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Role of the alkali labile sites, reactive oxygen species and antioxidants in DNA damage induced by methylated trivalent metabolites of inorganic arsenic

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Abstract

In the last decade arsenic metabolism has become an important matter of discussion. Methylation of inorganic arsenic (iAs) to monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) is considered to decrease arsenic toxicity. However, in addition to these pentavalent metabolites, the trivalent metabolites monomethylarsonous (MMA^{III}) and dimethylarsinous acid (DMA^{III}) have been identified recently as intermediates in the metabolic pathway of arsenic in cultured human cells. To examine the role of oxidative damage in the generation of DNA strand breaks by methylated trivalent arsenic metabolites, we treated human lymphocytes with both metabolites at non-cytotoxic concentrations. We further tested whether these effects are sensitive to modulation by the antioxidants ascorbate (Vitamin C) and selenomethionine (Se-Met). Both trivalent metabolites produced oxidative stress related DNA damage, consisting of single strand breaks and alkali-labile sites, with MMA^{III} being more potent at low concentrations than DMA^{III}. Neither MMA^{III} nor DMA^{III} induced DNA-double strand breaks. The oxidative stress response profiles of the metabolites were parallel as determined by lipid peroxidation induction. MMA^{III} induced peroxidation from the lowest concentration tested, while effects of DMA^{III} were apparent only at concentrations above 10 μM. The antioxidant Se-Met exhibited a more pronounced inhibition of trivalent arsenic metabolite-induced oxidative-DNA damage than did vitamin C. The present findings suggest that DNA damage by methylated trivalent metabolites at non-cytotoxic concentrations may be mediated by a mix of reactive oxygen and nitrogen oxidized species.

Abbreviations: iAs – inorganic arsenic; iAs^{III} – arsenite; MMA^{III} – monomethylarsonous acid; MMA^V – monomethylarsonic acid; DMA^{III} – dimethylarsinous acid; DMA^V – dimethylarsinic acid Se-Met-selenomethionine; ALS – alkali labile sites; LMPA – low melting point agarose; Et-Br – ethidium bromide; Tris – Tris[hydroxymethyl]aminomethane; DMSO – dimethyl sulfoxide; FDA – 5,6-carboxiacetate of fluoresceine

Introduction

Inorganic arsenic (iAs) exists ubiquitously in our environment, and various forms of arsenic circulate in soil, water, air and living organisms. Adverse health effects caused by arsenic compounds have long been recognized, including neurotoxicity, liver injury, peripheral vascular disease (known as Blackfoot disease), and increased risk of several cancers (Chen *et al.* 1988). Significant exposures to iAs occur in a variety of workplaces such as smelters, glass work shops,

computer manufacturing factories and agricultural fields that use pesticides and herbicides. Even though the use of arsenicals has been reduced, high levels of arsenic have been found in the drinking water of many geographic regions including Argentina (Astolfi *et al.* 1981), Chile (Borgoño *et al.* 1977), Taiwan (Tseng *et al.* 1968; Chiou *et al.* 1995), the USA (Alpert 2001), India (Das *et al.* 1994, 1995; Chowdhury *et al.* 2000; Mazumder et al. 2001), Inner Mongolia and México (Cebrian *et al.* 1994).

In humans, like many mammals, the metabolism of trivalent inorganic arsenic (iAs^{III}), also known as arsenite, involves at least 4 metabolites that can exert toxic effects. The scheme for the stepwise conversion of (–iAs–) into mono- and dimethylated products is as follows:

$$\begin{aligned} As^{III}O_3^{3-} + CH_3^+ &\rightarrow CH_3As^{V}O_3^{2-} + 2e \\ &\rightarrow CH_3As^{III}O_2^{2-} + CH_3^+ \\ &\rightarrow (CH_3)_2As^{V}O^{2-} + 2e \\ &\rightarrow (CH_3)_2As^{III}O^{-}. \end{aligned}$$

The two-step metabolic process involves (1) reduction to trivalent species and (2) oxidative methylation by which iAs is converted to mono and dimethyl arsenic forms (MMA, DMA, respectively). Thus, both pentavalent and trivalent methylarsenic forms are intermediates or products of this pathway (Lin *et al.* 2002; Thomas *et al.* 2004). However in the last years, another players had been involved in this process such as arsenic–glutathione complexes (Kala *et al.* 2000; Hayakawa *et al.* 2005).

Because DMA^V is cleared from cells more rapidly than MMA^V or iAs^{III}, and methylation reduces the amount of arsenic retained in tissues by increasing the water solubility of arsenite, methylation of iAs to monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) has been considered a process to decrease iAs toxicity (Patrick 2003). In support of this assumption Moore *et al.* (1997) demonstrated in the L5178Y/TK ± mouse lymphoma assay that MMA^V and DMA^V are less cytotoxic, mutagenic and clastogenic than are arsenate and arsenite. In contrast, there is some data in rats evidencing a carcinogenic capacity of DMA^v (reviewed in Kenyon & Hughes 2001). Besides, we found altered cell cycle kinetics and DNA single strand breaks in human lymphocytes exposed to DMA^V and MMA^V (Sordo *et al.* 2001). However, relative to iAs, the trivalent methylated arsenicals, MMA^{III} and DMA^{III}, have more potent cytotoxicity (Styblo *et al.* 2000) and exhibit stronger inhibition of enzymes with antioxidant functions (Styblo *et al.* 1997; Lin *et al.* 2001). Nonetheless, scant data are available regarding the genotoxicity of the trivalent methylated metabolites. Mass *et al.* (2001) demonstrated that DMA^{III} and MMA^{III} are more potent than iAs in generating DNA strand breaks in human lymphocytes. In 2003 Kligerman *et al.* showed that trivalent methylated arsenicals could induce chromosomal aberrations but not gene point mutations.

Moreover, Schwerdtle *et al.* (2003), working with isolated PM2 DNA found that only DMA^{III} produce DNA damage. However, in HeLa cells, the authors found that both trivalent methylated metabolites could produce a concentration-dependent induction of oxidative DNA damage. Furthermore, there is evidence that DMA^{III}, MMA^{III}, and others arsenic metabolites like dimethylarsine and trimethylarsine can nick isolated DNA without enzymatic or chemical activation in a manner that involves generation of reactive oxygen species (ROS) (Mass *et al.* 2001; Ahmad *et al.* 2002; Nesnow *et al.* 2002; Andrewes *et al.* 2003). The aims of this study were firstly to investigate the capacity of trivalent arsenic species at non-cytotoxic concentrations to generate DNA damage associated with oxidative stress and secondly to explore the capacity of different antioxidants to diminish the induction of DNA strand breaks.

