Studies on toxicological effects of lead in animals for evaluation of worldwide environmental lead pollution

(環境中の鉛汚染評価のための生物における鉛の毒性影響に関する研究)

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List of abbreviations

AIDS: Acquired immunodeficiency syndrome

ALAD: Delta-aminolaevulinic acid dehydratase

ALP: Alkaline phosphatase

ANOVA: Analysis of variance

ATSDR: Agency for Toxic Substances and Disease Registry

Bcl-2: B-cell lymphoma-2

BLL: Blood Lead Levels: BLLs

BUN: Blood Urea Nitrogen

CAT: Catalase

CoxI: Cytochrome oxidase subunit I

CDC: Centers for Disease Control and Prevention

cDNA: Complementary deoxyribonucleic acid

DDT: Dichloro-diphenyl-trichloroethane

DTT: Dithiothreitol

FET: Fish embryo toxicity testing

G: Relative centrifugal force

g: Gram

GBD: Global burden of Disease

GOT: Glutamic oxaloacetic transaminase

GPT: Glutamic pyruvic transaminase

GPX: Glutathione peroxidase

HO-1: Heme oxygenase-1

IL-8: Interleukin-8

ILA: International Lead Association

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

IQ: Intelligence Quotient

IU: International Unit

kg: Kilogram

km: Kilometer

L: Litter

LDH: Lactase dehydrogenase

LA-ICP-MS: Laser Ablation -Inductively Coupled Plasma Mass Spectrometry

mL: Millilitres

M: Molarity

mRNA: Messenger ribonucleic acid

mM: Micromolar

µg/dL: Microgram per decilitres

µL: Microlitres

NCBI: National Center for Biotechnology Information

OD: Optical density

OECD: Organization for Economic Co-operation and Development

Pb: Lead

Pb-B: Lead in blood

Pb-T: Lead in teeth

PbAc: Lead acetate

PBS: Phosphate buffered saline

$PM_{2.5}$: Particles that have diameter less than 2.5 micrometres

qRT-PCR: Reverse transcription quantitative polymerase chain reaction

RNA: Ribonucleic acid

ROS: Reactive oxygen species

rpm: revolutions per minute

SADC: The Southern African Development Community

SD: Standard deviation

SEM: Standard Error of Mean

TNF-α: Tumor necrosis factor alpha

TP53: Tumor protein 53

Tuba1: Tubulin 1a

T-bil: Total bilirubin

U: Unit

Ucp-2: Uncoupling protein-2

UA: Urea Acid

UNZAREC: University of Zambia Research Ethics Committee

USEPA: United States Environmental Protection Agency

Zn: Zinc

Preface

In this PhD thesis, studies on the toxicological effects of lead in animals for evaluation of worldwide environmental lead pollution are reported and discussed.

Lead is a toxic metal that is known to affects most systems in fish, animals, humans and both domesticated and wild birds. Lead poisoning is a good example of a man-made problem as it naturally occurs in very small amounts in the natural environment and its presence in large quantities in the environment is directly related to human activities. Ironically, lead as metal has no known biological functions in the human body and so its use is based on its special properties such as poor conductibility, malleability, ductility, resistant to corrosion that has kept it in use. Among these activities, mining, manufacturing of automobile and recycling of lead acid batteries are the major pollution sources. Besides the lead mining related activities, lead by-products contribute lead salts to the lead-cycle. These salts gain access to the environment via automobile exhausts. The heavier particles tend to drop to the ground contaminating the waters and soils, whilst the lighter particles traverse much longer distances via the air and remain suspended in the atmosphere. Thus, lead is a worldwide problem affecting humans, terrestrial animals, birds, plants and fishes in the natural and aquatic environments, respectively. The toxic effects of lead alter several systems and primarily the; (1) the central nervous system, (2) immune, (3) hematopoietic, (4) renal, (5) reproductive, and (6) hepatic systems of animals and humans. It has been further acknowledged that lead in wildlife and domestic animals may disrupt their survival and health, their fecundity and the ability to resist stressors including pathogens which threatens the biodiversity and ecosystem balance. Although lead related pollution and poisoning has gone down over the years in most developed nations due to stringent environmental protection regulations, it remains a problem in some developing nations with history of lead mining, smelting or extensive lead acid recycling. A good example of a developing nation with perennial lead poisoning in children and adults is Zambia in one of her towns, Kabwe which had a long history of lead-zinc mining that was in operation for over 90 years without adequate environmental concern.

In response to the Kabwe lead situation, the present study was aimed at investigating the toxicological effects of lead exposure in fish, animals and humans as my contribution to the impact of the worldwide lead pollution. The proportion of lead in lead-zinc mining wastes (soils), its levels in water were investigated in *in-vivo* exposure studies using zebrafish and laboratory rodent models. Wild rat teeth and human blood and plasma samples from Kabwe, Zambia were also used.

The thesis comprises five (5) chapters as outlined below:

In chapter 1, a general introduction of the lead situation from a global perspective to the Kabwe situation is briefly described.

Chapter 2: Describes the effects of Kabwe water lead levels on embryos and larval zebrafish (Danio rerio) under the following study themes. 2.1. Environmentally relevant lead concentrations induce cardiovascular, neuromuscular and oxidative stress responses in zebrafish (Danio rerio) embryos. 2.2

Acute exposure to environmentally relevant lead levels induces oxidative stress and neurobehavioral alterations in larval zebrafish (Danio rerio). *Aquatic toxicology Volume 227, October 2020. https://doi.org/10.1016/j.aquatox.2020.105607*.

Chapter 3: Describes effects of lead with zinc in rats and the application of wild rat incisors teeth in environmental lead exposure assessment under the following study themes. 3.1 Effects of lead exposure with zinc co-administration on multi-organ lead uptake, ALAD and oxidative stress genes responses in liver, kidney and brain in Sprague-Dawley rats. 3.2 Wild rat crown incisor as an indicator of lead (Pb) exposure and Pb incisor mapping using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS).

Chapter 4: Describes effects of chronic lead exposure in human. The impact of chronic environmental lead exposure on proinflammatory biomarkers cytokines profiles in males and females of reproductive age from Kabwe, Zambia.

In chapter 5, a general discussion and future perspectives are given based on all the results. The cited references are listed following this chapter. The overall abstract in English and Japanese languages are attached at the end.

CHAPTER:1 Introduction

General background

Global Perspective of Environmental Pollution

Pollution refers to the discharge of elements, compounds or energy into the natural environment in quantities that disrupts or impairs its ability to support biological life of humans, animals or other living organisms (Fernández-Luqueño et al., 2013). Environmental pollution occurs through natural means following natural disasters such volcanic eruption, rock weathering albeit in small amounts. However, large quantities of pollutants in the environment are introduced through anthropogenic activities such as mining and manufacturing processes (Marx et al., 2016). The commonly notable environmental pollutants include but not limited to toxic metals (lead, cadmium, mercury, arsenic), persistent organic pollutants (polychlorinated bipsheyls, polychlorinated dibenzo-*p*-dioxins, organochlorine pesticides such as dichloro-diphenyl-trichloroethane (DDT), phthalates) and air pollutants (particulate matter 2.5 ($PM_{2.5}$), carbon monoxide, ozone, nitrogen dioxide) among others (Wasi et al., 2013).

Globally, pollution has been implicated as one of the largest causes of morbidity and premature mortality (Sohrabi et al., 2020). It is estimated that in 2015 alone, around 9 million premature deaths were linked to pollution which translated to 16% of all deaths worldwide, three times more than a combination of deaths that arose from acquired immunodeficiency syndrome (AIDS), tuberculosis and malaria combined (Landrigan et al., 2018). Notwithstanding the notable wide spread of environmental pollutants and their deleterious effects on human health and welfare losses estimated at US$4.6 trillion per year, environmental pollution remains a neglected issue especially among developing nations (Landrigan et al., 2018). This scenario makes environmental pollution a serious issue especially that a link has been recognized to exists among the major ills of pollution, poverty, ill health and social injustice (Hajat et al., 2015).

Global lead Pollution

Lead as a metal exist as part of the outermost part of planet earth and naturally occurs in soils, plants and water in very low amounts. The introduction of large quantities of Pb in the environment and ecosystems is linked to human related activities such as mining of the lead ores, its processing and its use and by products (Needleman, 1999). It is widely recognised that the use of lead spans over 6000 years and its mining predated both the Bronze and Iron ages (Yahalom-Mack et al., 2017). Whereas its toxicity has been well documented as far back as 2000 years ago, the inherent properties of lead as metal such as poor conductibility, malleability, resistance to corrosion among others have kept it in use (Needleman, 1999). Consequently, at the global scale, the pollution of soil, water and air with lead seems to persist. Currently, it is estimated that the global lead production has more than doubled with the increased manufacturing of automobile and mobile phone batteries (ILA, 2020).

The Global Burden of Disease (GBD) Study estimates that lead accounted for half a million premature deaths and 9.3 million disability-adjusted life year (DALYs) in 2015 in the adult population (GBD, 2015). Although episodes of child mortality due to acute lead exposure has been reported in low-income and middle-income countries such as in Africa particularly in Nigeria and Senegal, impaired cognitive development and intelligent quotient (IQ) seems to be the major challenge in children (Landrigan et al., 2018). These cognitive impairment even at very low levels of lead have prompted the Centers of Disease Control and Prevention (CDC) to revise the reference level that can be classified as exposed in terms of lead blood level to 5 µg/dL in children during screening (CDC, 2012).

Another notable global impact of lead pollution has been reported in scavenging birds and waterfowls. The use of lead ammunition and fishing tackles has resulted in accidental Pb poisoning of non-target birds (Ishii et al., 2018). Lead poisoning related mortalities in wild birds, such as waterfowls, raptors and other scavengers having been reported around the world in Japan resulting in dwindling number of endangered species in some cases (Pain et al., 2019).

Kabwe Lead Situation

Kabwe town, formerly Broken Hill is a provincial capital of the central province of Zambia in Africa (Fig. 1.1) and is home to about 230,000 inhabitants (Yabe et al., 2020). The town has a lead-zinc mining history whose early active years had an indirect linkage to the Industrial Revolution in Britain and the Mineral Revolution in South Africa around the 17th Century and second half of the 19th Century, respectively (Mufinda, 2015). The extensive mining of lead and zinc without accompanying mine waste disposals regulations for 9 decades until it was abandoned in 1994 has left behind a legacy of extensive lead pollution of soils and general environment (Tembo et al., 2006). The presence of loose lead metallic dust from the mine site and waste dumps remains as continuous source of lead pollution (Mwandira et al., 2019).

The dispersion of the lead containing dust particles by strong winds and lead wastes run-offs from soils during the rainy season has led to the introduction of lead into residential areas and as a consequence the majority children have blood Pb levels above the least level of concern of 5µg/dL (Bose-O'Reilly et al., 2017; Yabe et al., 2020, 2015). Several authors have cited extension of lead pollution to domestic and non-domestic animals such as cattle, goats, chickens and wild rodents within the vicinity of the closed mine (Nakata et al., 2016; Nakayama et al., 2011; Yabe et al., 2013, 2011). Moreover, water has been reported to be contaminated by lead within the mine and surrounding areas (Kribek et al., 2009; Nachiyunde et al., 2013). Nachiyunde et al. (2013) reported that the lead levels in the natural water bodies and ground water in boreholes ranged from 0.01 to 94 µg/L of lead within the vicinity of the abandoned mine. The reported levels of lead in water in some sites were below the

50 µg/L lead in water set as the regulatory and permissible level by the regulatory body, the Zambia Bureau of Standards (Nachiyunde et al., 2013).

The Kabwe lead pollution situation is characterized by two aspects that are unique and peculiar. The first one is the chronic lead exposure situation in Kabwe. Chronic lead pollution that was initially mostly occupational lead exposure that directly affected the workers during the time the mine was operational to a more extensive environmental exposure that affects all residents within the vicinity of the abandoned mine (Mufinda, 2015). The Kabwe lead pollution poisoning predates the existence of the town itself based on evidence that show the earliest human settler, "Broken Hill Man" (*Homo heidelbergensis*) formerly *Homo rhodesiensis* who lived between 25,000 and 300,000 years ago may have suffered from lead poisoning (Lacy, 2014). This aspect makes Kabwe town an ideal place to study chronic lead exposure related toxicity in humans as most of the residents may have been exposed to lead from early childhood into their adulthood. The second one is a presence of both lead and zinc in large amounts in the soils above the benchmark values (Ettler et al., 2020;. Nakayama et al., 2011; Tembo et al., 2006). This second scenario implies that lead exposure especially in children with hand-to-mouth activities (Yabe et al., 2015) may exist as lead and zinc co-exposure. Furthermore, the oral bioaccessibility study revealed that both lead and zinc were highly bio-accessible implying that both would be easily taken up by the body following accidental ingestion due to hand-to-mouth activities especially in infants and toddlers (Ettler et al., 2020).

Objectives of my PhD Thesis

Whereas the widespread of chronic lead poisoning in Kabwe, Zambia has been appreciated in soils, water, animals and humans both children and adults, the toxic effects of this chronic exposure as it relates to the Kabwe situation remains unclear. Therefore, a combination of experimental and field studies is needed to understand the impact of the lead situation in Kabwe in fish, animals and humans. The main objectives of this doctoral thesis were to:

I. Investigate the effect of the Kabwe water lead levels on aquatic life and its toxicity effects on developing fish using embryo and larval zebrafish (*Danio rerio*)
II. Investigate the effects of lead and zinc combinations as they occur in Kabwe in animals using rodent model (Sprague-Dawley rats) exposed to lead and zinc.
III. Investigate the use of rodent tooth in Kabwe wild rodent tooth as a tool for environmental assessment and as an indirect marker for lead exposure in human
IV. Investigate the impact of chronic environmental lead exposure on proinflammatory and immunomodulatory tumor necrosis alpha and interleukin-8 biomarker cytokines in males and females of reproductive age from Kabwe, Zambia

My PhD study will contribute to updated body of knowledge around the Kabwe lead poisoning situation in fish, animals and humans. It will further add knowledge on the effect of the widespread lead pollution in animals and humans. It is expected therefore that a better understanding of the toxic effects of the lead in water, animals and humans will help different policy makers including government, non-governmental organizations (NGOs) make and lobby for better policies to address the situation. My ultimate desire is to update the knowledge n the effects of the extensive lead pollution in Kabwe and ensure generate interests and policies that will ensure a good socioeconomic and health benefits for human and animal life as my contribution to one health.

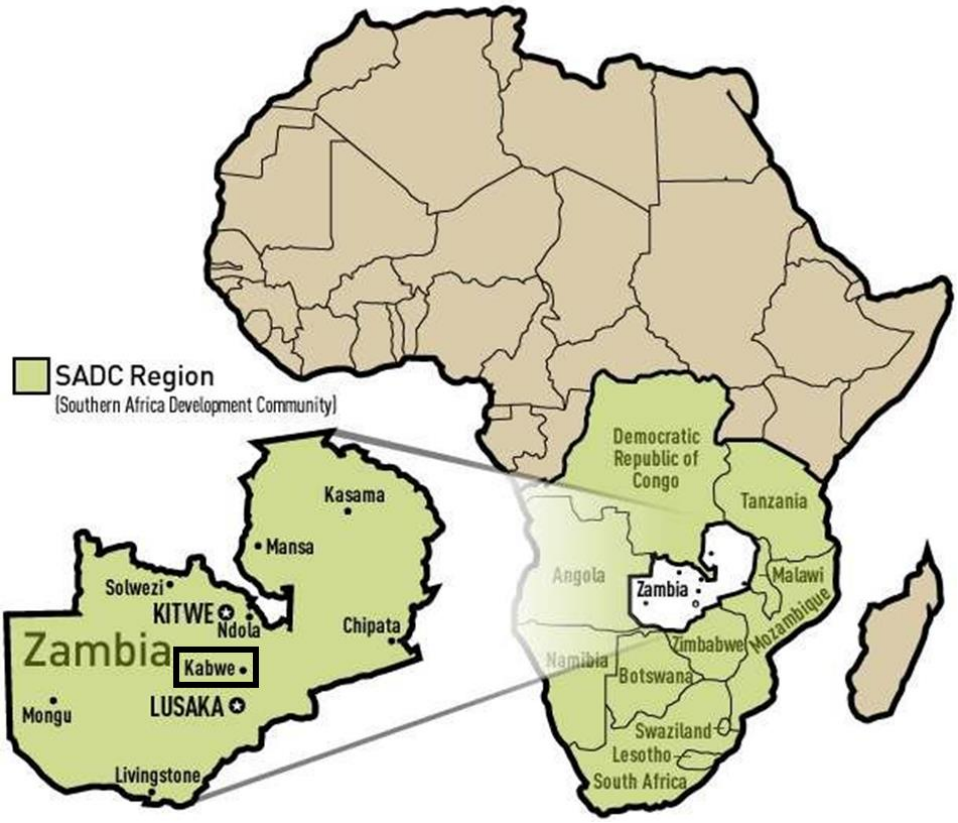

Fig.1.1. Map of Zambia and its location on the African continent in the southern part of African with the neighbouring Southern Africa Development Community (SADC) countries and showing Kabwe town marked with a rectangle. The distance between Kabwe and Zambia's capital city, Lusaka is approximately 110.8 km (Lubasi et al., 2012).

CHAPTER 2:

Effects of Kabwe water lead levels on fish using embryos and larval zebrafish (*Danio rerio*)

2.1 Environmentally relevant lead concentrations induce cardiovascular, neuromuscular and oxidative stress responses in zebrafish (Danio *rerio*) embryos

Abstract

Ubiquitous environmental lead (Pb) contamination is related to human activities with mining as one of the major contributors. A long history of lead-zinc mining in Kabwe, Zambia extensively polluted the environment and some isolated water bodies with lead. To this end, I explored the negative effects of the current Kabwe lead water levels on aquatic life using 2.5 hours post fertilization (hpf) old embryos zebrafish (*Danio rerio*) exposed to a wide range of Pb concentrations (1-5000 µg/L Pb) until 96 hpf. Exposure negatively impacted the development and survival of zebrafish embryos by inducing embryo coagulation mortalities, reduced hatching rates, lack of embryo hatching in a concentration dependant manner. At 24 hpf, elevated embryo burst activity at lowest level of exposure (1 µg/L Pb) and decreased burst count per minute beginning at 500 µg/L were recorded. At 100 – 500 µg/L Pb, exposure induced cardiovascular toxicity by reducing heart rate and increasing blood flow activity concurrently. At 72 hpf involuntary muscular twitching that increased in number and duration were recorded at 50-500 µg/L Pb. The Pb toxicity dysregulation of the oxidative stress system evidenced by downregulation of catalase, and upregulation of heme oxyeganse-1, glutathione-S-transferase, cytochrome c oxidase subunit I mRNA gene expressions. The proapoptotic gene, tumour protein 53 expression level were downregulated across exposed groups. In contrast, the antiapoptotic gene, B-cell lymphoma 2 were upregulated at the lowest exposure spectrum (1-25 µg/L) and downregulated from 50-500 µg/L Pb. The findings suggest that the developmental Pb exposure in zebrafish may be accompanied with deleterious effects within environmentally and regulatory Pb levels.

Keywords: fish; lead; heart rate; gene expression, apoptosis; spasm

Introduction

Lead (Pb) is a metal that naturally occurs in small amounts in the environment (Hailegnaw et al., 2015). High quantities of Pb in the environment is traceable to anthropogenic activities such as mining, Pb products production processes and their use (Komárek et al., 2008). An example of a heavily Pb polluted town with mining activities as a source is Kabwe, Zambia following 9 decades of an unregulated waste managements at the now closed lead-zinc mine (Yabe et al., 2018). The extensive Pb pollution of the Kabwe town has plunged the majority of its residents into perennial Pb poisoning especially children having blood Pb levels above the minimum Pb reference value of 5 µg/dL (Yabe et al., 2020, 2015). Domesticated animals, free range chickens including free roaming dogs inside the region of the closed mine have been found with marked Pb levels in blood (Nakayama et al., 2011; Toyomaki et al., 2020; Yabe et al., 2013, 2011). Moreover, water sampled from natural water bodies and boreholes inside the region of the closed mine were found to have a mean Pb level of 3 µg/L and the upper limit Pb concentration of 94 µg/L against the country's regulatory limit of 50 µg/L Pb in water (Nachiyunde et al., 2013).

Lead exposure to developing fish embryos is has been linked to undesirable effects including delayed in the hatching, premature hatching, malformations to mortalities of larvae (Jezierska et al., 2009). In addition, Pb poisoning causes an imbalance of the antioxidants and eventual dysregulation of the oxidative stress system (Kim and Kang, 2017). Lead exposure in *in-vivo* fish studies have demonstrated that one of the toxic mechanisms of Pb induced toxicity is through the dysregulation of the antioxidant responses through the increased generation of reactive oxygen species (ROS) following exposure (Kim and Kang, 2017). The review of several Pb exposure studies revealed that the younger the developmental stage in fish, the more sensitive the organism is to metal exposures such as Pb (Sfakianakis et al., 2015). Lead exposure in concentrations that ranged from 100 to 10000 µg/L (Sfakianakis et al., 2015) revealed that developing and young stages of fish were more susceptible to Pb induced toxicity than adult fish.

However, a dearth of data on the unfavourable impacts of the Kabwe water Pb levels on the cardiovascular, neuromuscular and ROS toxicity of developmental stages of fish exists. To bridge this information gap, I investigated the undesirable effects of the Kabwe water Pb levels on fish health by means of the fish embryo toxicity testing (FET) protocol. Zebrafish embryos due to their been transparent in early life stages and their susceptibility to environmental pollutants due to their ability to absorb compounds in water have become preferred model for chemicals in water (Yin et al., 2017). In addition the FET test was employed to inform on: 1) how safe are the water Pb levels reported from Kabwe, Zambia (mean Pb - 3 µg/L and upper limit- 94 µg/L), respectively to developing aquatic organisms including fish?; and 2) Is the country's regulatory limit Pb water (50 µg/L) as reported by Nachiyunde et al., (2013) supportive to aquatic life?

Materials and Methods

Fish husbandry and embryo collection

All experimental procedures were conducted with the due approval and strict adherence to the guidelines by the AnimCare animal research ethics committee (ethics approval number: NWU-00269-16-A5) at the North-West University. Wild-type zebrafish breeding stock kept at 26 – 28 ºC on a 14-hr light and 10-hr dark cycle in a ZebTec (Tecniplast, Italy) system at the North -West University's National Aquatic Bioassay Facility (NABF), South Africa. All the experiments were carried out following and in strict adherence to research guidelines mandated by the North-West University AnimCare Ethics Committee (Approval number NWU-00269-16-A5). The breeding process and the collection of embryos was carried out as previously described by Kataba eta al., (2020).

Lead (Pb) stock solution preparation and concentrations selection

A 10 mg/L Pb stock solution was prepared using pure grade (99.5%) lead acetate trihydrate in lead-free ultrapure water. From the Pb stock solution, ten dilutions (1, 10, 25, 50, 100, 500, 1000, 2000 and 5000 µg/L) were prepared using the embryo development medium. The selection of the primary first six exposure concentrations were chosen to reflect the range reported in water samples from Kabwe, Zambia and the permissible Pb water level by the national authority body (Nachiyunde et al., 2013). The rest of the Pb concentrations were included in adherence with the Fish Embryo toxicity (FET) test protocol recommended by the Organisation for Economic Co-operation and Development (OECD) guidelines for the testing of Chemicals (Busquet et al., 2014).

Fish Embryo Toxicity (FET) test and embryo activity analysis

Fertilized zebrafish embryos (2.5 hpf) within the same developmental stage, following sorting under a Zeiss stemi microscope, were assigned to 10 treatments of Pb at various concentrations (0, 1, 10, 25, 50, 100, 500, 1000, 2000 and 5000 µg/L) diluted with the embryo media in plastic 6-well plates (total volume 5000 µL/ well). A positive control with 3,4-dichloroaniline test solution was prepared. Six replicates (n = 5 embryos per well) for the control of 30 total embryos, five replicates of total 25 embryos per treatment (1-1000 µg/L), four replicates (n = 20) for 2000 µg/L and two replicates (n = 10) for 5000 µg/L were performed without renewal of the treatment solutions. The plates were sealed with self- adhesive, oxygen-permeable sealing film (BRAND®, Sigma Alrich, Madrid, Spain) to prevent evaporation of the test solution. The embryos that were incubated at 28 °C for 96h and the survival rate monitored every 24 h interval. Normal embryo morphology referencing was as described by Kimmel et al. (1995).

Sub-lethal embryo activity, cardiology and twitching

A non-invasive video recording technique of assessing embryos within their chorions or hatched larvae was used. To assess embryo activity at 24 hours post-fertilization (hpf) ,10 embryos per group were recorded for 1 min using a stereomicroscope (Zeiss, Oberkochen, Germany) using a remote-controlled microscope camera, and a digital tablet. Videos were assessed using DanioScope V1 Software (Noldus Information Technology, Wageningen, Netherlands). The mean burst activity and the burst count/min were computed as representative embryo activity. The mean burst activity represents the percentage of time (from total measurement duration) the embryo was moving, and the burst count / per minute represents the number of movements per minute.

At 72 hpf when embryos had hatched, blood flow and heart rate were assessed by way of video recording followed by analysis. Individual zebrafish larvae were picked with a pipette and placed in drop of the exposure media on a glass slide and videos were taken using a stereomicroscope using a remote-controlled microscope camera, and a digital tablet. Heart rate videos were taken with the heart in view while the larvae lay in lateral recumbency and recorded for 30 seconds. The videos were imported in DanioScope and automatically the number of beats per minute (BPM) were calculated. The blood flow videos were taken focusing on the caudal haemopoietic region just above the cloaca (anal) opening in view for 30 seconds. The blood flow was presented as an activity percentage.

Involuntary muscular twitching (spasms) of the larvae were assessed using the heart rate recording as an indicator for involuntary movements. The full video was used, and movements were confirmed by visually watching the video as well as output graphs. A peak indicated a count in a twitch and the peak width was used to determine the duration of the twitch over the 60 second time interval. The raw data was exported and used for time scale determinations (DanioScope V1 Software, Noldus Information Technology, Wageningen, Netherlands). For classification purposes, we defined a twitch peak as any activity percentage of 5% and above generated by a muscle twitch and excluded swimming attempt or pectoral fins beats initiated zebrafish larval body activity as shown in Fig. 2.1.1 below. The red line shows activity percentage that was regarded as twitch in representative exposure level in 50 ,100 and 500 µg/L Pb groups.

RNA extraction and real-time PCR analysis

An RNA isolation protocol was used where 5-10 pooled hatched embryo samples were homogenized in TRI Reagent® (SIGMA Life Sciences, ST. Louis, MO, USA) with a zirconia bead using a tissue lyser; chloroform was added, and samples were vortexed and then centrifuged at 13,000 g for twenty minutes at 4 °C. The supernatant was then mixed with 350 µL of 70% ethanol and then placed in the FastGene® RNA binding column. Afterwards, the standard FastGene® RNA Basic kits protocol was followed for the rest of the steps. The RNA was eluted from the membrane using RNase

free ultrapure MilliQ water. The RNA quality was assessed by spectrophotometry (OD 260:280 ratio) using a NanoDrop 1000A Spectrophotometer (Delaware, USA). The first strand TOYOBO cDNA synthesis kit ReverTra Ace-α (TOYOBO Co., Ltd., Life Science Department, Osaka, Japan) was used for cDNA synthesis according to manufacturer instructions.

The real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis (StepOnePlus Real-Time PCR System, Applied Biosystems, USA) was performed using a 10 µL PCR reaction mixture containing 5 µL of Fast SYBR Green Master Mix (Applied Biosystems, USA), 0.4 µL of 5 µM forward and reverse primers (Thermo Fisher Scientific, Life Technologies Japan Ltd., Japan), 20 ng of cDNA of each pooled zebrafish larvae samples, and 2.2 µL of distilled water. The qRT-PCR condition for all target genes was 95 ℃ for 20 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C, 62 °C or 65 °C for 30 s depending on the annealing temperature of a given primer set as shown in Table 2.2.1. Most of the primers used were obtained from published works (Jin et al., 2010; Shi and Zhou, 2010; Stancová et al., 2015) and some were generated using the NCBI and Primer3 tools. The gene expressions of various oxidative stress related genes were quantified with the relative absolute method using tubulin 1a (*Tuba I*) as a house keeping reference gene (Table 2.1.1). The choice of *Tuba I* as a housekeeping gene was based on its stability and constant expressions in both control and Pb exposed groups after preliminary validation when compared with beta actin, which is in agreement with (Mccurley and Callard, 2008) in zebrafish at various developmental stages following chemical treatment. The primer efficiency range was from 96.5% to 100.4%. The normalization, and the expression levels calculations were done using the comparative ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001).

Verification of Pb in exposure concentrations

The Pb exposure concentrations were quantified for verification of the exposure levels. Fresh Pb exposure concentrations were prepared from 99.5% lead acetate trihydrate and the recovered actual Pb test solutions in which had zebrafish embryos were sampled for analysis. After acidification, concentration of Pb was measured using the inductively coupled plasma mass spectrometry (ICP-MS, 7700 series; Agilent Technologies, Tokyo, Japan). The instrument detection limit for Pb was 0.001 µg/L.

Data Analysis

The FET test data statistics were generated from the TOXRAT® software. GraphPad Prism software (Prism 7 for Windows; Version 5.02, California USA) was used to perform the rest of the data analysis including embryo activity, blood flow, heart rate, twitching and gene expression. The data were first tested for normality using Kolmogorov–Smirnov test. For normally distributed data, a one-way analysis of variance (one-way ANOVA) and the differences among test groups were assessed

using Tukey's test. A non-parametric Kruskal–Wallis test followed by a Mann–Whitney test (all other comparisons) were applied for non-normally distributed data were used. The data were reported as mean and standard error of the mean (SEM). The hatching and overall survival rates proportions differences between the exposed and control groups were analysed by MedCalc® which uses the "N-1" Chi-squared test (Campel, 2007). The difference between groups were considered at two levels of significance and were marked at $p < 0.05$ (*). The graphical presentation of data was done using GraphPad Prism software).

Results

The theoretical exposure concentrations that were prepared (0, 1, 10, 25, 50, 100, 500, 1000, 2000 and 5000 µg/L) and used in the current study verified and were found to be were within 3% below and 10% above range (0, 1.1, 9.7, 25.2, 49.6, 100.7, 500.4, 1005.6, 2028 and 5003.7 µg/L, respectively). From the verification process, Pb concentrations analyzed using ICP-MS showed recoveries of over 95% (97 -110 % recovery).

Lead induced developmental toxicity in zebrafish embryo toxicity

The overall survival and hatching rates in the control and exposed groups are shown in Table 2.1.2. Lead exposure reduced the overall survival and hatching rates of exposed zebrafish embryos in a concentration dependent manner in in significantly. Lead-induced embryo coagulation mortalities between 24 and 72 hpf, that gradually increased with the increase in Pb exposure concentration (Table 2.1.2). The lowest exposure level seen with significant mortalities was 1.0 µg/L Pb (i.e. 16%). The mortalities increased with the increase in concentrations, i.e. 70% and 80% mortality at 2000 and 5000 µg/L Pb exposed groups, respectively. The hatching rates decreased with an increase in the Pb exposure concentrations from 84% in the 1.0 µg/L to 40% in the 500 µg/L Pb exposed group. No hatching was observed in the 1000, 2000 and 5000 µg/L Pb exposed groups. The overall survival rates in the present study followed a similar trend with the hatching rates and reduced with an increase in Pb exposure level. The major developmental anomaly recorded in the exposure groups were mildly and severely curved spines malformations in the 500 µg/L Pb exposed group only with 56.25% of the hatched larvae malformed.

