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Differential Sensitivities of Bone Marrow, Spleen and Thymus to Genotoxicity Induced By Environmentally Relevant Concentrations of Arsenite

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Abstract

It is known in humans and mouse models, that drinking water exposures to arsenite (As^{+3}) leads to immunotoxicity. Previously, our group showed that certain types of immune cells are extremely sensitive to arsenic induced genotoxicity. In order to see if cells from different immune organs have differential sensitivities to As^{+3}, and if the sensitivities correlate with the intracellular concentrations of arsenic species, male C57BL/6J mice were dosed with 0, 100 and 500 ppb As^{+3} via drinking water for 30 d. Oxidation State Specific Hydride Generation-Cryotrapping-Inductively Coupled Plasma- Mass Spectrometry (HG- CT- ICP- MS) was applied to analyze the intracellular arsenic species and concentrations in bone marrow, spleen and thymus cells isolated from the exposed mice. A dose-dependent increase in intracellular monomethylarsonous acid (MMA^{+3}) was observed in both bone marrow and thymus cells, but not spleen cells. The total arsenic and MMA^{+3} levels were correlated with an increase in DNA damage in bone marrow and thymus cells. An *in vitro* treatment of 5, 50 and 500 nM As^{+3} and MMA^{+3} revealed that bone marrow cells are most sensitive to As^{+3} treatment, and MMA^{+3} is more genotoxic than As^{+3}. These results suggest that the differential sensitivities of the three immune organs to As^{+3} exposure are due to the different intracellular arsenic species and concentrations, and that MMA^{+3} may play a critical role in immunotoxicity.

Keywords

immunotoxicity; arsenic; genotoxicity; intracellular arsenic species; bone marrow; spleen; thymus

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1. INTRODUCTION

Arsenic (As) contamination in food and drinking water is a world-wide public health issue. The trivalent inorganic form of As, arsenite (As^{+3}), is the most prevalent form in the environment. Exposure to As is associated with multiple diseases such as skin lesions, diabetes, cardiovascular diseases, and cancers (Argos et al., 2010; Schuhmacher-Wolz et al., 2009; Vahter et al., 2008). Once As^{+3} gets into the body, it is metabolized into monomethyl and dimethyl trivalent and pentavalent species (Aposhian and Aposhian, 2006). Monomethylarsonous acid (MMA^{+3}) has been shown to be more toxic than As^{+3} both *in vivo* and *in vitro* (Petrick et al., 2001; Styblo et al., 2000). MMA^{+3} can be further metabolized to dimethylarsonous acid (DMA^{+3}) or dimethylarsinic acid (DMA^{+5}) and excluded from the body.

Arsenic induced immunotoxicity has been studied by many groups both *in vitro* and *in vivo* (Ahmed et al., 2014; Nadeau et al., 2014; Biswas et al., 2008; Vahter et al., 2008; Soto-Peña et al., 2006; Gonsebatt et al., 1994). However, only a few studies have addressed the toxicity of As exposure to immune cells at environmentally relevant concentrations. Previous studies in our laboratory showed that *in vivo* drinking water exposure to As^{+3} at very low concentrations suppresses mouse bone marrow and spleen cell functions (Ezeh et al., 2014; Li et al., 2010). Human peripheral blood mononuclear cells (HPBMC) studies also showed a dose-dependent suppression of T cell proliferation at extremely low concentrations of As^{+3} (0.1–10 nM) in some individuals, with virtually all individuals susceptible to T cell immunosuppression by MMA^{+3} (Burchiel et al., 2014). Therefore, lymphocytes are extremely sensitive to As^{+3} exposure at environmentally relevant levels. However, it is unclear whether immunotoxicity is likely due to As^{+3} or MMA^{+3}.

T cells are generated in bone marrow and transferred to the thymus for development, while B cells develop in bone marrow. The spleen is a critical immune organ for the storage of both T and B cells and for systemic immune responses. In a previous study on the genotoxicity induced by As^{+3} in mouse thymus cells, we showed that mouse thymus cells are extremely sensitive to As^{+3} induced DNA damage, which is correlated with the inhibition of a base excision repair factor, Poly (ADP-ribose) polymerase (PARP) (Xu et al., 2016b). In the present study, the sensitivities of the three immune organs, bone marrow, spleen and thymus to As induced genotoxicity were compared both *in vivo* and *in vitro*. The species and levels of intracellular arsenic contents in the cells from the three immune organs were also determined and compared.