Materials and methods

Chemical reagents

Normal agarose, LMPA, Et-Br, Tris, Na_2EDTA, DMSO, FDA, sodium *m*-arsenite ($NaAs^{III}O_2$) and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hydroxide and trichloroacetic acid were purchased from Merck (Germany). The trivalent methylated arsenicals, methyloxoarsine ($MMA^{III}O$, $CH_3As^{III}O$) and iododimethylarsine of DMA^{III} ($DMA^{III}I$,

$(CH_3)_2As^{III}I$) were synthesized by Dr. William R. Cullen (University of British Columbia, Vancouver, Canada) using previously described methods (Cullen *et al.* 1984; Styblo *et al.* 1997). Identity and purity of the synthesized arsenicals were confirmed by 1H NMR, mass spectrometry, and hydride generation–atomic absorption spectrophotometry (HG–AAS) as described previously (Hughes *et al.* 2000). In aqueous solutions, $MMA^{III}O$ and $DMA^{III}I$ are presumed to form MMA^{III} and DMA^{III}, respectively. Working standards of these arsenicals (1 μg of As/ml) were prepared daily from stock solutions. Thiobarbituric acid was purchased from ICN Biomedicals Inc (Ohio, USA) and hydrochloride acid was purchased from Baker (Xalostoc, Mexico).

Blood samples

Human peripheral blood was taken by venipuncture from two healthy male non-smoking donors (aged 19 and 25 years). The blood samples were drawn in accordance with an Institutional approved human studies protocol. Each human blood sample was divided into two aliquots.

Human lymphocytes were isolated by a fycoll hypaque gradient as described by Sanderson *et al.* (1977). Each aliquot of human blood was combined with 0.2 ml of EDTA (10%) in order to prevent coagulation. Plasma was separated from cells by centrifugation in a Stratagene PicoFuge for 5 min at 4000 rpm. DNA damage in human lymphocytes was evaluated by Single Cell Gel Electrophoresis (SCGE) assay at pH > 13, pH 12.1, and neutral conditions. Oxidative damage to membranes was estimated with a lipid peroxidation test.

Treatments

All treatments were performed exposing the cells for 4 h at 37 °C. The final concentrations of trivalent metabolites, MMA^{III} and DMA^{III} were 2.5, 5, 10, 20, 40, 80 and 100 μM. Negative controls were included in all experiments. Ethyl methane sulphonate (EMS) 0.1 μM was used as a positive control for DNA damage, and hydrogen peroxide (100 mM for 10 min) for lipid peroxidation. Experiments were done in triplicate at a minimum.

Viability

Cell viability was measured by the dual stain FDA method, as described by Hartmann and Speit (1995). Briefly, cells were mixed with a fluorochrome solution containing 0.02 μg/μl of Et-Br and 0.015 μg/μl of FDA. Cells were then analyzed under a fluorescence microscope (Olympus BMX-60 with a UM61002 filter). Dead cells appeared red in color and live ones green. One hundred randomly chosen cells per condition were evaluated and the results are expressed in percentages.

Alkaline Single Cell Gel Electrophoresis assay

The alkaline SCGE assay was performed as described previously (Rojas *et al.* 1999), with some modifications. An appropriate number of lymphocytes (~500, 000) were obtained and mixed with 75 μl of 0.5% of LMPA. The mixture was dropped on a slide precoated with 150 μl of 0.5% agarose and then immediately covered with coverglass in order to make a microgel. Slides were placed in a chilled steel tray for 1 min to allow agarose to gel. The coverglass was removed, and another 75 μl of LMPA was applied.

Slides were immersed in chilled lysing solution pH 10 (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris; pH 10 fresh 10% DMSO and 1% Triton X-100). After lysis (4 °C for at least 1 h), two versions of the alkaline single cell gel electrophoresis assays were performed: one at pH > 13 as described by Tice *et al.* (2000), and the other at pH 12.1 (Rojas *et al.* 2000). Slides were placed in a horizontal electrophoresis chamber with cold running buffer solution (300 mM NaOH, 1 mM Na_2EDTA at pH > 13 or pH 12.1) and then allowed to remain in the buffer for 10 min to allow the DNA to unwind. Electrophoresis was conducted for 20 min at 25 V and 300 mA for pH > 13 or pH 12.1 (0.8 V/cm).

All technical SCGE steps were conducted under very dim indirect light. After electrophoresis, the slides were gently removed and the alkaline pH was neutralized with 0.4 M Tris, pH 7.5. Slides were dehydrated with two 10-min periods under 100% ethanol.

Neutral Single Cell Gel Electrophoresis.

A neutral version of the SCGE assay was performed as described previously (Rojas *et al.* 1999). Briefly, slides were prepared as described above and then immersed in chilled lysing solution (0.03 M Na_2EDTA and 2.5% of sodium dodecyl sulfate (SDS). After lysis (4 °C for at least 1 h), slides were placed in a horizontal electrophoresis chamber with cold running buffer solution (90 mM of boric acid, 5 mM Na_2EDTA, 117 mM Tris at pH > 7). The slides remained in the buffer for 2 h to allow DNA stabilization. Electrophoresis was conducted for 20 min at 0.57 V/cm.

All technical SCGE steps were conducted under very dim indirect light. After electrophoresis, the slides were gently washed for 15 min with a 0.4 M Tris solution, pH 7.5. Slides were dehydrated with two 10-min periods under 100% ethanol.

Evaluation of DNA damage

Et- Br (75 μl of a 2.0 μg/ml stock solution) was added to each slide and each sample was covered with a coverglass. Individual cells were visualized under 200× magnification on an Olympus BMX60 microscope with fluorescence attachments (excitation filter 515–560 nm and barrier filter 590 nm). The image for each individual cell was acquired immediately upon opening the microscope shutter, employing the Image Komet 3.0 software (Kinetic Imaging, UK).

For each experiment 100 cells per sample were scored. To assess DNA damage we measured the Olive tail moment, which represents the product of the amount of DNA in the tail (expressed as a percentage of the total DNA) and the distance between the center of the mass of the head and tail regions. We chose to measure this single parameter because we have found that it is tightly correlated with the other common parameters of the comet assay (tail length and percentage of DNA). The Olive tail moment was evaluated with Komet 3.0 software. The slides were analyzed blinded and randomized. As an internal control for each electrophoretic session, we used frozen lymphocytes with a known degree of DNA damage.