Lead exposure attenuated zebrafish embryo activity

Lead exposure affected the burst activity of zebrafish embryos, which represent the total activity during the recording time. Only the 1 µg/L Pb exposed group had significantly higher ($p < 0.05$) burst activity (1.8 ± 0.2 %) compared to the control group. Other groups showed a slight increase in activity except for the 1000 and 2000 µg/L Pb exposure treatments that displayed slightly reduced activity (Fig. 2.1.2A). The burst count per minute showed a statistically significant decrease ($p < 0.05$) in the

100 (0.24 ± 0.16 burst/min), 500 (0.49 ± 0.38 burst/min) and 1000 µg/L (0.49 ± 0.32 burst/ min) Pb exposure groups (Fig. 2.1.2B).

Lead exposure induced cardiovascular effects

Lead exposure caused changes in cardiovascular responses of 72 hpf zebrafish (Fig. 2.1.3). The heart rate was significanly lower ($p < 0.05$) compared to the control (Fig. 2.1.3A) at the highest exposure concentrations (100 and 500 µg/L). The blood flow at the same (100 and 500 µg/L) exposure concentrations was increased (Fig. 2.1.3B).

Lead induced exposure induced muscular twitching

In the present study, I observed some involuntary muscular twitching in the hatched zebrafish embryos. The twitching was observed in the exposure concentrations, in the 50, 100 and 500 µg/L Pb exposure groups only and were absent from all the other exposures and the control group (Fig. 2.1.4). The twitching increased from 3.5 ± 1.4 twitches per min in the 50 µg/L exposure group to 18.8 ± 2.4 twitches per minute in the 500 µg/L treatment (Fig. 2.1.4A). The duration of the twitches increased from 0.55 ± 0.16 seconds in the 50 µg/L Pb group to 0.62 ± 0.03 seconds in the 500 µg/L Pb groups (Fig. 2.1.4B).

Lead exposure affected antioxidant genes expression

Changes in antioxidant gene expression (*CAT*, *GPX*, *SOD*, *GST*, *HO-1* and *Nrf2*) following exposure to different Pb concentrations are shown in Fig. 2.1.5. Lead induced significant down regulation of *CAT* gene expression in 50 µg/L Pb group (0.6-fold change) and 500 µg/L Pb (0.4 -fold change) groups in relation to the control (Fig. 2.1.5A). The mRNA expression levels of *GPX*, *SOD* and *Nrf2* genes across the exposed groups remained unchanged when compared with the control (Fig. 2.1.5 B, C and F**,** respectively). The mRNA levels of *GST* were upregulated significantly in comparison with the control. The *GST* expression levels followed a concentration dependent pattern, i.e. 1 µg/L Pb (2.5-fold change), 10 µg/L Pb (4.9-fold change), 25 µg/L Pb (8.0-fold change), 50 µg/L Pb (7.9-fold change), 100 µg/L Pb (7.6-fold change), and 500 µg/L Pb (7.6-fold change), respectively (Fig. 2.1.5D). Furthermore, Pb exposure induced significant upregulation of *HO-1* mRNA levels at 100 µg/L Pb and 500 µg/L Pb groups with 2.2-fold change and 1.7-fold change, respectively (Fig. 2.1.5E).

Lead exposure affected pro-apoptotic and anti-apoptotic gene expression

Mitochondrial related electron transport reactive oxygen species (ROS) associated oxidative stress genes namely uncoupling protein-2 (*Ucp-2*) and cytochrome c oxidase subunit I (*Cox-I*) were

investigated. Lead exposure did not induce *Ucp-2* mRNA expression changes across the exposed groups (Fig. 2.1.6A). On the other hand, *Cox-I* mRNA levels were significantly upregulated across all the exposed groups with 7.8-fold change (1 µg/L Pb), 5.9-fold change (10 µg/L Pb), 5.9-fold change (25 µg/L Pb), 5.4-fold change (50 µg/L Pb), 7.3-fold change (100 µg/L Pb) and 6.3-fold change (500 µg/L Pb) compared to the control (Fig. 2.1.6B.)

The expression of the mRNA levels of the pro-apoptotic encoding protein tumour protein p53 (*TP53*) was significantly downregulated across all the exposure groups except in the 100 µg/L treatment when compared to the control (Fig. 2.1.6C). The Bcl-2 anti-apoptotic gene expression was significantly upregulated with 2-fold change (1 µg/L Pb), 1.8-fold change (10 µg/L Pb), and 1.7-fold change (25 µg/L Pb) when compared to the control. A significant downregulation of 0.8-fold at 50 µg/L Pb and non-induction gene expression at 100 µg/L Pb and 500 µg/L Pb was also observed (Fig. 2.1.6D).

Table 2.1.1: Primers used for real-time qPCR with annealing temperatures used and PCR product length

Target gene	Primer Sequence (5'-3')	Annealing Temperature (◦C)	Product length (bp)	Reference
Tubulin alpha I (*Tuba I*)	**F:** TTTGTGCACTGGTACGTGGG **R:** CCACACTCTCAGCTCCAACCTC	60	112	a
Glutathione-S-transferase (*GST*)	**F:** ACAACCTGTTCGATCTCCTGCTGA **R:** GTTGCCGTTGATGGGCAGTTTCTT	60	161	(Jin et al., 2010)
Superoxide dismutase (*SOD*)	**F:** CGCATGTTCCCAGACATCTA **R:** GAGCGGAAGATTGAGGATTG	60	100	(Stancová et al., 2015)
Catalase (*CAT*)	**F:** AGTGCTCCTGACGTCCAGCCA **R:** TGAAGAACGTGCGCACCTGGG	65	115	(Jin et al., 2010)
Glutathione Peroxidase (*GPX 4b*)	**F:** GGACGATCCAAGCGTGGTGGA **R:** CAGCCGTCACACGTCTGGGC	60	148	(Stancová et al., 2015)
Uncoupling protein 2 (*Ucp-2*)	**F:** TGGCTCAACCCACTGATGTA **R:** CAATGGTCCGATATGCGTC	62	102	(Jin et al., 2010)
Heme Oxygenase 1 (*HO-1*)	**F:** GGAAGAGCTGGACAGAAACG **R:** CGAAGAAGTGCTCCAAGTCC	62	107	(Shi and Zhou, 2010)
B-cell lymphoma 2 (*Bcl2*)	**F:** AGGAAAATGGAGGTTGGGATG **R**: TGTTAGGTATGAAAACGGGTGGA	62	83	(Jin et al., 2010)
Tumour protein p53 (*TP53*)	**F:** CCCAGGTGGTGGCTCTTGCT **R:** GAGTGGATGGCTGAGGCTGTTCT	62	113	a
Nuclear factor erythroid 2 (*Nrf2*)	**F:** GACAAAATCGGCGACAAAAT **R:** TTAGGCCATGTCCACACGTA	65	165	(Shi and Zhou, 2010)
Cytochrome c oxidase subunit I (*Cox-I*)	**F:** GGATTTGGAAACTGACTTGTG **R:** AAGAAGAAATGAGGGTGGAAG	60	105	(Jin et al., 2010)

Note: letter a represents the primer I designed using NCBI and Primer3. Letter R and F represents for reverse and forward primers, respectively

Table 2.1.2. The survival and hatching rates until 72 hpf during the exposure

Treatment (µg/L)	**Total introduced**	**Mortality 24 - 72 hpf**	**Mortality (%)**	**Hatched (Unhatched) 72 hpf**	**% Hatch 72 hpf**	**Overall survival rate (%)**
Control	30	2	6.7	28 (0)	93.3	93.3
1.0	25	4	16	21 (0)	84.0	84.0
10	25	13	52	12 (0)	48.0*	48.0
25	25	8	32	17 (0)	68.0*	68.0
50	25	8	32	17 (0)	68.0*	68.0
100	25	12	48	13 (0)	52.0*	52.0
500	25	18	72	10 (6)	40.0*	40.0
1000	25	11	44	0 (14)	0*	0
2000	20	14	70	0 (6)	0*	0
5000	10	8	80	0 (2)	0*	0

(* $p < 0.05$ between exposure groups and the control group, Chi-squared test)

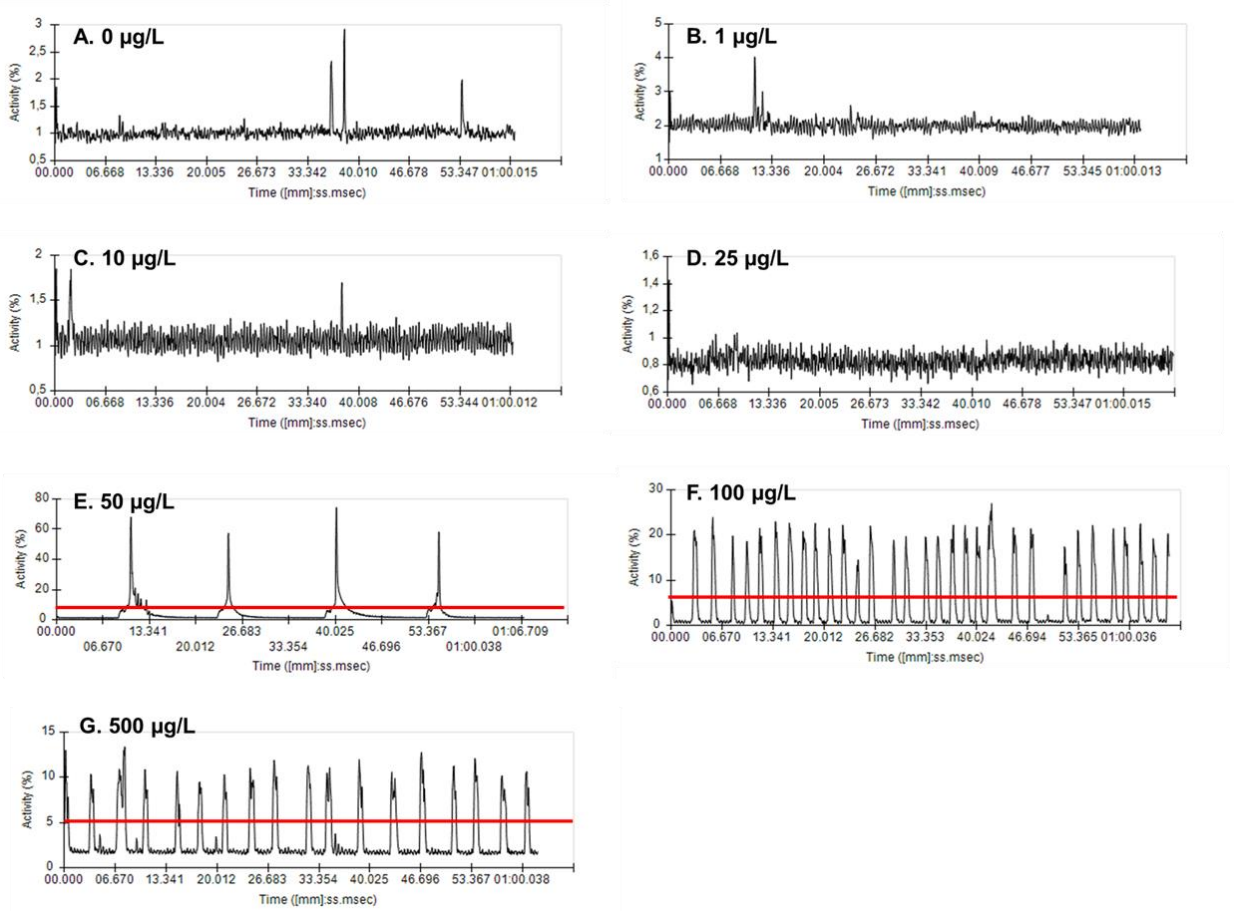

Fig. 2.1.1: Representative muscular activity plots across the Pb exposure concentrations (1-500 µg/L). The red line represents the 5% activity that was used as a cut-off point mark. Any activity less than 5% was taken as a normal muscular activity and such activity was not included in the computation of the number of twitches and durations of twitches /min shown in Fig. 2.1.4**.** Under results section. A. Control B. 1 µg/L C. 10 µg/L D. 25 µg/L. E. 50 µg/L F. 100 µg/L. G.500 µg/L

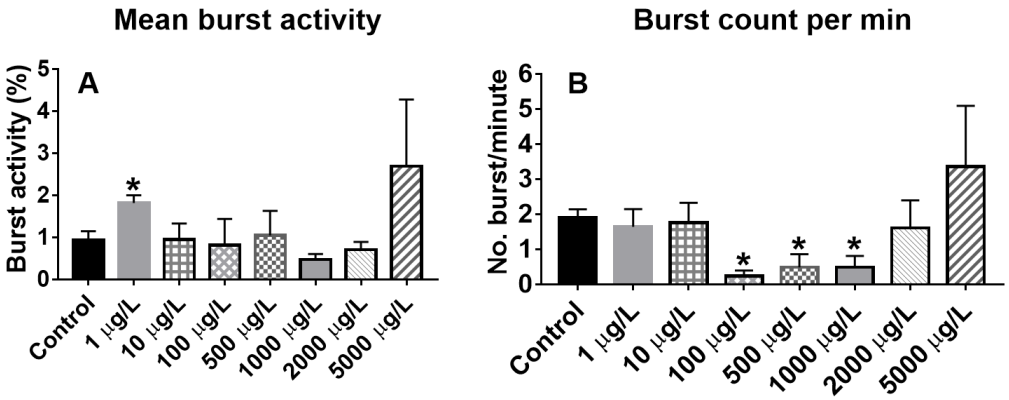

Fig. 2.1.2 Embryo activity at 24 hpf (n = 8) (A) mean burst activity (B) Burst count per minute. Values are presented as mean ± SEM. The asterisk (*) represents significant differences between exposed and the control group using Mann-Whitney test
(*$p < 0.05$).

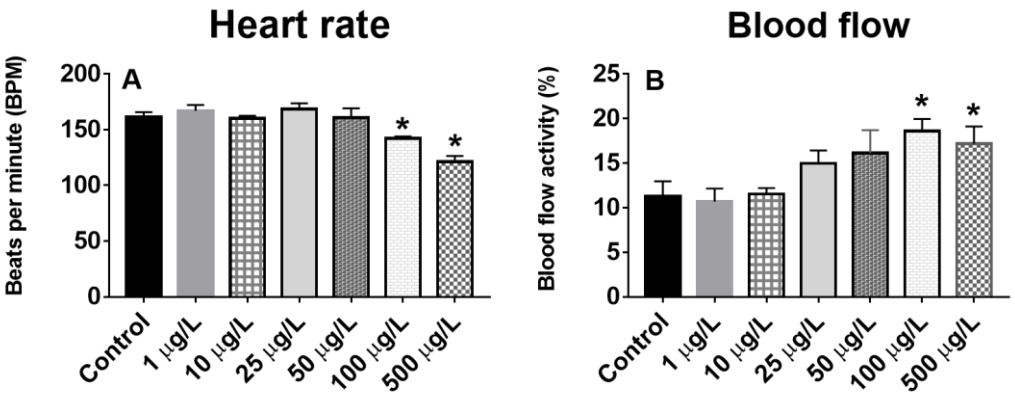

Fig. 2.1.3 Effects of Pb on the cardiovascular system of larvae (72 hpf). (A) Heart rate in beats per minute (n = 4). (B). Blood flow (n = 6 for all groups). Values are presented as mean ± SEM. The asterisk represents significant differences from the control using Mann-Whitney test (*$p < 0.05$).

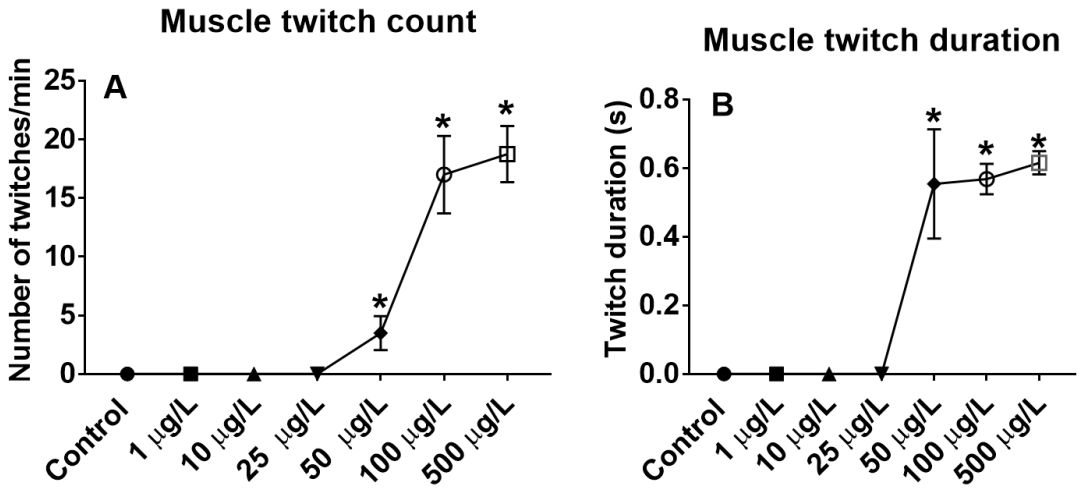

Fig. 2.1.4. Lead induced involuntary muscular twitching (n = 4): **A**. Muscle twitching (number of twitches/min); **B**. Twitch durations (s) Values are presented as mean ± SEM. The asterisk represents a statically significant difference from the control using Mann-Whitney test (*$p < 0.05$)

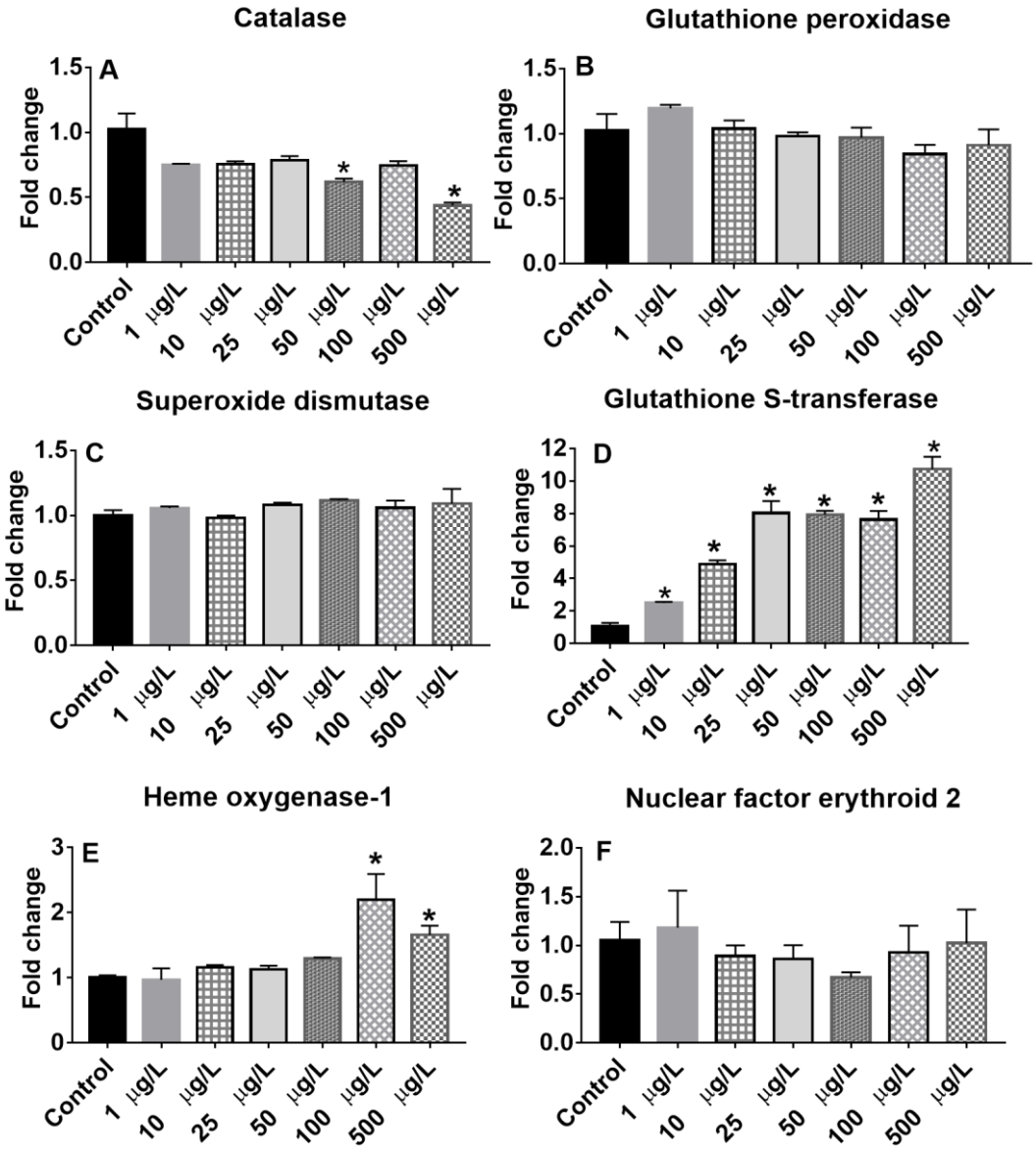

Fig. 2.1.5. Expression of antioxidant genes in the pooled samples (n = 4). Values were normalized against Tubulin alpha-1A (used as house-keeping gene) and represent the mean mRNA expression value ± SEM relative to those of the controls. The asterisk represents significant difference when compared with the controls (*$p < 0.05$; Mann-Whitney test)

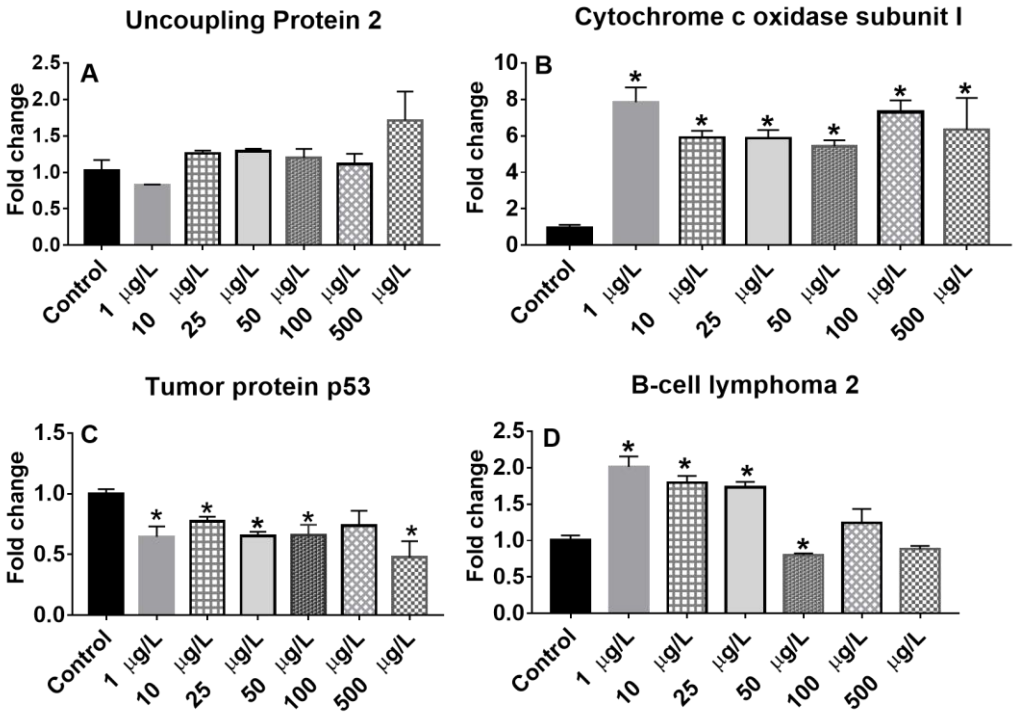

Fig. 2.1.6. Expression of Uncoupling Protein 2, Cytochrome C oxidase subunit I, Tumor protein p53 and B-cell lymphoma 2 (A, B, C and D) in the pooled samples (n = 4). Values normalized against Tubulin alpha-1A (used as house-keeping gene) and represent the mean mRNA expression value ± SEM (n = 4 pooled samples) relative to those of the controls. The asterisk represents a statistically significant difference when compared with the controls (*$p < 0.05$; Mann-Whitney test).

Discussion

The current study sheds some light on the negative effects of the environmental Pb water levels including the permissible value of 50 µg/L that were reported Nachiyunde et al., (2013) in Kabwe, Zambia on aquatic life. The permissible dissolved Pb in water Kabwe is comparable to the acute criteria for dissolved Pb of 54.1 µg/L at a hardness of 85 mg/L (as $CaCO_3$) recommended by the United States Environmental Protection Agency (USEPA) as recently reported by DeForest et al., (2017). However, nations and regulatory institutions around the globe seems not have a common criteria for water quality and aquatic toxicity (Li et al., 2019a). For instance, the dissolved in water for limit according to the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), USA is 15 µg/L (DeForest et al., 2017). On the other hand, DeForest et al., (2017) recently reported that concentration range of Pb that induced acute toxicity on aquatic organisms ranged from 20 to 1000 µg/L with chronic toxicity range of 0.3 to 40 µg/L Pb. The present study revealed deleterious effects of Pb exposure within the environmentally relevant levels as well as within the regulatory limits on the development of zebrafish embryos. Varying levels of Pb induced toxicity were recorded between our 1-5000 µg/L Pb FET test range with severity of toxicity increasing with increase in exposure concentration.

In addition, embryo behaviour activity, cardiovascular system and antioxidant system derangements that increased with the increasing exposure concentrations which were in agreement with other studies (Chen et al., 2012; Zhao et al., 2019**)** are discussed. Moreover, to the best my knowledge, the Pb induced neuromuscular toxicity exhibited by involuntary twitching in hatched embryos at 72 hpf from 50, 100 and 500 µg/L Pb were observed for the first time and have never been reported.

Failure to hatch or delayed hatching in zebrafish embryos that has been attributed to the dysregulation of the hatching gland's chorionase enzyme that digests the chorion (Jezierska et al., 2009) and embryo being unable to rupture the chorion (Fraysse et al., 2006) were recorded in the current study. Although, the mechanisms of delayed hatching may be complex, I observed some indirect connection between hatching rates and neuromuscular derangements in the present study. For instance, at 100 and 500 µg/L Pb exposures, the former which had a lower number of twitches with a shorter had 52% hatching rate, and the latter with more twitches with longer durations had 40% hatching rate. This pattern of hatching rate may in part be attributed to subtle twitches in the embryo, which do not manifest into a full burst of movement (Van Den Avyle et al., 1989) resulting in a failure of the zebrafish embryo to mechanically rupture the chorion.

The involuntary muscle twitching or spasms which have been suggested as neuromuscular toxic neuromuscular toxic effects of Pb (Van Den Avyle et al., 1989). In the present study, there seemed to have been a connection between muscular twitching observed at 72 hpf and embryo burst activity at 24 hpf in exposed groups suggestive of an early onset of early neuromuscular Pb toxicity. These effects

were observed beginning from the 50 µg/L with highest severity recorded at 500 µg/L Pb exposure. The current results were in tandem with those reported by (Chen et al. (2012) as spontaneous tail bends following exposure to 1000 µg/L lead acetate.

On the other hand, cardiovascular Pb induced toxicity appeared to have set in at 100 and 500 µg/L Pb exposed groups with significant heart rate reduction accompanied by a significant blood flow activity increase in the same group (Fig. 2A). However, the heart rate results were in contrast to a reported increased heart rate at 100 µg/L lead nitrate in an unhatched embryos that were acutely exposed up to 24 hpf (Tendulkar et al., 2015). The age and duration of exposure time may partly account for this difference as the younger the stage of the zebrafish, the higher the heart rate (Stoyek et al., 2018). On the other hand, the current results followed the pattern reported in zebrafish exposed to Pb at 3000 , 6000 and 12 000 µg/L Pb (Yin et al., 2017). The reduced heart rate may be linked to the antagonism between lead and calcium and the ability of Pb^{2+} ions to block calcium channels (Mattos et al., 2017). In addition, the increased blood flow activity in the 100 and 500 µg/L Pb exposure groups corroborated findings of increased risk of hypertension in humans after Pb exposure (ATSDR, 2019). Moreover, the lowest at which the cardiovascular toxicity observed in the present study is close to the upper limit environmentally reported water Pb concentration (94 µg/L Pb) in Kabwe, Zambia (Nachiyunde, 2013).

Lead exposure elicited an imbalance in oxidative stress responses considered as potential biomarkers of Pb induced toxicity in zebrafish (Kim and Kang, 2017). Catalase (*CAT*) was downregulated at levels (50 µg/L and 500 µg/L Pb) that had characteristic involuntary muscular twitching. This *CAT* downregulation pattern was similar to a report in zebrafish gills under acute copper induced oxidative stress response (Paul M Craig et al., 2007). The other two key antioxidant genes, *HO-1* and *GST* enzymes that play a critical anti-oxidative role in the detoxification and protection against Pb induced oxidative stress (Sun et al., 2016) were upregulated with the latter following a Pb concentration response. The *GST* gene expression pattern in the current study was similar to the one reported in salmon exposed to environmentally relevant cadmium (Espinoza et al., 2012), attributed to metabolic adaptation to tolerate the increased inorganic pollutant concentrations (Lopes et al., 2001).

The *Cox-I* gene expression, a terminal electron acceptor of the mitochondrial respiratory chain related to the generation of ROS (Sohal et al., 2008) was upregulated without an accompanying upregulation of *Ucp-2* expression. This was in contrast to what was reported in larval zebrafish exposed to acute environmentally relevant Pb levels (Kataba et al., 2020). The upregulation of CoxI gene expression in the absence of upregulation of *Ucp-2* and *Bcl-2* gene expression at 50 µg/L may have further exacerbated ROS Pb induced toxicity in the exposed groups as *Ucp-2* and *Bcl-2* genes are linked to quailing the ROS production as an initial defense mechanism (Paul M Craig et al., 2007). On the other hand, the *Bcl-2* and *TP53* gene expression pattern in the present study were dissimilar

with what was reported in larval zebrafish under acute Pb exposure (Kataba et al., 2020), the current exposed have a downregulated expression of both genes starting from 50 µg/L. The *Bcl-2* gene has been recognized as an apparent antioxidant against oxidative stress to prevent apoptosis (Jin et al., 2010). The prevention is achieved by neutralization of the pro-apoptotic proteins, p53 upregulated mediator of apoptosis (Pyati et al., 2007). The expression patterns of these genes above could have favoured the progression of apoptosis, a possible contributor to the neuromuscular involuntary twitching observed. Overall, the study has demonstrated the deleterious effects of the water Pb levels in Kabwe on zebrafish embryos including neuromuscular toxicity.

There are limitations to my study. Firstly, the lack of accompanying enzymatic assays and non-enzymatic such as lipid peroxidase and protein carbonyl compound analyses; and secondly, the scientific argument around gene expression fold change regarded as significant as an indicator of biologically significant changes (Mccarthy and Smyth, 2009). The first one could not be performed due to the limited sample volume of surviving larvae especially at highest end of our exposure spectrum. The second weakness invokes a limitation on interpretation of gene expressions with less than two times fold change. Notwithstanding, several reports have adequately demonstrated fold changes below 2 had accompanied significant biochemical alterations (Ahmadifar et al., 2019; Parolini et al., 2017; Safari et al., 2017) indicating that the gene expression changes in the mRNA in exposed groups compared to that of the controls could an adequate bioindicator

Conclusions

Lead dissolved in water poses a threat to aquatic life even at the lowest quantifiable amounts. Water lead concentrations of Pb as low as 10 to 50 µg/L Pb that are within the "permissible limit" could be detrimental to aquatic life especially at developmental stages evidenced by embryonic coagulation linked mortalities. Furthermore, the FET test concentrations provided additional insights on the Kabwe Pb water concentrations. Lead water concentrations of 50 to above 100 µg/L could even be more detrimental to developing fish embryos with a myriad Pb linked toxicities. Embryonic activity aberrations, cardiovascular toxicity (reduced heart rate, increased blood flow activity), oxidative stress system imbalance, antiapoptotic and proapoptotic balance and the neuromuscular toxicity (involuntary muscle twitching) are among the deleterious effects of environmentally relevant Pb levels. Further investigations on environmentally relevant Pb water levels and what may be considered as permissible or regulatory water Pb levels effects on aquatic life, are needed to ensure water bodies are safe to support aquatic life.