2. METHODS

2.1. Chemicals and Reagents

Sodium arsenite (CAS 774-46-5, $NaAsO_2$, Purity ≥ 90%) was purchased from Sigma-Aldrich (St. Louis, MO). Methylarsine iodide (MMA^{+3}) was obtained from Drs. Terry Monks and Todd Caminesch at the Southwest Environmental Health Sciences Center, University of Arizona. Penicillin/Streptomycin (Pen/Strep) and L-Glutamine were purchased from Life Technologies (Grand Island, NY). Dulbecco's phosphate buffered saline w/o Ca^{+2} or Mg^{+2} ($DPBS^{-}$) was purchased from Mediatech (Manassas, VA). Dimethyl sulphoxide

(DMSO), RPMI 1640 and Iscove's Modified Dulbecco's Medium (IMDM) base medium were purchased from Sigma Aldrich. Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Hanks Balanced Salt Solution (HBSS) was purchased from Lonza (Walkersville, MD). Sodium Hydroxide (NaOH) was purchased from EMD Chemicals Inc. (Gibbstown, NJ). 0.5 M EDTA solution was purchased from Promega (Madison, WI). The Comet Assay kit (Cat. No. 4252-040-ESK), hOGG1 FLARE™ Assay kit (Cat. No. 4130-100-FK) and the PARP activity kit (Cat. No. 4685-096-K) were purchased from Trevigen (Gaithersburg, MD). The BCA assay kit (Cat. No. 23225) was purchased from Thermo Scientific (Rockford, IL).

2.2. Mouse In Vivo Exposures

C57BL/6J male mice were purchased at 8 weeks of age from Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed following the protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center. Following one week of acclimation, mice (2–3 per cage) were exposed to As^{+3} at different concentrations via drinking water for 30 d. As^{+3} doses were prepared fresh weekly by weighing each water bag and determining the appropriate amount of stock As^{+3} solution to add to each bag to yield 100 and 500 ppb. No treatment was added to control bags. Water bags were weighed after each week and the change in weight was used to estimate the amount of water consumed by each group. The As^{+3} concentrations in drinking water were verified using Mass Spectrometry by Dr. Abdul-Mehdi S. Ali at Department of Earth and Planetary Sciences, University of New Mexico. Mice were fed 2020X Teklad global soy protein-free extruded rodent diet (Envigo, Indianapolis, IN) throughout the experiment.

2.3. Isolation of Bone Marrow Cells

Bone marrow cells were isolated according to the procedures described in Ezeh et al., 2014. Basically, mouse femurs were collected into HBSS medium in our animal facility and transferred to our laboratory to extract cells. Femurs were placed in petri dish containing 5 ml of cold sterile bone marrow medium (500 ml IMDM with 2% FBS, 2 mM L-glutamine, and 100 mg/ml Pen/Strep) and trimmed to expose interior marrow shaft of the femur, the end of the femur were then cut off. one cc syringe and 25 gauge needle were used to flush the bone marrow medium through the femur several times to release cells into the petri dish. The suspension was immediately transferred to a 15 ml centrifuge tube, centrifuged at 200 $\times g$ for 10 min, aspirated, and washed with bone marrow medium. The cell count and viability were determined by acridine orange/propidium iodide (AO/PI) staining on a Nexcelom Cellometer 2000.

2.4. Isolation of Thymus and Spleen Cells

Thymus and spleen were isolated following the sterile procedures described in Xu, et al., 2016. Basically, mouse thymus and spleen were harvested in our animal facility and transferred to the laboratory in HBSS on ice. Single cell suspensions of spleen and thymus cells were prepared by homogenizing the organ between the frosted ends of two sterilzed microscope slides (Fisher Scientific, Pittsburgh, PA) into a dish containing 5 mL of cold mouse medium (500 ml RPMI 1640 with 10% FBS, 2 mM L-glutamine, and 100 mg/ml

Pen/Strep). Suspended cells were centrifuged at 200 ×*g* for 10 min, aspirated, and washed with mouse medium. The cell count and viability were determined by AO/PI staining on a Nexcelom Cellometer 2000.

2.5. Oxidation state specific Hydride Generation- Cryotrapping- Inductively Coupled Plasma-Mass Spectrometry (HG- CT- ICP- MS)

The analysis of tri- and pentavalent As species was performed by HG-CT-ICP-MS as previously described (Currier et al., 2014; Matousek et al., 2013). Briefly, cell pellets were lysed in ice-cold deionized water. The trivalent species, As^{3+}, MMA^{3+}, and dimethylarsinite (DMA^{3+}) were measured in an aliquot of cell lysate directly, without pretreatment. Another aliquot was treated with 2% cysteine and analyzed for total inorganic As (As^{3+} + As^{5+}), total methyl-As (MMA^{3+} + MMA^{5+}), and total DMAs (DMA^{3+} + DMA^{5+}). Calibration curves were generated using cysteine-treated pentavalent As standards, (at least 98% pure) as previously described (Hernández-Zavala et al., 2008). The concentrations of pentavalent As species were determined as a difference between the values obtained for cysteine-treated aliquots and values from untreated sample aliquots. The instrumental LODs for As species analyzed by HG-CT-ICP-MS ranged from 0.04 pg As for methylated arsenicals to 2.0 pg As for inorganic arsenicals. All values are expressed as pg of As in each arsenic species.

