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# Effect of trivalent arsenicals on cell proliferation in mouse and human microvascular endothelial cells

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ABSTRACT

Chronic exposure to high levels of inorganic arsenic (iAs) has been associated with cancerous and non-cancerous health effects, including cardiovascular effects. However, the mechanism for a presumed toxic effect of arsenic on vascular tissue is not clear. Our working hypothesis is that inorganic trivalent arsenic and its methylated metabolites react with cysteine-containing cellular proteins and alter their function leading to adverse events such as cytotoxicity or proliferation. In this study, human microvascular endothelial cells (HMEC1) and mouse microvascular endothelial cells (MFP-MVEC) were exposed to arsenite ($iAs^{III}$), monomethylarsonous acid ($MMA^{III}$), or dimethylarsinous acid ($DMA^{III}$) for 72 h to evaluate cytotoxicity, and for 24, 48 or 72 h to evaluate cell proliferation. Both cell lines showed similar $LC_{50}$ values, from 0.1 to 2.4 μM, for all three trivalent arsenicals. The endothelial cells treated with1 nM to 1 μM concentrations of the three trivalent arsenicals did not show increased cell proliferation at 24, 48 or 72 h or increased rate of proliferation at 72 h of exposure. Overall, cytotoxicity of trivalent arsenicals to microvascular endothelial cells is similar to their cytotoxicity to epithelial cells, and that these compounds are not mitogenic.

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## 1. Introduction

As a natural component of the earth's crust, inorganic arsenic (iAs) is present in soil, water and food. Levels of inorganic arsenic in drinking water vary, and in some parts of the world the levels are as high as 900 ppb or higher [10]. Chronic exposure to high levels of inorganic arsenic has resulted in an increased risk of cancerous and non-cancerous effects [10]. Whether health effects can result from chronic exposure to low levels of iAs (<100 ppb) remains a matter of scientific debate [10,32].

Cardiovascular effects due to exposure to high levels of iAs, generally ≥100 ppb, are amongst the major investigated non-cancerous effects [10,23,28]. The incidence of cardiovascular disease (CVD) risk factors, such as diabetes and hypertension, are also suspected to be increased with exposure to high levels of iAs. Increased prevalence of markers of atherosclerosis, such as carotid plaques and increased thickness of carotid intima have been associated with exposure to high levels of iAs [23,24]. Risk of developing microvascular changes, such as renal disease, neurological diseases, and retinopathy were also reported to be greater with high iAs arsenic exposures in drinking water, and the concurrent presence of diabetes augmented these changes [7]. Most of the data are from regions of high iAs water contamination. There is little evidence with regard to any of the CVD-related effects at exposures to low levels of iAs (<100 ppb in drinking water) [23,28,32].

Animal studies have produced conflicting results [5,6,28]. In FVB female mice, chronic exposure to 100 ppb $iAs^{III}$ in drinking water for 22 weeks was shown to induce hypertension and cardiac hypertrophy [25]. Chronic exposure to 200 or 1000 ppb iAs in drinking water also showed development of atherosclerotic plaques in the macrovasculature of ApoE-/- mice [20]. Mice administered 20 mg/kg of sodium arsenite in water showed increased vascular leakage [5]. At a similar dose, iAs had anti-angiogenic activity in solid tumor models by decreasing expression of vascular endothelial growth factor (VEGF) [34].

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Soucy et al. [26] reported that vessel growth in fibroblast growth factor-2 (FGF-2)-containing Matrigel implants was increased in mice exposed to 5–500 ppb iAs in drinking water for 5 or 20 weeks, but the response decreased with time. However, $iAs^{III}$ alone did not induce neovascularization; rather, it enhanced the effect of FGF-2 in the Matrigel. In addition, the concentration of FGF-2 in the Matrigel was significantly higher than normally occurs in mice or in humans.

