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Exposure to arsenic via drinking water induces 5-hydroxymethylcytosine alteration in rat

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HIGHLIGHTS

- Arsenic accumulates in an organ-specific manner.
- No significant global 5mC alteration is observed in majority of organs.
- Arsenic induces 5hmC alterations in a dose-related fashion in most organs.
- 5hmC alteration appears to be a sensitive biomarker of arsenic exposure.

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ABSTRACT

Arsenic exposure has been implicated to alter DNA methylation process *in vitro* and *in vivo*, but it remains obscure whether it disrupts DNA demethylation process, which is pivotal for epigenetic regulation. The objective of this descriptive study was to investigate the relationship between arsenic exposure and 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC) alterations in various organs. In this study, we exposed male Sprague–Dawley rats to sodium arsenite (0.5, 2 or 10 ppm) via drinking water for 8 weeks. Spleen accumulated 2- to 3-fold higher arsenic levels than liver and heart. Lower arsenic levels were observed in the kidney, pancreas and lung. No significant arsenic-induced global 5mC alterations were observed in the majority of investigated organs. However, arsenic induced organ-specific alterations of 5hmC and/or 5hmC/5mC in some investigated organs, i.e. lung, heart, kidney, pancreas and spleen. Our observations suggest that 5hmC is a more sensitive biomarker of arsenic-induced impacts on epigenetic processes than 5mC. Moreover, demethylation via hydroxylation of 5mC appears to play a central role in the toxic mechanism of arsenic.

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1. Introduction

Arsenic is a toxic metalloid and classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC, 1987). Arsenic exposure can occur via diverse routes and among those, contaminated drinking water poses one of the major threats to human health (Nordstrom, 2002). It has been estimated that approximately 19.6 million people are at risk of adverse effects via consumption of arsenic-contaminated ground water in China (Rodríguez-Lado et al., 2013). Increasing evidence indicates that arsenic exposure is associated with cardiovascular disease (Navas-Acien et al., 2005), diabetes mellitus (Navas-Acien et al., 2006), neurological disorders (Jomova et al., 2011), reproductive effects (Shen et al., 2013) and genotoxicity (Basu et al., 2001). Furthermore, arsenic has been linked with the onset and progression of tumors in many organs, including lung, liver, kidney, skin and bladder (Bhattacharjee et al., 2013; Martinez et al., 2011). However, the molecular mechanisms of arsenic toxicity remain obscure.

Epigenetic alterations appear to be an underpinning mechanism of arsenic-induced toxicity (Reichard and Puga, 2010). Regulation of gene expression is pivotal for normal biological function and within this regulatory network, DNA methylation is a key control (Martin, 2013). Such methylation events are highly dynamic, heritable and reversible during mammalian embryogenesis and development (Jaenisch and Bird, 2003). Epigenetic mechanisms maintain many

Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; AS3MT, arsenite methyltransferase; DNMT, DNA methyltransferase; SAM, *S*-adenosylmethionine; TET, tetmethylcytosinedioxygenase.

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critical functions, including stable repression of target promoters, maintenance of genomic integrity, establishment of parent-specific imprinting patterns, and silencing of endogenous retrotransposon activity (Smith et al., 2012). Arsenic has the potential to alter DNA methylation at both genome-wide and gene-specific levels (Ren et al., 2011), but there is little consensus regarding the exact nature of these changes (Reichard and Puga, 2010). The diversity of model systems employed in such studies along with differing treatment settings or DNA methylation assays contributes to these seemingly conflicting findings. Arsenic can alter 5-methylcytosine (5mC) levels in rat liver (Reichard and Puga, 2010) and induce multiple organ toxicity (Mushak and Crocetti, 1995), but the alteration of DNA methylation in other organs remains unidentified. Recently, significant differences in arsenic-modified methylation and arsenite methyltransferase (AS3MT) levels were observed at 10 brain regions in mice (Flores et al., 2010; Sánchez-Peña et al., 2010). High-resolution methylation data also provide evidence that individual organs exhibit distinct 5mC profiles (Christensen et al., 2009; Illingworth et al., 2008). Such observations suggest that arsenic may alter DNA methylation in an organ-specific manner. However, it remains to be ascertained whether this is indeed the case.

There is some information on arsenic-induced DNA methylation alterations, but so far no data exist on its ability to modify demethylation processes which are characterized by conversion of 5mC to 5-hydroxymethylcytosine (5hmC) (Ficz et al., 2011; Globisch et al., 2010). Both methylation and demethylation processes play key roles in the dynamic balance of epigenetic regulation (Tahiliani et al., 2009). As a newly-defined base, the functions of 5hmC remain unclear, but both global levels and locus-specific distributions of 5hmC appear to be organ specific in healthy humans (Nestor et al., 2012). Because genomic 5hmC levels are proportional to those of pre-existing 5mC, the balance between these two cytosine modifications is likely to be an important epigenetic mechanism. A non-genotoxic carcinogen, phenobarbital, induced dynamic and reciprocal changes of 5mC/5hmC patterns across the promoter regions of several genes (Thomson et al., 2012), thus highlights the importance of 5hmC in epigenetic regulation. Hence, there is a close association between 5mC and 5hmC, but the question remains as to whether arsenic could modify the dynamics of this two-way process. Therefore, this study was designed to investigate arsenic accumulation and its effects on the profiles of 5mC, 5hmC and 5mC/5hmC ratios in various organs. This could provide an insight into the role of arsenic in modifying the dynamics of epigenetic regulation in specific organs.