2.6. In Vitro As^{+3} and MMA^{+3} Treatments

Bone marrow, spleen and thymus cells were isolated as described above from three 13-week old male C57BL/6J mice and pooled. Cells were washed and resuspended at 1×10^6 cells/ml in IMDM (for bone marrow cells) or mouse medium (for spleen and thymus cells). As^{+3} or MMA^{+3} were added to each wells to the final concentrations of 0 (control), 5, 50 and 500 nM. Cells were placed into a humidified 37 °C, 5% CO_2 incubator for 4 h. Treated cells were harvested by centrifugation at 200 ×*g* for 10 min and resuspended in cold $DPBS^-$. After another wash with cold $DPBS^-$, the cells were ready for Comet assay analysis.

2.7. Comet Assay (Single Cell Gel Electrophoresis Assay) and Fragment Length Analysis using Repair Enzymes (FLARE) Assay

Treated cells were immobilized in a bed of low melting point agarose on a Trevigen CometSlide™ following the Comet assay kit instructions. Cells were then lysed with Lysis Solution with 10% DMSO (Sigma-Aldrich) over night. On the next day, DNA in the lysed cells were unwound with basic buffer (8 g NaOH with 500 mM EDTA in 1 L of Milli-Q water, pH>13) at room temperature for 45 min. For FLARE assay, human 8-oxoguanine DNA glycosylase 1 (hOGG1) from the FLARE kit was diluted to 1:5 and applied to the wells. Slides were then incubated at 37 °C for 30 min before adding the unwinding buffer. For both assays, slides were electrophoresed in ice cold basic buffer with 21 volts for 30 min. Slides were washed, dried and stained with Sybr Gold (1:10000 dilution in TE buffer) and imaged using an epifluorescence microscope. Fifty randomly selected cells from each well were scored using CometScore software (TriTek Corp., Sumerduck, VA). DNA damage was reported by percentage of DNA in tail (Collins, 2004).

2.8. PARP activity assay

The PARP activity assay was performed using the Trevigen PARP activity kit. All reagents were supplied by the kit unless otherwise specified. The procedures were previously described by Sun et al., 2012. Basically, cells were lysed with the Cell Extraction Buffer supplied in the kit, and the protein concentration was determined by the BCA Protein Assay. Two hundreds ng of total proteins from each sample was combined with activated DNA and nicotinamide adenine dinucleotide (NAD) supplied by the kit, and then placed into a histone-coated strip well to formed PAR complex which was fixed to the bottom of the well. Anti-PAR monoclonal antibody was then added to the well to bind to PAR complex, followed by a Horseradish peroxidase (HRP) conjugated secondary antibody against the primary antibody. TACS- Sapphire™ was used a substrate to generate the chemiluminescence signal. An equal amount of 0.2 M HCl was added to stop the reaction. The signals were detected using SpectraMax® 340PC microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm wavelength.

2.9. Statistics

Data were analyzed using Excel 2013 and Sigma Plot v12.5 software. One-way analysis of variance (ANOVA) and Dunnett's t-test were used to determine differences between the control and treatment groups. Pearson Correlation and polynomial linear regression were used to analyze the correlations between intracellular As concentrations and DNA damage increases. All groups (Control and As^{+3} exposed groups) were used in the correlation analysis. For *in vivo* As^{+3} treatment, 5 animals (n = 5) were assigned to each treatment group. For the *in vitro* experiments, three replicates were performed and analyzed for each dose.

3. RESULTS

3.1. Intracellular arsenic species in the bone marrow, spleen and thymus cells of drinking water exposed mice

9-week old C57BL/6J male mice were exposed to 0, 100, and 500 ppb As^{+3} through drinking water for 30 d. No change in mouse body weight or water intake was observed during or after the exposure (Table 1). As^{+3} intake was calculated from the water intake of each mouse and the As^{+3} concentrations in the drinking water determined by mass spectrometry. Bone marrow, spleen and thymus were harvested from each mouse and the cells were isolated. Although not statistically significant, there was a trend of decrease in thymus weight, as well as the bone marrow and thymus cell recoveries (Table 1). The spleen weight and cell recovery were not affected by As^{+3} exposure. In order to measure the intracellular species and amounts of arsenic in these tissues from As^{+3} exposed mice, we used a very sensitive HG- CT- ICP- MS system to analyze the intracellular As levels in 5×10^6 cells pelleted from each tissue, as well as 50 μl plasma from each mouse (Table 2). A dose-dependent increase in total intracellular As levels was observed in bone marrow, spleen and plasma but not the spleen. Control mice showed detectable levels of As, presumably due to small amounts of As present in food and City of Albuquerque drinking water, which has As levels less than 5 ppb. Interestingly, the major increased As species in bone marrow and thymus was the trivalent methylated form, MMA^{+3}, which was almost undetectable in 500

ppb exposed spleen cells. Therefore, these results not only demonstrated that spleen is the least exposed tissue among the three immune organs, but also indicated that MMA^{+3} may be the major cause of genotoxicity in As^{+3} *in vivo* exposures. The concentrations of arsenic in plasma were an order of magnitude higher than those in cells from immune tissues; here DMA^{+3} and DMA^{+5} are the major metabolites.