Lipid peroxidation

Malondialdehyde (MDA), a product of lipid peroxidation, was measured according to the method of thiobarbituric acid reactive substances (TBARS) (Sinnhuber & Yo 1998) with some modifications. Briefly, 1 ml of human plasma was mixed with 2-thiobarbituric acid (TBA, 0.375%), 15% of trichloroacetic acid, contained in 0.25 N of HCl, making a final volume of 3 ml for each condition. Mixtures were brought to boiling for 15 min. Samples were then centrifuged at 3000 rpm for 3 min. The new supernatant was collected and its absorbance determined in triplicate at 532 nm in a Pharmacia Biotech Ultrospec 3000 spectrophotometer. The pink chromogen can be detected spectrophotometrically and has a molar absorption coefficient between 149,000 and 156,000 l/mol cm. The MDA concentration of samples was calculated using an extinction coefficient of 1.56×10^5 l/mol cm (Buege & Aust 1978). Results were given in nM of MDA per ml of plasma.

Statistical analysis

Results are expressed as means ± standard error (SE). The statistical significance in the comparisons between the experimental groups and control group was calculated by paired or unpaired two-tailed Student's *t*-tests. *P*-values < 0.05 were considered significant. In the case of treatment of antioxidants, the statistical comparisons were made between DMA^{III} and MMA^{III} and coexposition of arsenicals and antioxidant groups All analyses were performed using the statistical software Statistica 98 edition (Soft Inc., USA).

Results

Observing cells treated with dual fluorochrome stain (FDA and Et-Br) revealed that at concentrations of 40, 80 and 100 μM, but not 2.5, 5, 10 and 20 μM, trivalent methylated arsenic metabolites produced cytotoxic effects (Figure 1a and b). DNA damage at pH > 13 induced by arsenical trivalent species is shown in Figure 2a and b. Exposure to DMA^{III} and MMA^{III} metabolites resulted in an increase of DNA damage in both

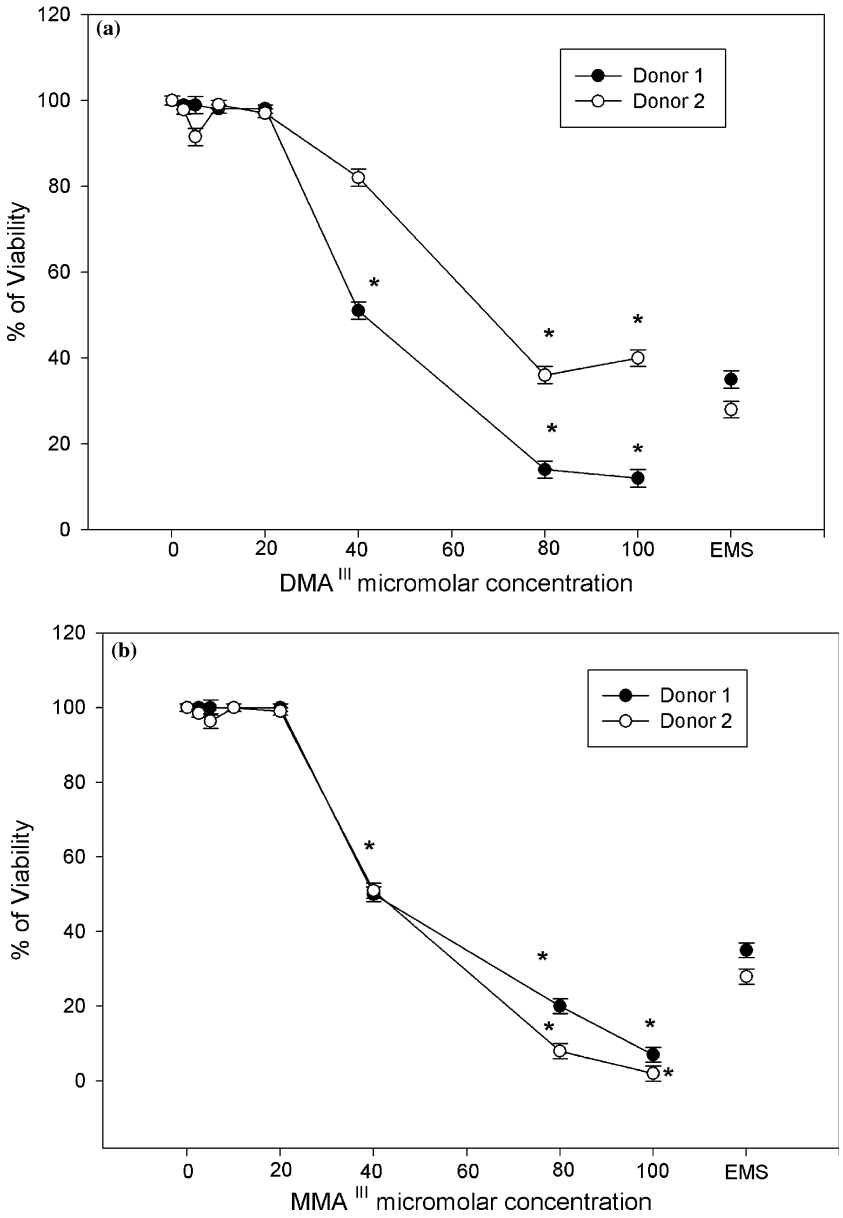

Figure 1. Human lymphocyte viability determined by a dual fluorochrome stain after treatment with DMA^{III} (a) and MMA^{III} (b) for 4 h at 37 °C. $^{*}P < 0.05$ *versus* control. Ethylmethane Sulfonate (EMS) 0.1μM as positive control.

donors. These results are in agreement with the findings of Mass *et al.* (2001), however we found that MMA^{III} is more genotoxic than DMA^{III} in the lower concentrations tested (2.5, 5 and 10 μM).

Only a small amount of DNA damage in $MMA^{III}$and DMA^{III} treated slides under pH 12.1 were found (Figure 3a and b). When subtracting net values of DNA damage determined at pH 12.1 from the values obtained under pH > 13, indirectly determined the alkali labile sites (ALS) contribution to DNA breaks generation. The values of ALS induced by both metabolites are shown in Figure 4a and b. ALS values were induced by exposure to both metabolites. These results implicate the participation of ROS generation in DNA damage induction of human lymphocytes. The results of the neutral comet assay showed no induction of DNA double strand breaks of human lymphocytes for either trivalent arsenic metabolite (Figure 5a and b).

