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ORIGINAL ARTICLE

Urinary arsenic speciation profiles in mice subchronically exposed to low concentrations of sodium arsenate in drinking water

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Abstract Arsenic is a proven human carcinogen. Although the mechanism of its carcinogenicity is still largely unknown, methylation is thought to have an important role to play in arsenic toxicity. In this study, urinary methylation profiles were investigated in female C57BL/6J black mice given drinking water containing 500 μg arsenate (As^V)/L, 250 μg As^V/L, or 100 μg As^V/L as sodium arsenate for 2 months. The concentrations of arsenic chosen reflected those in the drinking water often encountered in arsenic-endemic areas. Urine samples were collected from the mice at the end of the exposure period, and the arsenic species were analyzed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry. All detectable arsenic species showed strong linear correlation with the administered dosage. The methylation patterns were similar in all three groups with a slight decrease of dimethylarsinic acid/As^V ratio in the 500-μg/L group, which corresponded to the significantly higher arsenic retention in the tissue. The results indicate that urinary arsenic could be used as a good biomarker for internal dose and potential biological effects. Different doses of arsenic exposure could result in different degrees of methylation, excretion, and tissue retention, and this may contribute to the understanding of arsenic carcinogenicity.

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Introduction

Inorganic arsenic (iAs) is classified as Group I carcinogen primarily based on strong epidemiological data [1–3]. As ubiquitous environmental toxicants and carcinogens, arsenicals pose significant health impacts on both humans and animals. It has been estimated that up to 100 million people globally are at risk as a result of drinking arsenic-contaminated waters [4,5].

In mammals, iAs is methylated to its less harmful metabolites, monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V), which are then excreted in the urine together with the unmetabolized iAs, including arsenate (As^V) and arsenite (As^{III}). A simplified scheme of arsenic metabolism in mammals, including humans, is given in the work by Kitchin [6]. The reduction from pentavalent to trivalent arsenic states may occur enzymatically or non-enzymatically through glutathione. Oxidation and methylation are coupled in arsenic metabolism with the trivalent arsenic as the substrate and the more methylated pentavalent arsenic as the product. Monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) have been found in the urine of humans chronically exposed to iAs through drinking water [7,8]. Similar metabolites were also found in the bile of rats administrated with As^{III} intravenously [9]. MMA^{III} is more toxic to hepatocytes *in vitro*, whereas DMA^{III} is 380-fold more genotoxic than iAs [10]. This suggests that the previous thought of arsenic metabolism by stepwise methylation as a detoxification pathway is now questionable. However, the precise role of these toxic metabolic intermediates in arsenic carcinogenicity is yet to be elucidated. Recently, MMA^{III} was proved to be carcinogenic to mice [11].

The World Health Organization–recommended guideline value for arsenic in drinking water is 10 μg/L, whereas the value in Australia is 7 μg/L. However, many developing countries still have their standards set at 50 μg/L. It is generally regarded that levels greater than 50 μg/L of arsenic in drinking water is not protective for human health [12]. Notably, World Health Organization/Food and Agriculture Organisation of the United Nations has withdrawn its provisional tolerable weekly intake of 0.015 mg As/kg body weight (bw) at the 72nd Joint Expert Committee of Food Additives meeting recently [13].

There are substantial variations in the susceptibility to arsenic intoxication among individuals, which may probably be related to differences in their capacities to metabolize arsenic. Most population groups studied so far have an average of 10–30% iAs, 10–20% MMA, and 60–70% DMA in their urine samples, with considerable interindividual variations [14]. Significantly, recent studies have identified population groups with unusually low or high urinary excretion of MMA. It is conceivable that a genetic polymorphism may exist in the biomethylation of arsenic. Over and above these, the rates of methylation of arsenic have also been found to vary considerably among animal species [15,16].