3.2. Differential sensitivities of bone marrow, spleen and thymus in DNA damage and PARP activity to in vivo arsenic exposures

To compare the genotoxicity between lymphoid tissues, we performed Comet Assay on the cells obtained from the spleen, thymus, and bone marrow. Results showed a significant increase in DNA damage in both bone marrow and thymus cells starting at 100 ppb (Fig. 1A and 1C). No increase of DNA damage was observed in spleen cells (Fig. 1B). The activity of PARP was also measured as a common endpoint to see if the DNA repair ability was also inhibited. Arsenic is known to cause PARP inhibition, which has been shown to result in increased DNA damage (Qin et al., 2008; Xu et al., 2016b; Zhou et al., 2011). A dose-dependent decrease in PARP activity was seen in bone marrow and thymus cells (Fig. 2A and 2C). No change in PARP activity was observed in spleen cells (Fig. 2B). These results showed that bone marrow and thymus cells are more prone to genotoxicity compared to spleen cells, which may be the result of higher intracellular As and MMA^{+3} concentrations in *in vivo* exposures.

3.3. Correlations between DNA damage increase and intracellular arsenic concentrations in bone marrow, spleen and thymus cells

In vivo exposures to As^{+3} resulted a dose-dependent increase in total As and MMA^{+3} intracellular concentrations in bone marrow and thymus cells. We performed a regression analysis to reveal the relationships between total As or MMA^{+3} intracellular levels and the DNA damage in bone marrow, spleen and thymus cells. As shown, the amount of DNA damage correlated with the intracellular total As as well as the MMA^{+3} levels in bone marrow and thymus cells (Fig. 3A, 3C, 3D and 3F). There was no correlation between the DNA damage and intracellular As in the spleen cells (Fig. 3B and 3E). This analysis demonstrated that higher *in vivo* exposure to As^{+3} can increase the intracellular total As and MMA^{+3} in the bone marrow and thymus cells, which may induce or increase DNA damage in these immune tissues.

3.4. Differential sensitivities of bone marrow, spleen and thymus in DNA damage to in vitro arsenic exposures

In order to see if the differential sensitivities observed between spleen, thymus and bone marrow were due to different cell type susceptibilities, we directly added As^{+3} and MMA^{+3} to primary cultures of these cells. Cells were isolated and treated with 5, 50 and 500 nM As^{+3} or MMA^{+3} *in vitro* for 4 h and then were assessed for DNA damage using Comet assay. As shown in Fig 4A, bone marrow cells were sensitive to As^{+3} at concentrations as low as 5 nM. Spleen and thymus cells were only sensitive to As^{+3} treatment at the high concentration (500 nM, Fig. 4B and 4C). However, cells from all the three organs were sensitive to MMA^{+3} starting at the 5 nM concentration, indicating that MMA^{+3} is more genotoxic than As^{+3} *in vitro*. It was also observed that bone marrow cells seemed to be the

most sensitive among all the three organs. However, the sensitivities of spleen and thymus were comparable *in vitro*, indicating that the lower sensitivity in spleen cells observed in the *in vivo* experiment was due to lower As accumulation. The FLARE assay is a modified type of Comet assay that uses hOGG1 to enhance its sensitivity to detect oxidative DNA damage (Smith et al., 2006). We performed the FLARE assay with same samples used for the Alkaline Comet assay following 4 h incubation with As^{+3} and MMA^{+3}. The results obtained from the FLARE assay were similar to the Comet assay, except that a significant increase in DNA damage was observed at high doses of As^{+3} and MMA^{+3} only in thymus cells (Fig. 4C). This observation is consistent with our previous findings that As^{+3} induced oxidative stress in thymus cells at high concentrations (Xu et al., 2016b). These results demonstrated that bone marrow, spleen and thymus cells are more sensitive to MMA^{+3} induced genotoxicity than As^{+3} *in vitro*, indicating that the higher sensitivities of bone marrow and thymus cells in *in vivo* exposures were likely due to the significantly increased MMA^{+3} intracellular concentrations.

4. DISCUSSION

As^{+3} is known to cause multiple and complicated biological effects. There have been many studies focusing on the toxicity of As^{+3} over the years. However, two basic questions are often ignored when we set up the biological models and expose animals or cells to As^{+3}. What is the actual level of exposure and what chemical species are responsible for immunotoxicity in various lymphoid tissues? From previous studies, we know that various inorganic and organic arsenic species have differential toxicities (Akter et al., 2005). From studies in our laboratory, we have found that cells from different immune organs and tissues have differential sensitivities to As (Ezeh et al., 2014, 2016; Li et al., 2010; Xu et al., 2016a, 2016b). Therefore, it is important to determine if lymphoid organs and tissues, such as bone marrow, spleen and thymus have differential sensitivities to As^{+3} in terms of genotoxic and non-genotoxic actions both *in vivo* and *in vitro*.