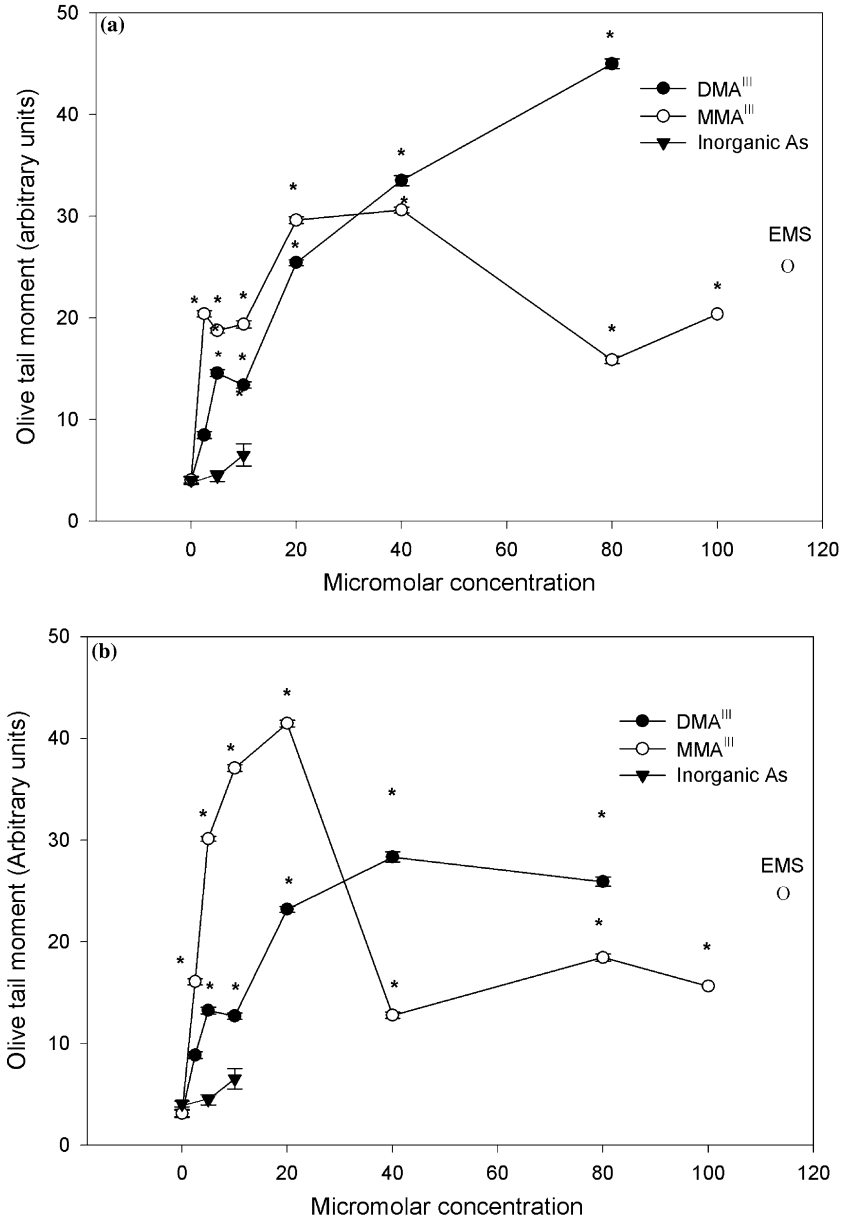

Figure 2. DNA damage determined by comet assay at pH > 13 modality in human lymphocytes for donor 1 (a) and donor 2 (b) after 4 h at 37 °C of trivalent methylated arsenical treatment (5, 10, 15, 20, 40, 80 and 100 μM) and iAs (5 and 10 μM). *$P < 0.05$ *versus* control. Ethylmethane Sulfonate (EMS) 0.1μM as positive control.

Lipid peroxidation levels in plasma from whole blood samples exposed to trivalent methylated metabolites of iAs are summarized in Figure 6. Levels of MDA did not were statistically different from control after treatment with DMA^{III}, with concentration below 10 μM meanwhile lipid peroxidation induction by DMA^{III} was observed from 10 μM, as shown in Figure 6a. In case of MMA^{III} exposure lipid peroxidation was generated since 2.5 μM concentration (Figure 6b).

There was a positive relationship between the oxidative stress DNA-marker (ALS) and the lipid oxidative marker (MDA) for DMA^{III} and MMA^{III} (Figure 7a and b, respectively). This relationship suggests that DNA single strand breaks induced by the trivalent arsenic metabolites are due to ROS