The pathogenesis of atherosclerosis has traditionally fallen under two overarching hypotheses: one theory stresses intimal cellular proliferation while the other premise emphasizes recurring thrombi organized over time into clinically significant plaques [28]. Various cellular mechanisms for arsenic-induced effects on the microvasculature have been suggested [2,3,22,29], including increased vascular oxidative stress, increased vascular inflammatory response, induction of endoplasmic reticulum stress, activation of G-protein coupled receptor activation, and stimulation of vascular redox signaling [29]. Pro- and anti-angiogenic responses have been reported when mouse aortic endothelial cells were exposed to high doses of iAs *in vitro* [22,29]. Stimulation of cell proliferation by low concentrations of iAs has been observed in endothelial cells, specifically, in porcine aortic endothelial cells *in vitro* [2], human microvascular endothelial cells (HMEC1) [13], and human lung microvascular endothelial cells [17], possibly secondary to oxidative stress. However, observations of oxidative stress *in vitro* secondary to exposure to arsenicals are not always expressed in a similar manner *in vivo* [10]. Furthermore, a recent study showed that in Bangladesh, in a high arsenic exposed population, an association with markers of oxidative stress was not found [14].

Liver sinusoidal defenestration was reported in a mouse model treated with 10–250 ppb iAs in drinking water [30]. However, the response in the mouse liver appeared to be transient, and furthermore, none of the hepatic sequelae, such as steatosis, fibrosis or cirrhosis, that usually are associated with these vascular changes were observed in longer term studies in mice [31,33].

The applicability to humans of these models in which the effects of iAs on the endothelium were evaluated, is questionable. Mouse models of cardiovascular abnormalities or other inflammatory changes in general do not appear to be representative of the disorders in humans [27,28] so that extrapolation of the results to human risk assessment must be made with caution.

Mammals metabolize inorganic arsenic to mono-, di- and trimethylated compounds of pentavalent arsenic, with mono- and dimethylated compounds of trivalent arsenic formed as intermediates. Numerous studies have shown that the trivalent forms, $iAs^{III}$, $MMA^{III}$, and $DMA^{III}$ are reactive and cytotoxic to several epithelial cell types, in contrast to the corresponding pentavalent forms that are not toxic [9]. Further, studies have shown that trivalent methylated forms ($MMA^{III}$, $DMA^{III}$) are somewhat more cytotoxic than $iAs^{III}$ to human keratinocytes, urothelial cells, and bronchial epithelial cells, the target tissues for iAs-induced cancer [11]. A similar toxicity pattern has been demonstrated for rat aortic macrovascular endothelial cells [15].

In the present studies, we examined the proliferative response of mouse and human microvascular endothelial cells to the trivalent arsenicals ($iAs^{III}$, $MMA^{III}$ and $DMA^{III}$) as well as the cytotoxicity ($LC_{50}$) of the trivalent arsenicals in the same cells.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Sodium arsenite ($NaAsO_2$, purity 99%) was purchased from Sigma (St. Louis, MO). $MMA^{III}$ and $DMA^{III}$ were synthesized by Dr. William Cullen (University of British Columbia, Vancouver, Canada), and supplied as the di-iodide and mono-iodide, respectively. The identity and purity of the chemicals were determined by NMR analysis at the University of British Columbia and accepted without further testing. $MMA^{III}$ and $DMA^{III}$ were stored desiccated in the dark at approximately 4 °C.

### 2.2. Cell culture

The human microvascular endothelial cell (HMEC1) line was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). HMEC1s were isolated from human foreskin dermal tissue and immortalized by the transfection of the transforming protein, SV40 large T antigen [1]. The HMEC1 line was grown in RPMI medium (Hyclone, Thermo Fisher Scientific, Rochester, NY) containing fetal bovine serum (5% v/v) (Atlanta Biologicals, Atlanta, GA), supplemented with 5 ml L-glutamine (MediaTech, Herndon, VA), 100 units/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen, Grand Island, NY) at 37 ± 1 °C in an atmosphere of 95% air and 5% $CO_2$.

The mouse mammary fat pad microvascular endothelial cell line (MFP MVEC) was created from cells isolated from the mammary fat pad (MFP) tissue of two female H-2Kb-ts-A58 mice (Immortomice, Charles River Laboratories, Wilmington, MA) [19]. Briefly, Immortomice carry a temperature-sensitive mutant of SV40 large T antigen under the control of the H-2Kb promoter (activated by interferon-γ), which can be used to conditionally immortalize isolated primary cells when grown at the permissive temperature of 33 °C [16]. MFP MVECs were cultured on 0.2% gelatin-(Sigma–Aldrich, St. Louis, MO) coated flasks in DMEM (Hyclone Laboratories, Logan, UT) supplemented with 2 mM GlutaMax™, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 1x MEM vitamins, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Fungizone™), 40 μg/mL gentamicin (all from Invitrogen, Grand Island, NY), and 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals) in an atmosphere of 95% air and 5% $CO_2$ at 33 °C for routine expansion and at 37 °C for at least 5 days before experiments.

