Genotoxic Effect of Arsenate and Arsenite in Human HaCat Cells in Culture Using Comet Assay

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Authors’ contributions

This work was carried out in collaboration among all authors. Author GB designed the study, performed the statistical analysis, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Authors EM and FMW managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Arsenic is an environmental chemical of toxicological concern today since it is a human genotoxin and chronic exposure is associated with development of cancers, including skin. Inorganic arsenate is metabolically reduced to arsenite by glutathione (GSH) prior to methylation. The aim of this study was to determine the relative toxic effects of arsenate and arsenite in HaCat cells (immortalized human keratinocytes) in vitro by measuring cytotoxicity, DNA damage, depletion of glutathione and apoptotic and necrotic events. HaCat cells were treated with arsenate and arsenite (10 μM) for DNA damage detection using Comet assay and cytotoxicity (10, 60 and 100 μM) all measured at 24 hr. In some experiment arsenate or arsenite (10 μM) was added at the same time as BSO 10 μM for 24 hr, and GSH levels were measured by HPLC with fluorescence detection. Flow cytometry was used to investigate apoptotic and necrotic events following arsenate and arsenite (10 μM) treatment for 24 hr. Arsenate and arsenite at 60 and 100 μM, but not 10 μM, reduced the number of adherent viable cells with time. Therefore, DNA damage could only be measured at 10 μM as at higher concentrations the cells did not produce classical Comets but showed fragmentation. DNA damage was significantly (p < 0.001) increased in cells treated for...
1. INTRODUCTION

Arsenic is widely distributed in the environment from natural geological sources, naturally occurring as arsenicals of copper, lead, silver, and gold, but high levels may also be found following combustion of coal especially low-grade brown coal, and in nonferrous metal industries. The Pentavalent inorganic compounds are arsenates such as sodium arsenate and trivalent arsenics such as sodium arsenite [1,2].

High levels of inorganic arsenic in the contaminated drinking water from natural sources can be found around the world, including Bangladesh, Taiwan, China, Chile, Mexico, India, as well as in several areas in United State and Europe can lead to a variety of toxic manifestations [3]. The majority of arsenic in surface water is as arsenate (As(V)) and in groundwater in deep anoxic wells as arsenite (As(III)). Skin lesions are one of the most common non-malignant effects related to chronic arsenic exposure via drinking water [4]. Trace elements from different regions of Tripoli city were analyzed for environmental control purposes, and compared them with the European and Canadian standards, this study showed that there are low concentrations of arsenic found in Tripoli city groundwater [5].

There are two main reactions in arsenic metabolism; reduction by glutathione (GSH) [6,7] and oxidative methylation reactions using S-adenosyl methionine (SAM) as a methyl donor [8,9]. The inorganic arsenic metabolites are less acutely toxic and more readily excreted into the urine than the arsenate and arsenite [10].

Arsenic compounds are used as pesticides and herbicides and as a wood preservative and this results in arsenic release into the environment. Sodium arsenate (Na$_3$AsO$_4$), and zinc arsenate (Zn$_2$AsO$_4$), are used as antifungal wood preservatives [11].

Arsenic can replace phosphate in many biochemical reactions [12], but trivalent arsenical forms are more toxic and react with thiol groups inhibiting important biochemical events which lead to toxicity [13].

Oxidative stress, chromosomal abnormality and altered growth factors are possible modes of action in arsenic carcinogenesis [14]. Arsenic induces DNA damage through mediation of reactive oxygen species (ROS) in human cells. Previous studies indicate that arsenic might inhibit the activities of catalase and glutathione peroxidase to produce accumulation of hydrogen peroxide [15], or stimulate the superoxide dismutase (SOD) to induce (O$_2^•$) and increase activity of heme oxygenase to release reactive ions [16]. Another study reported that arsenic could stimulate cell signaling and activate transcription factors to stimulate production of hydrogen peroxide and oxygen radical (O$_2^•$) [17]. Another study suggests that low concentrations of arsenite activates NADH oxidase to induce reactive oxygen species (ROS) in human smooth muscle cell tumor and trigger the oxidative DNA damage [18].