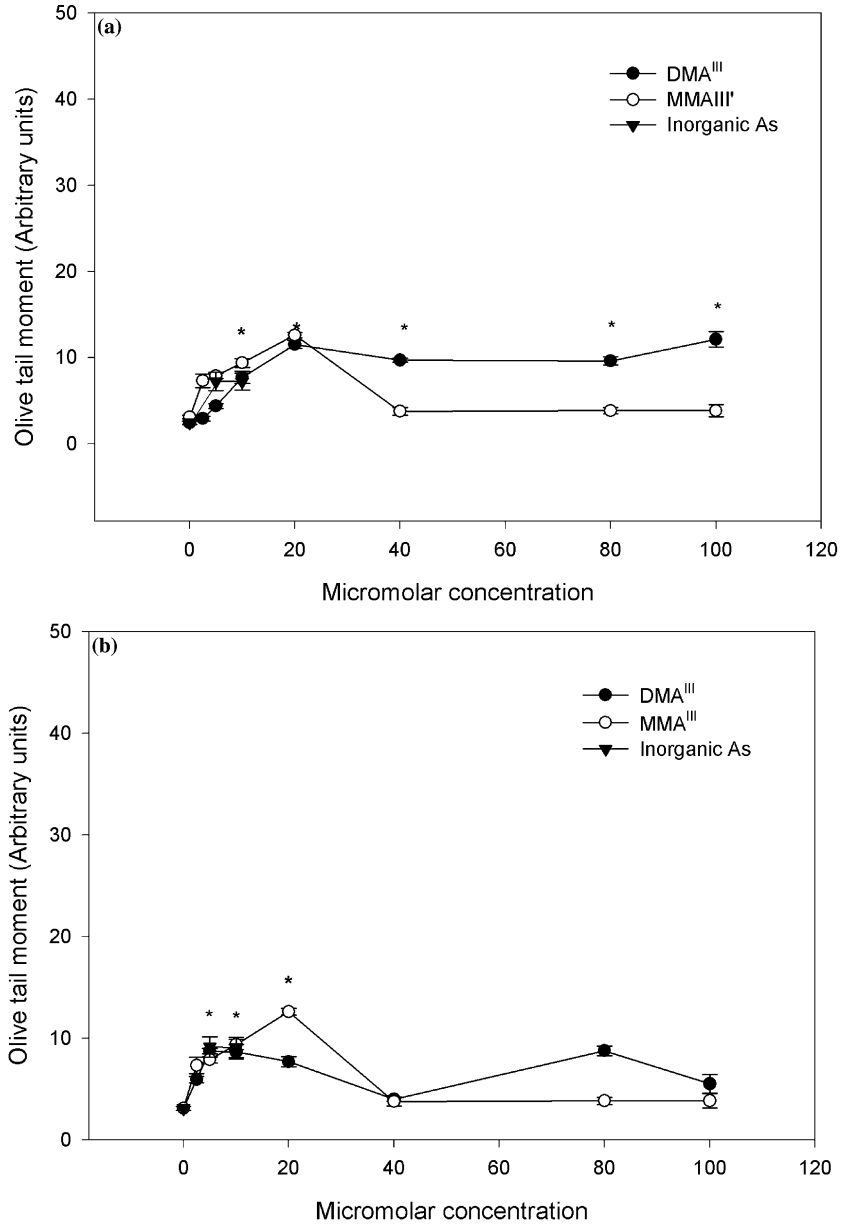

Figure 3. DNA damage determined by comet assay at pH 12.1 modality in human lymphocytes for donor 1 (a) and donor 2 (b) after 4 h at 37 °C of trivalent methylated arsenicals (5, 10, 15, 20, 40, 80 and 100μM) and iAs (5 and 10 μM) exposure. *$P < 0.05$ *versus* control.

related oxidative stress. In order to corroborate the role of oxidative stress in DNA oxidative damage caused by DMA^{III} and MMA^{III}, we administered co-treatments of trivalent arsenic metabolites and 40 μM of vitamin C or 2.8 μM of organic selenium [selenomethionine (Se-Met)]. DNA damage induced by DMA^{III}- and MMA^{III}-induced DNA damage was reduced by the antioxidant co-treatments, being the more potent antioxidant the Se-Met (Figure 8).

Discussion

Arsenic is a well established human carcinogen (Chen & Wang 1990), but the level at which it

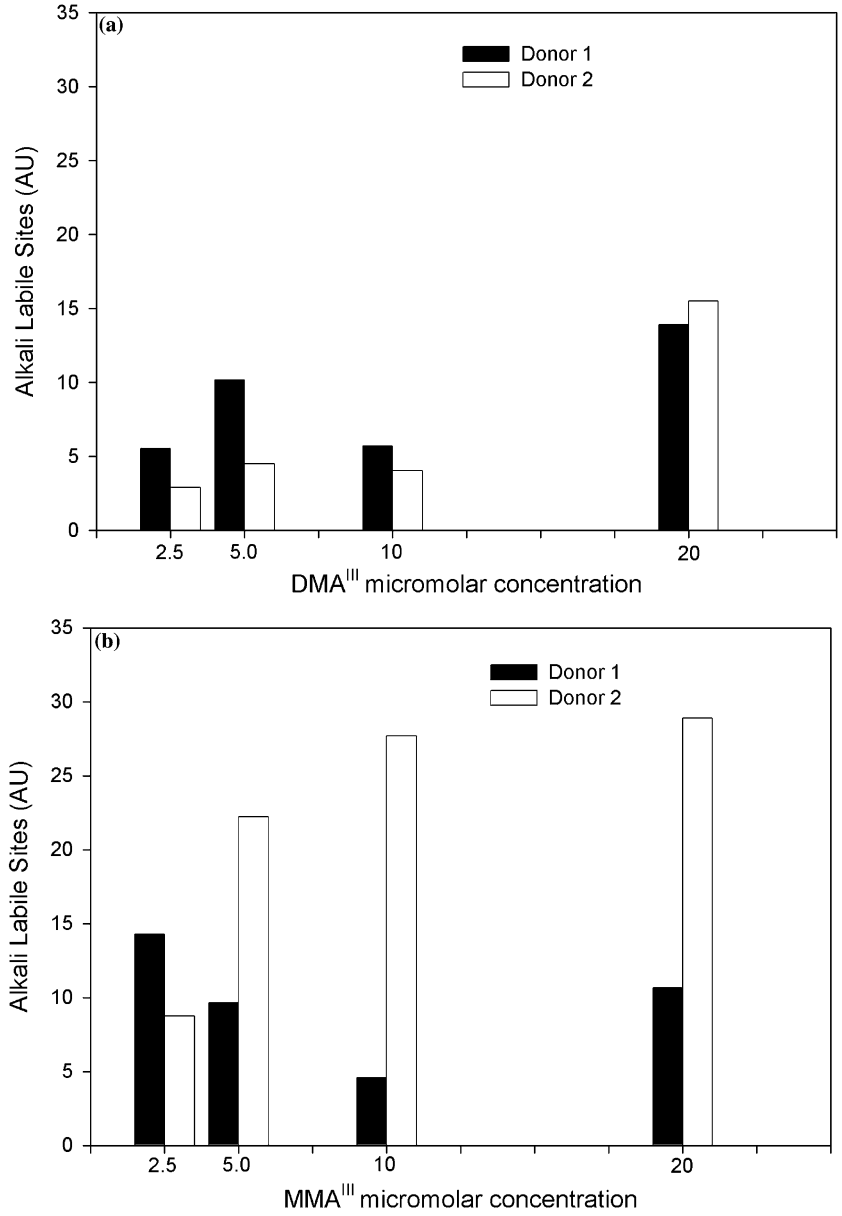

Figure 4. Alkali labile site (ALS) induction was evaluated for DMA^{III} (a) and MMA^{III} (b).

poses a measurable health risk has been the topic of considerable debate and its precise mechanism of action remains unknown. *In vivo* biomethylation and excretion of pentavalent arsenicals (iAs, MMA^{V} and DMA^{V}) has long been thought to be a major detoxification process because the pentavalent methylated metabolites are less reactive towards cellular macromolecules and they are eliminated more rapidly (Buchet *et al.* 1981; Vahter 1999).