For example, mice exposed to iAs excrete 90% of the administered dose in 2 days, of which almost 80% is DMA [17]. A similarly high degree of methylation has also been reported for dogs, rabbits, and guinea pigs exposed to As^V [18–21]. It is apparent that the efficient methylation of arsenic in these experimental animals may have led to faster tissue clearance and less toxic effects. For comparison, the biological half-time of arsenic in humans is in the order of 4 days [22]. It should be noted that the rats have efficient methylation capacity of arsenic, but most of the DMA produced is retained in the erythrocytes, resulting in a slower rate of excretion in the urine [23]. In contrast, some animal species (e.g. the marmoset monkey and the chimpanzee) appear to lack the ability to methylate iAs [20,24].

In this study, we examine the possibility of using the mouse as an animal model for elucidating the metabolism of subchronic arsenic exposure. In this context, the arsenic methylation processes in the mouse and human beings are compared. There is some information available from previous short-term studies in which relatively high arsenic dose was used. Thus, it would be instructive to study the urinary methylation profile in mice subchronically exposed to low doses of arsenic in the drinking water, as such exposure regimens/scenarios are similar to those of human exposure to arsenic in endemic areas.

Materials and methods

Reagents

Sodium arsenate and sodium arsenite were purchased from Ajax Chemical, Sydney, Australia. Mixed arsenic standards and internal standards for high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) analysis were purchased from Agilent Technologies, Victoria, Australia. All chemicals were of analytical-reagent grade unless stated otherwise. Mixed standards containing four arsenic species (As^{III}, As^V, MMA^V, and DMA^V) were prepared with concentrations of 1 μg/L, 3 μg/L, 5 μg/L, 10 μg/L, 15 μg/L, 20 μg/L, and 40 μg/L for arsenic speciation [25].

Animals and treatment

Animal care was provided in accordance with the institutional animal ethics–approved protocol (Animal Ethics Committee No.: NRC 2/99/19). Female C57BL/6J black mice, aged 4 weeks, were divided into groups of 15, with five mice per cage, and were given drinking water containing 500 μg As^V/L, 250 μg As^V/L, or 100 μg As^V/L as sodium arsenate *ad libitum* for 2 months. Groups of 15 control mice were given demineralized water containing less tan 0.1 μg As/L. The arsenic solution was prepared every 2 weeks and stored in polypropylene container under animal house condition. The arsenic concentration of the drinking water was monitored by HPLC-ICP-MS (see details later). The mice were fed with a commercial rodent diet (Norco, Brisbane, Australia). 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. The animal caring facility is operated at controlled temperature set at 21–23°C, 13 filtered air changes per hour, 12-hour/12-hour light and dark cycle, and year-round relative humidity of about 60%. The volume of the drinking water consumed and bw of each mouse were measured weekly. Each cage of five

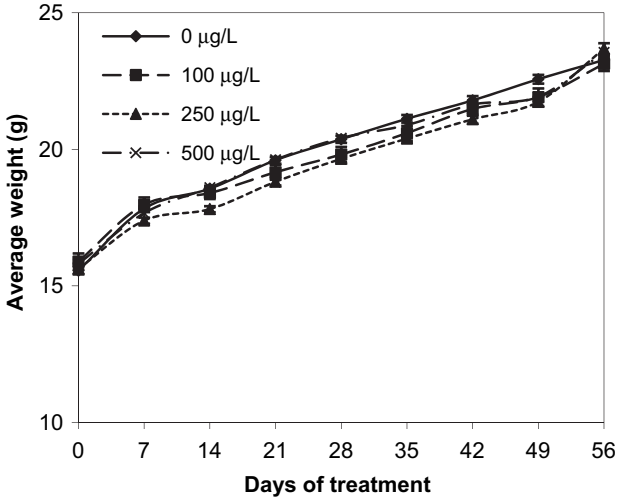

Figure 1. Changes in body weight (g; mean ± standard error) of female C57BL/6J mice continually exposed to different concentrations of arsenate in drinking water ($n = 15$ for each group).

mice was kept in a metabolic cage for 24-hour urine collection at the end of 2 months' exposure. All samples were stored at −80°C until analysis.