### 2.3. Determination of cytotoxicity ($LC_{50}$)

The number of cells required to be seeded on a 96-well plate to reach approximately 80% confluence at the end of 96 h of plating with a change of medium at 24 h after seeding was first determined. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were exposed to varying concentrations (HMEC1, 0.1–50 μM; MFP MVEC, 0.005–50 μM) of trivalent arsenicals at 24 h after plating and continued for 72 h. The cell viability after 72 h treatment was normalized to control. The data were analyzed by non-linear regression analysis (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA). The cytotoxic response of the trivalent arsenicals was compared based on the resulting $LC_{50}$ values.

### 2.4. Determination of cell proliferation by flow cytometry

The cells were plated in 6-well plates. To maximize the detection sensitivity, the cells were cultured in a low FBS-containing medium (1% FBS for HMEC1, 2.5% for MFP-MVEC). First, the number of cells required to be seeded in low FBS-containing medium that would reach 80% confluence at the end of 72 h was determined. The cells were seeded and immediately exposed to different concentrations of trivalent arsenicals (HMEC1, 1–1000 nM; MFP-MVEC, 1–500 nM). At 72 h after treatment, the cells were labeled with bromodeoxyuridine (BrdU) for 1 h to incorporate into DNA of cells. Cells were then harvested for flow cytometry analysis as

**Table 1**
$LC_{50}$ values of trivalent arsenicals on microvascular endothelial cells.

| Cell type | LC50 (μM) | | |
|---|---|---|---|
| | $iAs^{III}$ | $MMA^{III}$ | $DMA^{III}$ |
| HMEC1 | 2.3 | 0.74 | 2.0 |
| MFP-MVEC | 1.4 | 0.12 | 0.61 |

per BD Pharmingen™ BrdU flow kit (BD Biosciences, San Jose, CA) using a flow cytometer (FACSCalibur™, BD Biosciences, San Jose, CA). Cellular DNA was labeled with 7-aminoactinomycin D (7-AAD) and stained with fluorescent anti-BrdU antibody for flow cytometric cell cycle analysis. The number of cells in G0/G1, S and G2/M phases of the cell cycle were counted for at least 10,000 events. Cell proliferation was determined using the percent of cells in S-phase indicated the proliferation of cells actively synthesizing DNA.

### 2.5. Determination of cell proliferation by MTT assay

The cells were plated in 96-well plates in low FBS-containing (1% FBS for both cell types) medium and allowed to acclimatize for 24 h, followed by treatment with varying concentrations of arsenicals (1–500 nM). The cell survivability was determined at 24 h, 48 h and 72 h of arsenical treatment using the MTT assay. Any increase in cell number was expected to increase the MTT absorbance, and that was compared to control (1% FBS-containing media only) to determine the effect of treatment. Regular medium containing appropriate levels of FBS (5% for HMEC1 cells, 10% for MFP-MVECs) was used as a positive control.

### 2.6. Statistical analysis

Cell proliferation data obtained by flow cytometry were analyzed by one way analysis of variance followed by Dunnett's test. $P$ values less than 0.05 were considered significant. All statistical analyses were performed using Prism 5.03 (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. Cytotoxicity of trivalent arsenicals for microvascular endothelial cells

The $LC_{50}$ values for HMEC1 cells were: 0.74 μM for $MMA^{III}$, 2.0 μM for $DMA^{III}$, and 2.3 μM for $iAs^{III}$; and for MFP-MVECs cells: 0.12 μM for $MMA^{III}$, 0.61 μM for $DMA^{III}$ and 1.4 μM for $iAs^{III}$ (Table 1). Both HMEC1 and MFP-MVECs cells were slightly more sensitive to $MMA^{III}$ compared to $iAs^{III}$ or $DMA^{III}$. Lower concentrations of $iAs^{III}$ appeared to slightly increase the cell viability in both cell types, with a greater effect observed in MFP-MVECs (Fig. 1).