In this study, we examined the DNA breakage associated with oxidative stress by trivalent methylated arsenic metabolites at non-cytotoxic concentrations. We found that trivalent arsenic metabolites induced DNA damage, under both alkaline versions of the comet assay (pH > 13 and pH 12.1) showing that the observed DNA damage could be produced by ALS, DNA single strand breaks and/or delayed repair. However we did not find evidence for DNA double strand breaks at

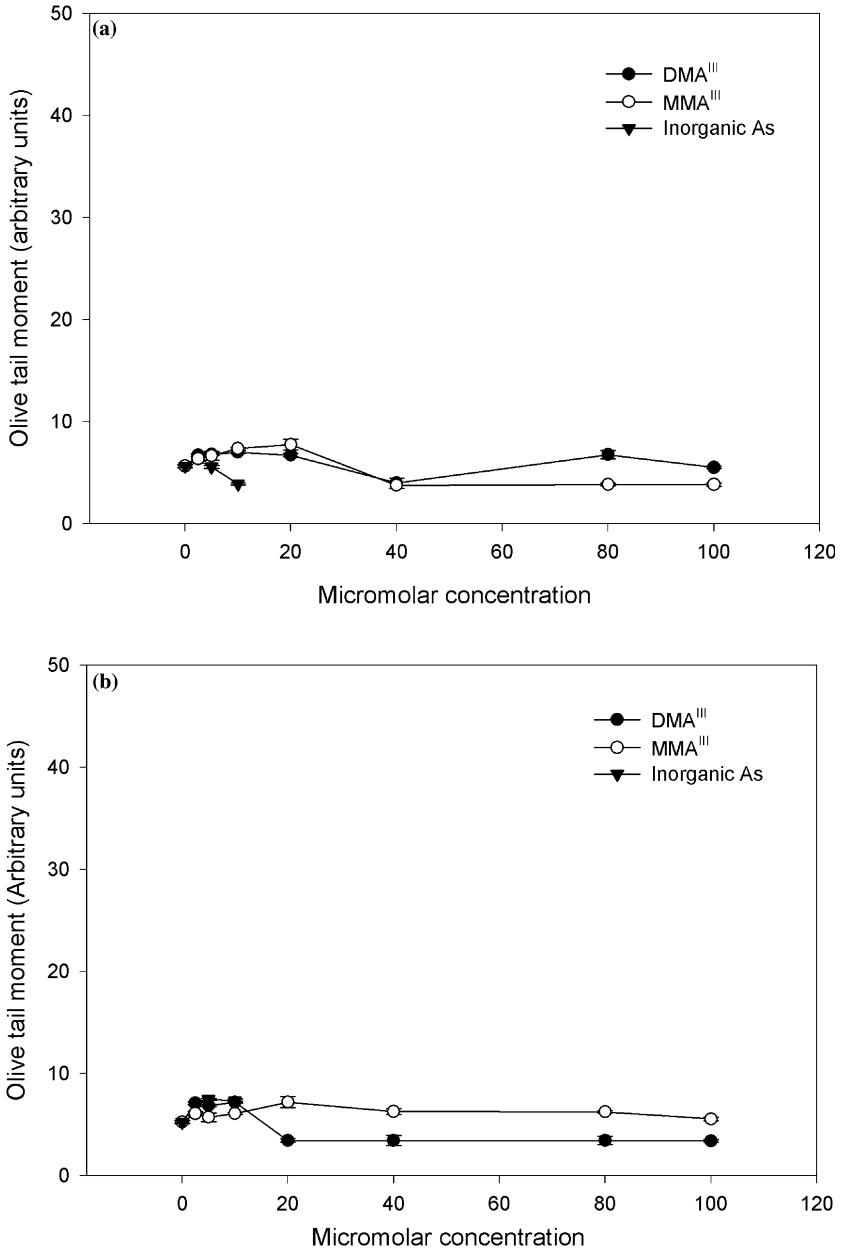

Figure 5. Comet assay evaluation of DNA double strand breaks in human lymphocytes of donor 1 (a) and donor 2 (b) after 4 h at 37 °C and neutral pH modality.

low concentrations. At doses above 10 μM, our pH 13 SCGE results are in agreement with the findings of Mass *et al.* (2001). However, it is worth noting that we observed that MMA^{III} is more genotoxic than DMA^{III} at lower concentrations (2.5, 5 and 10 μM).

The pH 12.1 SCGE data clearly demonstrate that both trivalent methylated metabolites of arsenic produce increased DNA damage. The DNA damage is consistent with the view that the trivalent arsenic metabolites have some capacity to directly induce DNA strand breaks. However, a DNA repair delay could also produce or contribute to the increased DNA damage (Rojas *et al.* 1999). Additional research is required to resolve this question.