Large portion of arsenite-induced DNA strand breaks come from excision of oxidative DNA adducts and DNA-protein cross-links [19].

The glutathione system may play a major role in protecting cells against exposure to inorganic arsenic. Glutathione has high electron donating capacity (high negative redox potential) and this combined with high intracellular concentration generates great reducing power [20]. In conditions of increased oxidative stress, cellular GSH is oxidized to GSSG and the GSSG/GSH ratio may be a sensitive indicator of oxidative stress [21]. Depletion of hepatic GSH in rats and
hamsters by buthionine sulphoximine (BSO) was observed to decrease the methylation of inorganic arsenic [22].

Apoptosis, or programmed cell death (PCD), is a highly regulated pathway that is important in normal developmental processes as well as in disease. Cells undergoing apoptosis are identifiable by a number of characteristics, including transport of phosphatidylserine (PS) to the membrane surface, activation of caspase proteases and DNA fragmentation in the nucleus. A critical stage of apoptosis involves the initiation of cell surface changes by dying cells that eventually results in recognition and uptake of these cells by phagocytosis in vivo [23].

In human HaCat cells, arsenic was shown to induce apoptosis by receptor mediated through the Fas/Fas ligand pathway which is not depending on the mitochondrial status [24]. Thus, arsenic may able to trigger both mitochondria-mediated and receptor-mediated apoptosis. Both mechanisms of apoptosis require caspase-3 activation [25]. Previous studies showed that the p53 activation was not necessary [25], however, in both human glioblastoma and human gastric cell lines, the inhibition of p53 suppressed arsenic-induced caspase activation, suggesting an involvement of p53 in arsenic-induced apoptosis [27].

The aim of this study was to evaluate the relative toxic effects of arsenate and arsenite in HaCat cells in vitro by measuring cytotoxicity, DNA damage, depletion of glutathione and apoptotic and necrotic events.

2. MATERIALS AND METHODS

2.1 Chemicals

Comet lysis buffer (2.5 M sodium chloride, 100 mM EDTA [pH 8.0], 1% sarkosyl, 10 mM Tris-HCl [pH 8.0], 10% DMSO, 10% Triton X-100). Comet alkali solution (0.3 M sodium hydroxide, 1 mM EDTA). Comet neutralising buffer (0.5 M Tris-HCl, pH 7.5). Cryopreservation medium (50% fetal calf serum [FCS], 10% DMSO, 40% Dulbecco’s Modified Eagle’s Medium [DMEM]). Resuscitation medium (50% FCS, 10% dextrose, 40% DMEM). Culture medium for HepG2 cells (DMEM, 10% FCS, 50 U/ml penicillin, 50 U/ml streptomycin, 2 mM L-glutamine). Sodium arsenite solution 0.05 M, sodium arsenate ≥98.0% and buthionine sulfoximine. All chemicals were purchased from Sigma Chemicals Co., Dorset, UK.

2.2 Cell Culture

HaCat cells were obtained from the European Cell Culture Collection (ECCC, UK). HaCat cell are an epithelial keratinocyte cell line that was isolated from the periphery of a melanoma on the upper half of the back of a 62 year old male [28, 29].

2.3 Arsenic Treatment

Multi-well plates were seeded with HaCat cells (approximately 2.5x10^5) in DMEM with 10% fetal calf serum. The cell monolayers were washed and then arsenate or arsenite (10 µM, 60 µM and 100 µM) were added in 1 ml DMEM for 24 hr.

2.4 DNA Damage Detection Using the Comet Assay

Single cell gel electrophoresis assay (Comet assay), provides a simple and effective method for evaluating DNA damage in cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA fragments migrate slower and remains within the confines of the nucleotide when a current is applied. Evaluation of the DNA comet tail shape and migration pattern allows for assessment of DNA damage. The technique was performed as described by [30].