Urinary arsenic speciation by HPLC-ICP-MS

Equipment

Arsenic species were measured using an HPLC-ICP-MS system based on a previously published method [25]. The system is composed of an Agilent 1100 series solvent delivery unit, an ion-exchange column (G3154A, 150 × 4.6 mm, 3-μm particle size; Agilent Technologies) and Agilent 7500CS ICP-MS series connected by Agilent LC-ICP-MS connection kit (Agilent Technologies).

Mobile phase

The mobile phase used consisted of 2 mM H_3PO_4 and 0.2 mM EDTA-Na, with pH adjusted to 8.0 with 1 M NaOH, filtered and degassed before analysis.

Sample preparation

An aliquot of 1-mL urine samples was filtered through a 0.45-μm membrane filter (Nalgene, Rochester, New York, USA). An aliquot of 0.2 mL of the filtered sample was obtained and was diluted with four volumes of Milli-Q water before analysis.

HPLC-ICP-MS analysis

Briefly, the column was equilibrated with the mobile phase at a flow rate of 1.5 mL/min for at least 1 hour before any sample injection. For sample analysis, an aliquot of 20 μL of urine sample was injected with replication. The concentration of each detected arsenic species was calculated from an external standard calibration curve.

Statistics

Results are presented as means ± standard errors. Statistical analysis was performed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Differences in the level of urinary arsenic species, composition percentage, and retention percentage were analyzed by one-way analysis of variance and post-hoc analysis least significant difference.

Results

Exposure to the three different As^V dosages in the drinking water had no significant effect on the growth of the mice compared with that in the control animals (Fig. 1). The mice drinking the As^V-containing water consumed a similar amount of water compared with the mice drinking Milli-Q water and appeared to be equally active and healthy. On average, arsenic intakes were 13.0 μg/kg bw/d, 32.5 μg/kg bw/d, and 65 μg/kg bw/d corresponding to the increasing doses.

Concentrations of urinary arsenic species are summarized in Table 1. Levels of As^V, DMA^V, and total arsenic were all significantly dose related. Both As^{III} and MMA^V were under the detection limit. Two chromatograms overlaying a standard solution of four arsenical compounds at 20 μg/L and a mouse urinary arsenic speciation are shown in Fig. 2.

There were strong linear correlations among all the urinary arsenic species and the original As^V concentration in the drinking water. Correlation coefficients were 0.99, 0.97, and 0.98 for As^V, DMA^V, and total As, respectively (Fig. 3).

The relative percentages of urinary As^V and DMA^V are shown in Table 2. There was no statistical difference in the percentage of neither DMA^V nor As^V. The ratio of DMA^V/As^V, an index of methylation capability, appeared to show a slight decrease in the 500-μg/L group compared with those of other groups. However, there was no statistically significant difference.

Water consumption (2.6 ± 0.2 mL/animal/d) was quite constant over the duration of the 2-month exposure. Total As intakes could be derived by multiplying the original As^V

Table 1 Concentrations of various arsenate metabolites in the urine of control and arsenate-exposed mice

Dosage (μg/L)	Animal numbers	As^V (μg/L)	As^{III} (μg/L)	Total inorganic As (μg/L)	DMA^V (μg/L)	MMA^V (μg/L)	Total As (μg/L)
0	15	<1	<1	<1	9.76 ± 2.00	<1	9.76 ± 4.01
100	15	6.76 ± 1.46[a]	<1	6.76 ± 1.46[a]	121.76 ± 7.70[a]	<1	128.53 ± 17.50[a]
250	15	12.28 ± 5.56[a]	<1	12.28 ± 5.56[a]	194.34 ± 28.84[a]	<1	206.62 ± 56.59[a]
500	15	27.37 ± 7.04[a]	<1	27.37 ± 7.04[a]	329.37 ± 30.55[a]	<1	356.74 ± 83.25[a]

Values are means ± standard errors.
[a] Means significantly dose-related trend.
As^{III} = arsenite; As^V = arsenate; DMA^V = dimethylarsinic acid; MMA^V = monomethylarsonic acid.