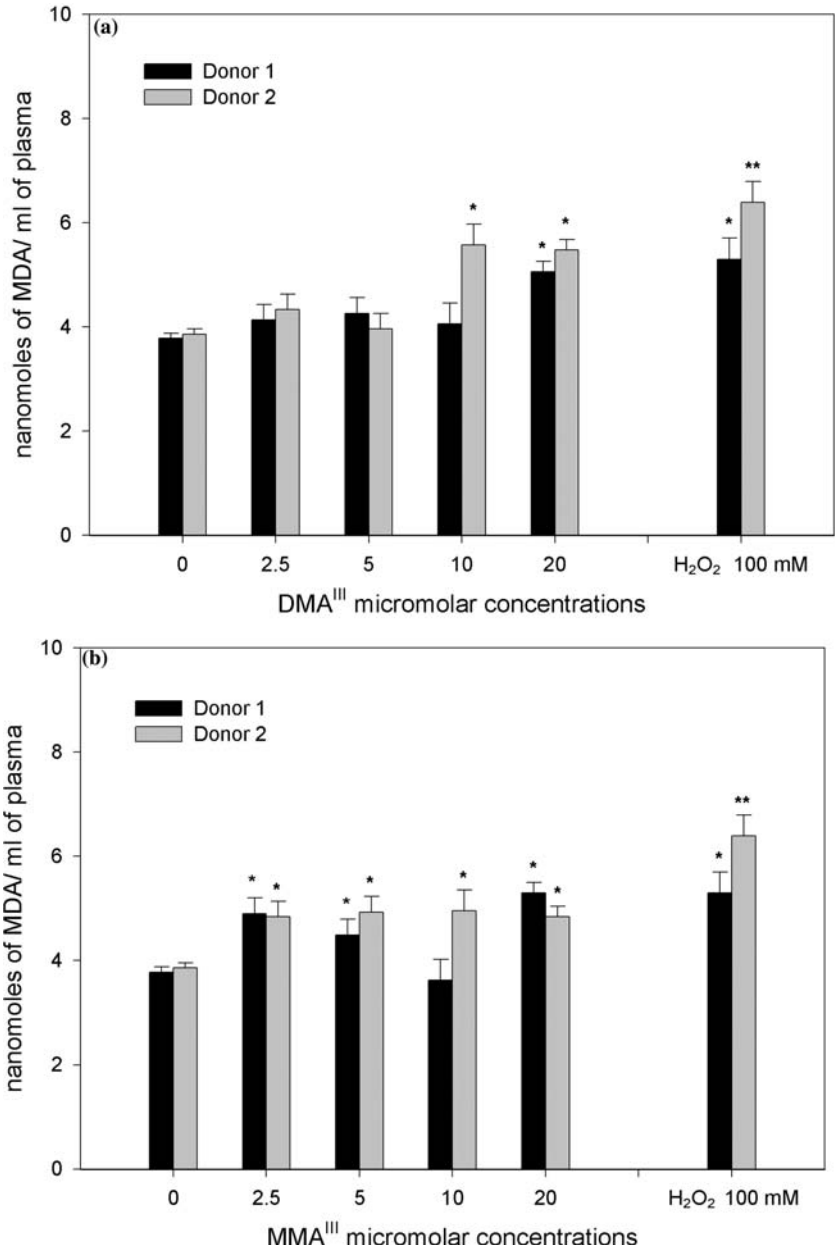

Figure 6. Levels of malondialdehyde (MDA) as lipid peroxidation marker in human plasma after whole blood treatment of DMA^{III} (a) and MMA^{III} (b) for 4 h at 37 °C. $^{*}P < 0.05$, $^{**}P < 0.005$ *versus* control as determined by student's *t*-test.

The higher induction of DNA damage at the more basic pH (> 13 *versus* 12.1) in addition to the ALS generated by MMA^{III} and DMA^{III} (Figures 4a and b) indicate that these metabolites induce DNA-damage through ROS generation as suggested by Schwerdtle *et al*. (2003). We also found an increase in lipid peroxidation induced by both of the tested arsenic metabolites. When we compared the indices of oxidative stress we found a relationship that reflects the generation of ROS by arsenic trivalent metabolites at non-cytotoxic concentration in human lymphocytes.

Two hydrosoluble antioxidant agents, vitamin C and Se-Met, were used to evaluate whether they could provide any protection against oxidative DNA toxicity caused by DMA^{III} and MMA^{III}.

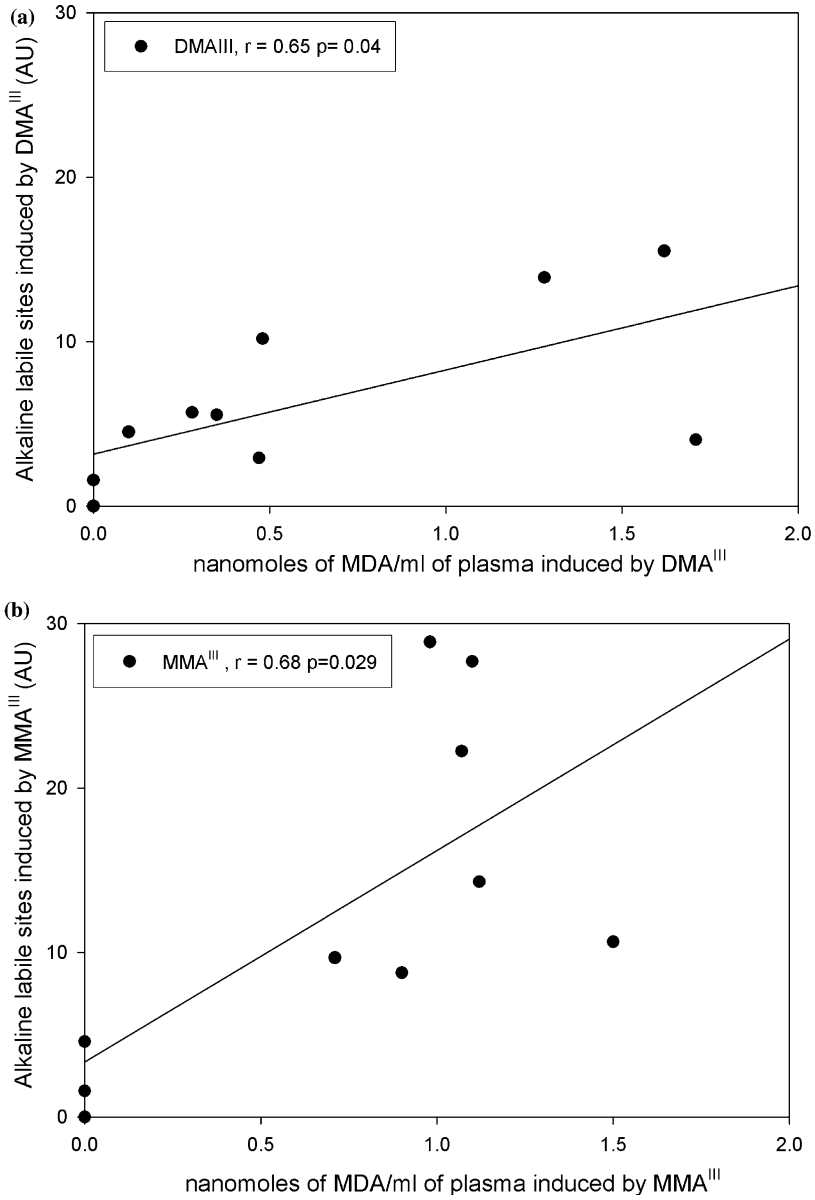

Figure 7. Correlation between the induction of ALS and lipidperoxidation marker induced by DMA^{III} (a) and MMA^{III} (b). The difference in DNA damage values between pH > 13 and pH 12.1 was taken as ALS value. Lipid peroxidation was calculated as the amount of nanomoles of MDA/ml of plasma. Each line represents the result of two independent experiments.

