, terutamanya metabolisme asid arakidonik dan linoleik. Penurunan metabolit asid docosahexaenoic (DHA) mengakibatkan penyusutan diameter mata, kanta dan retina, dan mikroridge terjejas bagi pendedahan As_2O_3 dan $HgCl_2$. Kerosakan jangka panjang pembelajaran melalui pendedahan As_2O_3 terbukti dengan kekurangan tindak balas keutamaan arah dan warna dalam ikan zebra dewasa sepadan dengan penyusutan kedua-dua asid arakidonik dan metabolit DHA. Kemandirian yang lebih rendah dalam larva ikan terdedah $HgCl_2$ berbanding As_2O_3 boleh dikaitkan dengan tindak balas keradangan dan pengembungan yang tidak normal pada pundi renang. Penilaian transkrip *adsl, shank3a, tsc1b* dan *nrxn* memperlihatkan hubungkait antara pendedahan embrio kepada As_2O_3 dan $HgCl_2$ dengan gangguan spektrum autisme (ASD). Dalam kajian ini, ikan zebra berjaya dibangunkan sebagai model haiwan untuk penilaian kesan keneurotosikan jangka panjang bahan toksik dengan

menggabungkan ketoksikan, tingkah laku, metabolomik dan ekspresi gen untuk memahami asas gangguan tingkah laku yang berkaitan dengan NDD. Oleh itu, menonjolkan risiko pendedahan kepada kepekatan rendah As_2O_3 dan $HgCl_2$ untuk tempoh jangka panjang. Mengambil kira taburan As_2O_3 dan $HgCl_2$ dalam persekitaran, kajian ini membuktikan bahawa arsenik dan merkuri sebagai salah satu faktor risiko NDD, terutamanya ASD dalam populasi manusia.

Kata kunci: zebrafish; merkuri; arsenik; tingkah-laku; metabolomik

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
As_2O_3	Arsenic trioxide
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism spectrum disorders
˚C	Celsius
CNS	Central Nervous System
dpf	Day post fertilisation
DNT	Developmental Neurotoxicity Testing
Na_2HPO_4	Disodium phosphate
g/cm^3	Gram per cubic centimetre
g/L	Gram per litre
hpf	Hour post fertilisation
kg	Kilogram
$HgCl_2$	Mercury (II) chloride
ID	Intellectual disabilities
$MgSO_4$	Magnesium sulphate
MeHg	Methylmercury
NDDs	Neurodevelopmental disorders
µg	Microgram
µg/L	Microgram per litre
µm	Micrometer
µM	Micromolar
mg/kg	Milligram per kilogram
mg/L	Milligram per litre

mM	Millimolar
min	Minute
KH_2PO_4	Monopotassium phosphate
ng/g	Nanogram per gram
nM	Nanomolar
pM	Picomolar
KBr	Potassium bromide
KCl	Potassium chloride
s	Second
$NaAsO_2$	Sodium arsenite
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium chloride
SEM	Standard Error Mean
t	Time
VPA	Valproic acid

CHAPTER 1

INTRODUCTION

1.1 Research background

Neurodevelopmental disorders (NDD) are heterogeneous disorders with irreversible brain dysfunction that result in deficits in motor function, social behavior, cognition, and communication (Díaz-Caneja et al., 2021; Thapar et al., 2017). NDD typically occur in infancy or childhood include Attention-Deficit/Hyperactivity Disorder (ADHD), Autism Spectrum Disorder (ASD), and Intellectual Disability (ID), among many others (Bowles, 2013; Micai et al., 2020).Epidemiological studies reported an increase in NDD prevalence in children aged 3-7 years from 2009 to 2017 from 16.2% to 17.8% (Saito et al., 2020; Zablotsky et al., 2019) and had impaired cognition (Arora et al., 2018). Besides genetics (Ishizuka et al., 2020; Liu et al., 2021a; Macchiaiolo et al., 2020), exposure to environmental chemicals has been renowned as a risk factor for NDD (Campbell et al., 2021). The magnitude of low level exposures are usually subtle for an individual child and potentially had later-onset (Bellinger, 2012). In Japan, adults who suffered arsenic poisoning in childhood exhibit neuropsychological dysfunctions (Yorifuji et al., 2016). Another study showed that children with high mercury concentrations in their hair ≥ 1 μg/g were 1.58 times more likely to have lower IQ scores (Feng et al., 2020) and performed worse on neuropsychological tasks (Santos-Lima et al., 2020). To date, however, little has been discussed about the possible long-term effects of low-level exposure to arsenic (As) or mercury (Hg) at environmental concentrations on developing human beings. Even less, the association between metals of interest with cognitive deficits as one of the main criteria of NDD is remain elusive. Due to etiology of NDD is multi-factorial, complex and poorly understood, animal models have been used to evaluate the causes and develop new therapies for NDD (Gerlai, 2010; Swaiman et al., 2017).

