THE EFFECTS OF CHRONIC LOW DOSE ORGANIC ARSENIC EXPOSURE ON THE KIDNEY: MECHANISM OF INJURY AND MICROSCOPIC CHANGES

BY

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ABSTRACT

Chronic exposure to inorganic arsenic has been linked with multiple medical conditions, which shifted the use of inorganic to the organic-based herbicide, monosodium methyl arsenate (MSMA). However, with increasing numbers of chronic kidney disease of unknown causes (CKDu), chronic exposure to herbicide is believed to be one of the potential explanation. To date, studies on the effects of organic arsenic exposure on the kidney are limited. Therefore, this study aimed to investigate the effect of chronic oral organic arsenic exposure on the rat's kidney. Thirty-six Sprague Dawley rats (N=36) were randomly divided into MSMA exposed, and its corresponding control groups for 2-,4- and 6-month, each with six animals per group. The exposed groups were given oral MSMA at 63.20 mg/kg body weight, while control groups received distilled water. At the end of each duration, the serum was collected for the creatinine level. The kidney tissues were harvested for arsenic level measurement, histopathological, immunohistochemistry, real-time PCR analysis and ultrastructural analysis. Genes expressions were done for kidney injury marker gene (*KIM-1*), oxidative stress genes (*Catalase*, *GSR*, *NOS1*), apoptosis genes (*Tp53*, *Caspase-3* and *Caspase-9*) and inflammatory genes (*Interleukin-6* and *Interleukin-8*). Serum creatinine was not significantly different between exposed and control groups. Tissue arsenic level was significantly higher in exposed groups as compared to that of the control group. All gene expression markers were downregulated at 2-month and upregulated at 4-month except for *Catalase* which remained downregulated. At 6-month, only *KIM-1*, *GSR* and *Caspase-3* remained upregulated. Histological, immunohistochemistry and ultrastructural findings showed chronological changes in the glomeruli and proximal tubules with increased expressions of malondialdehyde (MDA) staining, *Caspase-3* and TUNEL staining with the duration of exposure. Therefore, chronic oral exposure to low dose organic arsenic has demonstrated evidence of kidney injury in rats possibly due to oxidative stress.

خلاصة البحث

ارتبط التعرض المزمن للزرنيخ غير العضوي بالعديد من الحالات طبية، مما أدى إلى التوجه لاستخدام مبيد الأعشاب العضوي، أرسونات ميثيل أحادي الصوديوم، بدلا من المبيدات غير العضوية، ومع تزايد المصابين بمرض الكلى المزمن مجهول الأسباب، فإنه يُعتقد أن التعرض المزمن لمبيدات الأعشاب هو أحد التفسيرات المحتملة. الدراسات المتعلقة بآثار التعرض للزرنيخ العضوي على الكلى محدودة إلى الآن. ولذلك فقد هدفت هذه الدراسة إلى التحقيق في آثار التعرض المزمن للزرنيخ العضوي المعطى عن طريق الفم على كلى الفئران. تم تقسيم 36 من جرذان السبراق داولي بشكل عشوائي إلى مجموعات معرضة لأرسونات ميثيل أحادي الصوديوم ومجموعات ضابطة مقابلة لها، لمدة 2شهرين، و 4 أشهر، و 6 أشهر، وفي كل مجموعة ست جرذان. تم إعطاء أرسونات ميثيل أحادي الصوديوم للمجموعات المختبرة عن طريق الفم بجرعة 63.20 مجم/كجم من وزن الجسم، بينما أعطيت المجموعات الضابطة الماء المقطر. في نهاية كل فترة تم جمع أمصال الدم لقياس مستوى الكرياتينين. تم حصاد أنسجة الكلى لقياس مستوى الزرنيخ، وللتحليل الهيستوباثولوجي، والتحليل الكيميائي النسيجي المناعي، وتحليل تفاعل البوليميراز المتسلسل اللحظي، وتحليل التركيب الدقيق. تم القيام بالتعبير الجيني للجين المشير لإصابة الكلى (KIM-1)، وجينات الإجهاد التأكسدي (الكاتالاز، و GSR، و NOS1)، وجينات موت الخلايا المبرمج (Tp53، و Caspase-3 و Caspase-9) وجينات الالتهاب (Interleukin-6 و Interleukin-8). لم يكن الكرياتينين في الدم مختلفا بشكل كبير في المجموعات المعرضة مقارنة بالمجموعة الضابطة. وكان مستوى الزرنيخ في الأنسجة أعلى بشكل ملحوظ في المجموعات المعرضة مقارنة بمستويات المجموعات الضابطة. تم ملاحظة التنظيم التخفيضي في جميع مؤشرات التعبير الجيني في مجموعة الشهرين، والتنظيم الرفعي في مجموعة الـ4 أشهر باستثناء جين الكاتالاز الذي نظم تخفيضيا. في مجموعة الـ 6 أشهر، تم التنظيم الرفعي فقط في جينات KIM-1، و GSR، و Caspase-3. أظهرت نتائج التحليل الهيستوباثولوجي والتحليل الكيميائي النسيجي المناعي وتحليل التركيب الدقيق تغيرات كرونولوجية في الكبيبات والأنابيب القريبة مع زيادة تعبير تصبغ المالونديالديهايد وتصبغ Caspase-3 و TUNEL تناسبيا مع مدة التعرض. وبهذا فإن التعرض المزمن عن طريق الفم لجرعات منخفضة من الزرنيخ العضوي قد أظهر علامات على الإصابة الكلوية في الجرذان وذلك بسبب الإجهاد التأكسدي على الأرجح.