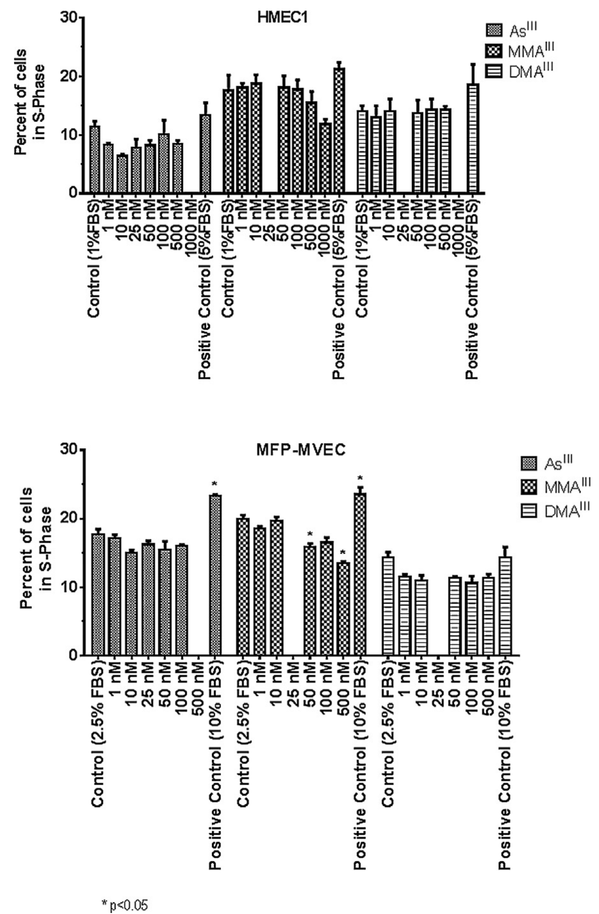


**Fig. 2.** Flow cytometry analysis for S phase of endothelial cells treated with trivalent arsenicals for 72 h at concentrations ranging from 1 nM to 1000 nM.

### 3.2. Trivalent arsenicals and endothelial cell proliferation

#### 3.2.1. Endothelial cell proliferation measured by flow cytometry

Both HMEC1 and MFP-MVECs exposed to trivalent arsenicals showed no increase in rate of cell proliferation measured at the end of 72 h treatment (Fig. 2). Specifically, the trivalent arsenicals did not increase the percent of cells in S-phase of the cell cycle as measured by flow cytometry..

#### 3.2.2. Endothelial cell proliferation measured by MTT assay

The effect of trivalent arsenicals on endothelial cell proliferation was also measured at 24, 48 and 72 h of treatment using the MTT assay (Fig. 3a and b). The MTT assay indirectly represents the number of living mitochondria, which correspond to the number of living cells. None of the three trivalent arsenicals induced increased

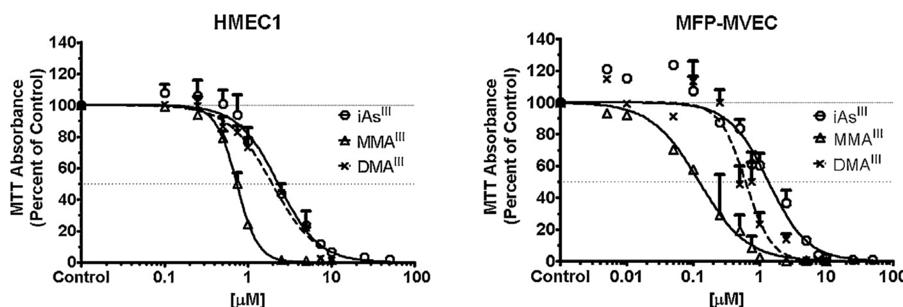


**Fig. 1.** Cytotoxicity evaluation determined by MTT assay of endothelial cells exposed to trivalent arsenicals for 72 h to estimate the LC50.