Although rodents have been conventionally utilized for this purpose, this model has proven to be laborious, high cost for large-scale screening and incompatible to study the long-term effects of chemicals at low concentrations (Sachana et al., 2021; Tsuji & Crofton, 2012). Alternatively, zebrafish are increasingly popular as model species in neurobehavioral research because of their small size, short embryonic duration, good permeability to small molecules, cost-effectiveness, transparent embryo, and larval zebrafish that can be easily characterized anatomically (Legradi et al., 2015; Martin & Plavicki, 2020). With these attributes and established genetic and molecular tractability, the zebrafish is on par with the traditional invertebrate animal models, *Drosophila melanogaster* and *Caenorhabditis elegan*s (Burne et al., 2011). Furthermore, as a vertebrate, zebrafish is a better candidate for modelling phenotypic abnormalities in mammals because it is comparable to more sophisticated animal models such as rodents (Gerlai, 2010). Zebrafish

exhibit a wide range of complex behaviors (motor, social, learning) and thus informative for modelling NDD (Homberg et al., 2016a; Stewart et al., 2014a). Since zebrafish embryo develop externally, their behavioral repertoire can be consistently studied at different life stages, making them ideal for developmental neurotoxicity studies (Kalueff et al., 2013; Zhu et al., 2020).The availability of various behavioral tests (Benvenutti et al., 2021) that can be performed repeatedly over time to determine the progression, duration, and reversibility of neurotoxic injury (Gerlai, 2020; Moser, 2011), makes it a distinctive experimental model.

In this study, zebrafish were used as a model organism to understand the developmental effects of chronic embryonic exposure to 30 µM arsenic trioxide (As_2O_3) and 100 nM mercury chloride ($HgCl_2$). The selected As and Hg concentrations is in line with our previous findings (Abu Bakar et al., 2017a; Abu Bakar et al., 2022). Based on these findings, the selected concentration are the highest non-toxic throughout the exposure period. Moreover, the concentrations of As and Hg were within the range reported in the water supply (Ahmed et al., 2021), edible fish (Ahmad et al., 2015; Hajeb et al., 2010) as well as hair sample of school children (Abdul Samad et al., 2017). To specify the potential underlying mechanisms of locomotion deficit and alteration in anxiety responses induced by these concentrations require studies with sophisticated behavioural and microscopy techniques, metabolomics, and gene expression. An automated behavioural platform and advanced microscopy method provides opportunity to capture minute alteration. Metabolomics and gene approach allows identification of affected metabolites and genes and potential pathways in relation to NDD (Kim et al., 2021)

1.2 Problem statements and objectives

The anticipated burden of NDD in children in Malaysia is considerably high with 3.3% of infants in Malaysia reported to have developmental delays (NHMS, 2016) and 4.7% children born with disabilities (NHMS, 2019). Besides genetics (Liu et al., 2021), exposure to environmental chemicals has been renowned as a risk factor for NDD (Campbell et al., 2021) which usually subtle for an individual child and potentially had later-onset (Bellinger, 2012). A recent global risk assessment predicted that 94-220 million people worldwide, 85-90% of whom live in South Asia, are possibly exposed to high levels of these toxicants in groundwater from their domestic water supply (Podgorski & Berg, 2020), even at low concentrations (Grandjean & Landrigan, 2006). Based on Malaysian Ministry of Environment and Water, and World Health Organization (WHO) permissible limit for As in water is 10 µg/L and Hg is 0.04 µg/L (Ahmed et al., 2021; DOE, 2015). Agricultural activities with extensive use of pesticides for palm oil plantation at Langat Basin, Malaysia considered as the major sources of As contamination in Langat River (0.98-21.94 µg/L). Meanwhile, elevated Hg levels were detected in seawater (0.21-1.49 µg/L) of Kampung Pasir Puteh, Johor, which is surrounded by petrochemical and industrial activities (Mahat et al., 2018). As (0.3-4.4 mg/kg = 0.3-4.4 mg/L) and Hg (0.4-1.7 mg/kg = 0.4-1.7 mg/L) had been reported in edible fishes from Peninsular

Malaysia (Ahmad et al., 2015; Hajeb et al., 2008; Mohamad et al., 2015). Alarmingly, fin mackerel and longtail tuna fish from Peninsular Malaysia had Hg contents (0.055–2.537 mg/kg = 0.055–2.537 mg/L) (Ahmad et al., 2015), and farmed sea bass from Setiu Lagoon with As contents (3.29 ± 0.65 mg/kg = 3.29 ± 0.65 mg/L) exceeded the permissible limits of Malaysian standards and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) guideline (As > 1 mg/kg = 1 mg/L, Hg > 1 mg/kg = 1 mg/L) (Tengku Nur Alia et al., 2020). Due to As and Hg widespread detected in the environment and the high frequency of fish consumption among Malaysian population (168 g/day fish) (Ahmad et al., 2016), raises a potential alarm for long-term toxic effects at low concentrations (Ahmad et al., 2022).

This study examined the long-term effects of chronic embryonic exposure to low concentrations of As (30 µM = 5.935 mg/L) and Hg (100 nM = 0.02715 mg/L), using zebrafish as a model organism to mimic human exposure at environmentally relevant concentrations. To assess the long-term toxic effect of As and Hg at functional level, NDD-like behaviours (motor, anxiety, social, and cognitive responses) measured at different life stages. With the aim to elucidate the underlying mechanism upon chronic embryonic exposure to As_2O_3 or $HgCl_2$ at environmentally relevant low concentration and its association with NDD, untargeted liquid chromatography-mass spectrometry (LCMS)-based metabolomics and gene expression were performed.

The objectives of this project are:

a) To evaluate effects of embryonic exposure to As_2O_3 or $HgCl_2$ on motor, anxiety, social, and learning responses in zebrafish larvae.
b) To elucidate the toxicity effects of As_2O_3 or $HgCl_2$ on development of zebrafish eye, swim bladder, and their survivability into adulthood.
c) To evaluate the effects of embryonic exposure to As_2O_3 or $HgCl_2$ on motor and learning behavior in adult zebrafish.
d) To profile metabolites and gene changes associated with NDD in the zebrafish after embryonic exposure to As_2O_3 or $HgCl_2$ using LCMS and qPCR platforms.

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