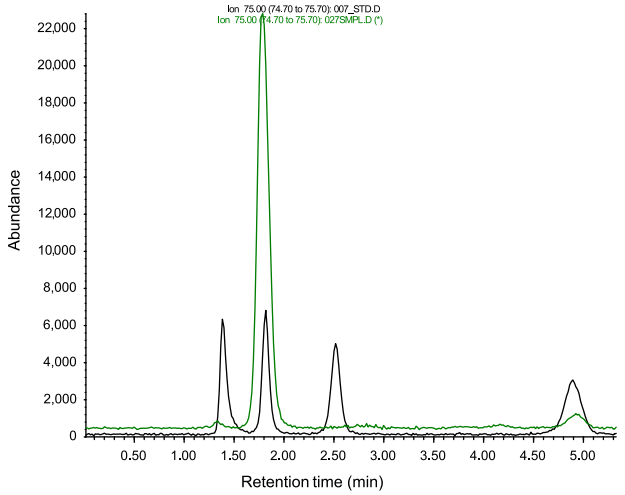

Figure 2. Two overlaying chromatograms obtained from a solution containing mixed standards of As^{III}, DMA^{V}, MMA^{V}, and As^{V} (bottom chromatogram), and a mouse urine sample showing the DMA^{V} and As^{V} peaks (top chromatogram). As^{III} = arsenite; As^{V} = arsenate; DMA^{V} = dimethylarsinic acid; MMA^{V} = monomethylarsonic acid.

concentrations in drinking water with the volume consumed, which were calculated to be approximately 260 ng/animal/d, 650 ng/animal/d, and 1300 ng/animal/d. The 24-hour total urinary arsenic excretion was calculated by multiplying the total urinary As concentration by the total urine volume collected in the metabolic cage for each individual animal. Percentage of total urinary arsenic excretion was significantly lower in the 500-μg/L group as compared with that in the groups exposed to lower dosages (Table 3). Correspondingly, the potential retention percentage was, therefore, significantly higher (Fig. 4).

Discussion

The level of exposure to iAs in animals or humans is often estimated by the concentration of its metabolites in the urine [26]. It has been argued that the urinary level of arsenic species metabolically related to iAs is more reliable than the total urinary arsenic, which could contain high levels of organoarsenic compounds derived from the dietary intake of seafood [27]. Indeed, for the control mice, there was still detectable arsenic in the urine that originated from the diet. Although urinary arsenic reflects exposure predominantly over the last 1–2 days [28], it has been shown that the fraction of the various arsenic metabolites in the urine are remarkably stable over a period of about a week, indicating that an individual's methylation of iAs is stable over this time frame [29]. Assuming that the methylation patterns of the mice used in the present experiments were stable over the study period, the results showed that all arsenic metabolites increased with increasing amounts of arsenic in the drinking water.

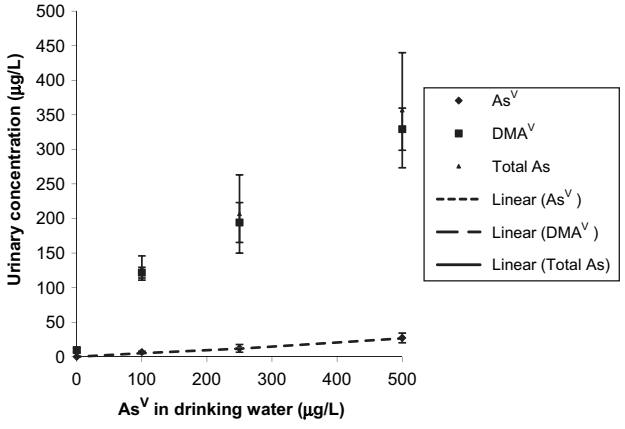

Figure 3. Linear correlation among urinary arsenic speciation levels with original dosage. Data are expressed as mean with standard error bar.