In this study, intracellular As concentrations and species were measured in lymphoid cells isolated from three immune organs following As^{+3} 30 d drinking water exposure. A correlation between increased DNA damage and increased intracellular MMA^{+3} levels was found in both bone marrow and thymus cells (Fig. 1 and Table 2). Spleen cells were not as sensitive as bone marrow and thymus cells *in vivo*, and the intracellular MMA^{+3} levels in the spleen were not as high as seen in bone marrow and thymus, which can be the reason that caused the sensitivity differences. T and B cells in the spleen are more mature than those in the bone marrow and thymus, which might be a factor that accounts for the differential intracellular As concentrations. One of our previous studies showed that the thymus cells at the earlier stage (double negative) are more prone to As^{+3} induced toxicity than the later stage (double positive) (Xu et al., 2016a). Therefore, it is possible that more mature cells are resistant to As toxicity, due to a lower intracellular As concentration or less toxic intracellular As species. Another observation that supports this hypothesis is that bone marrow cells were sensitive to even 5 nM As^{+3} exposure *in vitro* (Fig. 1A). Bone marrow consists of multiple types of early progenitor cells and the very early stage of pre-T cells that migrate to the thymus. It is important to understand potential mechanisms responsible for the susceptibility of early lymphoid cells to As toxicity. Also, in the Comet assay

experiments, we noticed that it is very common for bone marrow cells to have higher background DNA damage from *in vivo* exposures, which may also due to the fragility of the progenitor cells in the bone morrow.

MMA^{+3} has been shown to be more toxic than As^{+3} in lymphoid and non-lymphoid tissues. MMA^{+3} is formed *in vivo* mostly in the liver, kidneys, and lungs under the influence of the AS3MT enzyme (Chen et al., 2011). We have found little enzyme expression of AS3MT in mouse lymphoid tissue, and have found little evidence for metabolism of As^{+3} in lymphoid tissues (unpublished data). Thus we believe that the presence of MMA^{+3} in lymphoid tissues is a reflection of MMA^{+3} uptake from the blood. From Table 2, we know that although the pentavalent forms of As species in the plasma are MMA^{+5}, DMA^{+3} and DMA^{+5}, the MMA^{+3} concentration was also significantly increased. In Supplementary Table 1, we found that 500 ppb exposure of mice resulted in a 477 nM intracellular concentration of total As species in thymus cells, which is in the concentration range for our *in vitro* studies (Figure 4). MMA^{+3} was the most prevalent form in high dose exposed bone marrow and thymus cells. Also, there was As^{+5} in cells from all three tissues, but As^{+3} was almost undetectable in all three tissues. Therefore, we think that the differences in bone marrow and thymus compared to spleen may result from a difference in the ability of cells either to import or export As^{+3} and MMA^{+3} or to convert MMA^{+5} to MMA^{+3}. Our current efforts are aimed at measuring these differences.

Regarding the mechanism of DNA damage produced by MMA^{+3}, it has been found that MMA^{+3} may produce stronger inhibition than As^{+3} of PARP, a zinc finger protein that is required for base excision repair, (Sun et al., 2012; Zhou et al., 2014). In our previous studies, we demonstrated a correlation between PARP activity inhibition and the increase of DNA damage in mouse thymus cells (Xu et al., 2016b). Therefore, the DNA damage increase observed in the mouse bone marrow and thymus cells following *in vivo* As^{+3} exposure appears to result from suppression of the DNA repair system.

In summary, the present study showed differential sensitivities of the cells from three important immune organs, bone marrow, spleen and thymus, to As^{+3} induced genotoxicity *in vivo*. The *in vivo* sensitivity was shown to be correlated with an increase in the trivalent methylated arsenic species, MMA^{+3}. Bone marrow cells were found to be the most sensitive tissue *in vitro*, which may relate to the immaturity of the various cell types. These studies stress the importance of measuring lymphoid tissue exposure and performing speciation analyses for arsenic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- MMA^{+3} is the main species in bone marrow and thymus in As^{+3} exposed mice.
- Bone marrow is the most sensitive lymphoid tissue to arsenic-induced genotoxicity.
- Increase in DNA damage is correlated with more intracellular MMA^{+3} in bone marrow and thymus.
- An *in vivo* exposure to 100 ppb As^{+3} induced genotoxicity in bone marrow and thymus.
- MMA^{+3} is more genotoxic than As^{+3} *in vitro*.