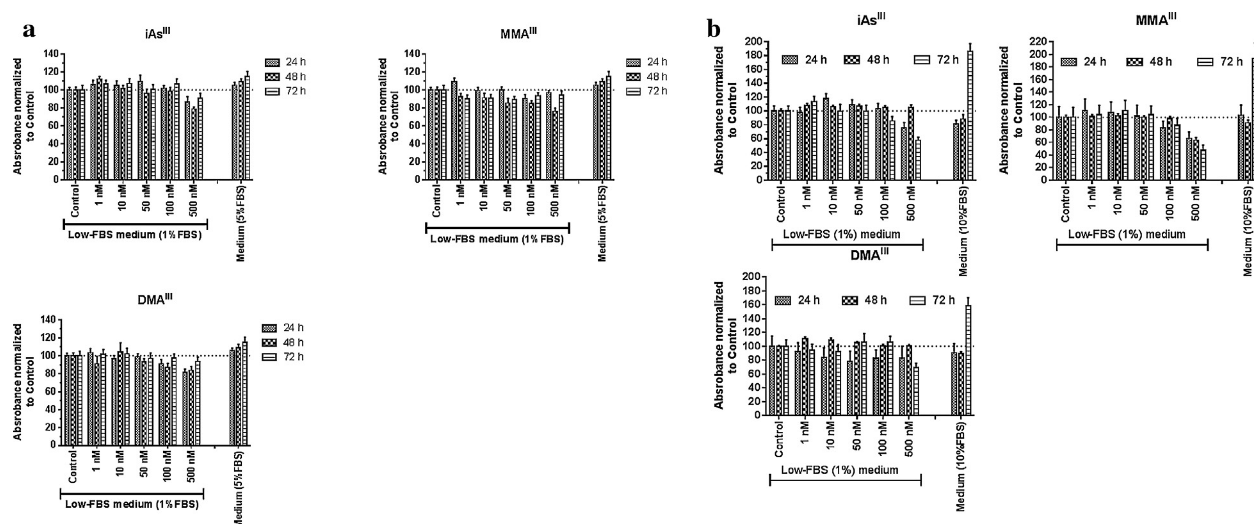


**Fig. 3.** Time course analysis of cell survival determined by MTT assay after exposure to trivalent arsenicals for 24, 48 or 72 h. (A) HMEC1 endothelial cells, (B) MFP MVEC endothelial cells.

endothelial cell proliferation by this assay measured at any time point.

## 4. Discussion

In the present study, none of the trivalent arsenicals increased the rate of cell proliferation or showed a cell proliferation response in either cell type. Importantly, this finding does not support arsenic causing CVD by means of endothelial cell proliferation.

Similar to earlier reports for epithelial cell types, $MMA^{III}$ was relatively more cytotoxic to both endothelial cell lines than $DMA^{III}$ and $iAs^{III}$. In mouse cells, $MMA^{III}$ was 5 fold more toxic than $DMA^{III}$ and 11 fold more toxic than $iAs^{III}$, and in human cells $MMA^{III}$ was 2 fold more toxic than $DMA^{III}$ and 3 fold more toxic than $iAs^{III}$. The mouse endothelial cells were more sensitive, with lower $LC_{50}$ values, for the three trivalent arsenicals, and the human cells were more sensitive for $iAs^{III}$ and $MMA^{III}$ compared to the results of our previous studies in human urothelial 1T1 cells, human keratinocytes and human bronchial epithelial cells [8,11]. The $LC_{50}$ of 2.3 μM for $iAs^{III}$ in HMEC1 cells is similar to the $LC_{50}$ of 2.4 μM reported by Graham-Evans et al. [13] who conducted a similar test using different medium. In that study, endothelial cells were most sensitive to $iAs^{III}$, with the lowest $LC_{50}$ value compared to other cell types, including keratinocytes (HaCaT), melanocytes (CRL 1675), dendritic cells, dermal fibroblasts (CRL1904), and monocytes (TIB202).