DNA damage was measured using the comet assay and expressed as Olive Tail Moment (OTM) which is defined as the tail length and the fraction of total DNA in the tail [31]. Results are given as mean±SEM for the 50 cells (25 per slide), and analyzed using the kinetic Komet version 5.5 software.

2.5 Cytotoxicity

To determine cell viability trypan blue exclusion assay performed, 100 µl of cell suspension was mixed 1:1 with trypan blue stain (0.4% w/v, Biowhittaker) and pipetted into a Neubauer haemocytometer (VWR Scientifics, West Chester, PA). The survival rate of the cells treated with arsenate and arsenite 10 µM was >90% after culturing and before starting the comet assay [32].
2.6 Detection and Measurement of Reduced Glutathione Using HPLC with Fluorescence Detector

The cells were seeded and grown to 80% confluence. Medium was removed and cells were treated with the 10 µM arsenate and arsenite with and without BSO (10 µM) in 1 ml medium. After the incubation, this solution was removed, 100 µL of 3mM monobromobimane in (50 mM N-ethylmorpholine) and then 10 µL 100% trichloroacetic acid (TCA) was added to stop the reaction, then harvested and contents of each well placed in a separate eppendorf tube. Glutathione standards were prepared fresh on the day and pipetted into eppendorf tubes. These were then incubated in the dark for 30 minutes. All samples were centrifuged and 0.5mL of the supernatant was removed into an amber HPLC vial which were sealed and analyzed immediately.

A mobile phase of 10% Acetonitrile and 0.25% Acetic acid was prepared and the HPLC was set up and allowed to equilibrate for 30 minutes. Before running samples, a standard curve was run to determine sensitivity and specificity of the machine. Standards used were in the range of GSH expected in the samples. Concentrations used were: 0, 5, 10, 15, 20, 40 nmol/ml on the column. Once this curve had been established samples could be prepared and injected onto the column, ready for analysis. The run time was 25 minutes and the GSH peak eluted at approximately 15 minutes. Peaks were analyzed by integrating the peak area. A Kontron system with a 420 pump, 425 gradient former, 360 autosampler and SFM25 fluorescence detector was used. The system was controlled by a Kontron Data System 450, software version 3.30 and the column used was a 150 mm×4.6 mm Hypersil 3ODS with a waters C18 guard column and a flow rate of 1.3ml/min. Glutathione levels were expressed per nmol/mg protein.

2.7 Detection and Measurement of Apoptosis and Necrosis Using Flow Cytometry

Flow cytometry (BD LSR II) is a technology that allows a single cell to be measured for a variety of characteristics, determined by looking at how they flow in liquid. The cells were treated with arsenate and arsenite (10 µM) for 24 hr. Cells were washed twice with cold phosphate buffer solution (PBS) and then approximately 1X 10^6 concentration of cells/ml immersed in binding buffer (0.1 mM Hepes/NaOH, pH 7.4, 1.4 mM NaCl, 25 mM CaCl_2). Then 100 µL of the diluted cell suspension (1 X 10^6 cells) was transferred to a culture tube that contained 5 µL of Annexin V-FITC and 5 µL of PI (Propidium iodide). Cells were fully vortexed and incubated for 15 min in the dark at room temperature. 400 µL of binding buffer was added and analyzed by flow cytometry (FACS) within 1 h. At least 10,000 events per sample were collected.

2.8 Statistical Evaluation

Data are expressed as mean±SEM. One-way ANOVA was performed when more than two groups were compared with a single control. Differences between individual groups were assessed by a Dunnett post hoc test, using Prism software (version 4).