Section bridge

- In the previous subchapter, section 2.1, the effects of the environmentally relevant lead levels in Kabwe water bodies induced reduced survival rates, cardiovascular toxicity, increased embryo activity and latter depressed burst count per minute. Lead induced neuromuscular toxicity was also observed from 50 µg/L Pb concentration.

- In the next subchapter, section 2.2, the effects of the Kabwe Pb water levels were investigated using 120 hours post fertilization old larval zebrafish acutely exposed to Pb (0, 3, 91 and 250 µg/L Pb) for short time period of 30 minutes with alternating dark/ light illumination. I asked the following questions and wanted to know what would be the impact of exposure on: swimming neurobehaviour pattern activity under dark/light illumination? If there were neurobehavioural changes, would these changes be accompanied by oxidative stress responses in acutely exposed larvae?

2.2 Acute exposure to environmentally relevant lead levels induces oxidative stress and neurobehavioral alterations in larval zebrafish (*Danio rerio*)

Abstract

The ubiquitous contamination of environmental lead (Pb) remains a worldwide threat. Improper Pb mine waste disposal from an abandoned lead-zinc mine has recently unearthed a widespread Pb poisoning in children in Kabwe Zambia. Although the adverse effects of Pb on human health have begun to receive attention, the ecotoxicological effects on aquatic vertebrates still need further investigation. In addition, there is paucity in the knowledge on the behavioural and molecular subcellular responses in larval zebrafish exposed to Pb within the range of environmental relevant concentration (average 3 µg/L with maximum of 94 µg/L) on aquatic organisms such as zebrafish. The adverse effects of environmentally relevant levels of Pb on larval zebrafish was evaluated by measuring swimming behaviour under alternating dark and light conditions. Larval zebrafish acutely exposed to environmentally relevant Pb exhibited neuro-behavioural alteration including enhanced hyperactivity under light conditions evidenced by increased distanced covered and speed compared to the control. The alteration of entire behavioural profiles was further associated with the disturbed expression patterns of mRNA level of key genes associated with antioxidant (heme oxygenase-1, Uncoupling protein-2 and cytochrome oxidase sub unit I), proapoptotic gene (tumor protein 53), and antiapoptotic gene (B-cell lymphoma-2). To the best of my knowledge, this is the first report on the effects of environmentally relevant Pb levels from Kabwe, Zambia and their adverse neurobehavioural effects and subcellular molecular oxidative responses in larval zebrafish acutely exposed within a 30 minutes period. The current results would be beneficial in understanding of the effects of low Pb levels acutely discharged into an aquatic environment and the life of aquatic organisms.

Keywords: lead; zebrafish larvae; behavioural toxicology; hormesis; hyperactivity

Introduction

Lead is known to be deleterious to almost all living organisms even in smaller amounts (Pokras and Kneeland, 2008). In humans, especially children, deficits in cognitive and academic skills have been reported in blood Pb concentrations lower than 5 µg /dL (Lanphear et al., 2000). In both young animals and humans, the nervous system, especially the brain, has been widely reported as the most vulnerable to Pb toxicosis due to its rapid growth that may incorporate Pb and the immature blood brain barrier (Flora et al., 2012). Furthermore, associations between subclinical Pb toxicosis and altered behaviour such as delinquent, antisocial and aggressive behaviours in humans have been reported in several studies (Nevin, 2000; Olympio et al., 2010; Sciarillo et al., 1992). Zebrafish are increasingly being used as a model organism due to their close homology (71%) with the human genome (Howe et al., 2014) . In zebrafish, Pb exposure to various levels and at different stages of the zebrafish development has been known to induce an array of neurobehavioral derangements such as memory deficit, altered coloured preferences, altered responses to environmental stimuli such as locomotor activity patterns under light and dark illumination and sensorimotor responses among others (Chen et al., 2012; Dou and Zhang, 2011; Fraysse et al., 2006; Lefauve and Connaughton, 2017; Xu et al., 2016; Zhao et al., 2019). This neurotoxicity is suggested to arise due to direct damage to the nervous tissue through oxidative stress or alteration on the neurotransmitters and or their receptors (Lee and Freeman, 2014a; Tu et al., 2018; Weber et al., 1997). For example, Pb^{2+} arouses Ca^{2+} and calmodulin to stimulate and modulate the release of neurotransmitters in neurons (Zhong et al., 2017). In addition, Pb exposure disturbs the balance of pro-oxidants and antioxidants, causing oxidative stress and Pb poisoning (J. H. Kim and Kang, 2017). In vivo studies have suggested that Pb exposure might induce increases in antioxidant responses in fish through the production of reactive oxygen species (ROS) (Kim and Kang, 2017; Maiti et al., 2010). Lead exposure in fish also has toxic effects on membrane structure and function owing to its high affinity to red blood cells, which increases susceptibility to oxidative stresses (Gurer and Ercal, 2000).

My results in the zebrafish embryo exposed to Pb developmentally were characterised by cardiovascular toxicity and neuromuscular toxicity within the Kabwe water lead levels and agreed with other environmental studies at low Pb exposure which had altered social behaviours (Pokras and Kneeland, 2008). Although numerous studies involving zebrafish exposed to low Pb level have shown negative impact on embryos and adult fish behaviours (Chen et al., 2012; Lee and Freeman, 2014a, 2014b; Lefauve and Connaughton, 2017; Li et al., 2019a, 2019b; Tu et al., 2018; Weber et al., 1997; Zhao et al., 2019), no acute studies have examined the effect of very-low Pb exposure like the Kabwe water lead levels (range 0.01-94 µg/L (Nachiyunde et al., 2013)).

The paucity of information on the adverse effects of Kabwe water Pb levels, Zambia on aquatic life especially young larval fish was my motivation to investigate the effects of low environmental Pb levels on behavioural and oxidative responses in larval zebrafish. To this end, I conducted this study

and asked the following questions: Do acute exposure to levels of Pb, as they occur in Kabwe, causes locomotor pattern activity change under dark/light illumination? If so, are the resulting neurobehavioural changes accompanied by oxidative stress responses?

Materials and Methods

Fish husbandry and larviculture

Adult zebrafish from a wild-type laboratory strain specifically kept as a breeding stock were used. Fish were maintained at 26–28 ºC on a 14-hour light and 10-hour dark cycle in a ZebTec (Tecniplast, Italy) flow-through, reconstituted water system in the National Aquatic Bioassay Facility (NABF) at North -West University, South Africa. All experimental procedures were conducted in accordance and adherence to guidelines approved by the North–West University AnimCare Ethics Committee (Ethics number NWU-00269-16-A5). Zebrafish were bred in a 60 L iSpawn breeding tank with a 1/8" nylon mesh false bottom to protect fertilized eggs from being consumed by the adults. Eggs were collected ≤ 2 hours post fertilization (hpf), counted, and placed into Pb-free, glass culture dishes containing an embryo development medium (E3 medium: each litre contains 0.875 g NaCl, 0.038 g KCl, 0.120 g $MgSO_4$, 0.021 g KH_2PO_4, and 0.006 g Na_2HPO_4). The unfertilized embryos were removed under a Zeiss stemi microscope and the viable embryos were kept in an incubator at 28 ºC in Pb-free embryo media. After 24 hpf again the embryos were examined under the stemi microscope to remove any coagulated embryos. Half of the embryo medium (E3 medium) was replaced daily with freshly oxygenated medium until the test started at 120 hours post fertilization (hpf). Larvae were not fed throughout the test.

Lead (Pb) stock solution and exposure protocols

Lead acetate trihydrate (PbAc; Purity 99.5%) was purchased from Fluka chemika (Sigma-Aldrich), Buchs, Switzerland. A Pb stock solution of 10 mg/L was prepared from PbAc in ultrapure water and stored at 4 °C. The exposure doses of Pb were prepared from the stock after appropriately diluting with Pb-free embryo media to 3 µg/L, 91 µg/L and 250 µg/L Pb concentrations, respectively. The 3 µg/L was based on the average Pb levels in water and 91 µg/L was estimated based on the maximum Pb sampled nearest to the point source (Nachiyunde et al., 2013) and 250 µg/L was the included as highest level of exposure approximately three times the maximum Pb level in Kabwe water bodies.

Acute Pb exposure, recording and analysis of locomotor behaviour in 120 hpf old larval zebrafish

Larval zebrafish, 120 hpf, reared in Pb-free embryo media were exposed to Pb at three concentrations (3 µg/L, 91 µg/L and 250 µg/L) and a control group (0 µg/L). Our choice of the age of the zebrafish larvae (120 hpf) was based on standard protocol that recommends that all behaviour

studies are done between day 5-7 days post fertilization when fish are free swimming but feeding on the yolk sac (Strähle et al., 2012). The experimental design consisted of a negative control (n = 12) and exposure concentrations; 3 µg/L (n = 12), 91 µg/L (n = 12) and 250 µg/L (n =12). One larva per well was transferred to the 12 well testing plate using a plastic pipette with each well containing 3 mL of the respective exposure solution. The acute exposure was replicated four times in order to achieve twelve replicates (n=3 per group per run). The 12-well plate was placed in a Noldus DanioVision chamber (Wageningen, Netherlands) and recorded at 25 frames per second. Experiments were performed at 28 °C in embryo media at a 10-minute light: dark cycle intervals for a total time of 30 minutes. I excluded the first 2 minutes as habituation, and selected the 5th minute under the first dark phase and 15 min time point in the light phase as the statistical behaviour reference point as previously described by Li et al., (2019a). In addition, zones (center and outer) within each well were setup by digitally dissecting zones per plate using Noldus EthoVision XT15 software. The exposure and recording were repeated four times while changing the position of each treatment to minimize the effect of positioning of the larvae in the DanioVision chamber. Thereafter, the larval zebrafish were sacrificed in cold ice water as approved by the North West University ethics committee and immediately preserved in 1.5 mL Eppendorf tubes with cold RNAlater® (Ambion, Johannesburg, South Africa). To obtain adequate pooled samples (5-10 larvae) for RNA extraction based on pre-experimental trials I had earlier conducted, additional exposures were carried out across the groups (n=24) using same conditions and concentrations of test compound. The samples were stored at -80 °C prior to RNA extraction.

RNA extraction and real-time PCR analysis

An RNA standard isolation protocol was used. For the initial RNA extraction, 5-10 pooled larvae samples were homogenized in TRI Reagent® (SIGMA Life Science, St. Louis, MO, USA) with a zirconia bead using a tissue lyser; chloroform was added, and samples were vortexed and then centrifuged at 13,000 g for twenty minutes at 4 °C. The supernatant was then mixed with 350 µL of 70% ethanol and then placed in the FastGene® RNA binding column (NIPPON Genetics Co. Ltd., Tokyo, Japan). Afterwards, the standard FastGene® RNA Basic kits (NIPPON Genetics Co. Ltd., Tokyo, Japan) protocol was followed for the rest of the steps. The RNA was eluted from the membrane using RNase free ultrapure MilliQ water. The RNA quality was assessed by spectrophotometry (OD 260:280 ratio) using a NanoDrop 1000A Spectrophotometer (Delaware, USA). The TOYOBO first strand cDNA synthesis kit ReverTra Ace-α (TOYOBO Co., Ltd., Life Science Department, Osaka, Japan) was used for cDNA synthesis according to manufacturer instructions.

The real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis (StepOnePlus Real-Time PCR System, Applied Biosystems, USA) was performed using a 10

µL PCR reaction mixture containing 5 µL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 µL of 5 µM forward and reverse primers (Thermo Fisher Scientific, Life Technologies Japan Ltd., Japan), 20 ng of cDNA of each pooled zebrafish larvae samples, and 2.2 µL of distilled water. The qRT-PCR condition for all target genes was 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C, 62 °C or 65 °C for 30 s depending on the annealing temperature of a given primer set as shown in Table 2.2.1. Most of the primers used were obtained from published works (Jin et al., 2010; Shi and Zhou, 2010; Stancová et al., 2015) and some were generated using the NCBI and Primer3 tools. The gene expressions of various oxidative stress related genes were quantified with the relative absolute method (Livak and Schmittgen, 2001) using tubulin 1 alpha (*Tuba I*) as a house keeping reference gene (Table 2.2.1**)**. The choice of *Tuba I* as a housekeeping gene was based on its stability and constant expressions in both control and Pb exposed groups after preliminary validation when compared with beta actin, which is in agreement with (Mccurley and Callard, 2008) in zebrafish at various developmental stages following chemical treatment. The primer efficiency range was from 96.5% to 100.4%.

Measurement of Pb in exposure test solutions

The Pb in exposure media were measured for verification purposes. Freshly constituted Pb exposure dose solutions using 99.5% lead acetate trihydrate and the actual Pb exposure solutions that remained in wells after sacrificing the zebrafish larvae during the study were collected and analyzed. After acidification, concentration of Pb was measured using the inductively coupled plasma mass spectrometry (ICP-MS, 7700 series; Agilent Technologies, Tokyo, Japan). The instrument detection limit for Pb was 0.001 µg/L.

Data Analysis

The larval locomotor behaviour data and gene expression data were analysed using GraphPad Prism software (Prism 7 for Windows; Version 5.02, California USA). The data were reported as mean and SEM (standard error of the mean). Data were tested for criteria of normality using the Kolmogorov–Smirnov test and homogeneity of variance using Levene's test. If data were normally distributed an analysis of variance (one-way ANOVA) was performed and the differences among test groups were assessed with the Tukey's test. For non-parametric data the Kruskal–Wallis test and Dunn's Multiple Comparison test or Mann-Whitney U test was used. Statistically, the difference between groups was considered to be significant at $p < 0.05$ (*).

Results

Concentration of Pb in exposure test solutions

The nominal Pb dose represents the theoretical doses upon which the exposure solutions were prepared (0, 3, 91 and 250 µg/L) using pure grade 99.5% lead acetate trihydrate. The actual Pb concentrations detected by ICP-MS (100 - 106.7% recovery) in the exposure solutions were within an 8% range (0, 3.2, 93 and 252.6 µg/L, respectively).

Effect of acute Pb exposure on locomotor behaviour and molecular subcellular responses in larval zebrafish (120 hpf old Danio rerio larvae)

Under locomotor behaviour, I investigated the mean distance covered, the mean speed, cumulative mobility, cumulative immobility, mobile frequency, immobile frequency, durations (time) spent in the center/outer of the plate (Fig. 2.2.1), dark/light illumination related locomotor behaviour with emphasis on the distance covered and speed as shown in Fig. 2.2.2. and Fig. 2.2.3

Lead effects on the mean distance covered and speed of larval zebrafish

The mean total distance travelled across the exposed group was significantly higher (Tukey test, $p < 0.01$) than that of the control group. The mean distance for the control, 3 µg/L, 91 µg/L and 250 µg/L were 51,891 ± 3054 mm,77,857 ± 5108 mm, 88,623 ± 4737 mm and 85,287 ± 3545 mm, respectively as shown in Fig. 2.2.1A. However, there was no significant difference in the mean distance covered between the Pb exposed groups. Regarding the mean swimming speed, I observed similar trend with the pattern of the mean distance covered. The exposed groups had significantly higher speed implying faster speed compared with that of the control (Tukey test, $p < 0.05$). The mean speed for the control, 3 µg/L, 91 µg/L and 250 µg/L were 28.8 ± 1.7 mm/s, 43.3 ± 2.8 mm/s, 49.3 ± 2.6 mm/s and 47.4 ± 2 mm/s, respectively (Fig. 2.2.1B).

Lead effects on the cumulative mobility time

Significant differences in cumulative mobility (Dunn's multiple comparison test, $p < 0.001$) were observed in the control (133.6 ± 15.4 s) versus 91 µg/L (275.9 ± 11.9 s) and 250 µg/L (273.4 ± 15.3 s) as shown in Fig. 2.2.1C. Among the exposed groups compared with that of the control we observed a reduction in the time spent immobile by the larval zebrafish during the behavioural assessment period. The control spent statistically significant less time being immobile with mean of 1652 ± 17.9 s while the larval zebrafish in the 3 µg/L, 91 µg/L and 250 µg/L spent 1531 ± 21.8 s, 1466 ± 17.4 s and 1478 ±16.3 s respectively, as shown in Fig. 2.2.1D.

Lead effects on the mean mobile and immobile frequency

The total number of times the larval zebrafish spent mobile were significantly higher in the exposed groups compared to the control. The average mean frequencies were 1348 ± 158.1 s; 2115 ± 157.3 s; 2638 ± 11.9 s and 2580 ± 107.8 s for the control, 3 µg/L, 91 µg/L and 250 µg/L, respectively

(Fig. 2.2.1E). Regarding the immobility frequency, the control group recorded a significantly lower frequency of 1247 ± 139 s when compared to the exposed groups namely; 3 µg/L which recorded 1770 ± 118.2 s; 91 µg/L with 2184 ± 82.8 s and 250 µg/L with 2210 ± 90 s. Among the exposed groups, there was an increased pattern in the immobility frequency with an increased in Pb exposure dose. A significant difference was seen between 3 µg/L and 250 µg/L immobility frequencies (Fig. 2.2.1F).

Lead effects on the time spent by larval zebrafish in the centre/outer zones

At the lowest level of exposure (3 µg/L - mean time was 164.6 ± 22.5 s) no difference in the centre zone preferences to that of the control group (155.7 ± 29 s) were recorded. However, a significant reduction in the time spent in the center zones was observed at 91 µg/L (86.4 ± 19.3 s) compared to the control group **(**Fig. 2.2.1G**)**. The exposure groups spent more time in the outer zones of the well although not statistically significant when compared to the control group. Comparing the behaviour among the exposed groups only showed a significant ($p < 0.05$) outer zones preference between 3µg/L (90.9 ± 1.6 s) and 91 µg/L (95.2 ± 1.1 s) with the of the larval zebrafish spending more time in the outer zone at 91 µg/L (Fig. 2.2.1H)

Lead effects on the distance covered and speed of larval zebrafish in the dark/light phases

In general, the exposed groups covered longer distances in both the dark and light phase compared to that of the control group (Fig. 2.2.2). Mean distance recorded at 5 minutes during the first dark phase was significantly higher in 91 µg/L (2642 ± 193.1 mm) than that of the control (1655 ± 163.7 mm). Under the light phase, the control group (1199 ± 126.2 mm) had covered significantly lower ($p < 0.01$) distance at 15 minutes time bin compared to the 3 µg/L (2773 ± 177.6 mm), 91 µg/L (3156 ± 305.4 mm) and 250 µg/L (3289 ± 177.6 mm) as shown in Fig. 2.2.2 **(**A, B and C**)**, respectively. During the second dark phase, the distance covered by the control group (2182 ± 318.5 mm) was lower than the exposed groups and the difference was significant in the 91 µg/L group (3585 ± 242.7 mm) and 250 µg/L group (3396 ± 328.6 mm) at 25 minutes time bin (Fig. 2.2.2 B and C)**,** respectively**.**

For swimming speed, the trend was similar as observed with the distance covered. At 5 minutes time bin, the speed of the 91 µg/L group (44 ± 3.2 mm/s) was significantly faster ($p < 0.05$) than that of the control group (27.8 ± 2.7 mm/s) as shown in Fig. 2.2.3B. At the 15 minutes time bin of the light phase, the larval zebrafish exposed to Pb were significantly faster than the control group ($p < 0.01$). The mean speeds at 15 minutes time bin were 20 ± 2.1 mm/s, 46.2 ± 5.8 mm/s, 52.6 ± 5.1 mm/s and 54.8 ± 3 mm/s for the control, 3 µg/L, 91 µg/L and 250 µg/L, respectively (Fig. 2.2.3B and C**).**

Lead effects on the subcellular oxidative stress responses in larval zebrafish

The messenger ribonucleic acid (mRNA) level of SOD2 in the larval zebrafish samples showed a slight increased level of expression in the exposed groups with 1.6-fold change at 3 µg/L, 1.3-fold

change at 91 µg/L and 1.7-fold change at 250 µg/L levels of exposure though not statistically significant (Fig. 2.2.4A). On the other hand, acute effect of Pb was not accompanied by statistically significant *GST* mRNA across the exposed groups though a slight increase in the mRNA levels was seen at 250 µg/L with 1.5-fold change (Fig. 2.2.4B). Similarly, the mRNA for *GPX* expression levels showed a slight increase at 3 µg/L of 1.2-fold change and at 250 µg/L of 1.5-fold change though not statistically significant. At 91 µg/L, I observed a slight decrease in the mRNA levels of *GPX* of 0.8-fold change which was not statistically significant (Fig. 2.2.4C). Additionally, we observed that the mRNA levels of *HO-1* were upregulated in exposed groups when compared to the control with 2.8-fold change, 2.1-fold change and 1.8-fold change for the 3 µg/L Pb, 91 µg/L Pb and 250 µg/L Pb, respectively. The upregulation was statistically significant in the 3 µg/L exposed group compared to the control (Fig. 2.2.3D).

The mRNA levels of *Ucp-2* were significantly upregulated ($p < 0.05$) in exposed groups when compared to the control with 3.9-fold change, 3.5-fold change and 3.4-fold change for the 3 µg/L, 91 µg/L and 250 µg/L, respectively (Fig. 2.2.4E). The expression of the mRNA levels for the *Bcl-2* gene were significantly upregulated following the Pb exposure across the exposed groups. The fold changes were 4.6-fold change, 6.3-fold change and 5.5-fold change at 3 µg/L Pb, 91 µg/L Pb and 250 µg/L Pb, respectively (Fig. 2.2.4F**).** In addition, the expression of *Cox-I* which is involved in the mitochondrial respiratory chain and ATP synthesis was significantly upregulated in 3 µg/L Pb and 91 µg/L Pb groups when compared to the control with 3.9-fold change and 3.2-fold change, respectively (Fig. 2.2.4G**)**. The expression of the mRNA levels for the *TP53* gene were slightly upregulated following the Pb exposure across the exposed groups. However, only the mRNA levels in 250 µg/L Pb group with a 2.5-fold (Fig. 2.2.4H) change were statistically significant when compared to the control ($p < 0.05$).

Table 2.2.1: Primers used for real-time qPCR with annealing temperatures used and PCR product lengths

Target gene	Primer Sequence (5'-3')	Annealing Temperature (◦C)	Product length (bp)	Reference
Tubulin alpha I (*Tuba I*)	**F:** TTTGTGCACTGGTACGTGGG **R:** CCACACTCTCAGCTCCAACCTC	60	112	a
Glutathione-S-transferase (*GST*)	**F:** ACAACCTGTTCGATCTCCTGCTGA **R:** GTTGCCGTTGATGGGCAGTTTCTT	60	161	(Jin et al., 2010)
Superoxide dismutase (*SOD*)	**F:** CGCATGTTCCCAGACATCTA **R:** GAGCGGAAGATTGAGGATTG	60	100	(Stancová et al., 2015)
Catalase (*CAT*)	**F:** AGTGCTCCTGACGTCCAGCCA **R:** TGAAGAACGTGCGCACCTGGG	65	115	(Jin et al., 2010)
Glutathione Peroxidase (*GPX 4b*)	**F:** GGACGATCCAAGCGTGGTGGA **R:** CAGCCGTCACACGTCTGGGC	60	148	(Stancová et al., 2015)
Uncoupling protein 2 (*Ucp-2*)	**F:** TGGCTCAACCCACTGATGTA **R:** CAATGGTCCGATATGCGTC	62	102	(Jin et al., 2010)
Heme Oxygenase 1 (*HO-1*)	**F:** GGAAGAGCTGGACAGAAACG **R:** CGAAGAAGTGCTCCAAGTCC	62	107	(Shi and Zhou, 2010)
B-cell lymphoma 2 (*Bcl-2*)	**F:** AGGAAAATGGAGGTTGGGATG **R**: TGTTAGGTATGAAAACGGGTGGA	62	83	(Jin et al., 2010)
Tumour protein p53 (*TP53*)	**F:** CCCAGGTGGTGGCTCTTGCT **R:** GAGTGGATGGCTGAGGCTGTTCT	62	113	a
Nuclear factor erythroid 2 (*Nrf2*)	**F:** GACAAAATCGGCGACAAAAT **R:** TTAGGCCATGTCCACACGTA	65	165	(Shi and Zhou, 2010)
Cytochrome c oxidase subunit I (*Cox-I*)	**F:** GGATTTGGAAACTGACTTGTG **R:** AAGAAGAAATGAGGGTGGAAG	60	105	(Jin et al., 2010)

Note: a: Primer sequence I designed using NCBI and Primer3 tools. F and R represents forward primer and reverse primer sequences, respectively.

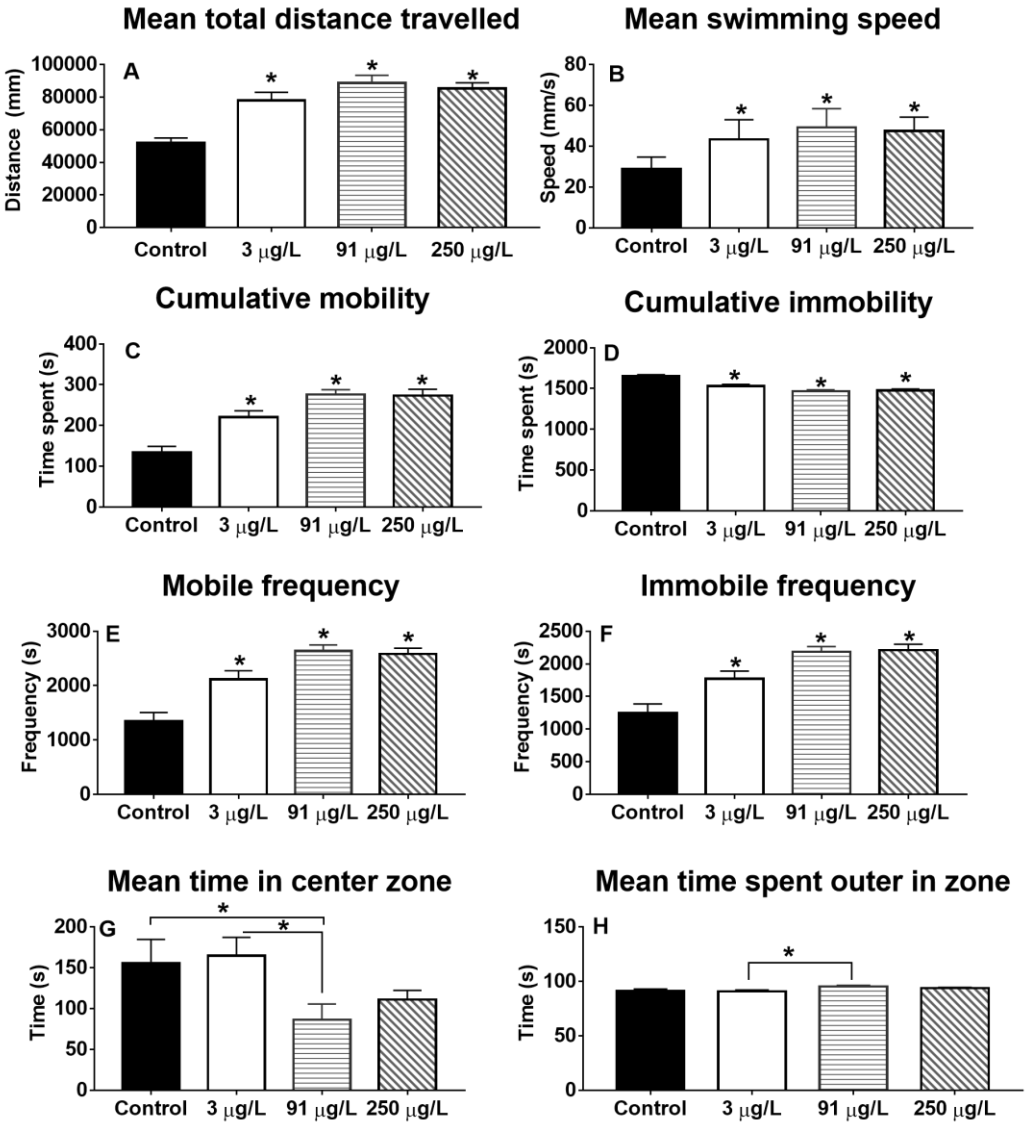

Fig. 2.2.1. Locomotor behaviour of larval zebrafish (120 hpf) exposed to 3 µg/L ,91 µg/L and 250 µg/L concentrations of Pb for 30 minutes during the test. For each exposure treatment n =12. Significance was regarded at $p < 0.05$(*).

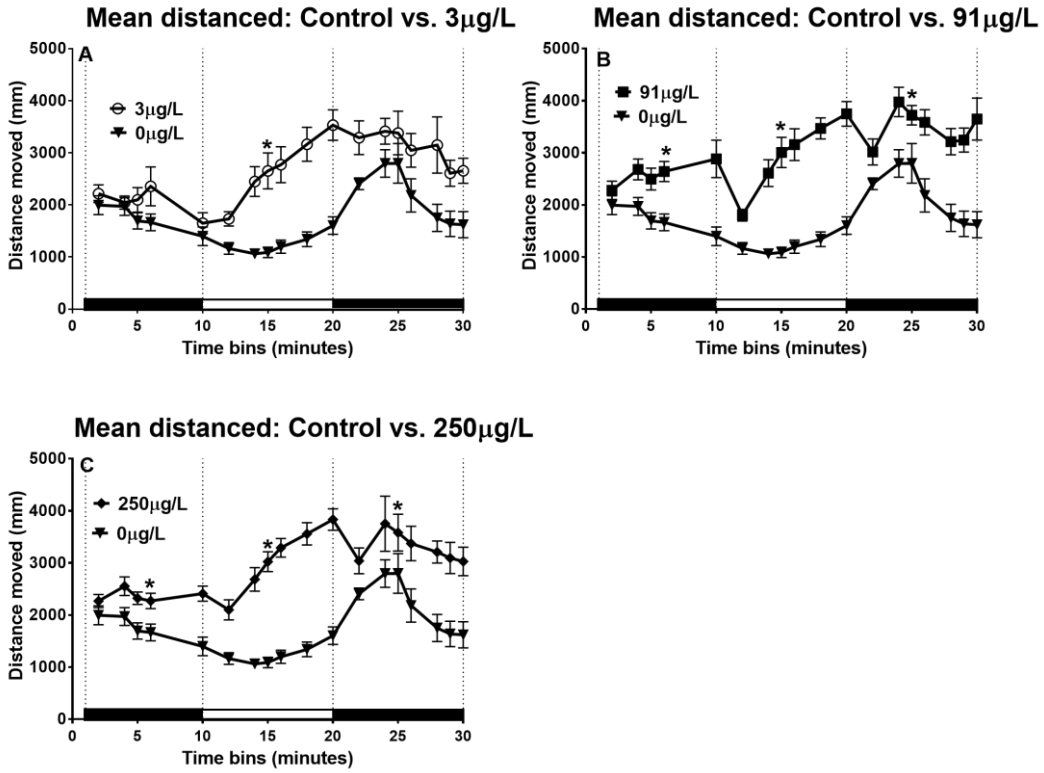

Fig. 2.2.2. Locomotor behaviour of larval Zebrafish (120 hpf) distance moved for exposed to 3 µg/L(**A**), 91 µg/L(**B**) and 250 µg/L (**C**) Pb concentrations and control for 30 minutes under dark/light transition illumination (5,15 and 25 minutes) during the test. The dark and white bar represents dark and light phases, respectively. For each exposure treatment n=12 Significance was regarded at $p < 0.05$ (*).

