Use of urinary porphyrin profiles as an early warning biomarker for Monomethylarsonous acid (MMA^{III}) exposure

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Abstract

Although it is well known that arsenic is toxic and that Arsenic is carcinogenic, the mechanism underlying this carcinogenesis is unknown. Our laboratories have established a model that produces multi-organ tumours in mice following extended exposure to arsenic in drinking water. Until recently the metabolism of arsenic was thought to be a detoxification process. Recent studies have shown that Monomethylarsonous acid (MMA^{III}) is the toxic intermediate of arsenic metabolism. It is a more potent cytotoxin and genotoxin than As^{III} and As^{V}, and is believed to be the proximal carcinogen. Exposure to arsenic is known to affect the activity of the enzymes of haem biosynthesis. We evaluated the use of urinary porphyrin profiles as an early warning biomarker for arsenic carcinogenicity. Young female mice were given drinking water containing arsenic as MMA^{III} *ad libitum*. 24h urine samples were collected at various time intervals for up to 48 weeks for urinary arsenic accumulation by HPLC-ICPMS and urinary porphyrin measurement by HPLC. Dimethylarsinic acid (DMA^{V}) was the major metabolite excreted and it showed significant dose-dependent increase at each time point and exposure dependent increase over 48 weeks. Porphyrin levels appeared to be age dependent. The results indicate that the urinary porphyrin concentration has the potential for use as an early warning indicator of chronic arsenic exposure prior to the onset of arsenic carcinogenesis.
Keywords: arsenic, porphyrins, haem synthesis, Monomethylarsonous acid, biomarkers, cancer.

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1 Introduction

Over one hundred million people globally are risk of developing cancer or other arsenic-related disease as a result of exposure to arsenic in drinking water or in food. The commonest form of drinking water exposure occurs when groundwaters become acidic, from acid sulphate soils, and dissolve environmental arsenic. Such exposures commonly occur in countries such as Bangladesh China and Taiwan. Studies show arsenic exposure causes non carcinogenic effects including black foot disease (NRC [1]), diabetes (Rahman et al. [2]) and ischemic heart disease (Tseng et al. [3]). Epidemiological evidence shows that arsenic exposure produces tumors of the skin, lung, and urinary bladder (ATSDR [4]; IPCS [5]; IARC [6]). Although arsenic is toxic to both humans and animals, and arsenic is carcinogenic, the mechanism underlying this is unknown.

Arsenic undergoes stepwise reduction of pentavalent arsenic to trivalent arsenic followed by oxidative addition of a methyl group to the trivalent arsenic (Cullen et al. [7]). Until few years back the methylation of inorganic arsenic was considered to be a detoxification mechanism (Vahter and Marafante [8]). Methylated arsenicals were thought to be less genotoxic than the inorganic arsenic (Moore et al. [9]). From recent studies by Petrick et al. [10] showing that MMA^{III} is more toxic than As^{III} and As^{V}, the hypothesis of methylation as a detoxification pathway has been questioned by Cullen and Dodd [11] who first demonstrated the toxicity of MMA^{III} in the yeast *Candida humicola*. MMA^{III} was more cytotoxic than inorganic arsenite in human hepatocytes (Petrick [10]). In a study by Mass et al [12] both MMA^{III} and DMA^{III} were 77 and 386 times more potent genotoxins than iAS^{III}, respectively.

Arsenic is also known to affect the activity of about 200 enzymes (Li and Rossman [13]), and some of these may have potential for use as biomarkers of effects. One of the important effect is that arsenic interferes with the function of the group of enzymes responsible for haem biosynthesis (Moore et al. [14]) including inhibition of coproporphyrinogen oxidase and haem synthase (Woods and Fowler [15]). Several studies have reported that exposure to arsenic affects the haem biosynthesis pathway in humans and animals (Woods and Fowler [15]; Ng et al. [16]). There were increases in protoporphyrin IX, coproporphyrin III, coproporphyrin I in the blood, liver, kidney and in the urine of rats 24-48hr after a single dose of arsenic (Wang et al. [17]). Urinary porphyrins were higher in the people of coal-borne arsenicosis endemic area in southwest of PR China. Greater increases in the urinary arsenic and porphyrins were found in women, children, and older people. The reason could be that they tend to spend more time indoors than the males (Wang et al. [18]). The synthesis of harderoporphyrin and the alteration of the porphyrin profile in the harderian glands of rodents is a highly sensitive biomarker for both single sub-lethal and chronic arsenic exposure (Ng et al. [19]). The aim of this study was to investigate arsenic metabolism and the alteration of urinary porphyrin profiles in mice chronically exposed to low doses of MMA^{III} and see whether these profiles could be used as biomarkers prior to the onset of carcinogenesis.