Se-Met co-treatment produced markedly better protection against single strand DNA breaks then did ascorbate co-treatment. This reduction could be explained by three different mechanisms. The protection may be achieved by the antioxidant molecules acting as chelators or substrate competitors of a detoxifying pathway. For example Se-Met may interact directly with arsenic trivalent metabolites or selenium may compete for glutathione (GSH) or S-adenosylmethionine during biomethylation of selenium (Biswas *et al.* 1999; Styblo & Thomas 2001). Consistent with the latter putative mechanism, levels of selenium have been reported to affect GSH concentration (Thompson & Clement 1991). And in a reduce GSH scenario, the glutathionylated arsenic complexes could be hydrolyzed and oxidized to MMA(V) and DMA(V), and these metabolites are

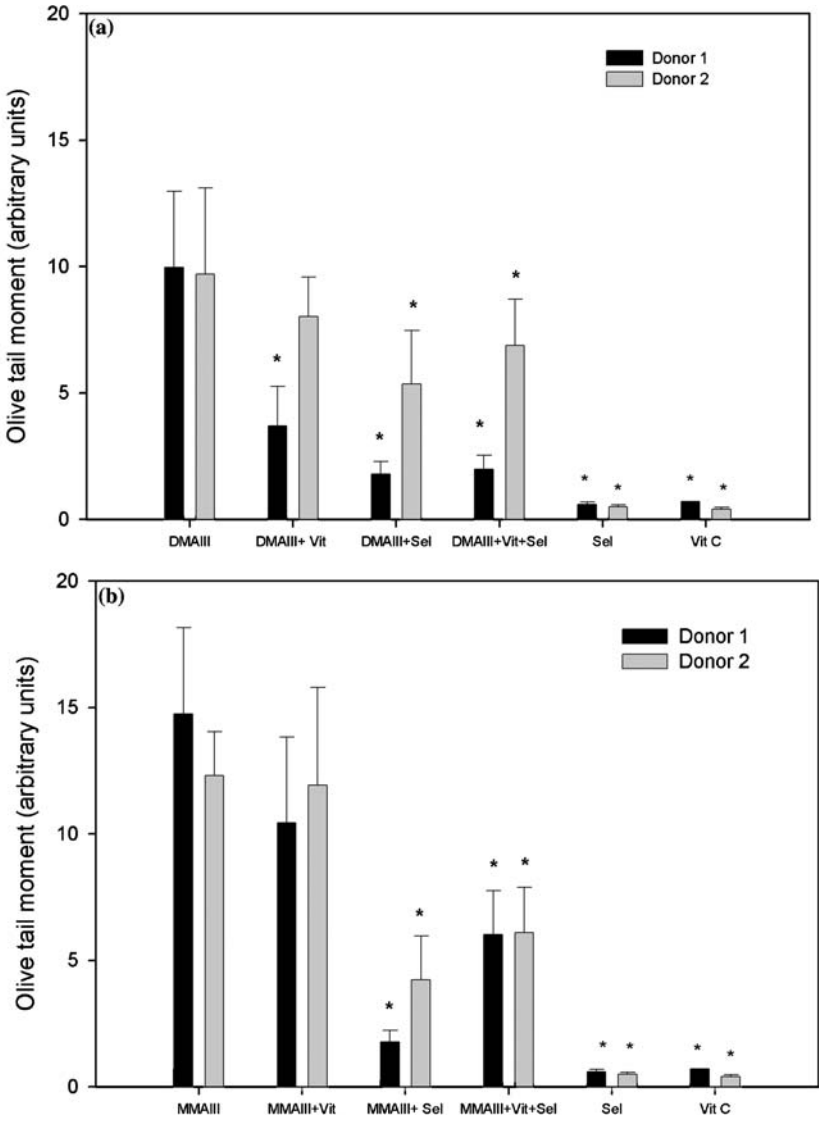

Figure 8. DNA damage reduction by exposure with 20 μM MMA^{III} and 20 μM DMA^{III} due to co-exposure with 2.8 μM selenomethionine and 40 μM Vitamin C. $^{*}P < 0.05$ *versus* without antioxidants.

less toxic than As trivalent species (Hayakawa *et al*. 2005)

Alternatively antioxidant protection may involve glutathione peroxidase (GPx) induction. Essential selenium is a critical catalytic component of GPx, which is a selenoenzyme with antioxidant function. Yeh *et al*. (2003) reported that selenium modulates arsenic effects on cytotoxicity, cell viability, and cell cycle in porcine endothelial cells and that this modulation is achieved through effects on GPx activity. In contrast, Styblo & Thomas (2001) reported that pre- or co-exposure to selenite (Se^{IV}) may enhance the toxic effects of iAs, increasing its retention in tissues and suppressing its methylation, a putative iAs detoxification process. The differences reported regarding the effects of selenium could be related to the metabolism and bioavailability of this metalloid in the organism. The metabolism and disposition of varies according to the form of selenium supplemented, Se-Met bioavailable than inorganic selenium forms (Mukhopadhyay *et al*. 1998).

A third possible mechanism for the observed antioxidant protection may involve selenium

activation of cellular signaling pathways. Seo *et al.* (2002a) showed that selenium can induce DNA repair and protect cells from oxidative damage. Furthermore, there is evidence that selenium activates p53 through a redox pathway that requires Ape1-Ref1 (Seo *et al.* 2002b). Studies in our laboratory are in progress to elucidate the mechanisms that may mediate of this effect.

In summary, the present data indicate that trivalent methylated arsenic metabolites at low concentrations can induce oxidative stress associated-genotoxicity in human lymphocytes, with MMA^{III} being more genotoxic than DMA^{III} at low concentrations and both metabolites could induce ALS, and that this effect can be modulated by the addition of Se-Met, and less efficiently by vitamin C. This difference in the antioxidant capacity suggests that, in addition to ROS, DMA^{III} and MMA^{III} could generate other kinds of reactive species, such as peroxynitrite (Burdenson *et al.* 2002) and that selenium protects the cell against this oxidant better than vitamin C (Rafferty *et al.* 2003; De Silva *et al.* 2004). Further studies are needed to elucidate which reactive species produce the DNA damage generated at non-cytotoxic methylated trivalent arsenic metabolites concentrations.

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