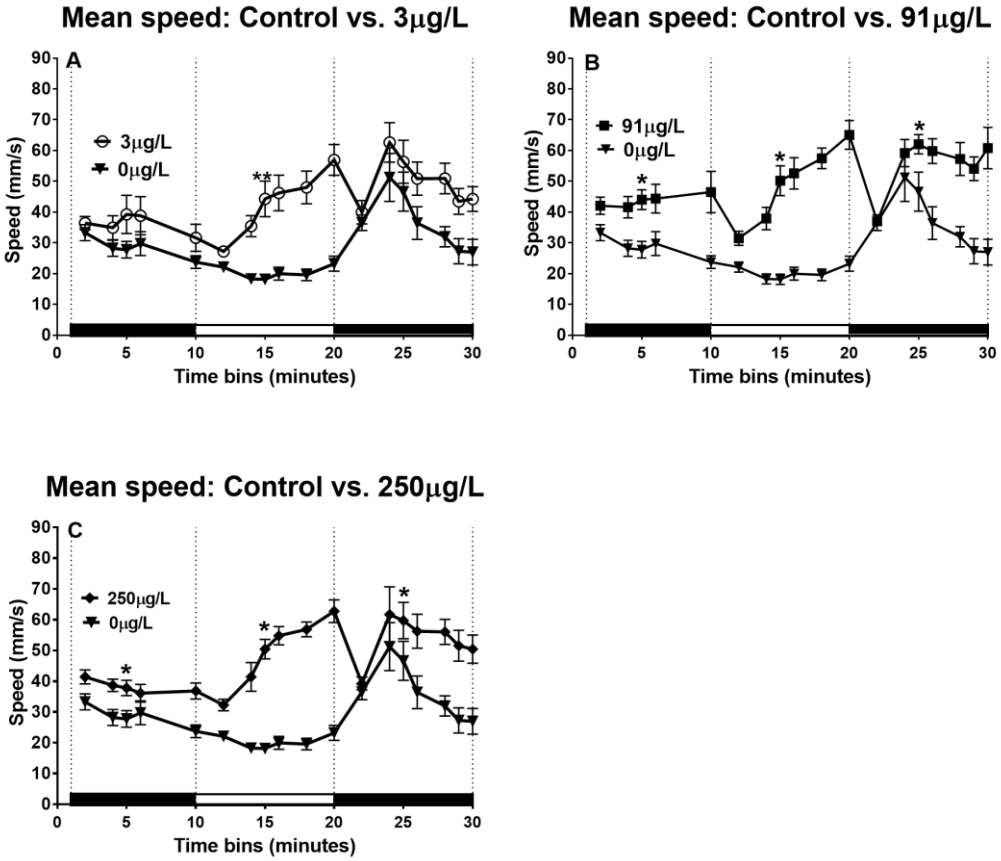

Fig. 2.2.3. Locomotor behaviour of larval Zebrafish (120 hpf) speed for exposed to 3 µg/L(**A**), 91 µg/L (**B**) and 250 µg/L (**C**) Pb concentrations and control for 30 minutes under dark/light transition illumination (5,15 and 25 minutes) during the test. The dark and white bar represents dark and light phases, respectively. For each exposure treatment n = 12. Significance was regarded at $p < 0.05$ (*)

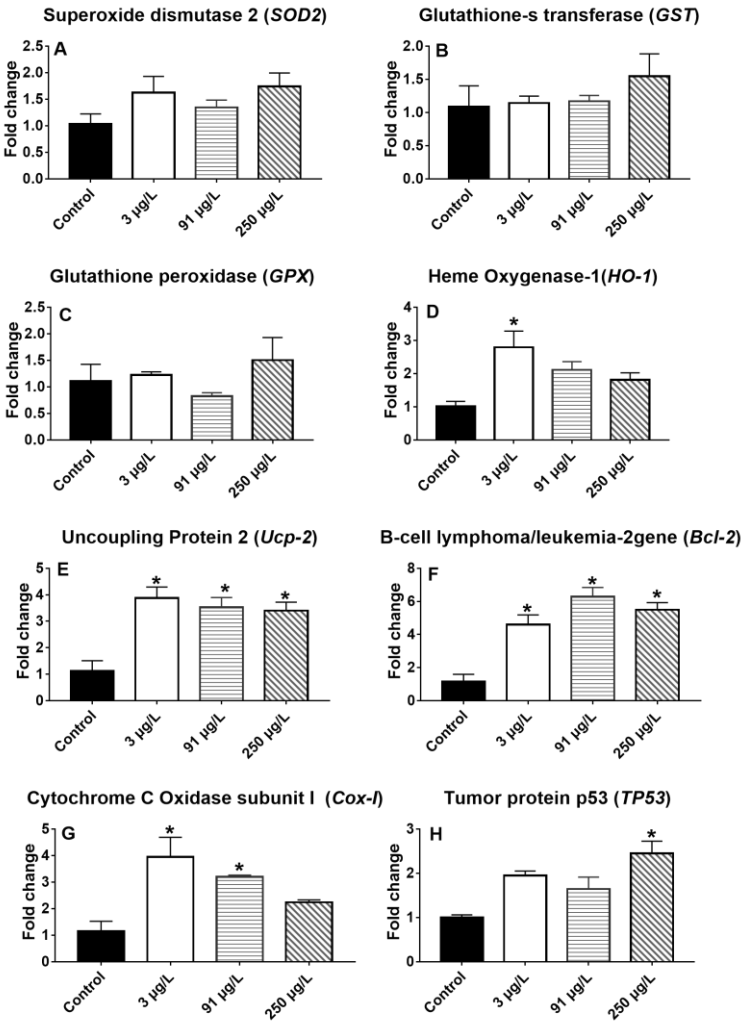

Fig. 2.2.4. Expression of *SOD2*, *GST*, *GPX*, *HO-1*, *Ucp-2*, *TP53*, *Cox-I* and *Bcl-2* (A, B, C, D,E,F,H and G) in the pooled samples from the larval zebrafish after the acute Pb 30 minutes dark/light illuminations (120 hpf old larval zebrafish) groups at 3 µg/L Pb, 91 µg/L Pb and 250 µg/L Pb concentrations and control. Values normalized against Tubulin alpha-1A (house-keeping gene) and represent the mean mRNA expression value ± SEM (n = 3 pooled samples) relative to those of the controls. The asterisk represents a statistically significant difference when compared with the controls *at $p < 0.05$.

Discussion

In this study, I investigated the effects of environmentally relevant levels of Pb (as reported in Kabwe town and Zambia (Nachiyunde et al., 2013) on ensuing subcellular oxidative stress responses of the primary antioxidant, pro-apoptotic and anti-apoptotic genes and locomotor behaviour of larval zebrafish. Acute Pb exposure to larval zebrafish for 30 minutes induced hyperactivity characterised by significant increases in distance covered, swimming speed and mobile frequency as well as increased distance covered and speed under light illumination. I observed that the hyperactivity was significantly increased in exposed groups under light conditions when the zebrafish are reported to assume relaxed state (Lee and Freeman, 2014b) indicating Pb's potentiating neurobehavioural effects. My findings were similar to the activity of zebrafish larval that were exposed as embryos to low Pb of 25 µg/L (Chen et al., 2012) and contrary to findings reported in other studies where they observed hypoactivity (Dou and Zhang, 2011; Lefauve and Connaughton, 2017; Li et al., 2019a).

The difference between some of the foregoing studies and the present study could have been related to the dose of Pb, the duration of exposure and the developmental stages of the zebrafish. For instance Li et al., (2019a) reported a concentration dependent reduction in locomotion of adult zebrafish exposed Pb over a 14 day period to 1 µg/L , 10 µg/L and 100 µg/L Pb, respectively. Dose and duration effects in Pb exposure in fish have been reported in the Mirror carp exposed to 50 µg/L of Pb over a 30 day period with hyperactivity reported in the first week and last week of exposure (Rehman, 2003). Other neuroactive chemicals such an cholinesterase inhibitor, paraoxon showed hyperactivity in a lower exposure range and hypoactive with a 100-fold increase in exposure concentration (Yozzo et al., 2013). This study confirms the responses that were obtained using the mammalian models, humans and rodents, exposed to chronic low Pb concentrations where hyperactivity was been reported as the neurobehavioural end point (Ma et al., 1999); thereby showing the application value of the zebrafish model to assess Pb exposure responses. While our experimental design was not aimed at explaining the mechanism behind this behaviour, our results demonstrated alterations in free locomotor activity manifested by hyperactivity, a recognized effect arising from Pb metal neurotoxicity (Atchison et al., 1987). In Mirror carp that exhibited Pb induced hyperactivity, a positive correlation between enhanced lipid peroxidation, a marker of oxidative damage in the brain and increased behavioural activity was reported (Rehman, 2003).

The abnormal behaviour of increased locomotor activity in larval zebrafish during daylight phases has ecological potential ecological implications in the form of increased predation. South et al. (2019) observed similar reduced anti-predator behaviour (i.e. increased locomotion) in mosquito larvae following exposure to low levels of the insecticide, dichlorodiphenyltrichloroethane (DDT). The authors indicated that the pollutant not only mediates the behaviour of the prey but also the interactions of the natural predator. This would inevitably result in potential alterations of the strength of trophic

interactions (South et al., 2019).

In case of the mRNA responses to acute exposure of environmentally relevant Pb in larval zebrafish, I observed a non-significant induction of the primary antioxidant enzymes apart from *HO-1* that was significantly upregulated at 3 µg/L. Functionally, *HO-1* catabolize free heme, that is, iron (Fe) protoporphyrin (IX), into equimolar amounts of Fe^{2+}, carbon monoxide (CO), and biliverdin (Gozzelino et al., 2010). In my study, the upregulation of *HO-1* at 3 µg/L Pb and its accompanying lack of significant upregulation at high levels (91 and 250 µg/L Pb) may have been a protective response against acute Pb exposure or a hormetic response (Calabrese et al., 2012). Heme oxygenase (*HO-1*) gene has been classified among the vitagene family exhibiting hormetic responses (Calabrese et al., 2004). According to the hormetic principles, low doses of drugs, toxicants, and natural substances may elicit a positive response in terms of adaptation to or protection from the stressor, whereas at higher concentration the toxic effect prevails (Calabrese et al., 2008). The hormetic dose – response can occur through different mechanisms: as a direct stimulatory response; after an initial disruption in homeostasis followed by the modest overcompensation response; or as a response to an "adapting" or "preconditioning" dose that is followed by a more challenging dose (Piantadosi, 2008).

Furthermore, a significant upregulation of the *Ucp-2* gene and *Bcl-2* across the exposed group was observed. The upregulation of *Cox-I* and the proapoptotic T*P53* genes seem to suggest an acute upregulation of ROS that led to an enhanced mobilization of the *Ucp-2* and *Bcl-2* genes to quail the ROS production as an initial protective mechanism (Paul M. Craig et al., 2007). *Ucp-2* functions, by an incompletely defined mechanism, to reduce the production of reactive oxygen species during mitochondrial electron transport (Giardina et al., 2008), while *Bcl-2* works to counter the effect of the *TP53* elevated gene expression and has been said to be an indicator of a conspicuous increase in ROS (Kowaltowski and Fiskum, 2005). Similarly, the significant upregulation of *Cox-I* expression in the acutely Pb exposed larval zebrafish points to the acute requirement for cellular responses to the generated ROS following exposure (Bourens et al., 2013).

While Pb has been reported as a neurotoxic element that causes behavioural dysfunction in fishes within days of exposure to sublethal concentrations (Weber et al., 1997), my study has demonstrated that effects in zebrafish larvae manifest within a very short period following exposure to environmentally relevant Pb levels. Taken together, my results showed that the lowest average Pb level of 3 µg/L Pb as found in Kabwe, Zambia may have deleterious effects to the same degree as higher exposure concentrations (91 µg/L and 250 µg/L) on aquatic life triggering a surge in ROS generation and hyperactive swimming behaviour as observed in the larval zebrafish.

The following limitations of using toxicogenomics in this study were identified. There is a long-standing debate as to what fold change in gene expression can be considered to be biologically significant (Mccarthy and Smyth, 2009). In this study I regarded a significant difference from the control to reflect those genes that are differentially expressed. It is however acknowledged that there

is a threshold for minimum fold gene change below, which differential expression is unlikely to be of any biological gene of interest. Therefore, this ad hoc approach probably provides an over estimation of the effects likely to occur following exposure to low levels of Pb. It is further acknowledged that the use of gene expression profiling as an indicator of sub-cellular changes needs to be verified through assessment of molecular and biochemical to reveal and confirm precise mechanisms of action (Fielden and Zacharewski, 2001). Due to the small sample volume I was unable to verify the increased antioxidant responses through analyses of enzymatic (e.g. superoxide dismutase and catalase) and non-enzymatic (lipid peroxidase and protein carbonyl) compounds. However, based on the studies (Ahmadifar et al., 2019; Parolini et al., 2017; and Safari et al., 2017) where similar fold changes were accompanied by significant biochemical changes, seem to support that the gene expression (i.e. upregulation of the anti-oxidant genes) results in increase of the enzymes thereby combatting ROS formation. It is then this premise that I postulated that the gene expression may be a good indicator (biomarker) of antioxidant responses against ROS formation.

Conclusions

Environmentally relevant concentrations of Pb as they occur in Kabwe could be detrimental to aquatic life especially in larval fish. Acute exposure to the environmentally relevant Pb levels attenuated larval zebrafish behaviour by inducing hyperactivity under dark/light illumination. This locomotor activity pattern alteration could be linked to altered neurobehavior via neurotoxicity mediated by oxidative stress or direct Pb neuro-intoxication due to lack of fully formed brain blood barrier. This has potential ecological ramifications through alterations in predator-prey interactions. However, the degree to which these observed effects following an acute exposure period to low Pb levels will persist during prolonged exposure needs to be investigated further.

CHAPTER 3:

Effects of lead in rats and the application of wild rat teeth in environmental lead assessment

3.1 Effects of lead exposure with zinc co-administration on multi-organ lead uptake, ALAD and oxidative stress genes responses in liver, kidney and brain in Sprague-Dawley rat

Abstract

In the natural environment lead (Pb) is mostly found together with zinc (Zn). The current study investigated the impact of Zn on Pb tissue accumulation and Pb induced toxicities. Sprague-Dawley rats were exposed to the following levels of Pb and Zinc (n = 6 per group) : control (0 Pb mg/l; 0 Zn mg/l), low Pb (100 Pb mg/l; 0 Zn), low Pb-Zn (100 Pb mg/l, 100 Zn mg/l), high Pb (1000 Pb mg/l, 0 Zn), and high Pb-Zn (1000 Pb mg/l, 500 Zn mg/l) orally for 8 weeks. The results showed significant body weight gain reduction in rats that received only Pb at high exposure relative to the control. Furthermore, Zn co-administration influenced Pb accumulation in soft and hard tissues. The testes and muscles accumulated less Pb in low Pb-Zn but accumulated much Pb in high Pb-Zn compared to non-Zn supplemented groups, respectively. On the other hand, bone Pb levels were significantly reduced in high Pb-Zn group. Lead exposure without Zn co-administration at high exposure significantly inhibited aminolaevulinic acid dehydratase enzyme activity. Furthermore, I observed antioxidants gene dysregulation in the liver, kidney and brain. Heme oxygenase-1 was downregulated in the kidney and brain in low Pb group. Liver glutathione peroxidase and thioredoxin reductase-1 were downregulated in the high Pb group. These findings suggest that Zn co-administration with Pb may confer some protective effects against Pb induced toxicities depending on the amount of Pb and Zn ingested.

Keywords: Lead, Zinc, oxidative stress system, toxicity

Introduction

Lead (Pb) is a globally recognized toxicant that persists in the environment and whose environmental presence is directly associated with human activities (Zhang et al., 2015). The most recognized marker of Pb exposure is its ability to adversely impair and inhibit the functions of delta aminolaevulinic acid (ALAD) enzyme that eventually affects haeme synthesis with anaemia as sequela (Wani et al., 2015). Ingestion of dust or soil containing Pb through hand to mouth activities has been suggested as major route of exposure in children (Yabe et al., 2015). Once taken into the body, lead is distributed throughout the body, in the blood, soft tissues such as kidney, liver and brain and hard tissues such as bones and teeth (ATSDR 2019).

The most common manifestation of lead toxicity in human populations are neurological effects in children and cardiovascular effects with high blood pressure and heart disease in adults. Infants and young children are especially are highly sensitive to even low levels of lead, which may result in behavioral problems, learning deficits and lowered intelligent quotient (IQ) among others (Rubin and Strayer, 2008). Lead has been linked to the disruption of the dopaminergic function in experimental studies and induction of oxidative stress (Hsu and Guo, 2002), which are hypothesized candidates in the aetiology of Parkinson, Alzheimer and other neurodegenerative age-related diseases in advanced age. Epidemiological studies suggest that lead may be probable human carcinogen (Wijngaarden and Dosemeci, 2006) .

In the natural environment, Pb occurs as a mixture with other toxic or essential metals such as but not limited to zinc, silver or copper implying that children ingest these mixtures of metals (Carocci et al., 2016). A unique setup has been reported in children under the age of 7 years with Pb blood levels above the 5 μg/dL reference value from Kabwe ,Zambia (Yabe et al., 2015) in an environment where both Pb and zinc (Zn) occur together in high amounts with high bioavailability (Ettler et al., 2020; Nakayama et al., 2011). The above situation suggest that children may be exposed to both metals at the same time in such an environment.

Some reports have shown that zinc co-administration may be protective against lead toxicities (Piao et al., 2007; Prasanthi et al., 2010; Zhai et al., 2018), coupled with some beneficial effects during chelation therapy of leaded rats (Flora and Tandon, 1990), studies of acute lead exposure with high zinc supplementation are limited. Moreover, other authors have demonstrated that zinc in certain amounts in humans may be toxic (Plum et al., 2010). Considering the foregoing, I conducted the current study to investigate the impact of zinc supplementation in mitigating lead toxicity. Specifically, I investigated tissue Pb accumulation, renal and hepatotoxicity using plasma biochemistry, total antioxidant capacity, and the molecular responses of the oxidative stress system of the liver, kidney and brain following acute Pb exposure in Sprague-Dawley rats.

Materials and Methods

Animal husbandry and exposure of lead and zinc

Animal husbandry and chemical exposures were conducted under ethical conditions approved by Hokkaido University (approval number: 16-0017). Seven weeks old Sprague Dawley male rats (n = 36) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The rats were kept and housed in 12 lead-free polypropylene cages in community of three rats (n = 3 per cage) with *ad libitum* access to both food (rodent chow, Labo MR Stock, Nosan Corporation, Yokohama, Japan) and distilled water and acclimatized for a week. Two cages with six (n = 6) rats were randomly assigned to one of the six exposure levels (control, low Pb (100 mg/L Pb), low Pb-Zn (100mg/L Pb; 100 mg/L Zn), high Pb (1000 mg/L Pb) and high Pb-Zn (1000 mg/L Pb; 500 mg/L Pb). There were no significant body weight differences among all the groups prior to exposure. Two different concentrations of Pb acetate: 100 and 1000 mg/L Pb (Wako Pure Chemical Industries, Osaka, Japan) and Zn acetate: 100 and 500 mg/L (Kishida Chemical Co., Ltd, Osaka, Japan) were administered via drinking water and gastric lavage every other day for eight weeks, respectively. The choice of Pb levels of exposure for the current study were based on the previous study in mice using that accumulated dose dependent Pb concentrations in their tissues at 100 and 1000 mg/L lead acetate (Togao et al., 2020). Body weight measurements were taken every two weeks and on the day of sacrifice and are shown in Fig. 3.3.1 under the results section. After fasting overnight, rats were euthanized under carbon dioxide with sevoflurane. After sacrifice, blood and other tissues namely; liver, kidney, brain, thyroid gland, spleen, femur (bone), muscle, and testis were collected for metal analysis. Some portion of blood samples were centrifuged at 3000 G for 10 minutes to obtain plasma for biochemical analyses. Small pieces liver, kidney and brain tissues were preserved in RNA Later (SIGMA Life Science, St. Louis, MO, USA) for molecular gene analysis. The rest of the tissues were stored at – 80 ◦C until analysis.

Lead tissue quantification

Blood and other tissues namely; liver, kidney, brain, thyroid gland, spleen, femur (bone), muscle, and testis analyzed for Pb metal analysis. The above soft and hard tissues were then section in 0.05- 0.2g in lead-free propylene weighing boats and were oven dried for 48 hours at 50 °C prior to microwave digestion. The dried samples were then digested as described below. In case of blood samples, 0.1 mL of the blood were measured and put in pre-washed digestion vessels. Then 5 mL of nitric acid (atomic absorption spectrometry grade, 30%; Kanto Chemical, Tokyo, Japan), and 1 mL of hydrogen peroxide (Cica reagent, 30%; Kanto Chemical, Tokyo, Japan) in readiness for digestion. The sample digestion was done using a ramped temperature program in a closed microwave system (Speed Wave MWS-2 microwave digestion system; Berghof, Eningen, Germany). The microwave system operating conditions used are given in Table 3.1.1. Following cooling, the sample solutions were

transferred into 15 mL polypropylene tubes and diluted to a final volume of 10 mL with ultra-distilled and de-ionized water.

The Pb concentration quantification was performed using the ICP-MS (7700 series; Agilent Technologies, Tokyo, Japan) as described by Nakata et al., (2016, 2015) with minor modifications. Additionally, quality control analysis was performed using DOLT-4 (dogfish liver; National Research Council of Canada) certified reference material. Replicate analysis of the reference material gave good recovery rates ranging 95-105%. The limit of detection (LOD) for Pb was 0.001 mg/L.

Plasma biochemistry analysis

Plasma kept at -80 °C was thawed for biochemical analysis. Twelve (12) parameters that included essential liver and kidney enzymes namely alkaline phosphatase (ALP), lactate dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and metabolites or metabolic by-products such blood urea nitrogen (BUN), triglycerides, albumin, uric acid, creatine, total cholesterol, total bilirubin, and total protein were measured using a combination of single and multiparameter SPOTCHEMTM II strips (Arkray Inc., Kyoto, Japan). A total volume of 100 µL of plasma per sample from 4 rats per group (n = 4) were used to measure using an Auto Dry Chemistry Analyzer SPOTCHEMTM SP- 4410 (Arkray. Inc., Kyoto, Japan) following manufacturer's instructions.

Delta-aminolaevulic acid dehydratase enzyme, catalase enzymes, the total antioxidant capacity and malonaldehyde (MDA) assays

Delta-aminolaevulinic acid dehydratase (ALAD) enzyme activity assay was carried out following the whole blood ALAD assay protocol according to Espín et al., (2015) and as previously reported by Nakata et al., (2021). From each whole blood sample 60 µL was obtained and was equally divided into 20 µL volumes and was placed into three separate 1.5 mL Eppendorf tubes namely; enzyme activity, blank and reactivated enzyme activity. To all the three tubes, 80 µL of 0.1% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA) lysate solution was added. Then to the enzyme's activity and reactivated activity tubes, 100 µL of 0.5 M phosphate-buffered saline (PBS) (pH 6.8) was added while 150 µL PBS was added to the blank tube. Then 50 µL of 60 mM 5-aminolevulinic acid (ALA) hydrochloride (Sigma–Aldrich, St. Louis, MO, USA) solution in PBS was added to the enzyme activity and reactivated tubes. Following, 50 µL of distilled water (DW) were added to the enzyme activity and blank tubes, respectively. To the reactivated tube only, 25 µL of 0.8 mM Zn acetate (Kishida Chemical Co., Ltd., Osaka, Japan) and 25 µL of 1 M dithiothreitol (DTT) (Wako Pure Chemical Corporation, Osaka, Japan) were added. All the three sample tubes were then incubated at 42 °C for 60 min after which the reaction was stopped by addition of 200 µL of 0.4 M trichloroacetic

acid (TCA) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 60 mM mercury chloride ($HgCl_2$) (Kanto Chemical Co). The samples were then centrifuged at 10000 rpm for 5 min and 400 µL supernatant was obtained and transferred to the new tubes. To the supernatant was added 750 µl of Ehrlich's reagent, (Nacalai tesque, Kyoto, Japan) in acetic acid (glacial; 99.7%, Thermo Fisher Scientific, Lancashire, UK) and perchloric acid (Kanto Chemical Co). The mixture was left to stand at room temperature for 10 min before reading the absorbance at 555 nm against the blank using an ultraviolet spectrophotometer (Shimadzu UV-2600, Shimadzu Inc., Kyoto, Japan). The ALAD activity was expressed in µmol porphobilinogen (PBG)/hr/L blood.

Catalase activity assay was performed using a modification of the colorimetric assay method described by Sinha, (1972). Briefly, 50 mg of rat liver tissue was homogenized in 50mM PBS (pH 7.0) and centrifuged at 16000 G for 45 min. Then the supernatant was obtained and used as the enzyme source. The reaction mixture of 2 mL PBS, 0.45 mL hydrogen peroxide (30% H_2O_2) (Kanto Chemical Co., Tokyo, Japan) and 0.025 ml supernatant was constituted. The absorbance was read at 570 nm against the blank using an ultraviolet spectrophotometer (Shimadzu UV-2600, Shimadzu Inc., Kyoto, Japan). The enzyme activity was expressed as micromoles (µmoL) of H2O2 consumed per minute.

The total antioxidant capacity was performed following a ferric reduction power (FRAP) method previously described by Benzie and Stain (1996). Pure grade and freshly prepared reagents were used. Firstly, 30mL of FRAP working reagent was prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 2,4,6- Tris(2-pyridyl)-S-Triazine)$_2$ abbreviated as TPTZ and 2.5 mL of ferric chloride hexahydrate ($FeCl_3$.6 H_2O). Then the assay was carried out in a 96 well plate. To each well, 65 µL 300 mM acetate buffer was added followed by 5 µL of ferrous sulphate ($FeSO_4.7H_2O$) standard solution and acetate buffer as a blank. Then 5 µL of plasma sample was added to the sample wells. And 100 µL of the FRAP working reagent was added across the wells. The absorbance was then measured using the enzyme-linked immunosorbent assay (ELISA) plate reader at 595 nm. The reaction was monitored for 8 minutes and readings at the 4th minute were taken as optimal values for calculation. A standard curve plot from the differences in absorbance at 595 nm was used to estimate the FRAP values for each sample expressed as µM Fe (II) equivalent.

RNA isolation and oxidative stress and related genes analyses in liver, kidney and brain tissues

To extract total RNA from the liver, kidney and brain tissues samples were homogenized in TRI Reagent® (SIGMA Life Science, St. Louis, MO, USA) with a zirconia bead using a tissue lyser; chloroform was added, and samples were vortexed and then centrifuged at 13,000 G for twenty minutes at 4 °C. The supernatant was then mixed with 350 µL of 70% ethanol and the rest of the steps were done using the NucleoSpin® kit (MACHEREY-NAGEL, Düren, Germany) with strict adherence to the. manufacturers instruction. The cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd., Life Science Department, Osaka, Japan). Most

of the antioxidant and related genes primer sets used in this study were obtained from previously published works (Albertini et al., 2007; Hamid et al., 2017) as shown in Table 3.1.2. The qRT-PCR (StepOnePlus Real-Time PCR system, Applied biosystems, Foster City, CA, USA) was performed by using 10 µl PCR reaction mixture containing 5 µL Fast SYBR Green Master Mix (Applied biosystems, Foster City, CA, USA), 0.4 µL of 5-µM forward and reverse primers (Thermo Fisher Scientific, Life Technologies Japan Ltd., Japan), 2 µL (20 ng of cDNA) cDNA template of the sample, and 2.2 µL of distilled water. The qRT-PCR condition for all target genes was 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C, forward and reverse primers (Thermo Fisher Scientific, Life Technologies Japan Ltd., Tokyo, Japan). The following qPCR conditions: 95 °C for 20 sec, followed by 40 cycles of 95 °C for 3 sec, 60°C for 30 sec were used. The gene expression levels were quantified using the relative absolute method (the ΔΔCT method) normalized with and beta-actin (Livak and Schmittgen, 2001).

Data analysis

The data analysis was performed using GraphPad Prism software (Prism 7 for Windows; Version 5.02, California, USA) and reported as mean and standard deviation (SD). Since the data did not follow a normal distribution, I log transformed the data for statistical analyses and retained the original values in the results and figures for easier interpretation and comparisons with other studies. In the present study, the difference between groups was deemed to be significant at $p < 0.05$ (*) using multiple Tukey's comparison test. Lower case alphabetical letters represented the difference (^{a,b,c} $p < 0.05$) The graphical representations were compiled using GraphPad Prism software.

Results

Lead exposure effects on body weight gain

The body weight changes in exposed and control rats is shown in Fig. 3.1.1. In general, the all the rats had steady weight gain that increased with the passage of time. From the 6th week, I observed a reduction in body weight gain. By the 8th week, the recorded body weight on in the high lead (1000 mg/L Pb) exposed group showed significant reduction when compared to that of the control ($p < 0.05$). No significant differences in body weight was observed among the exposed groups.

Lead tissue accumulation pattern

The Pb accumulation patterns following exposure are shown in Fig. 3.1.2. and Fig 3.1.3. In general tissue Pb accumulation followed a dose dependent pattern. Fig. 3.1.2. shows the Pb accumulation patterns in the blood, liver, kidney and brain tissues. The exposed groups significantly accumulated much Pb compared to the control group ($p < 0.05$; letters of the alphabet). Among the exposed groups,

the Pb accumulation levels increased with increase in the dose of Pb that was given (Fig. 3.1.2.). No significant differences were observed between the Pb and Pb with Zn co-administered groups. Fig 3.1.3. shows Pb distribution in other general soft and hard tissues. Significant Pb tissue accumulation between Pb only and Pb with Zn co-administered groups were observed in the testes and muscle where in both tissues, low Pb-Zn groups had much less Pb than the Pb only groups. At the high level of exposure, the zinc supplemented groups, in both muscles and testes accumulated much Pb than the high Pb only group (Fig 3.1.3). In case of bone for hard tissue, Pb accumulation was significantly different between the high Pb and high Pb-Zn groups, with the former accumulating much Pb (Fig 3.1.3). No significant differences in bone Pb accumulation were observed at the lower level of exposure.

Effects of lead on plasma biochemistry

In the present study as way of monitoring hepatic or renal damage following acute lead exposure with Zn co-administration, plasma biochemistry was assessed. Table 3.1.3. Shows the various parameters that were considered. No significant changes were observed in most of the parameters in among the exposed groups or exposed groups compared to the control group. The only significant change I observed was in the levels of blood urea nitrogen (BUN). The BUN levels were significantly reduced in the following order when compared to the control group: High Pb> High Pb-Zn > Low Pb-Zn.

Effects of exposure on ALAD enzyme, catalase enzyme, the total antioxidant capacity and malonaldehyde (MDA)

Aminolaevulinic acid dehydratase (ALAD) activity results are shown in Fig. 3.1.4A. At low exposure, no significant differences were observed. Significant ALAD activity inhibition was observed in the high Pb only group that was significantly different when compared with the control. Among the exposed groups, only the high Pb group had significantly reduced ALAD activity than the high Pb-Zn group only ($p < 0.05$).

Fig 3.1.4B shows catalase activity. The catalase activity was significantly elevated in the low-Pb and high Pb-Zn groups compared to the control ($p < 0.05$). Among the exposed groups, the catalase activity in the high Pb-Zn group was significantly upregulated than that of the high Pb-only group ($p < 0.05$).

The total antioxidant capacity in plasma samples is shown in Fig 3.1.4C. No significant differences were observed between the control and the exposed groups. Similarly, no significant differences were observed among the exposed groups.

Malondialdehyde (MDA) concentrations that were assayed as a measure of lipid peroxidation are shown in Fig 3.1.4D. In the present study, there were no significant differences in the MDA levels observed among the exposed groups and exposed groups relative to the control group.

Effects of exposure on antioxidant and related genes

Liver mRNA expression levels

Gene expression of essential antioxidant and related liver enzymes are shown in Fig. 3.1.5. The superoxide dismutase (*SOD1*) was upregulated in the low Pb-Zn group and in both the high and high Pb-Zn groups. The *Bax* gene mRNA levels were significantly upregulated in the low Pb-Zn group compared to the control group. On the other hand, the following genes were downregulated namely; *SOD1* (low Pb group), *TXNRD1* (low Pb-Zn and high Pb), *HO-1* (low Pb-Zn, high Pb and high Pb-Zn groups), *GPX* (high Pb), *Keap1* (high Pb group), *NRF2* (high Pb and high Pb-Zn groups), *Bax* (high Pb) and *NFKB* (high Pb and high Pb-Zn) as shown in brackets when compared with the control group. Comparatively, among the exposed groups between Pb only and Pb-Zn groups at high level of exposure, the following genes namely, *TXNRD1*, *GPX*, *Keap1*, *NFKB* and *Bax* were downregulated in the high Pb only group without Zn co-administration.