2 Materials and methods

2.1 Reagents

MMA^{III} was purchased from Bill Collin, British Columbia University, Canada. Mixed arsenic standards for HPLC-ICP-MS analysis including arsenobetaine (AsB), sodium arsenite (As^{III}), dimethylarsinic acid (DMA^{V}), disodium methyl arsenate (MMA^{V}) and sodium arsenate (As^{V}) were obtained from TRI chemicals Lab Inc. (USA), BDH chemicals (UK), Sigma (USA), Chem Service (USA) and Ajax Chem. Ltd. (Australia), respectively.

Porphyrin acid chromatographic marker kit (a mixture of standards) consisting of uroporphyrin III, 7-carboxyporphyrin, 6-carboxyporphyrin, 5-carboxyporphyrin and 4- carboxyporphyrin (coproporphyrin I), protoporphyrin IX, coproporphyrin III dihydrochloride, uroporphyrin I dihydrochloride and the internal standard meso-porphyrin IX dihydrochloride were purchased from Porphyrin Product (Logan, Utah, USA). All.

2.2 Animals and treatment

Animal experimental protocols were approved by the Institutional Animal Ethics Committee (AEC No. NRC 3/02/19). Female C57Bl/6J mice, aged 4-weeks-old, were divided into groups of 70, 5 mice per cage, and were given drinking water containing 100μg, 250μg or 500μgAs^{III}/L as monomethylarsonous acid (MMA^{III}) ad libitum for 12 months. A group of 105 control mice was given demineralised water containing <0.1μg As/L. The 50ppm MMA^{III} stock solution was prepared and kept at 4°C for a month and the working solution of 100, 250 and 500 ppb was prepared every 2 days. The animal care facility was operated at controlled temperature set at 21–23°C, 13 filtered air changes per hour, 12/12 h light/dark cycle and year round relative humidity of about 60%. All animals were kept in standard polypropylene cages with stainless steel wire-mesh tops equipped with polycarbonate plastic drinking bottles with stainless steel sip-tubes and given a commercial rodent diet ad libitum (Norco P/L, Brisbane, Australia).

2.3 Urinary arsenic speciation by HPLC-ICP-MS

The arsenic concentrations of the drinking water and feed were monitored by HPLC–ICP–MS. The volumes of the drinking water consumed by the mice in each cage and body weight of each mouse were measured weekly. Six cages of mice out of 14 cages from every group were selected and each cage of five animals was kept in a metabolic cage for 24 h pooled urine collection on time zero, week 1, 2, 4, 8 and every 8 weeks after that till 48 weeks. During urine collection, mice were kept in metabolic cages and given powdered rodent diet and water containing MMA^{III} at the original exposure level. Urine was collected on dry ice to preserve the urinary arsenicals and porphyrins in their excreted forms. Urine samples were stored away from light and at -80°C until analysis.

2.4 HPLC analysis of porphyrins in urine

Porphyrins were analyzed by HPLC with fluorescence detection (Wang et al. [17]). Briefly, an aliquot of urine sample (750 μL) with the addition of 10M hydrochloric acid (50μL) in a glass test tube was vortexed thoroughly and allowed to stand in the dark for 1/2 h. After centrifugation at 13,200 rpm in a bench top microfuge for 10 min, the supernatant (200μL) was diluted with 1M hydrochloric acid (800 μL) containing mesoporphyrin (73.32 nM) as the internal standard.

This solution was filtered using a 0.45 μm pore size syringe filter (Nalgene, USA), and the porphyrins were analyzed by injecting an aliquot (40 μL) onto the C18 reverse phase HPLC column (Radialpak, Novapak C18, 8mm × 100 mm, 4μm, and Novapak guard column, Waters Associates, USA) coupled to a Shimadzu HPLC system (LC-10A, Shimadzu, Japan); a fluorescence detector (LC-240, Perkin–Elmer, USA) set at 395 nm excitation wavelength and 615 nm emission wavelength for detection. The concentration of each porphyrin was calculated from a standard curve using the peak height mode.

2.5 Statistics

Statistical analysis was performed using SPSS 11.0 software. Differences in the level of urinary arsenic species and porphyrins were analyzed by ANOVA and post analysis LSD.