APPROVAL PAGE

The thesis of Wan Muhamad Salahudin Wan Salleh has been approved by the following:

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

As_2O_3	Arsenic Trioxide
CKD	Chronic Kidney Disease
CKDu	Chronic Kidney Disease of Unknown Causes
DMA	Dimethylarsinic Acid
GR	Glutathione reductase
GSH	Glutathione
GSR	Glutathione-Disulfide Reductase
H&E	Haematoxylin and Eosin
ICP-MS	Inductively coupled plasma mass spectrometry
MDA	Malondialdehyde
MMA	Methylarsonic Acid
MSMA	Monosodium Methyl Arsenate
PAS	Periodic Acid-Schiff
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND AND JUSTIFICATION

Human exposure to environmental arsenic remains a significant public health challenge. The World Health Organization (WHO) in 2018 had reported that 140 million people from 50 countries were exposed to arsenic above the recommended level of 10 µg/L (WHO, 2018). The primary sources of arsenic contamination were from anthropogenic activities such as agricultural, industrial and domestic activities. These activities include the usage of arsenic-based fertilizers, pesticides and herbicides, mining and refining, and efflux of industrial wastes into the water system (Smedley & Kinniburgh, 2002). Arsenic toxicity was suggested to be acquired by oral ingestion of drinking water from contaminated groundwater such as a tube or shallow wells (Mar Wai et al., 2019).

Chronic exposure to inorganic arsenic has been known to cause many medical conditions such as diabetes, hypertension, peripheral artery disease and various tumour formation in skin, lungs, bladder liver and kidneys (Robles-Osorio et al., 2015). Due to the adverse effects of inorganic arsenic on health, organic arsenical herbicides were introduced in the 1950s. Monosodium methyl arsenate (MSMA) is one of the most popular among organic arsenical herbicide due to its effectiveness, lower price and lower toxicity as compared to inorganic arsenic. Currently, MSMA is widely used for weed control in cotton and on turf and lawn (Matteson et al., 2014). After years of its usage, MSMA which was earlier thought to have less toxic chemical properties, was reported to be as toxic as inorganic arsenic in later studies. For example, the methylated arsenical, methylarsonous acid, $MMA^{(III)}$, is more toxic than inorganic arsenic species

(Styblo et al., 2000). Oral exposure of MSMA may produce more toxic metabolites such as dimethylarsinic acid (DMA) species in vivo including $MMA^{(III)}$ and DMA radicals (Albert et al., 2008a) and dimethylarsinic acid (DMA) may enhance prostate carcinogenesis (Suzuki et al., 2019).

In agricultural producing countries (i.e. Bangladesh, India), the reported cases of Chronic Kidney Disease of Unknown Causes (CKDu) is increasing (Ranasinghe et al., 2019). Many factors have been postulated as the potential causes such as physical exertion, heat stress, water quality and exposure to agrochemicals, (Weaver et al., 2015). Though no definite cause has been confirmed, the exposure to agrochemical such as MSMA has been identified to be one of the risk factors (Wimalawansa SJ, 2015).

To date, human studies to establish the association between chronic toxicity of arsenic and its effects on the kidneys are limited (Zheng et al., 2014). Meanwhile, animal studies were mostly focused on the outcome of inorganic arsenic over the acute and sub-acute duration. Only limited studies were found on chronic organic arsenic exposure particularly its effects on the rat's kidney (Albert et al., 2008b; An et al., 2020; Cohen et al., 2001; Perry et al., 2019; Rivas-Santiago et al., 2019; Zhang et al., 2019).