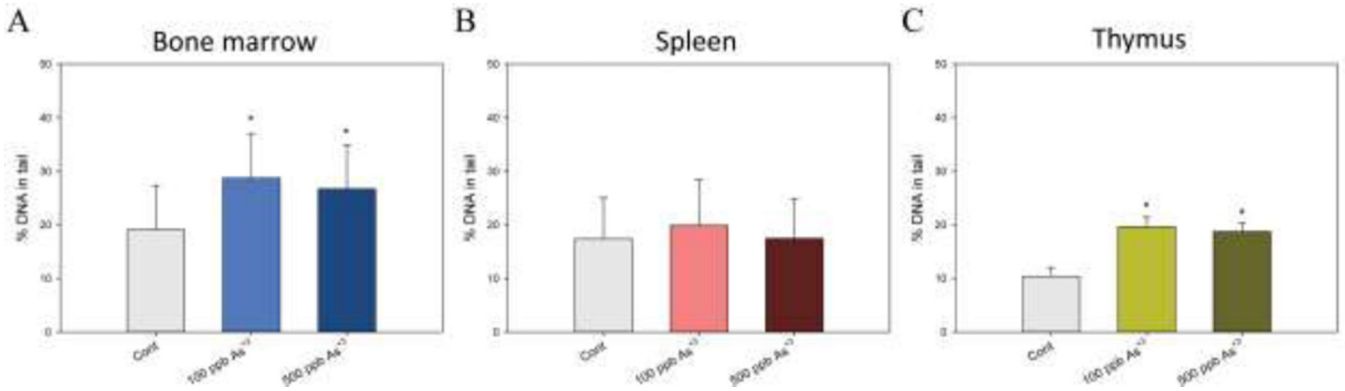

Figure 1.

DNA damage in bone marrow, spleen and thymus cells from 30 d As^{+3} drinking water exposed mice. 9-week old male C57BL/6J mice were exposed to 0, 100 and 500 ppb As^{+3} through drinking water for 30 d. Bone marrow, spleen and thymus cells were isolated and the DNA damage was measured by Comet assay. A, bone marrow. B, spleen. C, thymus. *Significantly different compared to control ($p<0.05$, n=5). Results are Means ± SD.

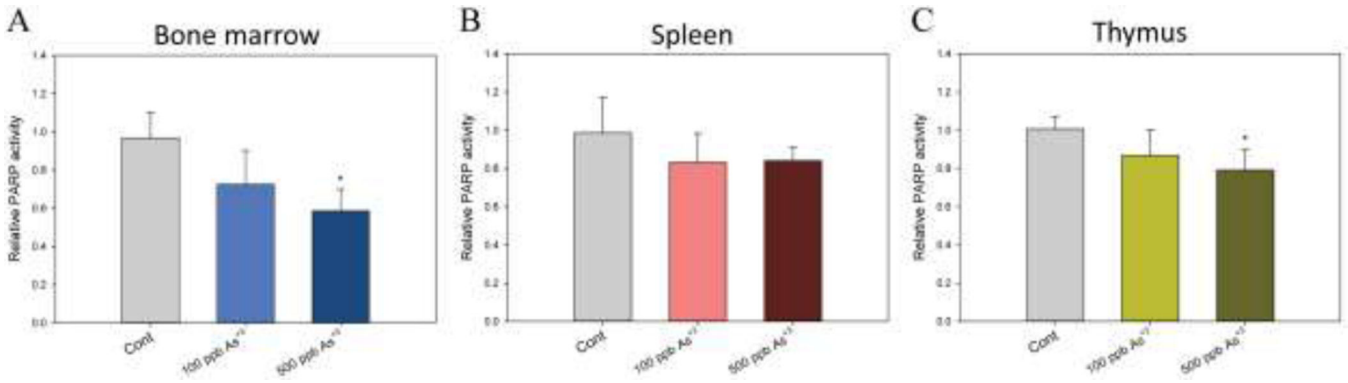

Figure 2.

PARP activity in bone marrow, spleen and thymus cells from 30 d As^{+3} drinking water exposed mice. 9-week old male C57BL/6J mice were exposed to 0, 100 and 500 ppb As^{+3} through drinking water for 30 d. Bone marrow, spleen and thymus cells were isolated and the PARP activity assay was used to measure the PARP activity in the cell protein lysates. A, bone marrow. B, spleen. C, thymus. *Significantly different compared to control ($p<0.05$, n=5). Results are Means ± SD.