3 Results

No tumors were observed in any of the animals in the control or treatment groups as expected. Previous study from our lab shows the earliest solid tumors observed in the C57BL6/J mice exposed to 500μg As/L in drinking water were after about 18 months of exposure. No abnormal appearance or behaviour was observed.

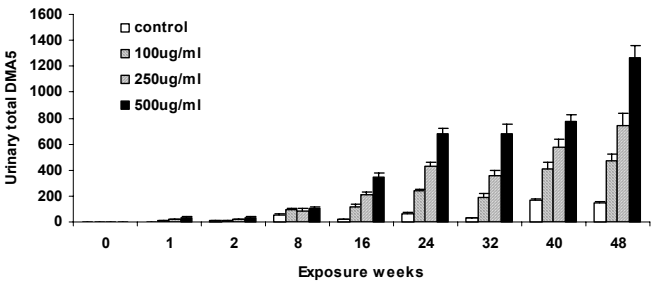

Figure 1: Average 24h pooled total urinary arsenic excretion (ng/24h) for the control and treatment groups. Vertical lines represent standard errors (N=5).

3.1 Urinary arsenic methylation profile

Twenty four hour urinary excretion pattern of DMA^V is shown in Fig.1. Limits of detection (LOD) for AsB, As^{III}, DMA^V, MMA^V and As^V were 0.5, 0.6, 0.6, 0.8 and 0.8 μg/L, respectively. AsB and MMA^V were below the LOD in all urine samples. There is a positive correlation between the urinary total arsenic and MMA^{III} concentrations in the drinking water. In addition, levels of total arsenic were significantly dose related within each age group.

3.2 Urinary Porphyrin profiles

Uroporphyrin showed dose-dependent increase after 4 and 16 weeks of exposure to MMA^{III}. There is a significant difference in all the test groups compared to the control after 8 weeks of exposure. The level of uroporphyrin in control group is significantly different from 250 and 500μg/L after 32 weeks and control group and 100μg/L group are significantly different from 500μg/L after 40 weeks of exposure.

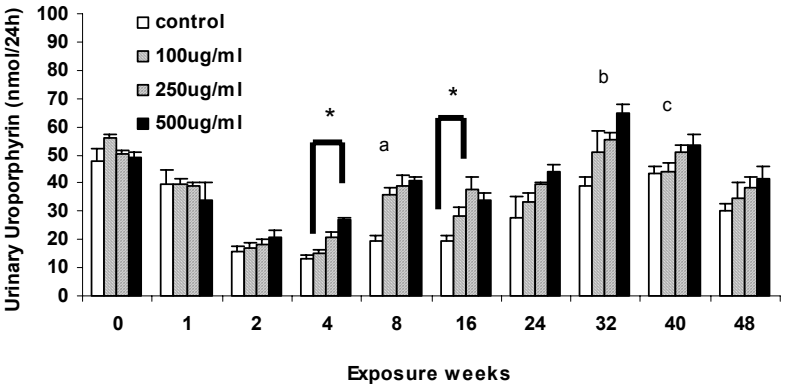

Figure 2: Average 24h pooled total urinary uroporphyrin excretion (nmol/24h) for the control and treatment groups. Vertical lines represent standard errors. * indicates a significant dose-response relationship ($p<0.01$). "a" - indicates a significant difference in the uroporphyrin level between control and test groups ($p<0.001$) "b" – indicates the level of uroporphyrin of control group is significantly different from 250 and 500 μg/L ($p<0.02$). "c" – indicates level of uroporphyrin of control and 100 μg/L groups are significantly different from 500 μg/L group ($p<0.05$) ($n=5$).

Urinary coproporphyrin-III concentrations also increased in most of the treatment groups compared to the control groups except time zero and week 1. The level of coproporphyrin III in the control group is significantly different from all the test groups after 8, 24, 32 and 40 weeks of exposure. Control, 100, and 500μg/L groups showed dose-response relationship after 24 months of exposure.