3. RESULTS

Arsenate and arsenite at 60 µM and 100 µM, but not 10 µM, reduced the number of adherent viable cells with time. Therefore, DNA damage could only be measured at 10 µM as at higher concentrations the cells did not produce classical Comets but showed fragmentation. DNA damage was significantly (p<0.001) increased in viable HaCat cells treated for 24 hr with 10 µM arsenate or arsenite (p<0.05, p<0.001 respectively) compared to control (Fig. 1). GSH levels were significantly increased in HaCat cells treated with 10 µM arsenate or arsenite (p<0.05, p<0.001 respectively) compared to control. Cells treated with BSO at the same time as 10 µM arsenate had increased GSH levels (p<0.001) but 10 µM arsenite did not significantly increased cellular GSH all compared to control (Fig. 2). Arsenate and arsenite (10 µM) increased apoptosis (AnV +ve, PI -ve), and arsenite increased necrosis (AnV +ve, PI +ve), although none of the values reached statistical significance (Fig. 3., Table 1). Arsenate and arsenite (10 µM) showed non significant differences in apoptosis (AnV +ve, PI -ve), and as well as in necrosis (AnV +ve, PI +ve) (Fig. 3., Table 1).

4. DISCUSSION

The mechanisms of arsenic carcinogenicity and other chronic effects in humans are not completely understood. The major modes of action of arsenic that have been reported are DNA damage, oxidative stress, chromosome aberrations, apoptosis, DNA methylation, signal
transduction and cell proliferation [33]. Generation of ROS is one of the major pathways for arsenic mediated genotoxicity and then it should be expected to induce specific DNA lesions consistent with oxidative damages [34,35].

**Fig. 1.** DNA damage in HaCat cells treated with (10 µM) arsenate and arsenite for 24h using Comet assay. OTM were recorded for 50 cells per each arsenic compound

***P<0.001 compared to control

**Fig. 2.** Effects of arsenate and arsenite (10 µM) on reduced GSH levels in HaCat cells at 24h using HPLC-fluorescence detection. Results are expressed as nmolGSH/mg protein

*p<0.05 compared to the control. **p<0.001 compared to the control

**Fig. 3.** Apoptosis and necrosis in HaCat cells induced by arsenate and arsenite (10 µM) at 24 hr
showed that 10 μM concentration of Arsenic and arsenate.

**Table 1. The effect of arsenate and arsenite (10 μM, 24 hr) on DNA damage, glutathione levels and apoptotic and necrotic events in HaCat cells**

<table>
<thead>
<tr>
<th></th>
<th>DNA damage (OTM)</th>
<th>GSH (nmol/mg protein)</th>
<th>Apoptotic/necrotic events (%) total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No BSO</td>
<td>BSO</td>
<td>An V −ve &amp; PI −ve</td>
</tr>
<tr>
<td>Control</td>
<td>0.5±0.1</td>
<td>61.1±2.3</td>
<td>29.2±12.4</td>
</tr>
<tr>
<td>Arsenate</td>
<td>2.5±0.7***</td>
<td>96.0±4.7</td>
<td>85.6±17***</td>
</tr>
<tr>
<td>Arsenite</td>
<td>5.8±0.7***</td>
<td>160.3±5.9***</td>
<td>50.0±7.2</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM
***p < 0.05 One-Way ANOVA compared to control

The aim of this study was to detect the genotoxic effect of arsenic compounds suggested by measuring DNA damage, depletion of glutathione and apoptotic and necrotic events. There have been a number of studies of the toxicity of arsenic to cells in culture but these have not addressed several aspects of toxicity in parallel. Here the effects of arsenic on cell viability, DNA damage and apoptosis were investigated in parallel using HaCat cells. The results in this study, was observed that arsenate and arsenite cause an increase in the mean comet tail-length. Ghazalla et al., (2016a) concluded that both arsenate and arsenite induced higher DNA damage in HepG2 cells. However, DMA arsenate metabolite is less toxic than its parent compound arsenate. Arsenate and arsenite are known to produce oxidative stress involving reactive oxygen species (ROS) formation by increase secretion of glutathione for cells protection which in agreement with our results [36].