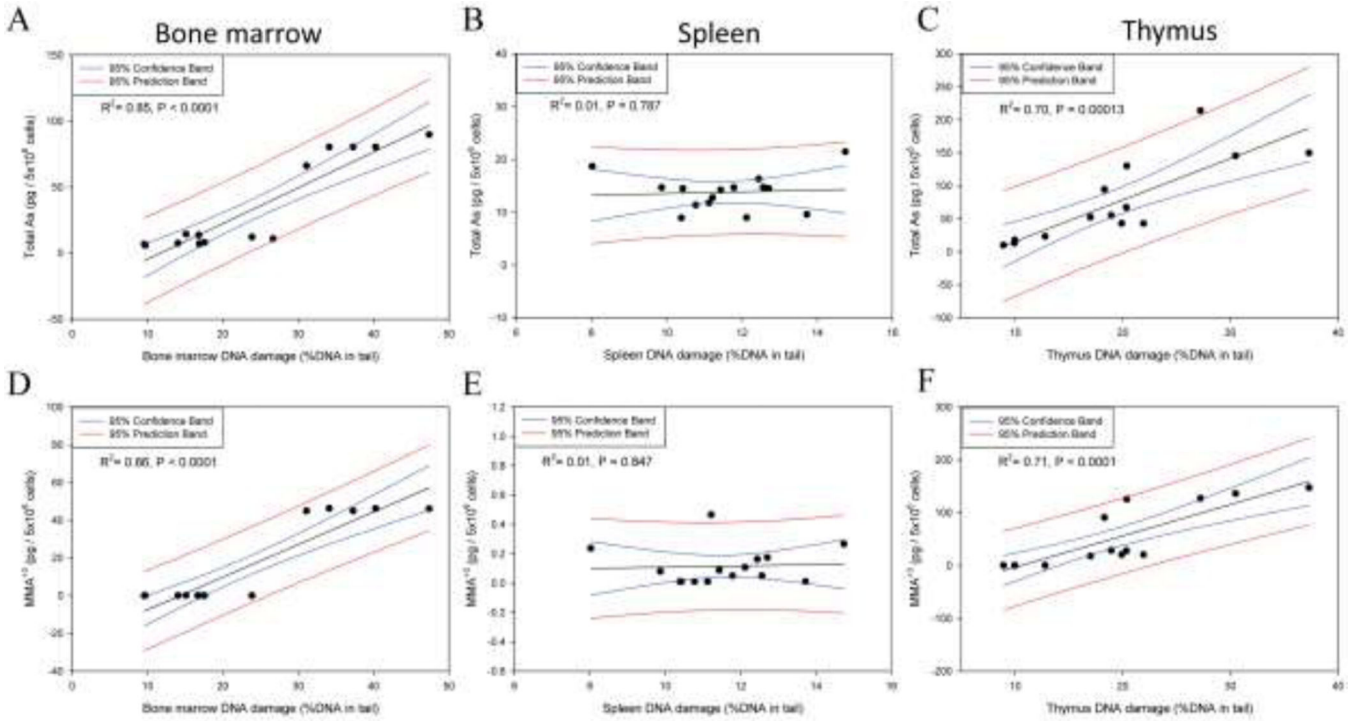

Figure 3.

Correlations between intracellular total As or MMA^{+3} concentrations and DNA damage in bone marrow, spleen and thymus cells from 30 d As^{+3} drinking water exposed mice. A, total As and DNA damage in bone marrow cells. B, total As and DNA damage in spleen cells. C, total As and DNA damage in thymus cells. D, MMA^{+3} and DNA damage in bone marrow cells. E, MMA^{+3} and DNA damage in spleen cells. F, MMA^{+3} and DNA damage in thymus cells.

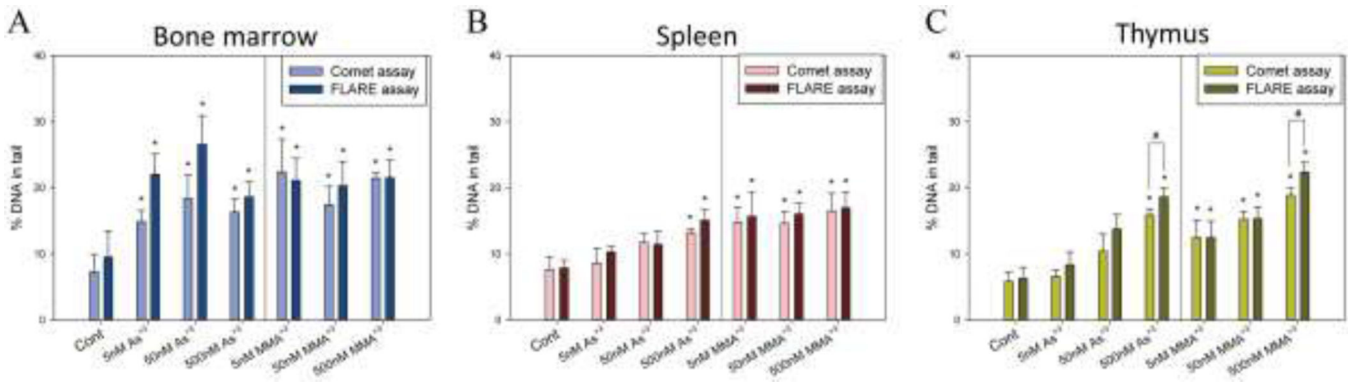

Figure 4.

DNA damage in bone marrow, spleen and thymus cells treated with As^{+3} and MMA^{+3} in vitro for 4 h. Cells were isolated from three 13-week old C57BL/6J mice and pooled. Bone marrow, spleen and thymus cells were then treated with 5, 50 and 500 nM As^{+3} or MMA^{+3}. Comet assay and FLARE assay were used to analyze the DNA damage and oxidative damage induced by the treatments. A, bone marrow. B, spleen. C, thymus. *Significantly different compared to control ($p<0.05$). #Significantly different compared to the Comet assay ($p<0.05$) Results are Means ± SD.

Table 1

Mouse body weight, water, arsenic (As) intake, tissue weight, cell recovery and viability of 30 d 0 (Control), 100 and 500 ppb As^{+3} *in vivo* drinking water exposed male C57/BL6 mice[1].