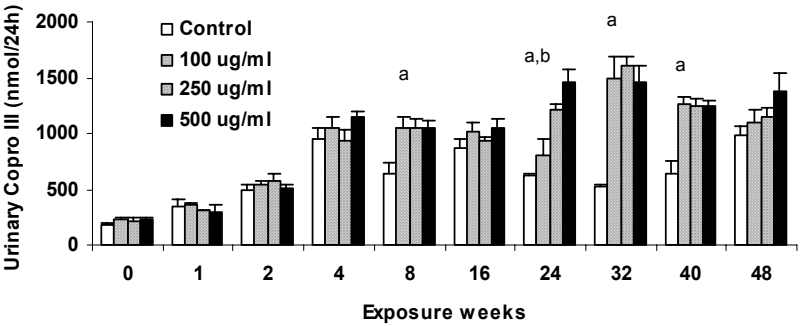

Figure 3: Average 24h pooled total urinary coproporphyrin III excretion (nmol/24h) for the control and treatment groups. Vertical lines represent standard errors. "a" - indicates the level of copro III in control group is significantly different from all the test groups ($p<0.01$). "b" - Control, 100 and 500 μg/L shows significant dose response relationship ($p<0.05$).

4 Discussion

Urinary levels of arsenic species can be used as reliable biomarkers of exposure to inorganic arsenic rather than total urinary arsenic, which often contains high levels of organo-arsenic compounds derived from dietary intake of seafood. Average arsenic from three representative samples of mouse food was found to be 385μg/kg, of which the inorganic As^{III} and As^{V} contents were 18.5% and 81.5%, respectively.

In animals a major interspecies difference in the arsenic methylation rate has been observed. Liver cystosol studies from marmoset and tamarin monkeys (Zakharyan et al. [20]) and guinea pigs (Healy et al. [21]) shows that these species are deficient in methyltransferase activity compared to other species like rabbit. Studies by Healy et al [22] using cystosol prepared from liver, lung, kidney and testes of male $B6C3F_1$ mice, shows all the tissues had the capacity to methylate arsenite. These results suggest that although the major part of arsenite methylation is happening in the liver, extrahepatic methylation may also be significant.

C57BL6/J mice exposed to 100, 250 and 500 μg arsenic as sodium arsenate showed DMA^{V} as a major urinary metabolite (Wu et al. [23]). In our experiment though the animals are exposed to MMA^{III} the major metabolite excreted in the urine was DMA^{V}. Studies have found monomethylarsonous acid (MMA^{III}) and dimethylarsenous acid (DMA^{III}) in human urine (Aposhian et al. [24]). Our result shows a dose-response relationship between the MMA^{III} concentrations in the drinking water and urinary DMA^{V} concentration. It also shows exposure dependent increase in the urinary excretion of DMA^{V}.

Since age is a confounding factor for the urinary porphyrin profile, it is important to use animals of similar age when conducting biomarker study. In our study urinary uroporphyrin showed dose-response relationship after 4 and 16 weeks of exposure period and a significant difference between control and test groups after 8, 32 and 40 weeks. This result agrees with the human study in Xing Ren of Guizhou Province of pr China where individuals chronically exposed to arsenic from burning arsenic contaminated coal for heating, cooking and drying food purpose had a significant increase in urinary uroporphyrin compared to the non-exposed population (Wang et al. [18]). In contrast, the same strain of mice (C57BL6/J) exposed to sodium arsenate at concentrations of 20mg/L (Woods and Fowler [15]) and 100, 250 and 500 ppb (Wu et al. [25]) showed no significant increase in uroporphyrin. However, C57Bl/6J male mice sub-chronically exposed to drinking water containing sodium arsenite or sodium arsenate showed significant increase in uroporphyrin (Garcia-Vargas et al. [26]). Significant increase in the protoporphyrin IX, uroporphyrin and Copro III were recorded in Wistar rats dosed by oral gavage with either a solution of sodium arsenite or sodium arsenate at a dose rate of 5 mg As/kg body weight but no significant increase in Copro I excretion (Wang et al. [17]).

Even though the concentration of MMA^{III} used was very low in this study, significant differences were observed between control and test groups for Coproporphyrin III. The findings here support our previous observations in mice which were chronically exposed to inorganic sodium arsenate. In conclusion, as shown in the human study of Ng and Qi [27] coproporphyrin and uroporphyrin could be used as biomarkers for arsenicosis in humans, particularly in the young age group (age < 20 years), our study also demonstrates that the Coproporphyrin III and uroporphyrin can be used as early warning biomarkers before the onset of cancer. Since MMA^{III} is a more potent cytotoxin, genotoxin and potential carcinogen than other arsenic species, it would be interesting to evaluate the overall porphyrin profile over the whole life span of these animals and the effect of MMA^{III} on enzymic control of haem biosynthesis.

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