Kidney mRNA expression levels

Fig. 3.1.6 shows the mRNA expression levels of the antioxidants and related gene following exposure in the kidney. Gene upregulation was observed in the *SOD1*gene in the low Pb-Zn, high Pb and high Pb-Zn groups. Both *Keap1* and *NFKB* genes were also upregulated in the low Pb-Zn group compared to the control. Lead exposure furthermore downregulated gene expression of *HO-1* in low Pb only, *Keap1*in high Pb, *NFKB* in high Pb and *NRF2* in both high Pb and high Pb-Zn groups compared to the control group.

Brain mRNA expression levels

Antioxidant and related gene expression in brain tissue are shown in Fig. 3.1.7. Exposure upregulated the mRNA levels of *HO-1* in the low Pb-Zn, high Pb and high Pb-Zn. The *Bax* gene was equally significantly upregulated in the low Pb-Zn group compared to the control. Furthermore, exposure downregulated *CAT* gene expression in the low Pb, high Pb and high Pb-Zn groups. Other genes that were downregulated included; *TXNRD1* (low Pb group), *Keap1* (low Pb and high Pb-Zn groups), *TGFB1* (low Pb and low Pb-Zn), and *NRF2* (low Pb group).

Table 3.1.1. Microwave operating conditions for teeth and blood digestion.

Tissues		Blood	
Temperature (ºC)	Time (min)	Temperature (ºC)	Time (min)
160	5	160	5
190	20	190	10
200	20	75	10
100	5		

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Table 3.1.2: List of Primers used for the antioxidant and related gene expression investigations

Gene	Accession No.	5'-Forward primer-3'	5'-Reverse Primer-3	Product size	Primer efficiency (%)	Reference
ACTB	V01217.1	AAGTCCCTCACCCCTCCAAAAG	AAGCAATGCTGTCACCTTCCC		98.4	[2]
SOD1	NM_017050.1	CTGAAGGCGAGCATGGGTTC	TCTCTTCATCCGCTGGACCG	120	104	NCBI
CAT	NM_012520.2	GCCCTCTTGCCTCACGTTCT	ACATCGGGTTTCTGAGGGGC	126	97.6	NCBI
GPX	NM_030826.4	TCCCGTGCAATCAGTTCGGA	GGTAAAGAGCGGGTGAGCCT	155	100	NCBI
HO-1	NM_012580.2	ACACGGGTGACAGAAGAGGCTAA	CTGTGAGGGACTCTGGTCTTTG	108	96.8	NCBI
TXNRD1	NM_001351984.1	GTCACACCAACTCCTCTCGG	TGTGTCCTCGAGTTTCCAGC	150	100	NCBI
Nrf2	XM_006234398.3	GCACATCCAGACAGACACCA	CTCTCAACGTGGCTGGGAAT	189	100	[13]
Keap1	NM_057152.2	CAGATTGACAGCGTGGTCCG	TGAAGAACTCCTCCTCCCCGA	166	94.5	NCBI
TGFB1	NM_021578.2	AGGGCTACCATGCCAACTTC	CCACGTAGTAGACGATGGGC	168	100	[13]
NFKB	NM_199267.2	CATACGCTGACCCTAGCCTG	TCACTGAGCTCCCGATCAGA	79	100	[13]
Bax	NM_017059.2	AGGACGCATCCACCAAGAAG	CAGTTGAAGTTGCCGTCTGC	166	98	[13]

Key: (*ACTB*): Beta Actin, (*SOD1*): Superoxide dismutase 1, (*CAT*): catalase; (*GPX*): Glutathione peroxidase; (*HO-1*):Heme oxygenase -1, (*TXNRD1*): Thioredoxin reductase 1, (*NRF2*): Nuclear factor erythroid 2-related factor 2; (*Keap1*): Kelch-like ECH-associated protein,1 ; (*TGFB1*):Transforming growth factor beta 1; (*NFKB*): nuclear factor kappa-light-chain-enhancer of activated B cells; (*Bax*): Bcl-2 Associated X-protein

.

Table 3.1.3.: Various plasma biochemistry parameters examined

Parameter/Group	Control	Low Pb	Low Pb-Zn	High Pb	High Pb-Zn
Total Bilirubin (mg/dl)	0.4 ± 0.12	0.5 ± 0.14	0.5 ± 0.10	0.4 ± 0.15	0.5 ± 0.29
GOT (IU/l)	57 ± 11	67 ± 16.4	75 ± 34.4	66 ± 14.2	88 ± 33.3
GPT (IU/l)	46 ± 9	54 ± 12.4	52 ± 22.5	38 ± 11.2	48 ± 23.4
LDH (IU/l)	627 ± 252.3	896 ± 365.8	852 ± 539.4	1064 ± 431.3	740 ± 234
ALP (IU/l)	425 ± 83.2	360 ± 162.3	435 ± 303.7	276 ± 43.6	354 ± 78.7
Triglycerides (mg/dl)	202 ± 43.6	166 ± 79.6	191 ± 68.03	214 ± 99.84	192 ± 77.8
Total cholesterol (mg/dl)	75 ± 6.5	66 ± 11.7	75 ± 11.8	51 ± 24.9	50 ± 12.6
Total Protein (g/dl)	6.3 ± 0.70	6.6 ± 0.77	6.9 ± 0.75	6.8 ± 0.35	6.4 ± 0.63
Albumin (g/dl)	3.7 ± 0.13	3.6 ± 0.30	3.7 ± 0.45	3.7 ± 0.19	3.7 ± 0.57
Uric acid (mg/dl)	5.9 ± 1.5	6.7 ± 2.2	6.3 ± 2.5	5.7 ± 1.9	6.6 ± 1.6
Creatine (mg/dl)	1.1 ± 0.16	1.2 ± 0.25	1.2 ± 0.15	1.0 ± 0.22	1.0 ± 0.26
BUN	23 ± 0.89^{a}	21 ± 1.83^{ab}	20 ± 0.98^{bc}	17 ± 1.47^{c}	18 ± 1.55^{cd}

Key: (ALP): alkaline phosphatase, (LDH): lactate dehydrogenase, (BUN): blood urea nitrogen, (GOT): glutamic oxaloacetic transaminase, (GPT): glutamic pyruvic transaminase. ($^{a, b, c, d}$ represents $p < 0.05$ Tukey's multiple comparison test)

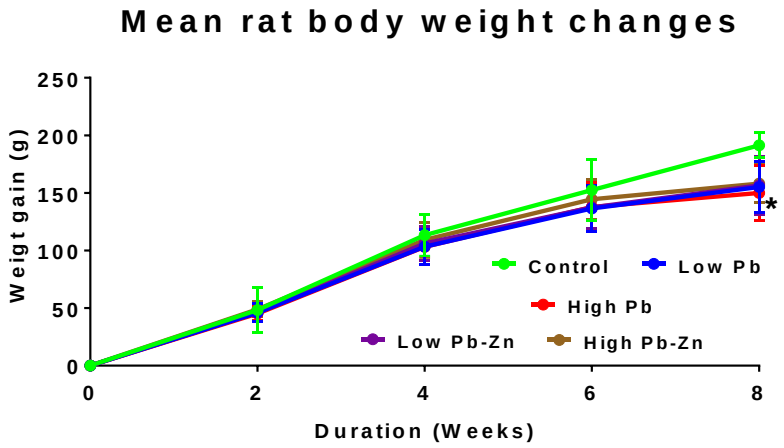

Fig. 3.1.1 Body weight changes in exposed and control rats during the 8 weeks exposure period (mean ± SD; n = 6, *p < 0.05; Tukey's multiple comparison test). Low Pb (100 mg/L Pb), Low Pb-Zn (100mg/L Pb; 100 mg/L Zn), High Pb (1000 mg/L Pb) and High Pb-Zn (1000 mg/L Pb; 500 mg/L Zn

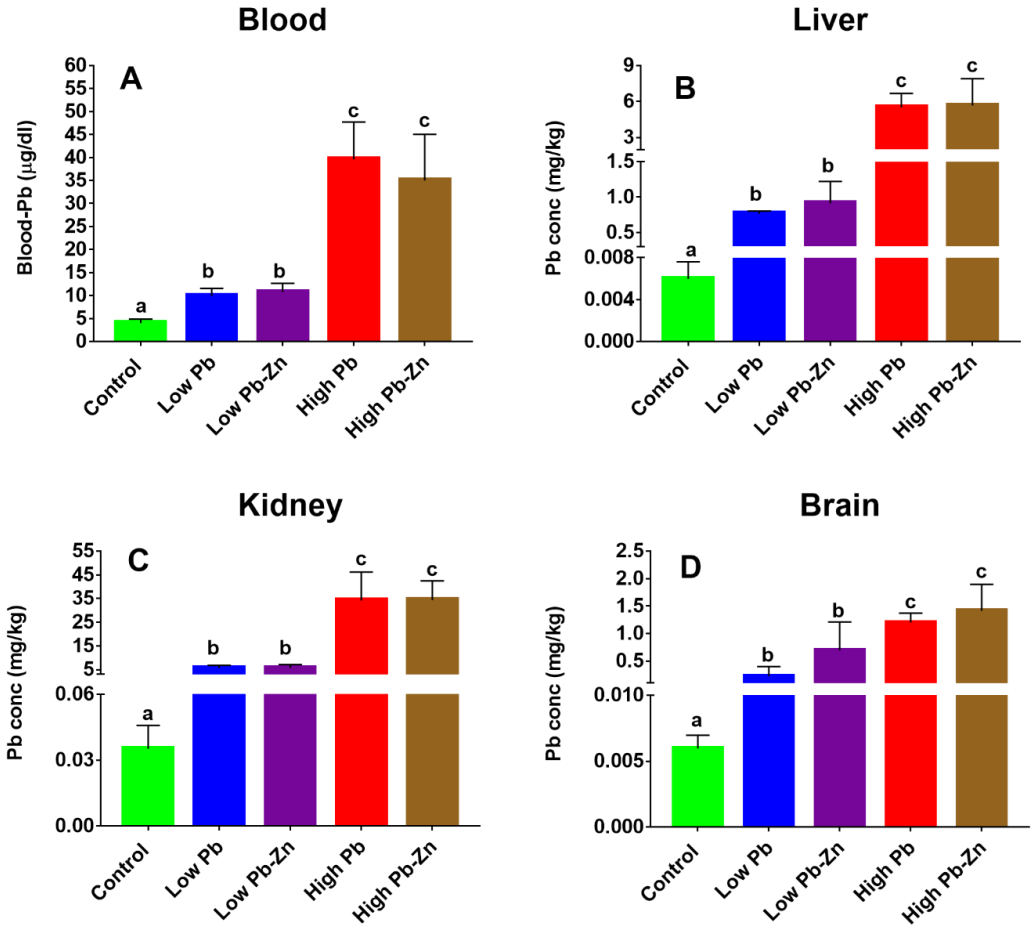

Fig. 3.1.2. Lead accumulation in blood (n = 6), Liver (mg/kg), Kidney (n = 3) and Brain (n =3). (Mean ± SD, n = 6, [a, b,c] $p<0.05$, Tukey's multiple test)

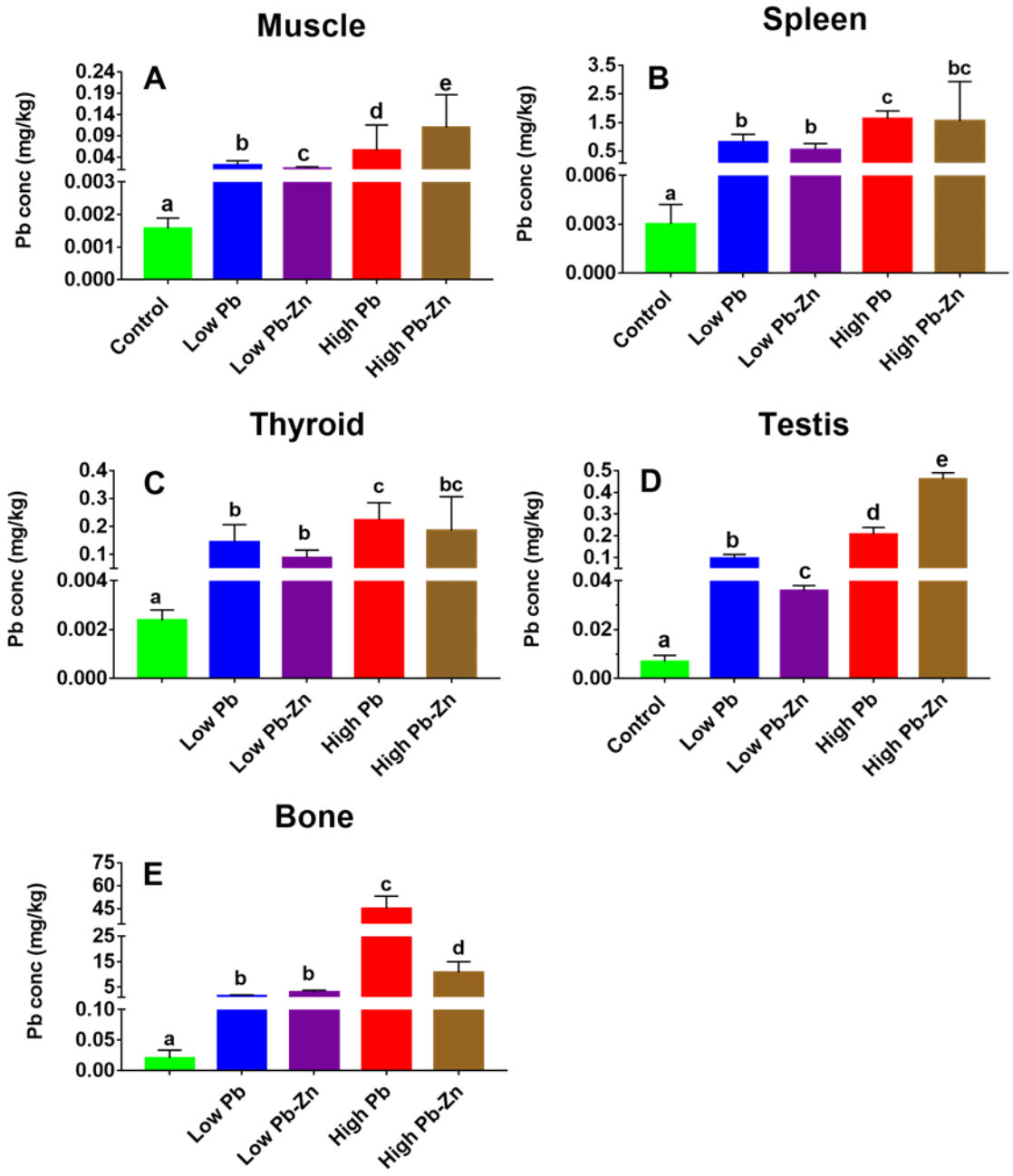

Fig. 3.1.3. Accumulation of Pb in the Muscle (n = 6), Spleen (mg/kg), Thyroid gland (n = 6), Testes (n = 3) and Bone (n =6). (Graph: Mean ± SD, n = 6, $^{a, b,c}$ $p < 0.05$; Tukey's multiple comparison test)

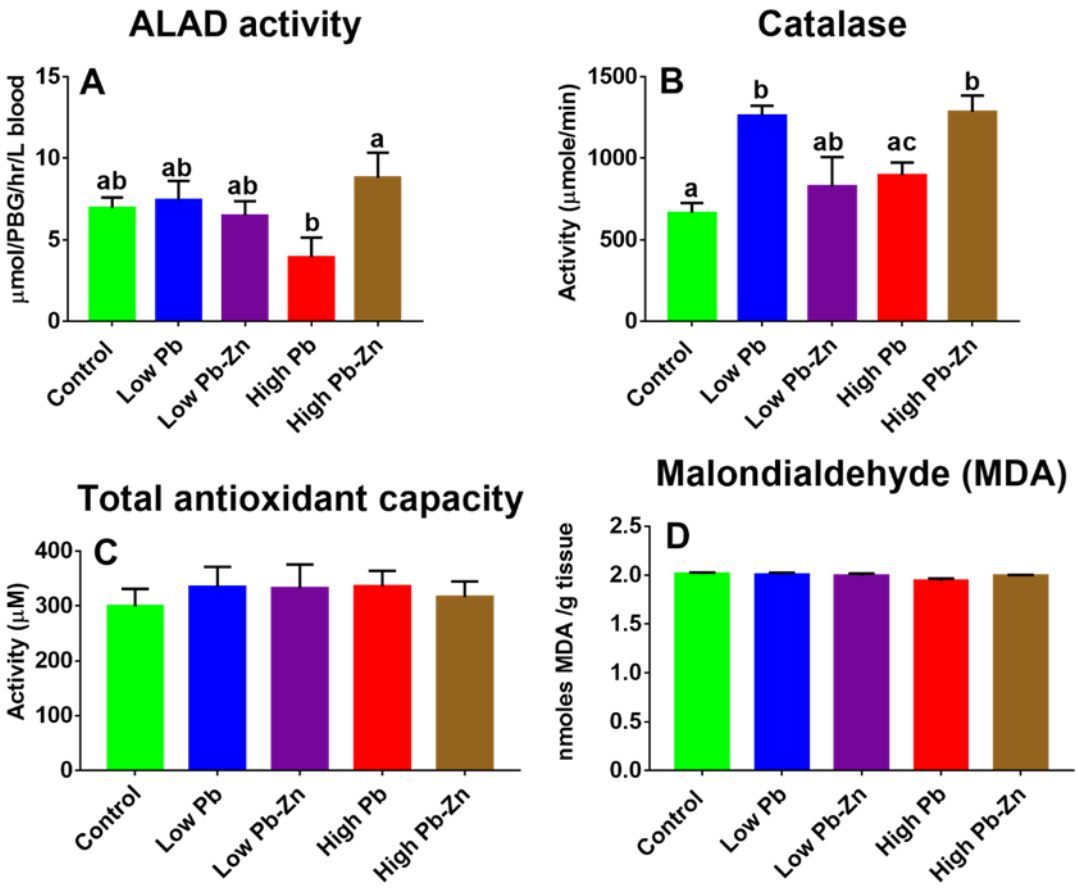

Fig. 3.1.4 A. Delta-aminolaevulinic acid dehydratase activity in whole blood (n = 5), **B**. Catalase activity in liver tissue (n = 3), **C**. Total antioxidant capacity of plasma of Sprague-Dawley rats exposed to lead and lead/Zn, **D**. Malondialdehyde levels in liver tissue (n =3). (All the data are presented as Mean ± SD, $^{a, b, c}$ $p < 0.05$, Tukey's multiple test).

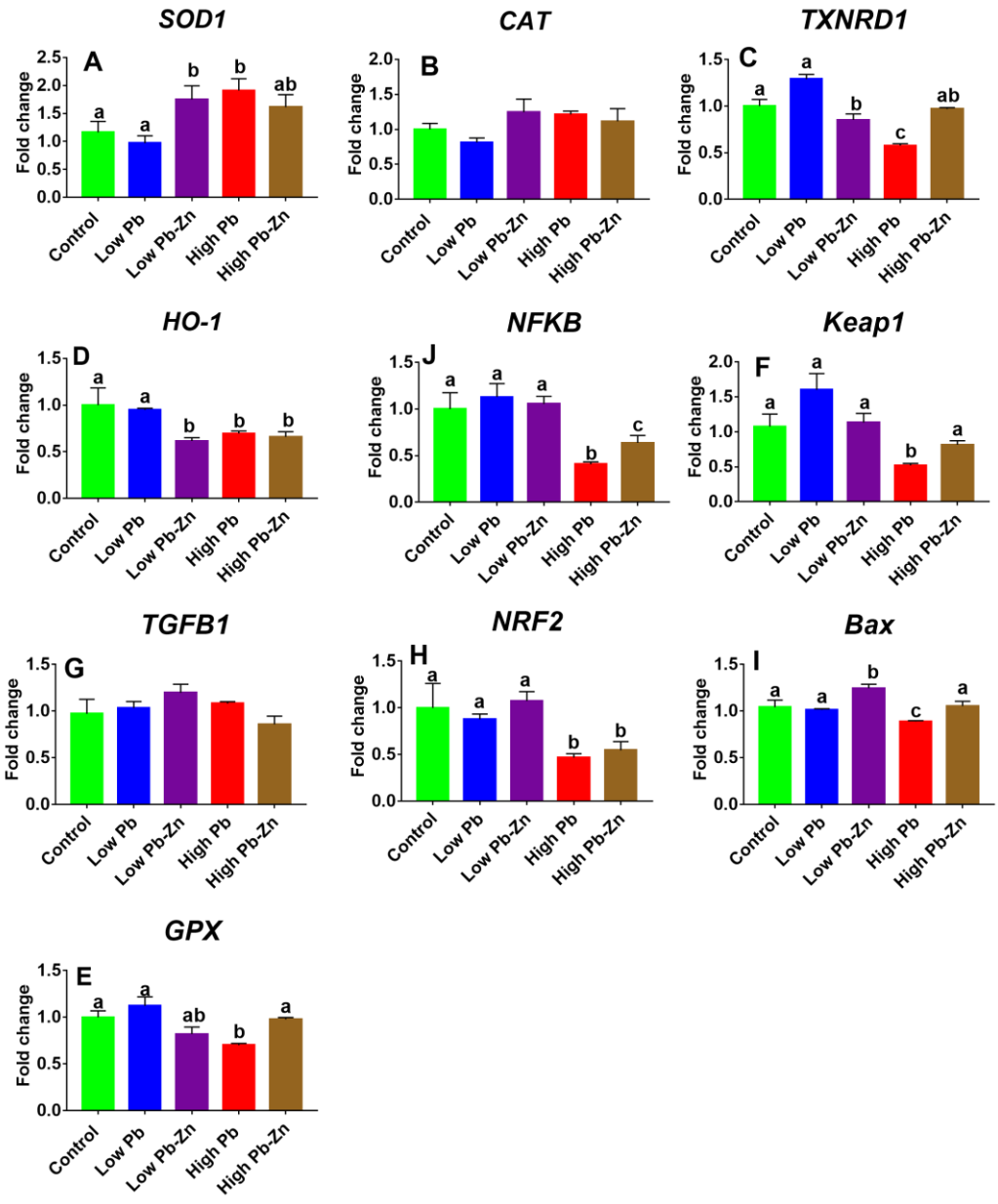

Fig. 3.1.5. Gene expression of antioxidant and related gene in the liver (n = 3; [a, b, c] $p < 0.05$). A. *SOD1* = superoxide dismutase 1, B. *CAT* = Catalase ;C.*TXNRD1*= Thioredoxin reductase 1, D. *HO-1*= Heme oxygenase -1, E. *GPX*= glutathione peroxidase;F.*Keap1*= Kelch-like ECH-associated protein,1, G. *TGFB1*= Transforming growth factor beta 1;H. *NRF2*= Nuclear factor erythroid 2-related factor 2, I. *BAX*= Bcl-2 Associated X-protein, J. *NFKB* = Nuclear factor kappa-light-chain-enhancer of activated B cells

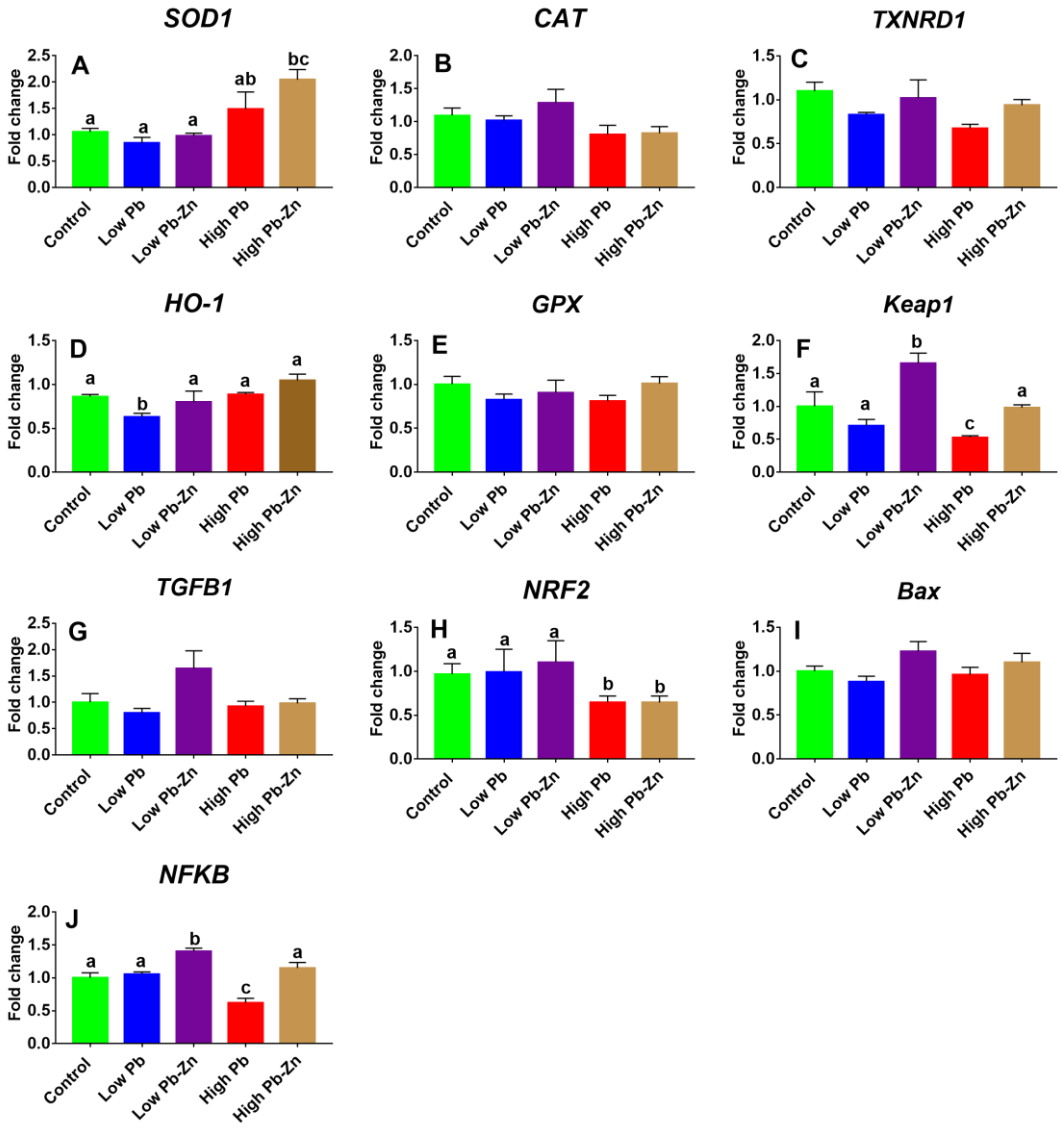

Fig. 3.1.6. Gene expression of antioxidant and related gene in the kidney (n = 3; [a, b, c] $p < 0.05$). A. *SOD1* = superoxide dismutase 1, B. *CAT* = Catalase ;C.*TXNRD1*= Thioredoxin reductase 1, D. *HO-1*= Heme oxygenase -1, E. *GPX* = Glutathione peroxidase;F.*Keap1*= Kelch-like ECH-associated protein,1, G. *TGFB1* = Transforming growth factor beta 1;H. *NRF2*= Nuclear factor erythroid 2-related factor 2, I. *Bax* = Bcl-2 Associated X-protein, J. *NFKB* = Nuclear factor kappa-light-chain-enhancer of activated B cells

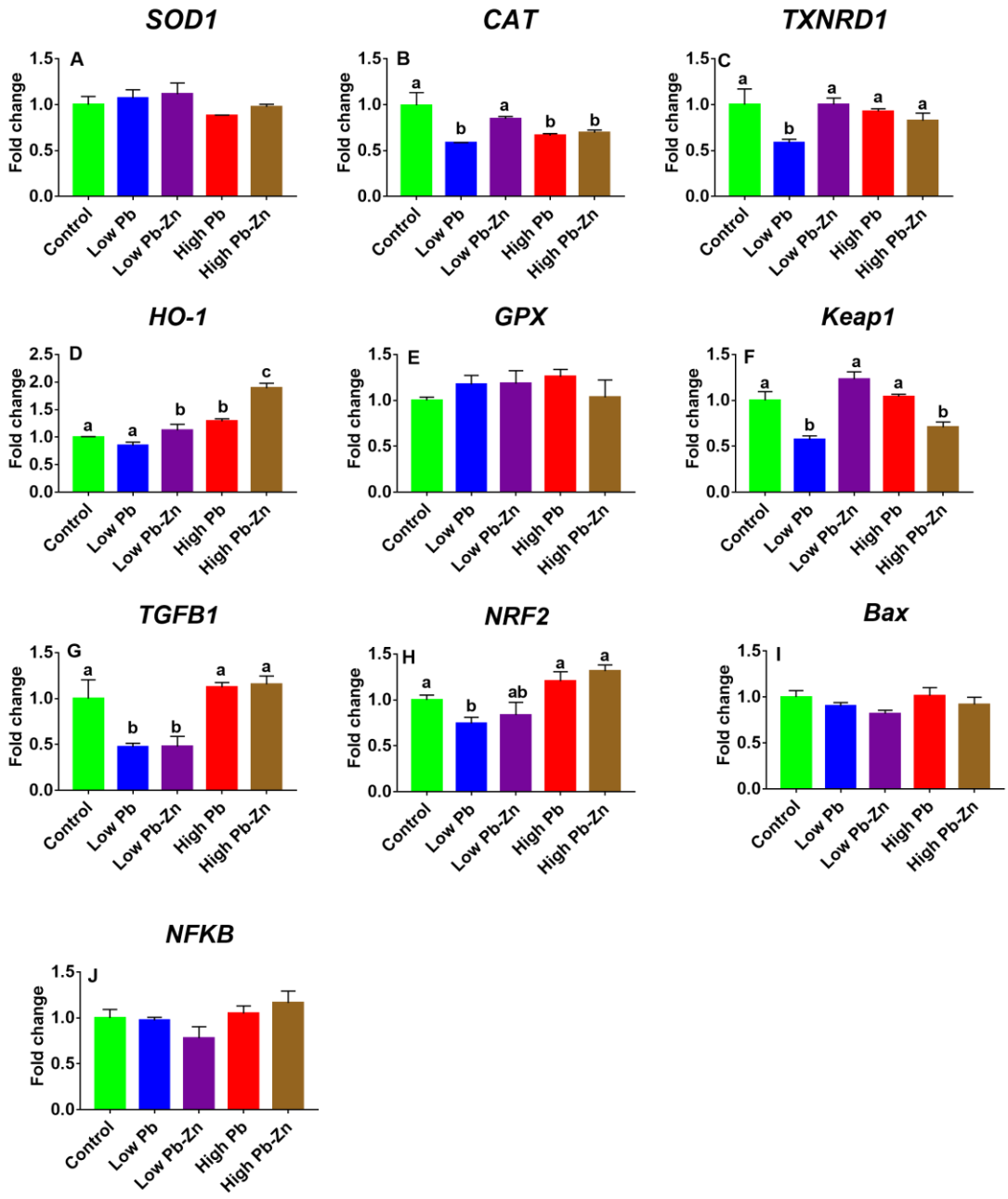

Fig. 3.1.7. Gene expression of antioxidant and related gene in the brain (n = 3; [a, b, c] $p < 0.05$).