Therefore, this study was carried out to investigate the effect of organic arsenical (MSMA) exposure on rat's kidney. The research provides a platform to describe the possible mechanism of chronic organic arsenic toxicity in the development of chronic kidney injury. Although epidemiological and experimental studies have linked acute inorganic arsenic toxicity to kidney injury, to the best of our knowledge, no study has investigated the possible link between chronic low dose organic arsenic exposure with chronic kidney injury. Thus, the understanding of the mechanisms involved in arsenic-induced kidney injury provides useful information on the management and prevention of the complications.

1.2 GENERAL OBJECTIVE

To study the effects of low dose chronic organic arsenic exposure on the rat's kidney.

1.3 SPECIFIC OBJECTIVES

i. To assess the renal function (serum creatinine) of the chronic low dose organic arsenic exposed rats.

ii. To assess the arsenic level in the kidney tissue of the chronic low dose organic arsenic exposed rats.

iii. To assess the histopathological changes of the chronic low dose organic arsenic exposed rats.

iv. To assess the tissue protein expression of selective immunomarkers representing kidney injury (*caspase-3, Tp53* and TUNEL staining) and oxidative stress (Malondialdehyde staining) of the chronic low dose organic arsenic exposed rats.

v. To assess the possible mechanism of kidney injury by investigating the expression of selected genes representing kidney injury (*KIM-1, caspase-3 and caspase-9),* oxidative stress (*catalase, GSR and NOS1)* and inflammation (i*nterleukin-6* and *interleukin-8*) of the chronic low dose organic arsenic exposed rats.

vi. To assess the ultrastructural changes of the kidney of the chronic low dose organic arsenic exposed rats.

1.4 RESEARCH HYPOTHESIS

i. There are significant differences in the renal function in the rats exposed to chronic low dose organic arsenic groups.

ii. There are significant differences arsenic level in the kidney tissue of the rats exposed to chronic low dose organic arsenic groups.

iii. There are histopathological changes of the kidney in rats exposed to chronic low dose organic arsenic groups.

iv. There are changes in immunomarkers of the kidney in rats exposed to chronic low dose organic arsenic groups.

v. There are significant differences in genes expression of the kidney in rats exposed to chronic low dose organic arsenic groups.

vi. There are ultrastructural changes of the kidney in rats exposed to chronic low dose organic arsenic groups.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 EPIDEMIOLOGY OF HUMAN ARSENIC EXPOSURE

Exposure to environmental arsenic remains a significant public health challenge. World Health Organization (WHO) in 2018 had reported that 140 millions of people from 50 countries are exposed to arsenic above the recommended level of 10 µg/L (WHO, 2018). Majority of people exposed to arsenic-contaminated groundwater are from South Asian countries (Parvez et al., 2006).

Before the year 2000, there were four significant areas of arsenic groundwater contamination identified in Asia, which were West Bengal, Bangladesh, India, and few sites in China. However, between 2000 and 2015, arsenic-contaminated groundwater problem has increased in various Asian countries, including Mongolia, Cambodia, Nepal, Afghanistan, Myanmar, Western Iran, Korea, Vietnam and Pakistan. Recently, several new sites of arsenic-contaminated groundwater have been reported worldwide, especially in Asian countries (Shahid et al., 2018). In Malaysia, it was reported that the level of arsenic in 19% of well water samples from Rosob village in Sabah exceeded the WHO health-based guidelines (Kato et al., 2010). There was also a report on increase arsenic level in soil samples, vegetables and fish samples in Malaysia (Ong et al., 2013). However, the palm oil plantation soil was uncontaminated by the arsenic (Ab Manan et al., 2018).

The primary source of human arsenic exposure was from the groundwater contamination either from tube wells or shallow wells, and the arsenic toxicity was suggested to be acquired through oral ingestion (Wai et al., 2019). The global

distribution of groundwater arsenic contamination, their concentration and source of arsenic are summarized in Table 2.1.

Table 2.1 Global Distribution of Groundwater Arsenic Contamination, Concentration and Their Sources of Arsenic