Table 2 Percentage of various arsenate metabolites and ratio of DMA^{V}/As^{V}

Dosage (μg/L)	Animal numbers	As^{V} (%)	DMA^{V} (%)	DMA^{V}/As^{V} (ratio)
0	15	—	—	—
100	15	5.2 ± 0.9	94.8 ± 0.9	20.7 ± 4.6
250	15	5.5 ± 1.9	94.4 ± 1.9	20.9 ± 5.7
500	15	7.2 ± 1.1	92.7 ± 1.1	14.1 ± 2.2

Values are means ± standard errors.
As^{V} = arsenate; DMA^{V} = dimethylarsinic acid.

Although many epidemiological studies have shown a good correlation between the intake estimate and different indices of urinary arsenic metabolites, the highest correlation between spot urinary indices and intake indices only reached a correlation coefficient of 0.67 even with careful questioning concerning consumption of fluids, well-characterized arsenic level in water, and adjustments for diuresis [28–31]. The strong linear relationships between levels of different metabolites and the original exposure dosage observed in this experiment confirm the validity of using the metabolites of arsenic as a biomarker of internal dose and potential biological effects.

Female C57BL/6J black mice appear to possess a highly efficient methylation capability; DMA^{V} accounts for more than 90% of the total urine arsenic, which is greater than the 80% reported in the male Naval Medical Research Institute mice after a singe dose of arsenate, 0.04 mg/kg bw, by intravenous injection [17]. In another study by Csanaky and Gregus [32], Carworth Farms Lane Petter mice were injected with a single dose of As^{V} (3.75 mg/kg bw, intravenously), and urine was collected immediately for 2-hour cumulative urinary excretion of arsenic metabolites. DMA^{V}, As^{V}, and As^{III} were 1.79%, 62.69%, and 35.30%, respectively. The observed difference may be caused by a difference in the dosage/exposure regimen used and/or interspecies variation. Other factors, such as gender and age, could also have an influence.

Many factors could influence the capability of arsenic methylation in animals. The possible effects of the dose level of arsenic on the methylation have been extensively reviewed [33], and it has been proposed that the enzyme methyltransferase could be saturated at high arsenic

Table 3 Percentage of urinary total As excretion and potential As retention in test groups

Dosage (μg/L)	Water consumption (mL/animal/d)	Average As intake (ng/animal/d)	24-hr total urinary As excretion (ng/animal/d)	Percentage of urinary excretion (%)	Percentage of retention (%)
100	2.6 ± 0.2	260	159.04 ± 9.89[a]	61.17 ± 3.80[b]	38.83 ± 3.80[c]
250	2.6 ± 0.2	650	170.02 ± 19.23[a]	26.16 ± 2.95	73.84 ± 2.95
500	2.6 ± 0.2	1300	360.36 ± 56.94[a]	27.72 ± 4.38	72.28 ± 4.38

Values are means ± standard errors.
[a] Significantly dose related ($p < 0.05$).
[b] Significantly greater than the other two groups ($p < 0.05$).
[c] Significantly lesser than the other two groups ($p < 0.05$).
As = arsenic.

exposure levels, which would lead to increased risk of health effects. Indeed, in cases of acute arsenic intoxication, the excretion of methylated metabolites of arsenic is delayed [34]. When comparing studies on urinary arsenic metabolites in people exposed to arsenic occupationally, experimentally, or through drinking water, with the general public exposed to much lower arsenic levels in the environment, there is no major difference in the average methylation efficiencies [35]. Most population groups studied so far have an average of 10—30% iAs, 10—20% MMA, and 60—70% DMA in their urine samples. Our results are consistent with this observation as the relative proportion of DMA^V and As^V are similar in the three groups. Thus, it can be concluded that the level of exposure to arsenic has little influence on the methylation efficiency.