A. *SOD1* = Superoxide dismutase 1, B. *CAT* = Catalase ;C.*TXNRD1*= Thioredoxin reductase 1, D. *HO-1*= Heme oxygenase -1, E. *GPX*= glutathione peroxidase;F.*Keap1*= Kelch-like ECH-associated protein,1, G. *TGFB1* = Transforming growth factor beta 1;H. *NRF2*= Nuclear factor erythroid 2-related factor 2, I. *Bax* = Bcl-2 Associated X-protein, J. *NFKB* = nuclear factor kappa-light-chain-enhancer of activated B cells

Discussion

The present study investigated the impact of zinc supplementation with lead on tissue lead accumulation and the ability of zinc to mitigate the toxicity of in. Lead exposure affected body weight gain in exposed rats. Interestingly, only the high Pb group had significant body weight gain reduction relative to the control and not the high Pb with zinc supplementation with other groups. The effect of lead toxicity on body weight loss has been reported in other studies as one of the common findings (Ismail et al., 2010). Whereas mechanisms behind lead exposure and body weight gain or loss remains unclear, perinatal lead exposure in mice demonstrated that Pb induced gut microbiota changes were linked to weight gain or weight loss in mice (Wu et al., 2016). The protective effect of Zn on weight at high exposure may have been due Zn's ability to protect gut microbiota by minimizing Pb uptake by gut microvilli and Pb induced gut inflammation among others (de Queiroz et al., 2014; Talpur et al., 2018).

Zinc supplementation in lead exposed animals has been known to partially minimize lead tissue uptake and mitigate specific lead induced clinical toxicity (Batra et al., 1998; Piao et al., 2007; Ugwuja et al., 2020). The specific mechanisms by which this is achieved are not very clear yet. Some authors indicate that the reduced uptake is due to the interaction of Pb and Zn divalent ions at absorption sites in gastrointestinal tract into systemic circulation and transportation into tissues (Batra et al., 1998; Ugwuja et al., 2020). The inherent antioxidant properties of zinc and it being part of most metalloenzymes in the body further confers zinc its protective capability against lead induced toxicity (Soussi et al., 2018). In the present study, zinc supplementation was associated with reduced accumulation of Pb in testes and muscles at low exposure which may have been protective as previously reported (Flora and Tandon, 1990; Piao et al., 2007; Zhai et al., 2015). On the other hand, Zn supplementation increased uptake of Pb in testes at high Pb exposure contrary to what was reported by Batra et al., (1998). Muscle tissue Pb uptake was equally elevated at high exposure suggesting that the positive effects of Zn supplementation against Pb may be limited depending on dose of exposure. In the present study, at both levels of exposure, the blood, liver, kidney and brain levels of Pb uptake were not affected by Zn co-administration. This suggests Zn supplementation may not fully prevent the distribution of Pb to other organs or tissues through systemic circulation (Piao et al., 2007). In case of bone tissue, Pb uptake was significantly reduced in Zn supplemented group at high level of exposure only. The reduced Pb uptake by bone tissue would further reduce the total Pb body burden and minimize endogenous Pb poisoning associated with metabolic remobilization of minerals such as calcium (Batra et al., 1998).

In the present study, Pb induced renal and hepatic toxicities were not very apparent due to lack of the changes in the biomarkers of liver or kidney related enzymes (lactate dehydrogenase, alkaline phosphatase, and glutamic oxaloacteic transaminase), general plasma biochemistry, malondialdehyde (MDA) and total antioxidant capacity (Ugwuja et al., 2020) . This suggest that the

period of exposure and the Pb tissue burden may not have been enough to elicit overt tissue damage. However, low BUN levels were observed across the exposed groups indicative of mild signs of Pb induced hepatotoxicity (Olayinka and Olukowade, 2010).

Lead possess a very high affinity for sulfhydryl (SH) functional groups (Hsu and Guo, 2002). Thus, the inhibition several essential antioxidant enzymes such as ALAD, catalase (*CAT*), glutathione peroxidase (*GPX*), superoxide dismutase (*SOD*) by Pb via its SH group attachment is postulated to be the main mechanism of Pb induced toxicity (Gurer and Ercal, 2000; Li et al., 2013; Zhai et al., 2015). And the ALAD enzyme which is key in heme synthesis through the conjugation of two δ-aminolaevulinic acid molecules into porphobilinogen is highly sensitive to Pb toxicity hence it is regarded a reliable biomarker of Pb exposure (Nakata et al., 2021). In the present study, Zn co-administration prevented ALAD inhibition activity in the high Pb-Zn group at high exposure level where the toxic effects of Pb on ALAD were observed. The lack of Pb induced ALAD effects at low exposure level agreed with Flora et al.,(2008) who reported lack of ALAD inhibition effects in rat blood with Pb levels below 15 μg/dL. On the other hand, at the high level of exposure, the observed ALAD effects in the present study were consistent with a report in humans that showed that inhibition of ALAD was more pronounced in blood Pb levels above 25 μg/dL (Patil et al., 2006). The protective effects of Zn against Pb inhibition has been reported in other studies and Zn ability to displace Pb from the SH groups coupled with its involvement as a component of ALAD enzyme is the plausible protective mechanism (Ahamed et al., 2007; Lamidi and Akefe, 2017; Zhai et al., 2018). Catalase activity was activated as a protective response at low Pb group and Zn further enhanced the protective response at the high level of exposure in the high Pb-Zn group. The protective effects are derived from catalase's ability to breakdown cellular hydrogen peroxide into water and oxygen generated during increased ROS production (Nandi et al., 2019).

In the present study, I investigated antioxidant and related genes' molecular responses of the liver, kidney and brain of genes. The former two tissues were chosen based on their high Pb accumulation and the metabolic and excretory functions, respectively (Nandi et al., 2019). The brain was chosen owing to its high susceptibility and pronounced cognitive dysfunction following Pb exposure (Ouyang et al., 2019). The protective effect Zn co-administration in Pb induced oxidative stress toxicity was observed at low level of exposure. Zn supplementation prevented the downregulation of essential antioxidant genes especially *SOD1*, *CAT*, *HO-1* and *TXNDR1* genes in liver, kidney and the brain. Zinc confers protection due to its functional role as a component of some enzymes such as superoxide dismutase, a Zn-Cu metalloenzyme or as a direct antioxidant for enzymes like catalase (Soussi et al., 2018). Moreover, co-administration was protective for other oxidative stress regulating gene Keap1 a cysteine-rich component of the Keap1-NRF2 complex regulating cyto-protection against reactive oxygen species (ROS) through its activation of the antioxidant responsive elements (Kansanen et al., 2013). The anti-pro apoptotic regulator gene, *Bax* gene expression non-

downregulation in Zn supplemented groups in liver at low exposure level suggest indirect Zn involvement in protection of tissues against Pb induced apoptosis (Ohtsuka et al., 2004). However, just like with tissue accumulation, the protective effects of zinc supplementation were mostly seen in low Pb dose and low Zn dose. This suggest that a threshold of Zn concentration exists at which the protective properties against Pb do not exist.

In conclusion, this study revealed that the protective effects of Zn in Pb and Zn co- exposure on Pb tissue accumulation and Pb toxicity may depend on the quantities of the metals ingested. Whereas, Zn supplementation may reduce tissue Pb accumulation in some tissues at low levels, the reverse may happen with increased level of exposure. Moreover, Zn co-administration at high level of exposure mitigated Pb ALAD activity inhibition toxicity. Notwithstanding, Zn supplementation may confer oxidative stress gene protection at molecular level of some genes. Further studies are required to investigate the Zn dose effect that seems to undo the beneficial effects of Zn supplementation in lead exposure. Furthermore, it must be noted that Zn in Pb exposure with Zn co-administration scenarios may partially mitigate against Pb induced toxicities and not fully protective.

Section bridge

- In the previous subchapter, section 3.1, the effects zinc co-exposure with lead on tissues Pb uptake and its toxicity on selected biomarkers and gene molecular expression of antioxidant genes in the liver, kidney and brain was reported. My motivation for this study was the presence of both lead and Zn in high levels in the Kabwe environment. Zinc showed mitigative and protective effects on Pb accumulation in the testes and muscle at low exposure. At high level of exposure where the effects on the ALAD activity was obvious, Zn co-exposure prevented the inhibition of ALAD activity. Protective antioxidant gene responses in the liver, kidney and brain was also observed.

- In the next subchapter, section 3.2, I explore the use the wild rodent incisors as indicator of lead exposure from rats sampled from residential areas within varying distances within the vicinity of the lead-Zinc mine. I analyzed lead accumulation in teeth and compared it to that of blood. I further did the lead mapping using Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS).

3.2. Wild rat crown incisor as an indicator of lead (Pb) exposure and Pb incisor mapping using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)

Abstract

Lead (Pb) is a metal toxicant of great public health concern. The present study investigated the applicability of the rat incisor in Pb exposure screening. The levels of lead in teeth (Pb-T) in the "crown" and "root" of incisors in experimentally Pb exposed Sprague Dawley rats were quantified using an inductively coupled plasma mass spectrometry and the "crown" accumulated much Pb-T than the "root". As an application of the Pb-T "crown" results in experimental rats, I subsequently analyzed the Pb-T in the "crown" incisors of environmentally Pb exposed wild rats (*Rattus rattus*) sampled from residential sites within varying distances from an abandoned lead-zinc mine. The Pb-T accumulation in the "crown" of incisors of *R. rattus* rats decreased with increased distance away from the Pb-Zn mine and vice versa. Furthermore, the Pb-T was strongly correlated ($r = 0.85$) with the Pb levels in blood. Laser ablation inductively coupled plasma mass spectrometry of lead in teeth (Pb-T) mappings revealed a homogenous distribution of Pb in the incisor with an increased intensity of Pb-T localized in the tip of the corona enamel surface in both Sprague Dawley and *Rattus rattus* rats. These findings suggest that Pb-T in the "crown" incisor may be reflective of the rat's environmental habitat, thus a possible indicator of Pb exposure.

Keywords: lead, incisor, biomarker, rodent, sentinel

Introduction

Lead (Pb) is a toxic metal known to cause a number of physiological and biochemical dysfunctions in animals and humans (Galal et al., 2019). Although Pb poisoning in children has considerably receded in developed countries (Dapul and Laraque, 2014) , chronic exposure to low levels of Pb remains a perennial phenomenon in some developing countries. A case in point is Kabwe town in Zambia, with a lead-zinc mine history legacy characterized by an alarming Pb poisoning in adults and children from residential areas within the radius of the closed mine and its tailing wastes (Bose-O'Reilly et al., 2018; Yabe et al., 2020; 2015). Moreover, acute fatal cases of Pb poisoning in over 400 children in Nigeria (Dooyema et al., 2012) and 18 children in Dakar, Senegal (Haeflinger et al., 2009) linked to anthropogenic activities have been reported. Thus, biomarkers of Pb exposure that are reliable and easy to obtain to facilitate continuous environmental Pb monitoring using sentinel animals sharing habitats with humans are required (Barbosa et al., 2005).

Traditionally, Pb in blood (Pb-B) has been widely used as a biomarker for Pb exposure in humans (Wang et al., 2016 and Augusto et al., 2016). However, Pb-B possess a short mean biological life of only around 30-40 days and may only reflect primarily both ongoing steady-state exposures and relatively recent exposures (Barbosa et al., 2005), Moreover, changing the conditions of exposure causes a Pb-B variation and typically the blood lead level reverts to normal once the exposure ceases (Rossi, 2008). The concentration of Pb in teeth (Pb-T) in contrast is a cumulative function of earlier exposure which allows for the identification of historic undetected cases of Pb exposure even after the other indices have returned to normal (Steenhout and Pourtois, 1981). Although the Pb-T in the whole tooth has been considered as a biomarker for teeth Pb exposure in children (Winneke and Brockhaus, 1982) and rodents (Arora and Hare, 2015), Pb accumulation maybe unevenly within the tooth because of different dental parts (Wang et al., 2016).

The use of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) as a technique have been applied to appreciate accumulation patterns of metals in the dentine and enamel aspects of the tooth (Cox et al., 1996; Evans et al., 1995; Ryu et al., 2003) . However, there are still limited reports on the distribution or mapping of Pb in rodent incisor teeth from environmentally or experimentally Pb dosed rats. Therefore, further investigations using techniques for advanced mapping of distribution Pb in teeth such as laser ablation inductively coupled plasma mass spectrometry are needed.

Wild rodents have been used as sentinel animals and biomonitors in environmental related pollution assessments of pesticides (Yohannes et al., 2017), asbestos (Ardizzone et al., 2014) and heavy metal pollution (Martiniakova´ et al., 2011). In the present study, the rodent incisor tooth was investigated in laboratory and wild rodents as biomarker of Pb exposure. I hypothesized that Pb accumulates differently within the incisor teeth and that the part with much accumulation may be sampled as biomarker of Pb exposure. To evaluate my hypothesis, I experimentally exposed Pb to SD

rats for a period of 8 weeks and quantified Pb-T levels in the “root” and “crown” subdivision of the upper and lower incisors and found that the crown accumulated much Pb-T. As an application of my results, I analysed Pb-T in the “crown” of the lower incisors (L2) of wild rodents trapped from areas known to be contaminated with Pb from Kabwe, Zambia in parallel with Pb-B in the same rats. To the best of my knowledge, the quantification of Pb-T in the “root” and “crown” subdivisions of incisor teeth in the experimentally Pb exposed rats as well as the use of the “crown” of environmentally exposed rats has not been reported. Lead distribution mappings were performed using LA-ICP-MS in both experimentally and environmentally exposed rats to augment the Pb-T quantification done using ICP-MS and ascertain the distribution of Pb on a rodent incisor.

Materials and Methods

Laboratory animals and exposure

Animal experiments were performed at the Faculty of Veterinary Medicine, Hokkaido University under supervision and with the endorsement of the Institutional Animal Care and Use Committee of Hokkaido University, Japan (approval number: 16-0017). Sprague Dawley male rats (n = 18) aged seven weeks were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The rats were kept in six lead-free polypropylene cages in community housing of three rats (n = 3 per cage). The animals were acclimated to the animal facility for one week prior to Pb exposure with access to lead-free food (rodent chow, Labo MR Stock, Nosan Corporation, Yokohama, Japan) and distilled water *ad libitum*. There were no significant body weight differences among all the groups. Two cages with six (n = 6) rats were randomly assigned to the three exposure levels (control, low, and high dosage exposure). Two different concentrations of Pb acetate: 100 and 1000 mg/L Pb (Wako Pure Chemical Industries, Osaka, Japan) were given in the drinking water for eight weeks to the low and high dosage groups, respectively. The control group received only distilled water for the same period. The choice of Pb levels of exposure for the current study were based on the previous study in mice using that accumulated dose dependent Pb concentrations in their tissues at 100 and 1000 mg/L lead acetate (Togao et al., 2020). After the end the of exposure, period rats were euthanized under carbon dioxide with sevoflurane. Blood and incisors teeth (upper and lower) were collected following extraction. For uniformity, we assigned the upper and lower incisors on left side of the jaws for quantitative Pb analysis using the inductively coupled plasma mass spectrometry and those on the right side were assigned for qualitative Pb mapping using laser ablation inductively coupled plasma mass spectrometry for each individual rat across all the groups. The blood and teeth samples were collected in lead - free polypropylene tubes stored at -80 ºC and -20 ºC prior to analysis, respectively.

Wild rat sampling and species identification

The sampling was done permission from the Zambian Ministry of Fisheries and Livestock, as well as the Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan (approval number: Vet-17010). A total number of 24 wild rats were collected from Kabwe, Zambia a town known for extensive Pb environmental contamination (Ikenaka et al., 2010; Nakayama et al., 2011; Yabe et al., 2020, 2015) between June and July 2017. Kabwe is situated at approximately 142º70'S and 28º260'E. A total number of 24 wild rats were captured using live traps in Kabwe as previously described by our research group (Nakayama et al., 2011). Six (n = 6) wild rats were captured from each of the four sites within varying distance from the closed Pb-Zn mine. The furthest distance away from the closed mine was used as the site control in Lukanga (LK; 5.90 km) and other sites taken as naturally exposed sites were in Makululu (MK; 2.11km), Chowa (CH; 0.86 km), and Mutwe Wansofu (MW; 0.81 km) townships , respectively. The sampling sites were located **(**Fig. 3.2.1**)** using a global positioning system (GPS).

The rats were euthanized with sevoflurane and blood and incisors teeth (lower) were collected following extraction. The samples were then immediately stored at -20 ºC before being transported to the Faculty of Veterinary Medicine, Hokkaido University under the cold chain systems after obtaining an international sanitary certificate from the Ministry of Fisheries and Livestock, Zambia (No. 11614). At Hokkaido University, the blood and teeth samples were stored at -80 ºC and -20 ºC in a deep freezer, respectively until analysis. The species of the wild rats sampled were identified using genomic DNA sequencing (Robins et al., 2007) and only the rats that were of *Rattus rattus* species (Table 1) were used in the current study. After the exclusion of the non- *R. rattus* rat species, the final sample sizes were; n = 2 (LK), n = 5 (CH), n = 5 (MK) and n = 3 (MW), respectively.

Digestion and quantitative analysis of lead in blood and teeth subdivisions

The Pb concentration in the blood and incisors teeth collected from the laboratory exposed Sprague Dawley rats and the wild rats from Kabwe, Zambia were quantified. In the current study, blood and teeth were acid digested using the method described by Nakata et al., (2016; 2015) with minor modifications. To minimize surface contamination of incisor teeth and remove excess blood in teeth, the teeth were firstly cleaned using a method described by Ishii et al. (2018) with some modifications. Briefly, the teeth samples were placed in an ultrasonic bath of L-cysteine (Cica reagent, 100mg/L; Kanto Chemical, Tokyo, Japan) for 5 minutes followed by rinsing in an ultrasonic bath of distilled water for 10 minutes prior drying. The samples were then dried in an oven at 54 °C for 48 hours. Each tooth was divided into two subdivisions using the yellow-orange pigmentation of enamel

on the crown part of the labial surface of the tooth (Mancinelli and Cappello, 2016) as a distinguishing reference mark illustrated (Fig. 3.2.2). Each lower (L) incisor was divided into two subdivisions namely; L1 ("root") and L2 ("crown"). The L1 is a part that is embedded in the jawbone and L2 for the part tooth visible in the oral cavity. Similarly, the upper (U) incisor was divided into two subdivisions namely; U1 ("root") for the upper part of the tooth embedded in the jawbone and U2 ("crown") for the part tooth visible in the oral cavity in live rodents as shown in Fig. 3.2.2.

In case of blood microwave digestion. 0.1 mL of the blood was measured and put in pre-washed digestion vessels. And then of 5 mL of nitric acid (atomic absorption spectrometry grade, 30%; Kanto Chemical, Tokyo, Japan) was added, and 1 mL of hydrogen peroxide (Cica reagent, 30%; Kanto Chemical, Tokyo, Japan) in readiness for digestion. The sample digestion was done using a ramped temperature program in a closed microwave system (Speed Wave MWS-2 microwave digestion system; Berghof, Eningen, Germany). The microwave system operating conditions used are given in Table 3.2.2. Following cooling, the sample solutions were transferred into 15 mL polypropylene tubes and diluted to a final volume of 10 mL with ultra-distilled and de-ionized water.

The Pb concentration quantification was performed using the ICP-MS (7700 series; Agilent Technologies, Tokyo, Japan) as described by Nakata et al., (2016, 2015) with minor modifications. The operating conditions of ICP-MS were as given in Table 3 below. The quality control was performed by analysis of DOLT-4 (dogfish liver; National Research Council of Canada) certified reference material. Replicate analysis of the reference material gave good recovery rates ranging 95-105%. The limit of detection (LOD) for Pb was 0.001 mg/L.

***Laser ablation inductively coupled plasma mass spectrometry analysis of incisor teeth of Sprague Dawley* and *Rattus rattus* rats**

The incisor teeth samples from laboratory Sprague Dawley and environmentally Pb exposed rats were processed and analysed according to the method previously described by Ishii et al., (2018) with some minor modifications. Briefly, excess tissues and surface contamination of the teeth were cleaned using an ultrasonic bath of L-cysteine for 5 minutes followed by cleaning in an ultrasonic bath of distilled water and drying at 54 °C for 48 hours. Samples were sliced into ~40 µm sections along the longitudinal axis with a diamond blade and polished. The teeth sections were systematically scanned by a focused laser beam with the following parameters; spot diameter: 100 µm, scan speed: 70 µm/sec using LA (NWR213; ESI, Portland, OR, USA)-ICP-QQQ-MS (8800 series; Agilent Technologies). Detailed analytical conditions are presented in Table 3.2.4. We reconstructed two-dimensional images from time resolved analysis data of LA-ICP-MS by iQuant2 (Suzuki et al., 2018); an in-house developed software. This software shows the localization of elements.

Data Analysis

The data analysis was performed using GraphPad Prism software (Prism 7 for Windows; Version 5.02, California, USA) and reported as mean and standard deviation (SD). The data was first tested for normality using Kolmogorov–Smirnov test. The data was not normally distributed. I log transformed the data for statistically analyses and retained the original values in the results and figures for easier interpretation and comparisons with other studies. In the present study, difference between groups was deemed to be significant at $p < 0.05$ (*) using multiple Tukey's comparison test. log transformation of Pb concentrations was also done for the Pearson's correlations analysis between Pb-B concentration and Pb-T levels and the correlations were considered be significant at $p < 0.05$. The graphical representations were compiled using GraphPad Prism software.

Results

Lead in blood (Pb-B) and incisors subdivisions of experimentally exposed Sprague Dawley rats

The Pb-B levels increased significantly with the increase in the dose of Pb given with the high Pb group having the highest mean concentration of 39.63 ± 8.09 μg/dL, followed by the low Pb group which had mean of 9.90 ± 1.71 μg/dL and the control had lowest with 4.01 ± 0.86 μg/dL (Fig. 3.2.3A). Both the high Pb and low Pb groups accumulated significantly higher Pb levels ($p < 0.05$) when compared to the control (Fig. 3.2.3 A). Further, the Pb-B levels between the low and high group were also significantly different ($p < 0.05$).

The accumulation of Pb in the teeth subdivisions (Pb-T) in L1 and L2 of lower and U1 and U2 upper incisors are shown in Fig. 3.2.3 B and C, respectively. The accumulation Pb-T in both subdivisions of the lower incisors was in a Pb dose dependent manner with the high Pb group accumulating higher Pb-T than the low Pb group and the control. The Pb-T in the L2 was significantly higher (Tukey test, $p < 0.01$) than that of L1 for both low Pb and high Pb groups. At low Pb, the Pb-T in L1 was 4.72 ± 2.10 mg/kg, and L2 had 90.17 ± 13.57 mg/kg. At high Pb exposure, L1 had 33.07 ± 15.51 mg/kg and L2 had 132.40 ± 47.33 mg/kg Pb-T, respectively. Similarly, Pb-T accumulation in the upper incisors and their subdivisions accumulated in Pb dose dependent manner. The Pb-T in the U1 (6.79 ± 2.03 mg/kg) was significantly lower ($p < 0.01$) than U2 (42.44 ± 16.58 mg/kg) in the low Pb group. Likewise, in the high Pb group, the U1 (26.13 ± 11.63 mg/kg) accumulated lower Pb-T than U2 (63.32 ± 24.89 mg/kg). In addition, I observed that L2 or U2 at lower exposure accumulated significantly higher Pb-T than the L1 or U1 at high exposure (Fig. 3.2.3 B and C).

The relationship between incisor teeth parts and blood in the experimentally Pb exposed Sprague Dawley rats

Fig. 3.2.4 shows the relationship between the Pb-T in the lower and upper incisors "root" and "crown" subdivisions with the Pb-B concentration in the experimentally Sprague Dawley rats. In the

present study, positive log transformed Pearson's correlations between the Pb-B and Pb-T across all the exposed groups in the root and crown of the lower and upper incisors were observed. Much stronger correlations between Pb-T and Pb-B were recorded in the root of both lower and upper incisors than in the crown. The correlations were significant namely, lower incisor (L1) Pb-T Vs Pb-B ($r = 0.91$, $p < 0.05$), lower incisor (L2) Pb-T Vs Pb-B ($r = 0.82$, $p < 0.05$), upper incisor (U1) Pb-T Vs Pb-B ($r = 0.91$, $p < 0.05$) and upper incisor (U2) Pb-T Vs Pb-B ($r = 0.85$, $p < 0.05$) as shown in Fig. 3.2.4 A, B, C and D.

Lead in blood (Pb-B) in the environmentally Pb exposed Rattus rattus rats

Fig. 3.2.5A**.** shows the Pb-B in wild rats sampled from different sites within varying distances from the closed Pb-Zn mine as a reference point as shown in Fig. 3.2.1. The accumulation of Pb-B differed significantly among the sites with the highest Pb-B levels seen in rat samples captured closer to the old mine and lowest in rat samples collected furthest from the mine (Fig. 3.2.5A). Quantitatively, the CH site group had mean Pb-B of 245.40 ± 161.90 µg/dL, the MW site group had 213.20 ± 14.22 µg/dL, the MK site had 40.11 ± 15.56 µg/dL and the LK site had 15.60 ± 0.99 µg/dL. Furthermore, in reference to the control site sample (LK), the Pb-B levels in known contaminated sites were significantly higher in MK ($p < 0.05$), MW ($p < 0.05$) and CH ($p < 0.05$) groups. No difference in Pb-B levels in the CH and MW group were observed (Fig. 3.2.5 A).

Lead levels in the lower incisors "crown" (L2) teeth and their relationship with Pb-B in the environmentally Pb exposed Rattus rattus rats.

Fig. 3.2.5B. shows the accumulation pattern of Pb-T in the crown part of the lower incisor (L2) of *R. rattus* rats exposed to Pb in their natural environment. The accumulation Pb-T was higher in the rats that were captured closer to the closed Pb-Zn mine had higher Pb-T levels than those captured further away from the closed Pb-Zn mine. Quantitatively, the CH group had highest levels with Pb-T of 383.60 ± 144.10 mg/kg, followed by MW group with Pb-T of 102.60 ± 91.60 mg/kg, MK with 32.66 ± 8.02 mg/kg and the least Pb-T was in the LK group with 3.17 ± 1.69 mg/kg. The Pb-T concentrations among the groups were statistically significant ($p < 0.05$) as shown by lower case letters (Fig. 3.2.5B). In addition, the exposed groups were significantly different from the assigned control (LK) group ($p < 0.05$). In the present study, the Pearson's correlation analysis between Pb-B and Pb-T of log transformed data in the crown of lower incisors in environmentally Pb exposed rats was positive ($r = 0.85$, $p < 0.05$) as shown (Fig. 3.2.5C).

Lead distribution in the incisor teeth of the experimentally exposed Sprague Dawley rats and environmentally exposed Rattus rattus rats using LA-ICP-MS

The local distribution of the Pb mappings using LA-ICP-MS in the incisor teeth of both experimentally and environmentally Pb exposed rats are shown in Fig. 3.2.6 A and B, respectively. A homogenous distribution of Pb was observed in the greater portion of the tooth from the root extending to the pulp and the dentine in both experimentally and environmentally Pb exposed rodent teeth samples (Fig. 3.2.6 A, B). On the other hand, an inhomogeneous distribution of Pb was found in the surface enamel near the tip of the corona incisors having an intense localized distribution of Pb. The distribution of Pb in both experimentally and environmentally Pb exposed rodent teeth were similar.

Table 3.2.1. Wild rat species

No.	ID	Species	Sex	Site
1	LK1	*Rattus rattus*	Male	Lukanga
2	LK2	*Rattus rattus*	Female	Lukanga
3	CH1	*Rattus rattus*	Female	Chowa
4	CH2	*Rattus rattus*	Female	Chowa
5	CH3	*Rattus rattus*	Male	Chowa
6	CH4	*Rattus rattus*	Male	Chowa
7	CH5	*Rattus rattus*	Male	Chowa
8	MK1	*Rattus rattus*	Female	Makululu
9	MK2	*Rattus rattus*	Male	Makululu
10	MK3	*Rattus rattus*	Female	Makululu
11	MK4	*Rattus rattus*	Male	Makululu
12	MK5	*Rattus rattus*	Male	Makululu
13	MW1	*Rattus rattus*	Female	Mutwe wansofu
14	MW2	*Rattus rattus*	Male	Mutwe wansofu
15	MW3	*Rattus rattus*	Male	Mutwe wansofu

Key: LK (Lukanga), CH (Chowa), MK (Makululu) and MW and (Mutwe wansofu), respectively.

Table 3.2.2. Microwave operating conditions for teeth and blood digestion.

Teeth		Blood	
Temperature (ºC)	Time (min)	Temperature (ºC)	Time (min)
160	5	160	5
190	20	190	10
200	20	75	10
100	5		

Table 3.2.3. Detailed analytical conditions of ICP-MS.

Parameter	Value
RF Power	1500 W
Argon gas pressure	600 kPa
Cell gas (Helium)	100 kPa
Peak pattern	1
Replicates	3
Sweeps/replicate	100
Stabilization time	30 s

Table 3.2.4. Detailed analytical conditions of LA-ICP-MS.

LA system (NWR213, ESI, Portland, OR, USA)	
Wavelength, nm	213
Pulse duration, ns	4
Fluence	2.7 J/cm^2
Repetition rate	10 Hz
Spot diameter	100 µm (zoomed version: 20 µm)
Scan speed	500 µm/ sec (zoomed version: 20 µm/sec)
Ablation mode	line scan
Carrier He gas flow rate	0.8 L/min
Make up Ar gas flow rate	0.8 L/min
ICP-QQQ-MS (8800 series, Agilent Technologies, Tokyo, Japan)	
RF power	1550 W
Plasma Ar gas flow rate	15 L/min
Auxiliary Ar gas	not used
Collision	not used
MS/MS	not used
Integration time	0.01 sec for ^{206}Pb, ^{207}Pb, ^{208}Pb, and 0.005 sec for other isotopes
Measured Isotopes	^{13}C, ^{25}Mg, ^{31}P, ^{43}Ca, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb, ^{207}Pb, ^{208}Pb

Fig. 3.2.1. Sampling sites for environmentally exposed wild rats. Fig. shows the closed Pb-Zn mine marked ”yellow” shape and the sites where wild rats were sampled from: Mutwe wansofu (MW; n = 6), Chowa (CH; n = 6), Makululu (MK; n = 6) and Lukanga (LK; n = 6).

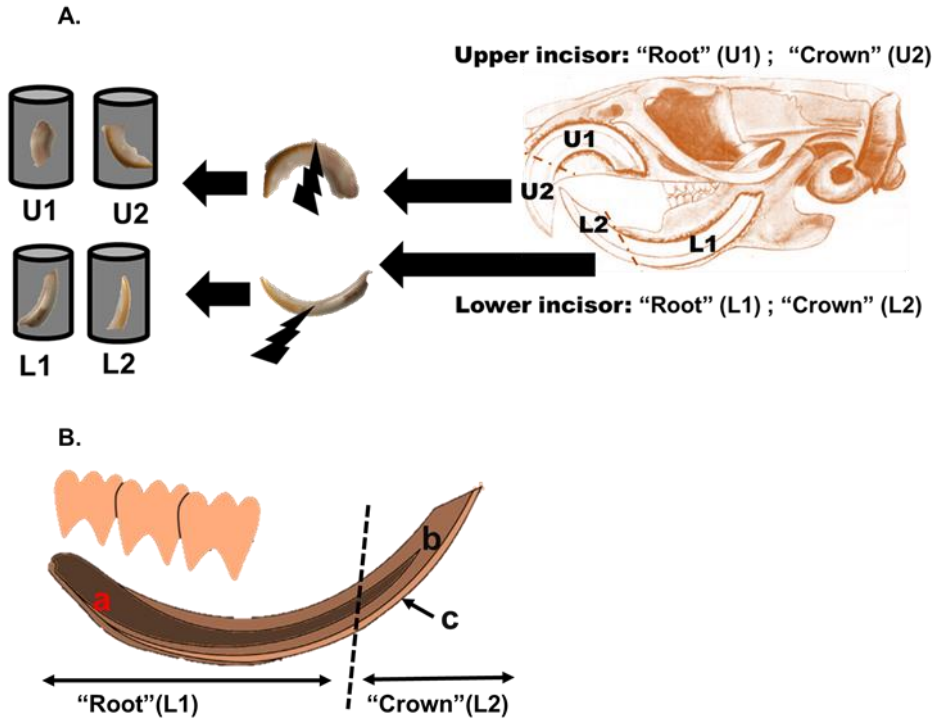

Fig. 3.2.2. Divisions of the incisor teeth and schematic illustration of the longitudinal section of incisor tooth. A. Lower and upper incisor teeth with the divisions that were processed for microwave acid digestion. Upper incisors: "Root" (U1) and "Crown" (U2) are as shown. Similarly, lower incisors: "Root" (L1) and "Crown" (L2) are shown. B. Illustrated copyright free schematic longitudinal section modified from Park et al. (2017) of lower incisor tooth sectioned for LA-ICP-MS analysis. The three major parts of incisor tooth are in view: a): dental pulp. b). dentine. c). enamel running only in the front part of the tooth. The dotted line is the imaginary division line used based on the obvious discolouration of enamel on the crown part of the tooth.