Increase in ROS was observed at 2.5 μM arsenite in HaCat cells following a dose-dependent response, leading to oxidative stress. The excessive ROS in cells affected the viability of the cells in an inversely dose dependent manner [37, 38]. This is in agreement with our recent study in the viability of the cells. Tom and Metka (2004) proposed that if generation of ROS is one of the major pathways for arsenic-mediated genotoxicity, then it should be expected to induce specific DNA lesions consistent with oxidative damage [39]. These findings are in consistent with our results which provide additional proof that reactive oxygen species (ROS) mediate the genotoxic response in mammalian cells on treatment with arsenite and arsenate.

In comparison, our study with some other studies showed that 10 μM concentration of Arsenic compound caused DNA damage of human keratinocytes cells in culture [40], pentavalent arsenicals (arsenate) are less genotoxic than trivalent arsenicals (arsenite) induced by the chromosomal aberration frequencies [41] as well as, arsenics induce different extent of DNA damage observed by using single-cell gel electrophoresis in human mononucleocytes, fibroblast, and HL60 cells [42], so that all these findings are in consistent with our findings.

Generally, antioxidants potentially can alter arsenic toxicity through reduction of ROS generation, enhancing antioxidant capacity. GSH also serves as the electron donor for the reduction of arsenate to arsenite [43]. However, in the measurement of GSH level arsenite showed highly significant increase in GSH content, but slightly significant increase was observed in cells treated with arsenate as well as the cytotoxicity of arsenite was higher than arsenate. Therefore, the genotoxicity induced by arsenate was less than the genotoxicity induced by arsenite. In comparison the cells treated with and without buthionine sulfoximine (BSO) in presence of arsenic compounds, there was decrease in cellular GSH level in cells treated with both arsenics at the same time with buthionine sulfoximine which is evidence that arsenic compounds produce DNA damage by production of reactive oxygen species.

Deneke reported that low concentrations of arsenite have been shown to induce a transient increase in cellular glutathione levels in bovine vascular endothelial cells. In our study the increase in GSH levels at low doses of arsenate and arsenite, and by arsenate even in the presence of BSO [44], support the study by Schuliga et al., who suggested upregulation of GSH in response to oxidative stress [45]. The upregulation is thought to be a secondary stress response directly regulated by the thiol reactivity...
of arsenic compounds [46,36]. These findings are consistent with our observations.

Our findings agree with others who suggested that arsenics can initiate cell differentiation, act as an apoptosis and necrosis, by affecting cell transformation or cause cell cycle arrest. In our present study, arsenate and arsenite increased apoptotic cells, and arsenate increased necrotic cells, although none of the values reached statistically significant. Dong, (2002) investigated apoptosis in JB6 Cl 41 cancer cells exposed to low (0.5-25 µM) and high (50-200 µM) concentrations of arsenite and arsenate [47]. At the lower concentrations cell differentiation took place, whereas apoptosis was evident at higher concentrations. Zhong-ying et al., (2003) studied indication of apoptosis in esophageal carcinoma cells (SHEE85) by arsenic trioxide at concentrations of 0.5 µM and 5.0 µM. At 0.5µM arsenic trioxide induced a small degree of apoptosis while 5µM induced morphological changes typical of apoptosis [48]. Yen-chou et al., reported that although arsenite induced apoptosis in mouse embryonic fibroblast cells (NIH 3T3) at 10, 20, 40, 80 µM in a concentration and time-dependent manner, arsenate did not induce apoptosis, even at 80µM. Arsenite was believed to induce apoptosis by generating hydrogen peroxide through activation of superoxide-producing enzymes (such as NADPH oxidase). Hydrogen peroxide acts as mediator to induce apoptosis through release of cytochrome c from mitochondria into the cytosol [49].

5. CONCLUSION

Arsenite was more cytotoxic than arsenate and also produced more DNA damage. Arsenate and arsenite are known to produce oxidative stress involving ROS formation and depletion of glutathione. Although arsenite appeared to have less effect on apoptosis and necrosis than arsenate, it is possible that the grossly damaged cells had fragmented (arsenite decreased the total number of cells), thus negating them from the analysis.

CONSENT AND ETHICAL APPROVAL

As per international standard or university standard guideline participant consent and ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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