Treatments		**Mouse body weight (g)**	**Water intake (ml/d)**	**As intake (ng/d)[2]**
Control		27.71 ± 1.66	3.34 ± 0.11	18.50 ± 0.77
100 ppb		27.53 ± 2.69	3.68 ± 0.27	420. 54± 12.32 *
500 ppb		27.50 ± 1.42	3.59 ± 0.34	2111.32 ± 323.19 *
		Tissue weight (mg)	Cell recovery (× 10^6 cells)	Viability (%)
Bone Marrow	Control		51.35 ± 12.75	86.16 ± 2.93
	100 ppb		42.78 ± 4.82	88.64 ± 1.72
	500 ppb		43.14 ± 8.12	87.46 ± 2.10
Spleen	Control	106 ± 6.60	186.6 ± 13.39	77.72 ± 3.19
	100 ppb	106 ± 6.04	183.4 ± 14.31	74.75 ± 1.71
	500 ppb	103 ± 10.03	180.8 ± 30.96	76.32 ± 2.65
Thymus	Control	52.32 ± 7.31	71.32 ± 14.50	87.16 ± 1.56
	100 ppb	46.24 ± 5.73	69.62 ± 17.24	87.68 ± 1.20
	500 ppb	40.88 ± 11.69	60.34 ± 16.85	87.98 ± 0.89

[1] Mice were 9-week old when the exposure started. 5 mice (n = 5) were treated for each single dose. Drinking water was changed each week and water intake was calculated from the change in the weight of the water. Cell recovery and viability were obtained using a Cellometer 2000 with AO/PI staining. Results are Means ± SD.

[2] As intake was calculated based on drinking water samples measured by Mass Spectrometry and daily water intake of each mouse.

* Significantly different from Control ($p < 0.05$).

Table 2

Arsenic (As) amounts in different species in bone marrow, spleen, thymus cells (5×10^6 cells), and 50 μl plasma in C57/BL6 male mice exposed to 0 (Control), 100 and 500 ppb As^{+3} *in vivo* for 30 d. Samples were analyzed by Oxidation State Specific Hydride Generation-Cryotrapping- Inductively Coupled Plasma- Mass Spectrometry (HG- CT- ICP- MS)[1].

Tissues	Exposure doses	As amount by species (pg)						Total As
		As^{+3}	MMA^{+3}	DMA^{+3}	As^{+5}	MMA^{+5}	DMA^{+5}	
Bone Marrow	Control	ND	ND	0.52 ± 0.10	5.46 ± 0.74	0.03 ± 0.01	0.88 ± 0.13	6.88 ± 0.63
	100 ppb	ND	0.07 ± 0.04	1.77 ± 0.73*	7.60 ± 1.78	0.34 ± 0.15*	2.02 ± 0.21*	11.80 ± 2.49*
	500 ppb	ND	45.70 ± 0.69	4.13 ± 0. 85*	11.70 ± 3.54*	10.35 ± 1.31*	7.52 ± 3.12*	79.44 ± 8.47*
Spleen	Control	0.29 ± 0.10	ND	0.16 ± 0.12	10.14 ± 1.90	0.43 ± 0.41	0.51 ± 0.07	11.21 ± 2.19
	100 ppb	0.11 ± 0.08	0.12 ± 0.05	0.97 ± 0.71	11.62 ± 2.89	0.29 ± 0.08	0.66 ± 0.33	13.73 ± 2.79
	500 ppb	0.51 ± 0.26	0.22 ± 0.17	1.83 ± 0.42*	11.33 ± 2.86	1.61 ± 2.13	1.15 ± 0.27*	16.45 ± 3.54
Thymus	Control	2.62 ± 0.91	0.13 ± 0.10	0.44 ± 0.18	11.86 ± 3.01	0.61 ± 0.86	0.22 ± 0.14	15.83 ± 3.06
	100 ppb	5.51 ± 2.49	25.70 ± 4.50*	0.81 ± 0.16	14.34 ± 6.40	8.08 ± 1.86*	0.63 ± 0.07*	54.42 ± 10.10*
	500 ppb	ND	125.43 ± 21.23*	3.17 ± 2.01*	NA	3.47 ± 3.59	NA	146.79 ± 43.34*
Plasma	Control	ND	3.79 ± 2.561	138.92 ± 13.00	NA	9.72 ± 9.86	226.76 ± 17.11	386.35 ± 21.54
	100 ppb	ND	2.40 ± 0.50	363.87 ± 131.87*	43.36 ± 21.12	28.59 ± 7.57*	639.43 ± 144.39*	1077.66 ± 381.32*
	500 ppb	ND	12.72 ± 4.92*	802.84 ± 66.46*	63.56 ± 25.92	57.07 ± 17.58*	2027.22 ± 358.66*	2963.41 ± 332.48*

[1] 5 mice (n = 5) were treated for each single dose. Sample lost happened during collections and injections. Values are in picograms (pg). Results are Means ± SD. All values are expressed as pg of As in each arsenic species.

NA, n < 3 due to sample lost.
ND, below detection range.

[*] Significantly different from Control ($p < 0.05$).