Notwithstanding, ratio of DMA^V/As^V, an index for the methylation capability, decreased slightly in the 500-μg/L group. Generally, a low fraction of DMA in the urine is associated with a low overall rate of excretion, and thus, a higher retention in tissues [33]. We found that the retention percentage of 500 μg/L group was significantly higher than those in the lower-dosed groups. Besides urine, arsenic could also be excreted through other pathways, such as feces, milk, hair, nail, and other body fluids, although they account for only a very small fraction.

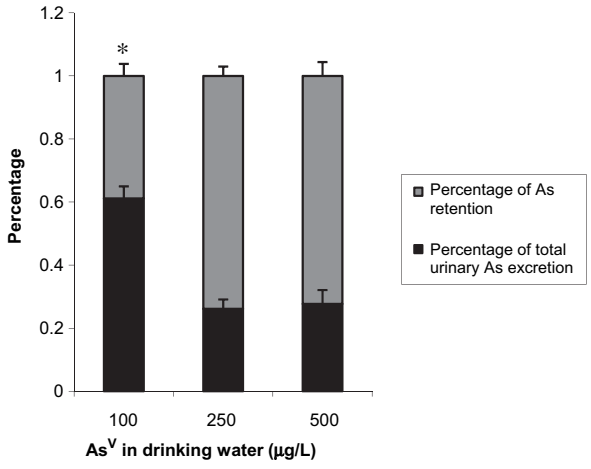

Figure 4. Relative percentage of total urinary As excretion and retention under different dosages of arsenate exposure. *$p < 0.05$. As = arsenic.

However, feces may be an important pathway when the arsenic bioavailability is low. We did not measure the arsenic excretion through these pathways, but the percentage could be an index of potential tissue retention. In contrast, other studies have indicated a slight decrease in the relative amount of DMA in urine and a corresponding increase in the relative amount of MMA with increasing exposure to arsenic through drinking water [36—38]. The study by Ng et al. [39] examined the metabolite profile of three groups of workers after occupational exposure to arsenic at three copper chrome arsenate timber treatment companies. One group of workers showed elevated levels of iAs in their urine, and the results suggested that the methylation capacity of these individuals was compromised. This was the first reported case of elevated concentrations of iAs in the urine profile of occupationally exposed workers. Interestingly, the levels reported in the workers were similar to those found in the urine of young children who lived in an arsenic-endemic area of northern Argentina [40]. Another study indicated that there were no significant differences in the total inorganic urinary arsenic levels between a control group and an exposed group of ion implanter maintenance engineers, but the MMA content was significantly higher in the exposed group [41]. It remains unclear whether compromised liver function, which could be at least in part caused by the exposure to arsenic, was responsible for the observed alteration in the arsenic methylation profile.

Other factors that may influence the methylation of arsenic in humans are age, gender, smoking, and nutrition. It was also found that women had a significantly higher DMA percentage and lower MMA percentage than men, suggesting that women may possess a more efficient methylation capability than men [25,42,43]. Elderly residents of the blackfoot disease—endemic area showed a poor methylation ability of arsenic species in their urine, and this was attributed to old age [42]. However, age was found not to be related to the capability to metabolize iAs in a study by Shraim et al. [25]. Nutritional status may also be an important factor. For example, higher selenium levels increased the disposition of arsenic and its methylation capability, resulting in an increase of total urinary arsenic and ratio of DMA/MMA. However, excessive intake of selenium can inhibit methyltransferase, and thus, may alter the disposition and methylation capability [25,44,45]. The difference in the methylation capabilities in different populations may sometimes be attributable to genetic

polymorphism. However, the specific genes for arsenic methylation are still unclear at this stage.

In conclusion, the urinary arsenic speciation profile could be used as a biomarker of internal dose as levels of all detectable arsenic species showed strong linear correlation with the original dosage. Methylation capability was similar in all three groups of mice with a slight decrease in the highest 500 μg/L group with respect to the ratio of DMA^V/As^V. This may result in greater arsenic retention in tissues and may cause subsequent severe adverse biological effects, especially cancer. The difference in methylation capacities between human beings and mice should be taken into account when interpreting or extrapolating experimental results obtained using mice as the animal model.

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