None of the three test compounds caused an increase in the percent of endothelial cells in S-phase of either human or mouse at any concentration, ranging from nM range to μM range for up to 72 h exposures. Similarly, there was no increase in cell viability at 24, 48 or 72 h with $iAs^{III}$, $MMA^{III}$ or $DMA^{III}$ treatment in a growth kinetics experiment, indicating no stimulation of cell proliferation. However, in the cytotoxicity assay, when viability was measured after 72 h exposure to the trivalents, there was a slight increase in cell viability (<15% in HMEC1, and <30% in MFP-MVECs) with lower concentrations of $iAs^{III}$. This is in contrast to the growth kinetics outcome (Fig. 3) which did not show increased viability at 72 h. The amount of FBS in the medium is the major difference between these two experiments. Low concentrations of $iAs^{III}$ in low-FBS containing medium did not stimulate cell proliferation (Fig. 3). Also, neither $MMA^{III}$ nor $DMA^{III}$ increased cell numbers (Fig. 3). There was a statistically significant decrease in the number of S-phase cells with $MMA^{III}$ treatment in MFP-MVECs at 50 and 500 nM concentrations. MF-MVECs are most sensitive to $MMA^{III}$ compared to $As^{III}$ or $DMA^{III}$ (Fig. 1, Table 1) and it is likely that $MMA^{III}$ is cytotoxic at these concentrations.

The findings of the current study, which show that trivalent arsenicals do not induce endothelial cell proliferation *in vitro* are in contrast to other studies which have suggested a weak biphasic response in HMEC1 cells [13] and porcine aortic endothelial cells [2,3]: a proliferative response at low concentrations of $iAs^{III}$ and a cytotoxic response as the concentration of $iAs^{III}$ is increased. These contrasting results may be due to differences in the media used to grow HMEC1 cells or in macrovascular or microvascular cells in the porcine experiments. Graham-Evans et al. [13] used endothelial basal medium, whereas RPMI-based medium was used in our study. Furthermore, there were differences in the amount of added growth factors in the medium. Barchowsky et al. [2] showed that $iAs^{III}$ slightly increased the rate of proliferation only in confluent cells but not in cells that were exponentially growing. In the current experiment, the number of cells seeded were approximately 80% confluence at the end of 72 h and therefore, the cells were growing throughout the experiment. Furthermore, endothelial cells from different tissues have different biological properties and likely respond to chemicals, such as trivalent arsenicals, differently, or even not responding at all.

A weak biphasic (hormetic) response has been observed in HMEC1 cells with other agents, such as Au@Fe3O4 Janus particles [18] and statins [4]. The hormetic response could represent an adaptive process of the system in response to the stressor by increasing expression of cell protective signaling, and is known to occur for a large number of cytotoxic agents in multiple cancer cell lines [4,21]. Cell protective signaling includes NF-kB signaling, Nrf2, kinases, including deacetylases, which in turn stimulate growth factor production [21]. This might explain why Barchowsky et al. [2] reported involvement of NF-kB signaling in the low-dose $iAs^{III}$-induced increase in the rate of cell proliferation in porcine aortic endothelial cells.

These various responses have all been investigated *in vitro*. However, it is unknown what the effect will be *in vivo* in animals or in humans.

The results of our current study do not support a direct mitogenic effect by trivalent arsenicals as the basis for endothelial effects of arsenicals at any concentration. *In vitro*, our results suggest that at sufficiently high concentrations (>0.1 μM) trivalent arsenicals can produce endothelial cytotoxicity. *In vitro*, this results in cell death. *In vivo*, the cell damage, including cell death, would

result in consequent effects, such as regenerative proliferation and/or other sequlae in endothelial cells or other vascular cells. The consequences could be the various cardiovascular effects associated with exposure to high levels of inorganic arsenic.

Whether or not an endothelial and consequent cardiovascular effect is produced in vivo in animal models or in humans will be dependent on the concentrations of the trivalent arsenicals in the endothelial cells being greater than the minimal amount required to produce an effect. Thus, a threshold is involved. To achieve tissue concentrations of trivalent arsenicals in humans >0.1 μM, the apparent threshold for a biologic effect in epithelial, endothelial and other cell types [10,12], requires exposure to inorganic arsenic in drinking water of approximately 100 ppb [10].

Multiple epidemiological studies have associated exposure to high levels of arsenic (≥100 ppb in drinking water) with cardiovascular changes [20,25,29,32]. However, evidence for vascular changes in humans induced by exposure below 100 ppb in drinking water is lacking [28]. Our results with mouse and human endothelial cells support a threshold effect for trivalent arsenicals and are consistent with that level being >100 ppb in the drinking water, which translates to a tissue level of >0.1 μM.

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