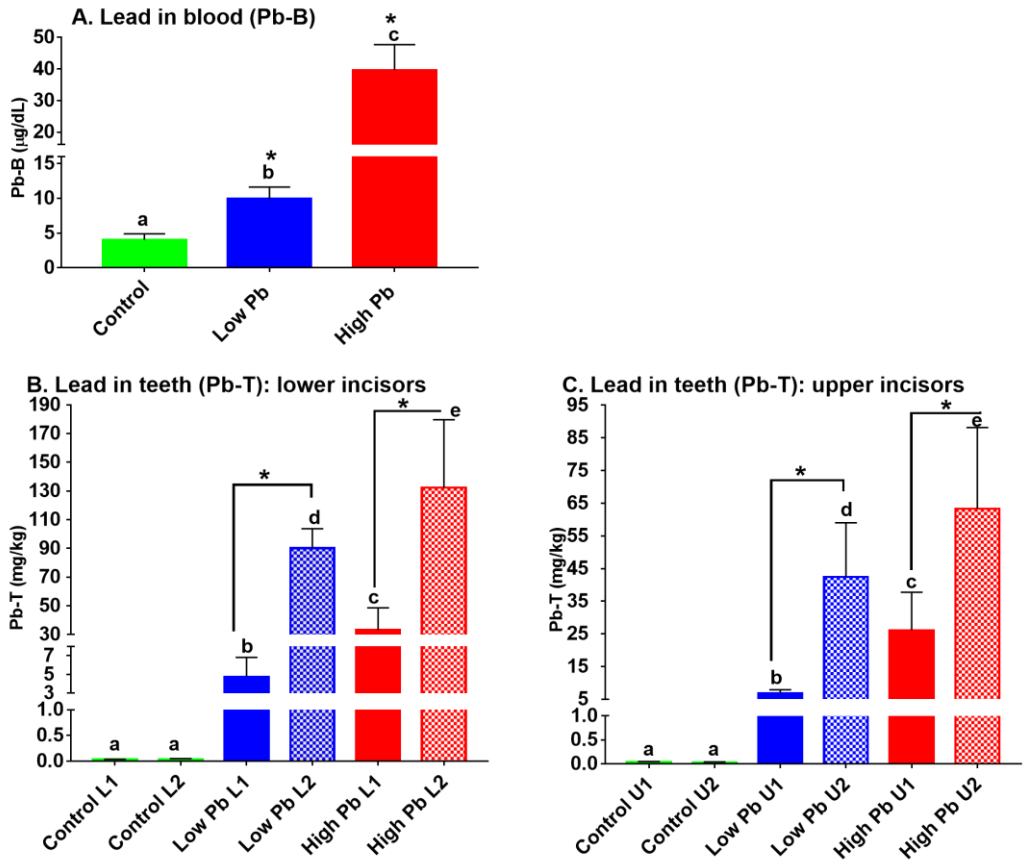

Fig. 3.2.3. Mean ± SD of Pb-B and Pb-T of experimentally Pb exposed Sprague Dawley rats. **A.** Pb-B in control (n = 6), low Pb (100 mg/L Pb; n = 6) and high Pb (1000 mg/L Pb; n = 6), **B**. Pb-T in the lower incisor divisions (L1 and L2) and; **C**. Pb-T in the upper incisor divisions (U1 and U2). ($^{a,b,\,c,\,d,\,e}$ represent $p < 0.05$, Tukey's multiple comparison test). Significant difference between the control and exposure groups, (*) at $p < 0.05$, Turkey's multiple comparison test.

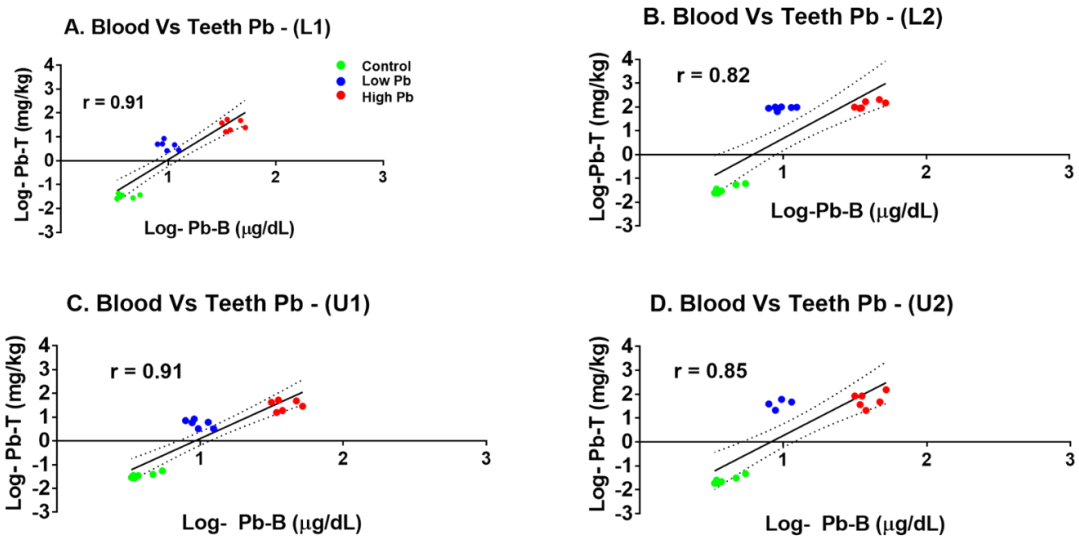

Fig. 3.2.4. Pearson's correlations of log transformed Pb-B and Pb-T "crown" and "root" of the lower and upper incisors in the experimentally Pb exposed Sprague Dawley rats. A. lower incisor (L1) Pb-B Vs Pb-T (r = 0.91, $p < 0.05$); B. lower incisor (L2) Pb-B Vs Pb-T (r = 0.82, $p < 0.05$); C. upper incisor (U1) Pb-B Vs Pb-T (r = 0.91, $p < 0.05$) and ; D. upper incisor (U2) Pb-B Vs Pb-T (r = 0.85, $p < 0.05$).

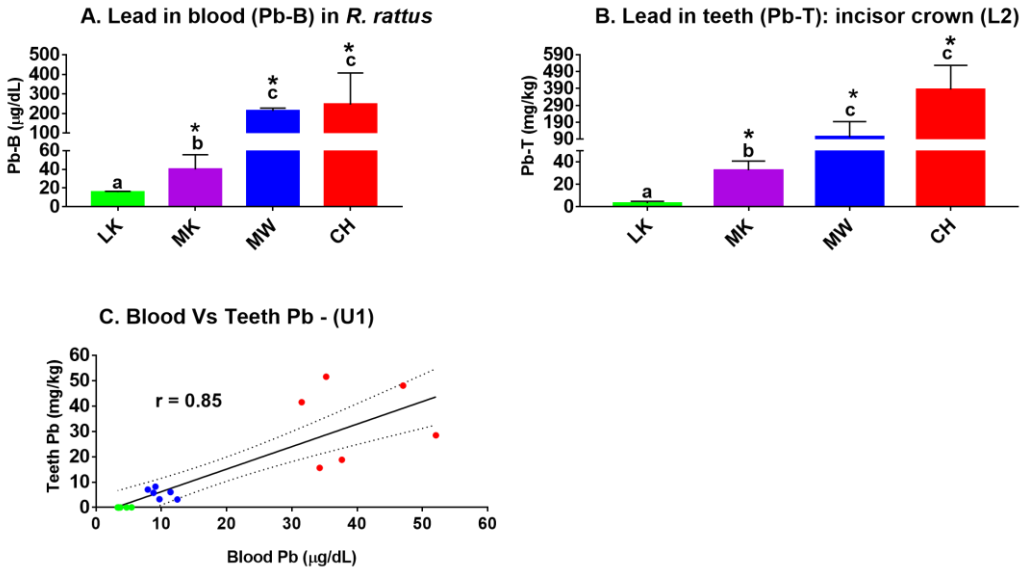

Fig 3.2.5. Mean ± SD of Pb-B and Pb-T of environmentally Pb exposed *Rattus rattus* rats. **A**. Lead level in blood (Pb-B) in rats sampled from Lukanga (LK; n = 2); Makululu (MK; n = 5); Mutwe Wansofu (MW; n = 3) and Chowa (CH; n = 5). **B**. Lead in teeth (Pb-T) in rats sampled from LK (n = 2); MK (n = 5); MW (n = 3) and CH (n = 5); ($^{a,b,\ c,\ d,\ e}$ represent $p < 0.05$, Tukey's multiple comparison test). Significant difference between the control and exposure groups, * at $p < 0.05$, Turkey's multiple comparison test. **C**. Log transformed Pearsons correlations of Pb-B Vs Pb-T in environmentally exposed *Rattus rattus* rats ($r = 0.85$, $p < 0.05$).

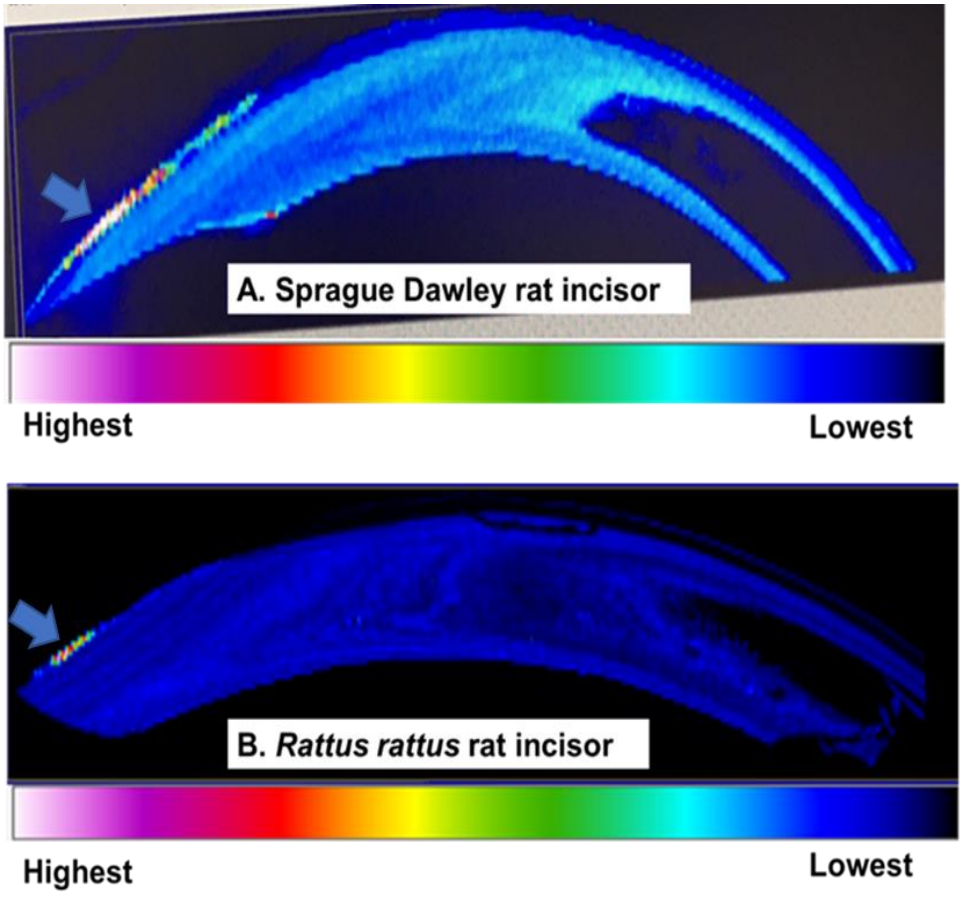

Fig. 3.2.6. Pb distribution in the incisor teeth of the experimentally exposed Sprague Dawley rats and environmentally exposed *Rattus rattus* rats using LA-ICP-MS. Homegenous distribution of Pb in the pulp and dentine of the with inhomogenous distribution of Pb-T characteristed by intense depositis of Pb in the exterior enamel (arrow). **A**. Sprague Dawley and; **B**. *Rattus rattus* rats in incisors .

Discussion

The present study focused on Pb quantities in the rodent incisor tooth as a biomarker of Pb environment assessment. The most striking observation was the high accumulation of Pb-T in the "crown" of the upper and lower incisor teeth than the "roots" across all levels of exposure in experimentally Pb exposed Sprague Dawley rats. The inherent incisor tooth structural variations between the "root and "crown" due to the larger part of the dental root pulp and higher dentine crown mass as well as dentine-enamel ratio could be the key factors in the accumulation of Pb-T differences (Rabinowitz, 1995). The rodent incisors are predominantly dentine with a thin layer of enamel located on the front part of the tooth (Mancinelli and Cappello, 2016). The influence of the large dentine mass in the "crown" of incisor teeth may have been one of the contributing factors behind the L2 or U2 in the low Pb group accumulating much Pb than the L1 or U1 in the high Pb exposure group. The current findings agrees with other studies that demonstrated that Pb is highly accumulated in the dentine part of the teeth (Bellis et al., 2007; Grobler et al., 2000; Kamberi et al., 2012; Steenhout and Pourtois, 1981).

The Pb-T in both the "root" and the "crown" of the lower incisors and upper incisor teeth and that of Pb-B accumulation in the Sprague Dawley rats following experimental Pb exposure were in a Pb dose-dependent pattern. These findings suggests that rodent incisors could be a useful indicator of exposure to Pb as was reported in goats (Bellis et al., 2007) and rats (Arora and Hare, 2015). Furthermore, strong positive correlations between Pb-B and Pb-T were found in the experimentally Pb exposed rats as has been reported in humans (Barton, 2010; Rabinowitz, 1995). Taken together, my results in the experimentally Pb exposed Sprague Dawley rats showed that Pb-T in the "crown" incisor analysis may provide some advantages in assessing Pb exposure.

In the *Rattus rattus* rat species used as sentinel animals around the closed Pb-Zn mine in Kabwe, Zambia, the accumulation of Pb-T in L2 and Pb-B were linked to the distance in reference to the point source. Moreover, there was a strong positive correlation between Pb-T in L2 and Pb-B in the blood of rats. This was in agreement to the pattern of Pb-T accumulation reported in deciduous teeth of children living near a lead-acid battery smelter (Johnston et al., 2019). Comparatively, my results were also in tandem with surface enamel Pb-T in children, were much Pb-T accumulation was reported in polluted areas than in less polluted areas (Costa de Almeida et al., 2007). Furthermore, the Pb-T in the "crown" of sentinel rats in the present study corroborated the findings of Pb concentrations in soils (Nakayama et al., 2011); and Pb-B in free roaming dogs (Toyomaki et al., 2020) and free range chickens (Yabe et al., 2013) sampled within the vicinity of the former Pb-Zn mine. Taken together, the current findings indicate that the rodent tooth incisor crown may be a useful tool for environmental Pb exposure monitoring.

The Pb-T in the crown incisors were relatively higher than the Pb-B corroborating reports that indicated that Pb-T were better indicator exposure and cumulative Pb body burden than Pb-B (Hegde

et al., 2010). The high Pb-B levels observed in wild rats were however not surprising as they were recorded from the Chowa and Mutwe Wansofu sampling sites that were near to the mine where children with very high Pb-B were previously reported. (Yabe et al., 2015). On the other hand, I observed that the lower the Pb-B level, the lower the Pb-T, which adds further merits to the use of L2 sample as biomarker of exposure. While, the duration of exposure in environmentally exposed *R. rattus* rats may not be clearly known, findings in experimental Pb exposed rats that were exposed for eight weeks suggest that the teeth may not only be useful for chronic Pb exposure but also in sub chronic exposure where blood levels are elevated.

The lead in teeth (Pb-T) distribution mapping using LA-ICP-MS in the lower incisors of the experimentally and environmentally exposed rats were performed for the first time. Interestingly, the distribution of Pb-T was homogeneously distributed in the dental pulp, dentine and the greater part of enamel except on the front tip part of the coronal enamel in both sets of rat incisors. The intense Pb localization seen near the upper corona enamel surface in rats were in contrast to other studies that demonstrated that calcified tissue layers in direct contact or in proximity to vascular tissues accumulated much Pb than those further away in bones (Ishii et al., 2018) and teeth around the circumpulpal dentine (Bellis et al., 2007; Cox et al., 1996; Hare et al., 2011). However, the present findings agreed in part with a report in human incisor teeth samples which had much of the Pb primarily deposited in the secondary dentine region close to the pulp and secondarily, at surface enamel (Wang et al., 2016). Moreover the observed high intensities of Pb in the outer part of incisors were only in the front side, the only side bearing enamel in rodent incisors (Mancinelli and Cappello, 2016). This phenomenon has been demonstrated in both erupted and non-erupted teeth that highly accumulated Pb in the outer enamel surface and with a gradual reduction of Pb in the deeper layers of the enamel (Cleymaet et al., 1991; Costa de Almeida et al., 2007; Olympio et al., 2018; Ryu et al., 2003). Taken together, the characteristic enamel surface Pb accumulation in the crown incisors further supports the use of the crown incisor as an alternative biomarker of environmentally Pb exposure in wild rats and laboratory rats.

The current study limitation bordered around wild rats sampling. The small number of samples for the Lukanga (n = 2) and Mutwe Wansofu (n = 3) after the exclusion of other species after genomic sequencing except the *R. rattus* species is the notable limitation. The logistics and travel restrictions could not permit re- sampling to increase the sample size of the *R. rattus*. Further validation of the rat incisor “crown” as suitable alternative biomarker to blood-Pb for environmental Pb exposure assessment with much larger sample size is recommended. Notwithstanding, the current study has revealed that the “crown” subdivision of the incisor rodents could be a suitable biomarker of Pb exposure. The use of the incisor crown subdivision has merits as it is easy to extract and is stable for preservation purposes (Barbosa et al., 2005). Besides, the inherent advantage of teeth over the blood sample matrix in its ability to retain of Pb after a month or more after the source is removed makes it

an attractive alternative biomarker of Pb exposure. In the current study, wild rodents were trapped from residential areas and sometimes in houses making them suitable sentinel marker for human exposure especially children with hand- to - mouth activities. Furthermore, targeting the “crown” incisor for Pb exposure assessment without targeting the whole incisor tooth will maximize time, resources and increase chances of detection of Pb in sentinel wild rats.

Conclusions

The “crown” and the “root” of both lower and upper incisor teeth Pb-T in experimentally Pb exposed Sprague Dawley rats accumulated Pb in a dose dependent manner with the “crown” accumulating much Pb-T than the “root”, suggesting that the “crown” maybe a superior marker of Pb exposure than the “root”. Furthermore, the Pb-T accumulation in the “crown” of the lower incisors (L2) of wild rats discriminated the varying distances of sampling sites in relation to the Pb-Zn mine point source in environmentally exposed *R. rattus* rats. In addition, the strong positive correlations between Pb-B and Pb-T observed in both experimental and environmentally Pb exposed rats support the possibility of rodent teeth as a useful tool for environmental assessment of Pb. The high Pb-T in the “crown” compared to the “root” and the high localised distribution of Pb in the coronal enamel as observed from the LA-ICP-MS mapping indicate that the “crown” subdivision of the incisor tooth may be adequate for its use in sentinel rodents for Pb exposure assessment. Further studies are required to validate the rat incisor “crown” as suitable alternative biomarker to blood-Pb for environmental Pb exposure assessment.

CHAPTER 4:

Effects of chronic lead exposure in human

4.0 The impact of chronic environmental lead exposure on proinflammatory and immunomodulatory tumor necrosis alpha and interleukin-8 biomarker cytokines in males and females of reproductive age from Kabwe, Zambia

Abstract

Lead (Pb) poisoning remains a great public health challenge globally that is known to induce a wide range of ailments in both children and adults. Globally, the cases of lead poisoning are reducing due to the enactment of laws and regulations around indiscriminate discharge of lead into the environment from mining and the use of leaded gasoline. However, in some developing countries, lead mining wastes from previously lead-zinc mines remain an environmental challenge that exposes residents to perennial Pb sources as case is in Kabwe, Zambia. In this study I investigated the impact of chronic environmental Pb exposure to adult males and females on immunomodulatory cytokines tumor necrosis factor alpha and interleukin-8 using plasma samples. The plasma samples were divided based on the paired blood lead levels (BLL). I categorized the subjects into low BLL (< 10 μg/dL) and high BLL group (> 10 μg/dL) according to sex and run cytokine assays using the standard human cytokine/chemokine Milliplex protocol. The results revealed that female subjects had increased levels of tumor necrosis factor alpha in the low blood lead group and a reduction in the tumor necrosis factor alpha levels in the high blood lead level. In male subjects, the impact of lead exposure on the levels of tumor necrosis factor alpha and interleukin-8 was not significant between the low blood and the high blood lead level groups. A negative correlation between blood lead levels and tumor necrosis factor alpha was found suggesting that increase in blood lead levels could lead to a reduction in tumor necrosis factor alpha and interleukin-8 levels, respectively. The reduced levels of circulating tumor necrosis factor alpha in female subjects suggest that chronic Pb exposure may precipitate immune and inflammation related disorders in females than their male counterparts. Further studies are recommended to investigate the impact arising from the effect of lead on immunomodulatory cytokines especially in females.

Keywords: lead, chronic, immunomodulatory, cytokines, sex-linked

Introduction

Lead (Pb) poisoning remains a great public health challenge globally that accounts for approximately 0.6% of the world total disease burden (WHO, 2010). Despite the advanced knowledge regarding Pb as toxicant with deleterious effects in animals and humans, the unique physical and chemical properties of Pb has kept it in use at both industrial and domestic levels (Mishra et al., 2003). Lead naturally occur in the environment as galena sulphide in very small amounts, large quantities of environmental Pb is attributed to automobile leaded gasoline use, industrial discharges from mining or manufacturing processes including lead battery recycling (Landrigan and Todd, 1994). Although, there is a global decline in Pb usage due to its known toxic effects, occupational and environmental Pb exposure remains a problem in both developed and developing countries (Tong et al., 2000).

Lead poisoning may take two forms namely, acute and chronic poisoning with the former being less common than the latter (Srosiri et al., 2010). Acute Pb poisoning is linked to accidental or occupational Pb exposure and is characterized by blood lead levels (BLLs) between 100 - 120 μg/dL (Flora et al., 2012) with a rapid onset of clinical signs such as constipation, abdominal pain, headaches, emesis seizures, coma and in an unfortunate circumstances death ensues (Dapul and Laraque, 2014; Marsden, 2003). The clinical manifestations of chronic Pb poisoning may include recurrent and persistent symptoms that accompany acute Pb poisoning with relative lower blood lead levels compared to typical acute Pb related clinical manifestations (Flora et al., 2012)**.** In addition, neurobehavioural impairments with behavioural and learning deficits as sequelae even at low Pb exposure in children are common (Wani et al., 2015).

On the other hand, chronic Pb poisoning in adults has been reported to elicit poor learning abilities and other maladies including cardiovascular, haematological, and renal related diseases (Landrigan and Todd, 1994; Srosiri et al., 2010). Moreover, in both adult males and females, chronic Pb poisoning is known to cause insidious reproductive and immune related derangements (Assi et al., 2016) . In males BLLs exceeding 40 μg/dL are associated with low sperm count, poor sperm morphology and motility and a reduction in the level of serum testosterone (Kumar and Devi, 2018; Wani et al., 2015). Lead induced reproductive disorders in females such as miscarriage, low birth weight and pre-term delivery have been reported in moderately low BLLs less than or equal to30 μg/dL (Chang et al., 2006). Whereas the mechanisms of Pb induced reproductive disorders are complex, Pb induced disruption of the antioxidant system, steroidogenesis and immunomodulatory factors such as cytokines among others have been implicated (Mishra et al., 2003; Srosiri et al., 2010; Turksoy et al., 2019). Cytokines are small secreted mediators and regulators of haematopoiesis, inflammation and immunity (Boulay et al., 2003) that are directly or indirectly by Pb exposure. In particular, the effect of Pb exposure on tumor necrosis factor alpha and Interleukin-8 and cytokines are linked to infertility in adults (Boskabady et al., 2018).

A classical scenario of chronic Pb poisoning in males and females of reproductive age has been reported in Kabwe, Zambia (Yabe et al., 2020). Based on the recent report by Yabe et al., (2020), over 50% of both men and women of reproductive age had BLLs that ranged between 5 and 44 µg/dL. Despite the reported BLLs being above the reference level of 5 µg/dL according to the Centers for Disease Control and Prevention (CDC, 2012), the impact of chronic Pb poisoning in adults of reproductive age in Kabwe remains unknown. Hence the objective of this study was to investigate the impact of chronic Pb exposure on immunomodulatory cytokines in adult males and female from Kabwe, Zambia. To the best of my knowledge, the findings reported in the current study forms a baseline data for immunomodulatory cytokines in chronically environmentally Pb exposed Kabwe residents.

Materials and Methods

Ethical consideration

The samples used in this study were drawn from a sampling that was approved by the University of Zambia research Ethics committee (UNZAREC; approval No. 012-04-16) and all participants were volunteers that gave written informed consent. Moreover, the sampling was duly approved and sanctioned by the Ministry of Health through the regulatory body, the Zambia National Research Ethics Board and the District Medical Office in Kabwe (Yabe et al., 2020).

Study target population

The study targeted adult males and females of reproductive ages from Kabwe town of Zambia. Huge piles of Pb contaminated soils dispersed by natural elements such as wind and water runoffs from the abandoned tailings and mine dumps has extensively polluted the town with Pb (Blacksmith institute 2013, Bose-O'Reilly et al, 2018). In this study, plasma samples obtained from blood samples that were collected from adult females and males volunteers that came from all corners of the town at health centres during the 2017, July and August period as was previously reported by Yabe et al. (2020) were used. The study population comprised of a wider section of Kabwe as shown in Fig. 4.1.

Sampling strategy

The plasma samples used in this study were randomly drawn based on the BLLs, age and sex clustering. The BLLs data used were based on the venous blood samples of adult Kabwe residents (male and female) sampled during the July-August, 2017 KAMPAI human broad survey that had been previously analyzed using an inductively coupled plasma mass spectrometry (ICP-MS) courtesy of Yamada et al. (2020). The sex, age, body mass index (BMI) and smoking status were extracted from the questionnaire and screening information that were recorded from participants during the July-August 2017 survey. Based on the Kabwe residents BLLs characteristics, the BLLs were divided into

two groups namely, low BLL and high BLL. The low BLL group represents the Centers of Disease Control and Prevention's low exposure threshold in adults set at less than 10 µg/dL (CDC, 2013). By based on the Centers of Disease Control and Prevention convention, BLLs above 10 µg/dL were classified as the high BLL group. As shown in Table 4.1, the BLL mean for the low BLL group were 3.76 and 4.13 µg/dL for female and male, respectively. And for the high BLL group, the mean BLLs were 23.5 µg/dL for the female group and 23.7 µg/dL for the male group. Age and smoking status were used as exclusion and inclusion criteria. Only healthy males and females of reproductive age of 18 years and above and with no history of smoking were included in this study. A total of 129 plasma samples (Low BLL female: n = 47; Low BLL male: n = 43; high BLL female: n = 21 and high BLL male: n = 18) were used for cytokines analysis were as shown in Table 4.1. The BMI and age characteristics were as listed in Table 4.1.

Human cytokine assay

Based on the pre-experiment cytokines assay I had earlier conducted on several cytokines (epidermal growth factor, transforming growth factor alpha, interferon gamma, interleukin-10, interleukin-15, interleukin-2, interleukin-4, interleukin-6, interleukin-7, interleukin-8, interleukin-1 beta, tumor necrosis alpha and vascular endothelial growth factor) only interleukin-8 and tumor necrosis factor alpha were consistently detected in Kabwe human samples. Thus, only TNF-α and IL-8 were assayed. The two plasma cytokines, interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) were assayed using MILLIPLEX ® MAP Kit-Human cytokine/chemokine Magnetic Bead Panel (HCYTMAG-60K) (Merck KGaA, Darmstadt, Germany) with strict adherence to the manufacturer's instructions. All reagents and standard solutions (3.2 to 2000 pg/ml) were provided and prepared according to the guidelines outlined in the manual. The frozen plasma samples that were kept at –20 °C were thawed prior to analysis at room temperature. For the protocol as an initial step, the 96 well plates were conditioned using 200 µL wash buffer on a plate shaker for 10 min at room temperature. Then the wash buffer was decanted, and all residual amounts were removed by inverting and tapping onto clean absorbent towels. The standards and the positive and negative control samples were added to appropriate wells, followed by the addition of 25 µL assay buffer to the background and sample well. The plasma samples (25 µL) were then added to the sample wells. Then 25 µL of the premixed beads were applied to all the wells. The plate was then sealed with a plate sealer and wrapped with aluminium foil and incubated with mild agitation overnight for 18 h at 4 °C. Following incubation, the well contents were decanted, and the plate was gently washed with 200 µL two times and the detection antibodies were added to each well and the well plate contents were incubated for 1 h at room temperature. And this was followed by the application of the fluorescent conjugate streptavidin-phycoerythrin to each well and incubation for 30 min at room temperature. The well contents were gently decanted, and the plate was washed two times using the wash buffer. Finally, 150 µL sheath

fluid was added before running the samples on Luminex® 200™ with xPOTENT ® software (Luminex Corporation, Austin, TX, USA).

Data analysis

The statistical analyses were all performed using GraphPad Prism software (Prism 7 for Windows; Version 5.02, California USA). The data were reported as mean ± SD (standard deviation). I assessed the data for normality using the Kolmogorov–Smirnov test and its homogeneity of variance using Levene's test. My data was not normally distributed, so I applied the Kruskal–Wallis test and Dunn's Multiple Comparison test or Mann-Whitney U test was applied to compare between two groups. Correlation analyses were performed using the Spearman non-parametric correlation coefficient test. The principal component analysis (PCA) was performed to explore association between plasma cytokines (TNF-α and IL-8) and age, BMI and BLL using JMP Pro version 15 (SAS Institute, NC, USA). All statistical analyses with p-value < 0.05 were considered significant.

Results

The blood lead levels and other characteristics of the studied adult female and male subjects

The general characteristics of the studied subjects are listed in Table 4.1. In this study, the BLLs levels were used as the principal determining characteristic for the randomly selected plasma samples and the categorization into low and high BLL groups. The female subjects age ranges were 47 – 69 years for the low and 21 – 68 years for high BLL group. The mean age between the two groups was not significantly different (p = 0.51). The BMI between the low BLL and high BLL groups in females was statistically similar between the two groups. In case of male groups, the age range for the low BLL was 19 - 72 years and 21 – 66 years in the high BLL group. The mean age between the low BLL and the high BLL as shown in Table 4.1 was statistically different (p < 0.01). The BMI mean was not statistically different. All the selected plasma samples were from non-smokers in both female and male groups.