Country / Region	Estimated exposed population	Arsenic Speciation	Concentration (mg/L)	Source
Asian				
Bangladesh	36 000 000	NA	0.001- 2.5	Natural
Chile	130 000 to 400 000	iAs (III) and iAs (v)	0.1 - 0.8	Natural
China	5 600 000	NA	0.22 - 4.40	Natural
India (west Bengal and Northern India	6 000 000	NA	0.22 - 3.2	Natural
Indonesia	NA	iAs (III)	0.000008 - 2.0	Natural and Industrial
Japan	NA	iAs (V)	0.01 - 0.400	Natural and Industrial
Malaysia	NA	NA	0.22	Natural
Philippines	NA	NA	0.1	Industrial
Taiwan (South – West Coast)	100 000 to 200 000	iAs (III) and iAs (V)	0.01 - 1.82	Natural
Thailand	14 085	NA	0.001 - 5.00	Industrial
Vietnam	> 1 000 000	NA	0.001 - 3.05	Natural
Africa				
Ghana	< 100 000	NA	0.01 - 0.17	Natural and Industrial
Americas				
Argentina	2 000 000	NA	0.05 - 9.9	Natural
Canada	NA	iAs (V)	0.10 - 3.00	Natural
Mexico	400 000	iAs (III) and iAs (V)	0.008 - 0.624	Natural
USA	13 000 000	iAs (III) and iAs (V)	0.05 - 1.7	Natural
Europe				
Hungary and Romania	400 000	NA	0.06 - 4.00	Natural
Poland	NA	NA	NA	NA
Spain	> 50 000	NA	0.001 - 0.1	Natural
Oceania				
New Zealand	5 600 000	iAs (III)	8.5	Natural

Source from (Adekunle, 2016) * N/A - not available, iAs - inorganic arsenic.

2.2 BRIEF INFORMATION ABOUT ARSENIC

2.2.1 Chemical Properties, Classification, Speciation and Toxicity of Arsenic

Arsenic is classified as metalloid due to its both metal and non-metal properties. Arsenic exists in both reduced and oxidized states. Arsenic has an atomic number of 33, an atomic mass of 75 with a density of 5.72 $g.cm^{-3}$. It occupies group V of the periodic table. Its most common commercial form is arsenic trioxide (As_2O_3) (Mana et al., 2017).

Arsenic compounds are classified into three major groups; inorganic arsenic compounds, organic arsenic compounds and arsine gas. Inorganic arsenic compounds consist of trivalent and pentavalent state. The most common inorganic trivalent compounds are arsenic trioxide, sodium arsenite and arsenic trichloride. Pentavalent inorganic compounds are arsenic pentoxide, arsenic acid and arsenate. Common organic arsenic compounds are arsanilic acid, methylarsonic acid, dimethylarsinic acid (cacodylic acid) and arsenobetaine (NRC, 1999).

Speciation referred to the existence of an element in different chemical forms, oxidation states and mineral phases. It may represents element toxicity and bioavailability in the soil (Abbas et al., 2018). The arsenic speciation (based on the oxidation state) and arsenic forms (organic or inorganic) in relation to the toxicity properties are also extensively studied to date. The most common studied arsenic species are the (+3) and (+5) arsenicals. It was reported that (+3) arsenicals are more toxic than the (+5) (Braeuer et al., 2020). The order of the toxicity for arsenicals based on previous findings began with arsenite, followed by monomethylarsine oxide, dimethylarsinic acid, dimethylarsonate, monomethylarsonate and arsenate (Vega et al., 2001).

The inorganic form of arsenic has attracted researchers' attention more than organic arsenic. Therefore, limited studies on organic arsenic as it was assumed to be

less toxic than inorganic arsenic (Cohen et al., 2006; Hughes et al., 2011). However, a few recent pieces of evidence showed that organic arsenicals are as toxic as inorganic arsenicals. For instance, the methylated arsenical, methylarsonous acid MMA(III) is as toxic as inorganic arsenic species and potentially induced oxidative DNA damage (Braeuer et al., 2020; Tokar et al., 2014). Oral exposure of MSMA also may produce more toxic metabolites such as dimethyl arsenic acid (DMA) and monomethyl arsenic acid (MMA) (Albert et al., 2008b).

2.2.2 History and Application of Arsenic

Historically, arsenic was previously used as a therapeutic agent in 400 BC, known as Fowler's solution, for the treatment of chronic bronchial asthma, leukaemia, psoriasis, spirochaetal and protozoal diseases. However, Fowler's solution has been banned in most of the countries due to its toxicity. Arsenic trioxide was also reported being used in the treatment of acute promyelocytic leukaemia due to its anticancer effects by inducing autophagy, thus apoptosis in cancer cells. It also could alter gene regulation patterns, which decreased cell growth in human liver cancer, bladder cancer and breast cancer cells. Other than that, arsenic has been widely used in agricultural industries for pesticides, herbicides (e.g. monosodium arsenate, cacodylic acid), wood preservative (e.g. copper chromium arsenate), and cotton desiccants (e.g. arsenic acid). Arsenic is also used as a decolouring agent in the glass industries. Elemental arsenic is used as an additive in the alloy's productions. In the livestock industry, organic arsenic is added to swine and poultry feed as an antimicrobial medication. Nowadays, arsenic has also been used in semiconductors and solar cells components (Nordberg et al., 2014).