2. Methods

2.1. Chemicals

Sodium arsenite was purchased from the National Research Centre for Certified Reference Material in China, 2′-deoxy-5-methylcytidine (i.e., 5mC) was from Tokyo Chemical Industry (Japan), deoxyguanine (G) was purchased from J&K and 5-hydroxymethylcytosine (i.e., 5hmC)-modified DNA standard was obtained from Zymo Research (USA). DNase I, FastAP™ alkaline phosphatase and exonuclease I were all purchased from Fermentas China. LC-grade methanol was obtained from Fisher Scientific (USA). Distilled water (18.2 MΩ) was obtained from a Milli-Q system (Millipore, MA).

2.2. Animals and arsenic treatment

This study was approved by the Institutional Animal Ethics Committee of Institute of Urban Environment, Chinese Academy of Sciences. All animals were carefully handled in compliance with guidelines. Thirty male Sprague–Dawley rats weighing approximately 200 g were sourced from SLAC Laboratory Animal (Shanghai, China). They were randomly divided into four groups: six in the control group and eight in each dose groups (0.5, 2 and 10 ppm). The highest dose was comparable to reported environmental arsenic levels of 9.9 ppm in the ground water of heavily polluted areas (Nordstrom, 2002). The animals were exposed to arsenic via drinking water; the controls received deionized water. Animals had access to both water and food *ad libitum* throughout. No arsenic contamination was detected via food.

After the exposure of 8 weeks, rats were sacrificed by laparotomy following anesthesia. Six organs (i.e., liver, kidney, heart, spleen, lung and pancreas) were carefully removed. The dissected organs were weighed and their coefficients (organ weight/body weight) were calculated. All samples were immediately frozen by immersing into liquid nitrogen, and then stored at −80 °C until further processing.

2.3. DNA extraction and digestion

DNA was extracted and purified using the Sangon Biotech DNA Mini Kit (Shanghai, China), according to manufacturer's instructions. RNase A was added onto the kit column to remove RNA residue. DNA was eluted from the column with 100 μl of DEPC-treated water. The hydrolysis method used was derived from Friso et al. (2002). Briefly, 1 ng DNA was denatured by heating at 100 °C for 3 min and subsequently chilled in an ice bath for 10 min; then one-tenth of the final volume of 0.1 M ammonium acetate (pH 7.5) and 2 units of DNase I were added. The mixture was incubated at 37 °C for 3 h. Two units of alkaline phosphatase were added and kept for an additional 3 h at 37 °C. Finally, the mixture was incubated overnight at 37 °C following an addition of 40 units of exonuclease I to ensure complete hydrolysis. A quality control (QC) was prepared by mixing aliquots of each sample to be analyzed; therefore, it broadly represented the entire sample set. To generate a standard for 5hmC, 5hmC-modified DNA strand was also digested employing the same procedure mentioned above.

2.4. 5mC and 5hmC determination

Nucleoside concentrations were determined using an Ultimate™ liquid chromatography (Dionex, USA) coupled to a 5500 QTRAP tandem mass spectrometer (ABI, USA). A 2.1 × 100 mm 2.6 μm Kinetex® core-shell C_{18} column (Phenomenex, USA) was used for reverse-phase separation. The column was maintained at 40 °C; injection volume was 5 μl. The mobile phase was a mixture of (A) H_2O and (B) methanol, with a programmed gradient as follows: 3% B increased to 5% B in 5 min, increased to 100% B in 2.5 min and held for 1 min, ramped to 3% in 0.1 min, and finally maintained at 3% for 3 min. The flow rate was 0.5 ml/min. A mass spectrometer equipped with an electrospray ion source was operated in positive ionization in the multi-reaction monitoring (MRM) mode: 242/126 for 5mC; 258/142 for 5hmC; and, 268/152 for guanine (G), respectively. Other instrumental parameters were: ion spray voltage (IS) = 5500 V, curtain gas (CUR) = 30 psi, collision gas (CAD) = medium, nebulizer gas (GS1) = 55 L/h, auxiliary gas (GS2) = 50 L/h, and source temperature = 500 °C. Optimal settings for collision energies (CE), de-clustering potential (DP) and entrance potential (EP) were determined for each analyte's MRM transitions. Nitrogen was used as the nebulizer and collision gas. Data acquisition and processing were performed using Analyst software. In order to reduce any bias associated with gradual changes in instrument sensitivity over a long time sequence, samples across all experimental groups were run in a randomized fashion.

To generate calibration curves, 5mC, 5hmC and G isolates digested from commercial DNA standards were employed. A series of mixtures containing different concentrations of the three standards were prepared and injected onto LC/MS/MS. Peak areas of each standard were determined using Analyst software. Linear equations were plotted using peak area vs. concentration with R^2-values of 0.9995 for 5mC, 0.9941 for G and 0.9923 for 5hmC, respectively. These linear equations were then used to calculate exact nucleoside contents in the genomic

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