Effect of environmental lead exposure on the plasma TNF-α and IL-8 cytokines in females

The plasma cytokines concentration in females ranged between 1.5 and 36.7 pg/mL with the mean of 17.7 ± 1.45 pg/mL for TNF-α in the low BLL group. In the high BLL group, the TNF-α range was between 1.88 and 39.1 pg/mL with a mean of 11.7 ± 1.80 pg/mL. A statistically significance (p < 0.05) reduction in the circulating TNF-α was observed as shown in Fig. 4.2. A slight but non-significant reduction in IL-8 cytokines levels were observed. Based on the Spearman's correlations (r = 0.288, p < 0.05) analyses, a positive correlation between TNF-α and IL-8 levels were observed (Table 4.2). A weak non-significant negative correlation (r = - 0.183) between age and BLL was observed

Effect of environmental lead exposure on the plasma TNF-α and IL-8 cytokines in males

The plasma cytokines concentration in males chronically exposed Pb are shown in Fig. 4.3. The TNF-α was slightly reduced in the high BLL group compared to the low BLL group with means of 12.6 ± 1.46 pg/mL and 14.6 ± 2.04 pg/mL, respectively. The plasma IL-8 were also not statistically different between the two groups. Table 4.3 shows the Spearman's correlations analyses for the plasma cytokines and other variables in males. A non-significant positive correlation between TNF-α and IL-8 was observed. Among the other variables, a positive significant correlation between BLL and age was observed.

The comparative effect of chronic Pb exposure on plasma TNF-α and IL-8 cytokines in adult female and males

Fig. 4.4. below shows the principal component analysis (PCA) results of the male and female plasma TNF-α and IL-8 cytokines and other variables. The first principal component (PC1) accounted for 27.6 % and PC2 accounted for 24.4 %. The age and BLL were the major contributor of the data variations that were in PC1. And age and BMI accounted for much of the variation in data for PC2. The overall associations in the cytokines relative to the age, BMI and BLL as estimated after PCA Row-wise method showed some associations. The TNF-α was positively correlated (r = 0.21) with the IL-8. Generally, the cytokines levels reduced with an increase in BLL in both male and female. On the other hand, the BLL was negatively correlated (r = -0.21) to both TNF-α and IL-8. No associations were observed between the plasma cytokines, and BMI and age.

The comparative effect of chronic exposure on plasma cytokines based on sex are shown in Fig. 4.5. The circulating plasma TNF-α cytokine was significantly reduced in the low BLL group of the male compared to that of the female in the low BLL. In the high BLL group, the female TNF-α was slightly lower than that of the male. The IL-8 showed a similar pattern to that of the TNF-α in a non-significant manner

Table 4.1 General characteristics of adult female and male subjects sampled

Gender: Female	**Low BLL (n = 47)** **Mean ±SD (Min-Max)**	**High BLL (n = 21)** **Mean ±SD (Min-Max)**	***p*-values**
BLL (µg/dL)	3.76 ± 2.21 (0.79-7.59)	23.5 ± 7.95(14.1- 42.5)	< 0.001*
Age (years)	37.7 ± 12.3 (47-69)	36.5 ± 14.6 (21-68)	0.511
BMI (kg/m^2)	24.6 ± 5.23 (15.2- 37.9)	24.1 ± 4.07 (18.4- 31.9)	0.695
Smoking status	Never smoked	Never smoked	
Gender: Male	**Low BLL (n = 43)** **Mean ±SD (Min-Max)**	**High BLL (n = 18)** **Mean ±SD (Min-Max)**	***p*-values**
BLL (µg/dL)	4.13 ± 2.12 (1.18–7.96)	23.7 ± 7.28 (15.5 – 40.7)	< 0.001*
Age (years)	39.3 ± 13.4 (19 -72)	47.7 ± 12.5 (21 - 66)	0.008*
BMI (kg/m^2)	24.1 ± 5.03 (18.6 – 38)	(21.9 ± 3.00 (16.2 – 29.7)	0.293
Smoking status	Never smoked	Never smoked	

Mann Whitney U test ($p < 0.05$). BMI (Body Mass Index), BLL (Blood Lead Level)

Table 4.2. Correlation coefficient of cytokines and independent variables in females (n = 68)

Variables	BMI	Age	IL-8	TNF-α	BLL
BMI (kg/m^2)				-	-
Age (years)	**0.322***				
IL-8 (pg/mL)	-0.091	0.084			
TNF-α (pg/mL)	-0.104	0.061	**0.288***		-
BLL (µg/dL)	-0.145	-0.183	-0.028	-0.150	

*Spearman's correlation coefficient $p < 0.05$. BMI (Body Mass Index), IL-8 (Interleukin-8), TNF-α (Tumour necrosis factor alpha), BLL (Blood Lead Level)

Table 4.3. Correlation coefficient of cytokines and independent variables in males (n = 61)

Variables	**BMI**	**Age**	**IL-8**	**TNF-α**	**BLL**
BMI (kg/m^2)				-	-
Age (years)	0.172				
IL-8 (pg/mL)	-0.048	-0.069			
TNF-α (pg/mL)	-0.035	-0.075	0.163		-
BLL (µg/dL)	-0.137	**0.372***	0.050	-0.133	

*Spearman's correlation coefficient $p < 0.05$. BMI (Body Mass Index), IL-8 (Interleukin-8), TNF-α (Tumour necrosis factor alpha), BLL (Blood Lead Level)

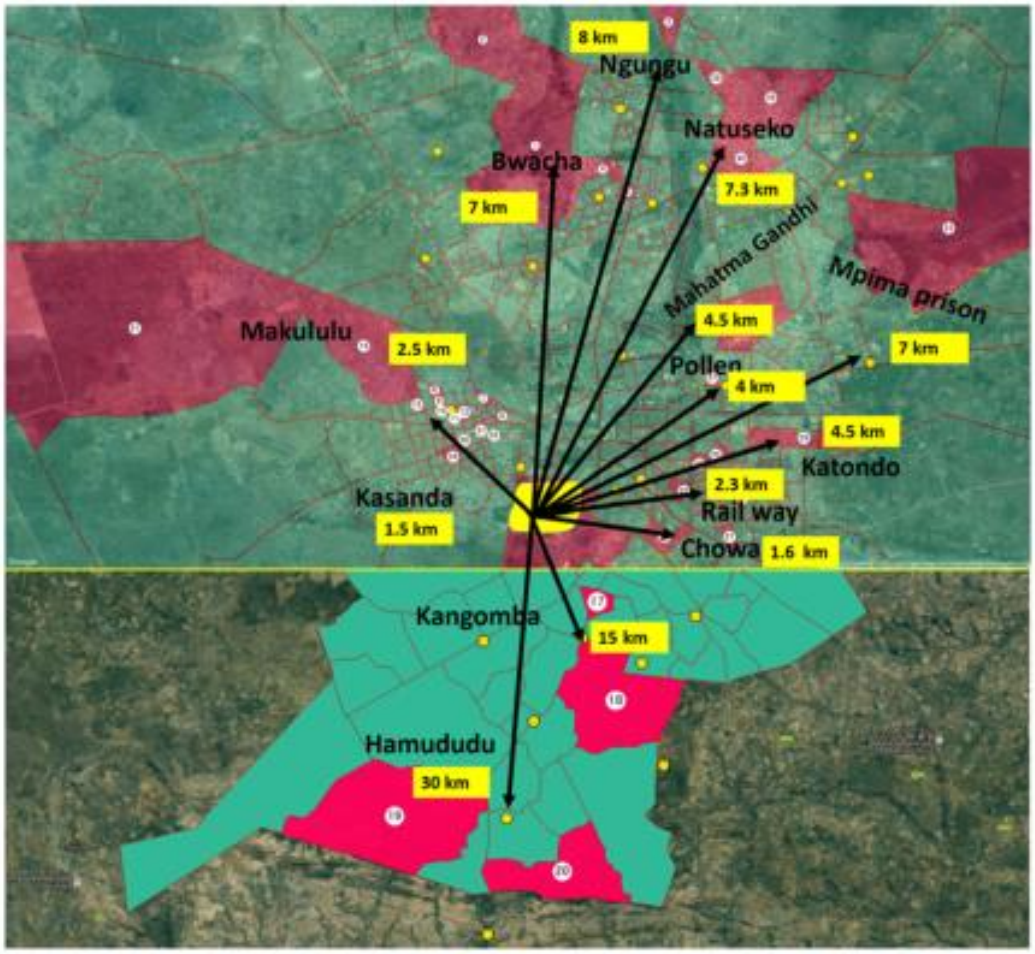

Fig. 4.1. Map of Kabwe showing the Standard Enumeration Areas (SEAs) that were selected for sampling across Kabwe town and the health centres used for sampling are shown in yellow in July and August 2017 (Yabe et al., 2020).

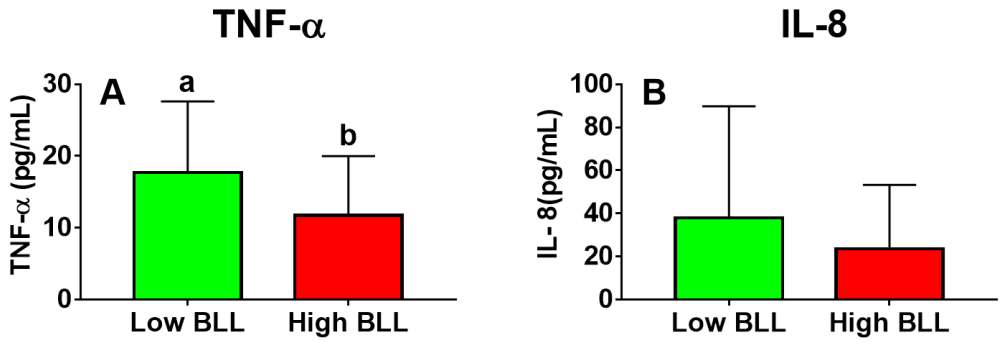

Fig. 4.2. Cytokines in adult female plasma samples (mean ± SD). **A.** Tumor necrosis factor alpha (TNF-α) and **B.** Interleukin-8 **(**IL-8). ($^{a, b}$ represents $p < 0.05$, Mann Whitney test).

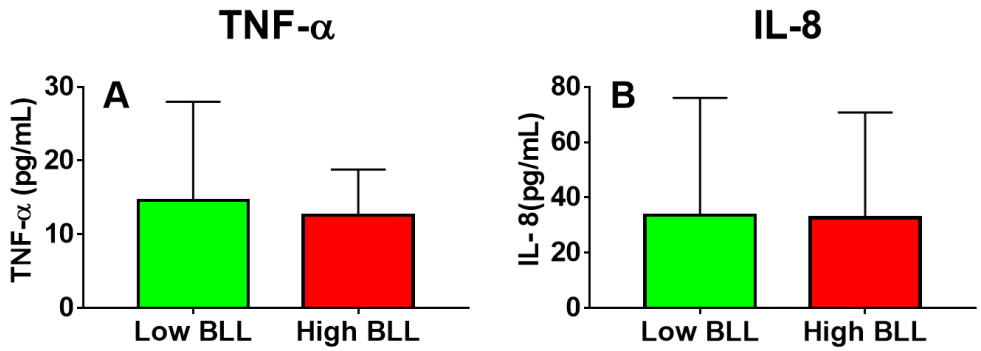

Fig. 4.3. Cytokines in adult male plasma samples in both low and high BLL groups (mean ± SD). **A.** Tumor necrosis factor alpha (TNF-α) and **B.** Interleukin-8 **(**IL-8).

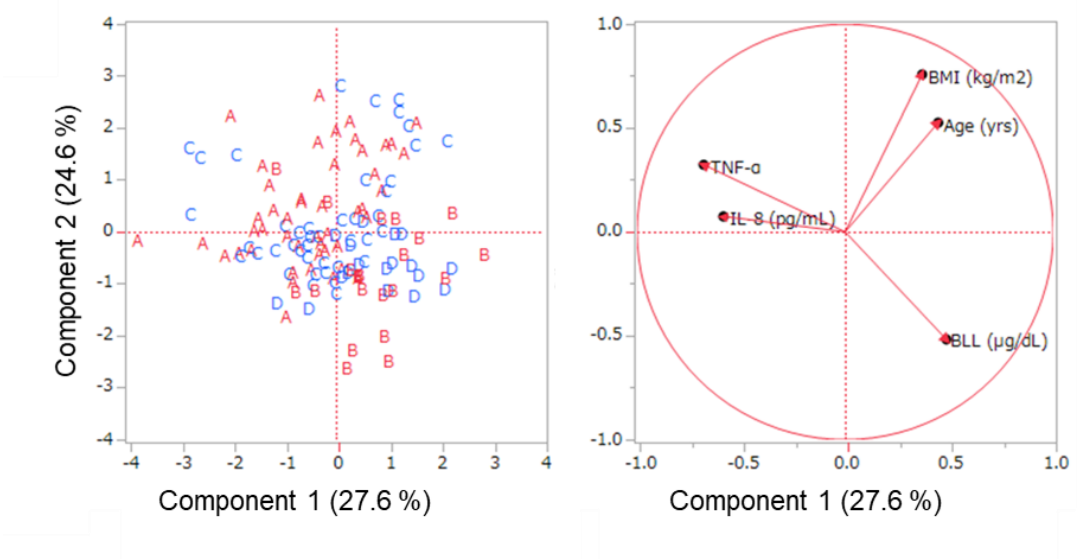

Fig. 4.4. Principal component analysis of the plasma cytokines, Tumor necrosis factor alpha (TNF-α) and Interleukin-8 (IL-8) and their relationship with the age, BMI, and BLL in the total samples analyzed (n = 129) in adult female and male (Alphabetical letters represents the four groups investigated in the study: **A**= Low BLL female, **B** = high BLL female; **C**= low BLL male and **D** = high male groups)

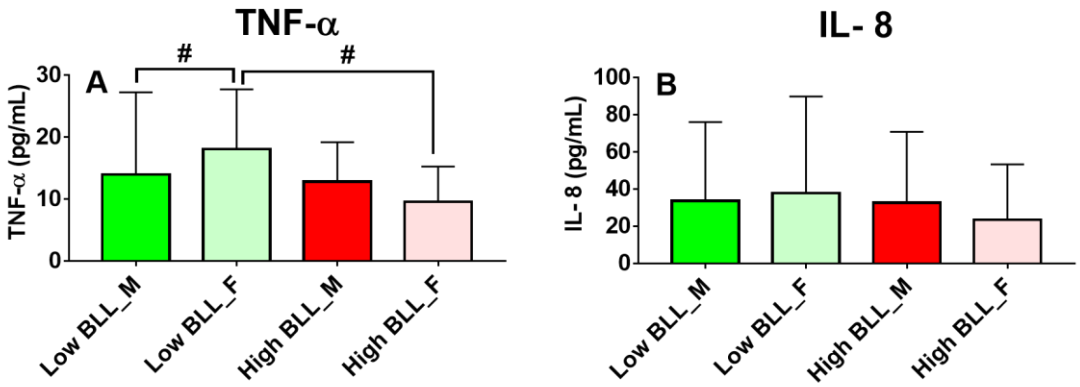

Fig. 4.5. Cytokines in adult female and male plasma samples across the two groups. Key: Low BLL_M = Low BLL group in males, Low BLL_F = Low BLL group in females, High BLL_M = High BLL group in male, High BLL_F = High BLL group in female (mean ± SD). **A.** Tumor necrosis alpha (TNF-α) and **B**. Interleukin-8 (IL-8). ($^{\#}$ represents $p < 0.05$, Mann Whitney test).

Discussion

In the current study, the first baseline findings on the effect of chronic environmental Pb exposure on immunomodulatory cytokines namely; tumor necrosis alpha and interleukin-8 in adult male and female residents of Kabwe, Zambia are reported. Immunomodulatory cytokines play a vital role in the ability of the body to combat both communicable and non-communicable diseases (Lee et al., 2010). The dysregulation of cytokines levels therefore can exacerbate ailments by enhancing pathological processes such bone and autoimmune maladies including cancer progression (Metryka et al., 2018) . Lead induced toxicities in adult females of reproductive age has been linked to insidious clinical manifestations such as miscellaneous miscarriages, pre-term birth and babies with low birth weight (Wani et al., 2015). Although the mechanisms behind the above clinical manifestations are complex, Pb induced inflammation and immune system dysregulation have been said to be indirectly or directly linked to immunomodulatory cytokines (Fenga et al., 2017).

In general the levels of both tumor necrosis alpha and interleukin-8 detected in plasma samples of adult males and females of reproductive ages under chronic Pb environmental exposure was similar to the levels that have been reported in plasma or serum samples in occupationally Pb exposed subjects (Turksoy et al., 2019). Moreover, in the current study, environmental Pb exposure impact on immunomodulatory cytokines revealed a sex dependent effect. In particular, the female's plasma samples revealed an increase in circulating tumor necrosis alpha cytokines at low blood lead level and reduction in TNF-α levels at high blood lead level with corresponding levels in interleukin-8. The male plasma cytokines showed a similar trend that were observed in the females though not statistically significant.

The differences observed in the levels of the plasma cytokines between male and females have also been reported by other authors who attributed the differences to the inherent differences in the genetic makeup and hormonal profiles (Baldaçara and Silva, 2017). Estradiol hormone has been implicated as one the factor that accounted for the sex-linked differences in cytokine levels in females and males (Esmailidehaj et al., 2020). In particular, high levels of 17β-estradiol were accompanied by reduced the concentrations of TNF-α in peripheral blood cultures (Rogers and Eastell, 2001). Notwithstanding, in the present study, I observed that females subjects with low blood lead levels had higher concentrations of TNF-α than the ones with high blood lead levels suggesting that chronic Pb exposure had significant impact on the concentrations of the immunomodulatory cytokines tumor necrosis alpha and interleukin-8.

The plasma tumor necrosis alpha and interleukin-8 levels were positively correlated in the female subjects in this study, a pattern that agreed with other authors that demonstrated that an increase in TNF-α was accompanied by a corresponding increase in IL-8 levels (Valentino et al., 2007). Moreover, the level of IL-8 is dependent on TNF-α as the latter is known as the inducer of the production of the latter in body cells such as macrophages (Turksoy et al., 2019). The relationship

between the two cytokines implies that an adverse impact related to Pb exposure through the reduction of the type I helper (Th1) cells that produce TNF-α will eventually impact the other (Valentino et al., 2007).

On the other hand, a negative correlation between blood lead level and the two immunomodulatory cytokines observed suggest that female Kabwe residents with high blood levels will have low levels of these cytokines. Considering the age of female participants in this study, the findings in this study imply that the deleterious effect of Pb on immunomodulatory cytokines could be among the factors that may negatively impact on fertility and the wellbeing of the foetuses. Based on the lack of association between the two cytokines and the body mass index and age in the current study implies that reducing the Pb exposure could be a mitigate measures against the low levels of circulating cytokines observed in subjects with elevated blood levels.

This study has some limitations. The first limitation was on the small sample size though the selection represented a broad section of the Kabwe residents. The lack of other analyses on the general blood and hormonal profiles is another limitation. And this being a cross section study naturally entails that follow up studies must be conducted to further explore the impact of the chronic environmental Pb exposure with a large sample size. I therefore recommend that further studies be conducted to ascertain the impact of the long standing environmental Pb pollution in adult residents in Kabwe, Zambia.

Conclusions

In conclusion, the chronic environmental lead exposure as it obtains in Kabwe, Zambia revealed negative effects on the levels of the circulating plasma TNF-α and IL-8 cytokines in adult female and male subjects. The female subjects showed increased levels of TNF-α in the low blood lead group and a reduction in the TNF-α levels in the high blood lead level. In male subjects, the impact of lead exposure on the levels of TNF-α and IL-8 was not significant between the low blood and the high blood lead level groups. A negative correlation between blood lead levels and TNF-α was found suggesting that increase in blood lead levels could lead to a reduction in TNF-α and IL-8 levels. Therefore, my present findings suggest that adult females under Pb exposure may be more vulnerable and susceptible to TNF-α and IL-8 dysregulation linked diseases. Further studies are recommended to investigate the impact arising from the effect of lead on immunomodulatory cytokines especially in females.

CHAPTER 5:

Concluding Remarks and Future Perspectives

In this PhD thesis, the effects of lead toxicity in fish, animals and humans with concurrent investigation of the sentinel wild rodent tooth for environmental lead pollution has been reported and discussed.

Lead poisoning remains a global socioeconomic and public health issue that is mainly a man-made problem as it naturally occurs in very small amounts in the natural environment and its presence in large quantities in the environment is directly related to human activities. The Kabwe lead pollution situation is a good example a long-standing issue of lead poisoning. The Kabwe lead pollution poisoning predates the existence of the town itself based on evidence that show the earliest human settler, "Broken Hill Man" (*Homo heidelbergensis*) formerly *Homo rhodesiensis* who lived between 25,000 and 300,000 years ago may have suffered from lead poisoning. This aspect makes Kabwe town an ideal place to study chronic lead exposure related toxicity in animals, aquatic organism and humans as most of the residents may have been exposed to lead from the early childhood into their adulthood. There is a presence of both lead and zinc in large amounts in the soils above the benchmark values. This second scenario implies that lead exposure especially in children with hand-to-mouth activities may exist as case of both lead and zinc co-exposure. Furthermore, the oral bioaccessibility study revealed that both lead and zinc were highly bio-accessible implying that both would be easily taken up by the body following accidental hand-to-mouth activities.

In my doctoral thesis, the studies on the effects of the effects of lead toxicity in fish, animals and humans with concurrent investigation of the sentinel wild rodent tooth for environmental lead pollution as relates to the Kabwe situation revealed the following summaries:

1. Lead dissolved in water poses a threat to aquatic life even at the lowest quantifiable amounts. Water lead concentrations of Pb as low as 10 to 50 µg/L Pb that are within the "permissible limit" could be detrimental to aquatic life especially at developmental stages evidenced by embryonic coagulation linked mortalities. Embryonic activity aberrations, cardiovascular toxicity (reduced heart rate, increased blood flow activity), oxidative stress system imbalance, antiapoptotic and proapoptotic balance and the neuromuscular toxicity (involuntary muscle twitching) are among the deleterious effects of environmentally relevant Pb levels. Further investigations on environmentally relevant Pb water levels and what may be considered as permissible or regulatory water Pb levels effects on aquatic life, are needed to ensure water bodies are safe to support aquatic life.

2. Environmentally relevant concentrations of Pb as they occur in Kabwe could be detrimental to aquatic life especially in larval fish. Acute exposure to the environmentally relevant Pb levels attenuated larval zebrafish behaviour by inducing hyperactivity under dark/light illumination. This locomotor activity pattern alteration could be linked to altered neurobehavior via neurotoxicity mediated by oxidative stress or direct Pb neuro-intoxication due to lack of fully formed brain blood barrier. The neurobehavioral attenuation reported in my study may have potential ecological ramifications through alterations in predator-prey interactions affecting the survival rates of

developing fish in the wild. However, the degree to which these observed effects following acute Pb exposure within a 30 minutes period to low Pb levels will persist during prolonged exposure needs to be investigated further.

3. The lead-zinc co-exposure situation in children in Kabwe, Zambia calls for special attention as zinc impact on toxicity may be partially protective or detrimental. The study on impact of zinc co-administration with lead acute exposure suggest that the protective effects of Zn in Pb and Zn co-exposure on Pb tissue accumulation and Pb toxicity may depend on the quantities of the metals ingested. Whereas, Zn supplementation may reduce tissue Pb accumulation in some tissues at low levels, the reverse may happen with increased level of exposure. Notwithstanding, Zn supplementation may confer oxidative stress gene protection at molecular level of some genes. Further studies are required to investigate the Zn dose effect that seems to undo the beneficial effects of Zn supplementation in lead exposure

4. The “crown” and the “root” of both lower and upper incisor teeth Pb-T in experimentally Pb exposed SD rats accumulated Pb in a dose dependent manner with the “crown” accumulating much Pb-T than the “root”, suggesting that the “crown” maybe a superior marker of Pb exposure than the “root”. Furthermore, the Pb-T accumulation in the “crown” of the lower incisors (L2) of wild rats discriminated the varying distances of sampling sites in relation to the Pb-Zn mine point source in environmentally exposed *R. rattus* rats. In addition, the strong positive correlations between Pb-B and Pb-T observed in both experimental and environmentally Pb exposed rats support the possibility of rodent teeth as a useful tool for environmental Pb exposure assessment. The high Pb-T in the “crown” compared to the “root” and the high localised distribution of Pb in the coronal enamel as observed from the LA-ICP-MS mapping indicate that the “crown” subdivision of the incisor tooth may be adequate for its use in sentinel rodents for Pb exposure assessment. Further studies are required to validate the rat incisor “crown” as suitable alternative biomarker to blood-Pb for environmental Pb exposure assessment especially as it relates to use as sentinel for human lead exposure.

5. The chronic lead exposure situation in Kabwe may have negative indirect effects in immune and proinflammatory cytokines in adults. The female subjects showed increased levels of TNF-α in the low blood lead group and a reduction in the TNF-α levels in the high blood lead level. In male subjects, the impact of lead exposure on the levels of TNF-α and IL-8 was not significant between the low blood and the high blood lead level groups. A negative correlation between blood lead levels and TNF-α was found suggesting that increase in blood lead levels could lead to a reduction in TNF-α and IL-8 levels. Further studies are recommended to investigate the impact arising from the effect of lead on immunomodulatory cytokines especially in females. Further studies are required to clarify the impact of this effect in age and non-communicable disease burden in adults residing in polluted areas of Kabwe.

Therefore, further research to explore the negative effects of Pb exposure on fish, animal and human life and public sensitization efforts on the negative impact regarding the effects of the Kabwe lead situation are highly recommended to ensure better quality of life for animals and people.

As future perspectives in combating the Kabwe situation the following actions are recommended:

a) Upscale lead screening in both adults and children presenting with any clinical conditions in the area as the presence of zinc with lead may exacerbate lead induced toxicity.
b) Children and expectant mothers need special lead screening and where applicable chelation therapy be applied
c) Promotion of voluntary screening tests for non-communicable and fertility related conditions in adults especially females that may be highly susceptible to lead induced cytokines dysregulation.
d) The regulatory water lead levels of 50 μg/L need to be revised to protect aquatic and ensure human public health through regulatory bodies such as the Zambia Environmental Management Agency (ZEMA) mandated to ensure a health environmental.

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English thesis abstract

Lead (Pb) is a metal toxicant of global concern whose environmental presence in large quantities is directly related to human activities such as mining without "good-for-earth" practices in place. The objective of the present work was to investigate the toxicological impact of lead exposure on fish, animal and human health. Lead exposure in water at concentrations reported in Kabwe, Zambia in Africa (0.01-94 µg/L) to embryos and larval zebrafish (*Danio rerio*) under alternating dark and light illumination revealed negative toxicological effects on fish life. Lead exposure induced low survival rates, cardiovascular toxicity and neuromuscular toxicity (involuntary muscle twitching) at 50 µg/L concentration Pb and above in embryos. On the other hand, acute Pb exposure attenuated the larval zebrafish behaviour and induced hyperactivity under dark/light illumination. Lead induced oxidative stress system imbalance and caused the downregulation of catalase mRNA and upregulation of glutathione S-transferase mRNA in zebrafish embryos. Exposure further induced upregulation of the apoptosis regulator protein mRNA of B-cell lymphoma 2 gene in embryos. Acute exposure in larval zebrafish led to the upregulation uncoupling protein-2, cytochrome c oxidase subunit I and B-cell lymphoma 2 genes. The co- exposure of Pb together with zinc (Zn) like the Kabwe environment in Sprague-Dawley rats (*Rattus norvegicus*) revealed that Zn partially mitigated the toxic effects of lead at low and not at high exposure through reduced tissue Pb uptake in both the testes and muscles. A reduction in bone Pb uptake was also observed in the Zn and Pb co-exposed group. No mitigative Zn effects were seen in other tissues. Zinc co-administration prevented Pb inhibition of blood delta aminolaevulinic acid dehydratase enzyme activity at high level of exposure. Furthermore, Zn prevented the downregulation of the catalase, superoxide dismutase, glutathione peroxidase and other vital signalling genes in the liver, kidney and brain tissues. As sentinel marker of Pb exposure tool, the investigation of use of the crown incisor of the wild rat (*Rattus rattus*) sampled from polluted areas of Kabwe, Zambia suggested that the accumulation of Pb in the "crown" incisor was reflective of the rat's habitat. In addition, the strong positive correlations between lead in blood and lead in teeth was observed suggesting that the "crown" incisor may be a useful tool in Pb exposure assessment. The investigation on the impact of chronic Pb exposure in adult male and female Kabwe residents on the levels of the plasma immunomodulatory protein cytokines revealed reduced levels of tumor necrosis factor alpha and interleukin-8 in female subjects only with blood lead levels above 10 µg/dL and a mean of 24 µg/dL. The increase in blood lead levels resulted in reduced concentration of plasma immunomodulatory protein cytokines in females.

Taken together, these findings suggest that the levels of lead in the Kabwe environment including the aquatic environment posed a negative impact on younger aquatic life, animals and humans. Further studies are required to ascertain the impact of chronic lead exposure in the presence of high levels of zinc especially in vulnerable groups such as expectant mothers, immunocompromised individuals and children living in lead polluted environment.

Japanese thesis abstract

日本語要旨

鉛（Pb）は世界的に懸念される毒性金属であり、環境に配慮の無い不適切な鉱山活動など人間活動が原因の環境中の鉛汚染が引き起こされる。本研究の目的は、鉛曝露が魚類、動物および人の健康に与える毒性影響を明らかにすることである。アフリカのザンビア・カブウェ鉛汚染地域の環境水で報告されている濃度（0.01-94 µg/L）の鉛曝露は、明暗交互照明下の室内実験においてゼブラフィッシュ（*Danio rerio*）の 胚と幼生に対して毒性影響を及ぼすことが明らかになった。50 µg/L の鉛濃度では、胚の生存率低下、心血管系毒性および神経筋毒性（不随意筋の痙攣）を誘発した。鉛の急性曝露はゼブラフィッシュ幼生の行動を減衰させ、明暗照明下での多動性を誘発した。ゼブラフィッシュ胚の酸化ストレス機構に関しては、鉛曝露により catalase 遺伝子の mRNA 発現量が低下し、glutathione S-transferase および B-cell lymphoma 2 遺伝子の mRNA 発現量が上昇した。また、ゼブラフィッシュ幼生の急性曝露では、uncoupling protein-2、cytochrome c oxidase subunit I 、B-cell lymphoma 2 genes 遺伝子の mRNA 発現量を上昇させた。Sprague-Dawley ラット(*Rattus norvegicus*)に対して亜鉛（Zn）と鉛の共曝露を行ったところ、亜鉛は精巣と筋肉における鉛の取り込みを減少させ、低濃度曝露では鉛の毒性影響を一部緩和したが、高濃度曝露では緩和しないことが明らかになった。また、亜鉛と鉛の共曝露群において、骨における鉛濃度の減少が観察された。他組織では鉛の毒性に対する亜鉛の緩和効果は認められなかった。高濃度の亜鉛投与により、鉛による血中の delta aminolaevulinic acid dehydratase 酵素活性の阻害が抑制された。さらに、肝臓、腎臓および脳における catalase、superoxide dismutase、glutathione peroxidase およびその他の重要なシグナル伝達遺伝子の mRNA 発現量の低下を抑制した。野生歩哨動物における鉛曝露のバイオマーカーとして、カブウェで採材した野生ラット（*Rattus rattus*）の頭頂部側の切歯で観察された鉛蓄積は、ラットの生息環境の鉛汚染を反映していることが示唆された。さらに、血中と歯の鉛濃度の間に強い正の相関関係が観察されたことから、頭頂部側の切歯が鉛曝露評価に有用であると考えられた。成人男女のカブウェ住民における慢性的な鉛曝露が血漿中の免疫系サイトカインに及ぼす影響に関しては、血中鉛濃度が 10 µg/dL 以上（平均値 24 µg/dL）では、女性における血漿中の tumor necrosis factor alpha および interleukin-8 のタンパク質濃度が低下しており、血液中の鉛濃度上昇は、女性における一部の血漿中サイトカインを減少させることが考えられた。

本研究で明らかにした知見は、カブウェの環境における鉛汚染が水棲生物、動物、人に毒性影響を与えることを示唆している。今後、高濃度の亜鉛の存在下での慢性的な鉛曝露の影響を解明するためには、妊婦や免疫不全を呈する患者、子供など調査対象を拡大した更なる研究が必要である。