Original Research Article

Genotoxic effect of arsenate and arsenite in human HaCat cells in culture using Comet assay

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 24 hr with 10 μM arsenate and arsenite compared to control. GSH levels were significantly increased in HaCat cells treated with10 μM arsenate and arsenite ($p < 0.05$, $p < 0.001$, respectively) compared to control. Cells treated with buthionine sulphoximine (BSO) at the same time as arsenate had increased GSH levels ($p < 0.001$), but arsenite and BSO did not increase cellular GSH. Arsenate and arsenite increased apoptosis, and arsenate increased necrosis, although none of the values reached statistical significance. Arsenite was more cytotoxic than arsenate. Arsenate and arsenite are known to produce oxidative stress involving ROS formation and depletion of glutathione. The increase in GSH levels at low doses of arsenate and arsenite, and by arsenate even in the presence of BSO.

K**ey words**
Arsenic, HaCat cells, DNA damage, Comet assay, glutathione**.**

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^{+5}) and in groundwater in deep anoxic wells as arsenite (As^{+3}). 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_3AsO_4), and zinc arsenate (Zn_2AsO_4), are used as antifungal wood preservatives (11).

Arsenate 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 (O2•) 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 (O2•) (17). Another study suggest 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 (26), 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

Materials and Methods

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 0.05M, sodium arsenate ≥98.0% and buthionine sulfoximine. All chemicals were purchased from Sigma Chemicals Co., Dorset, UK.

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 62year old male (28, 29)

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.

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 migrates 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.

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).

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 then10 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% Acetonitrite 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 auto-sampler 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 mmx4.6 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.

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 10^5 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.

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).

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 (figure 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 (figure 2). Arsenate and arsenite (10 µM) increased apoptosis (AnV +ve, PI -ve), and arsenate increased necrosis (AnV +ve, PI +ve), although none of the values reached statistical significance (figure 3, Table 1).

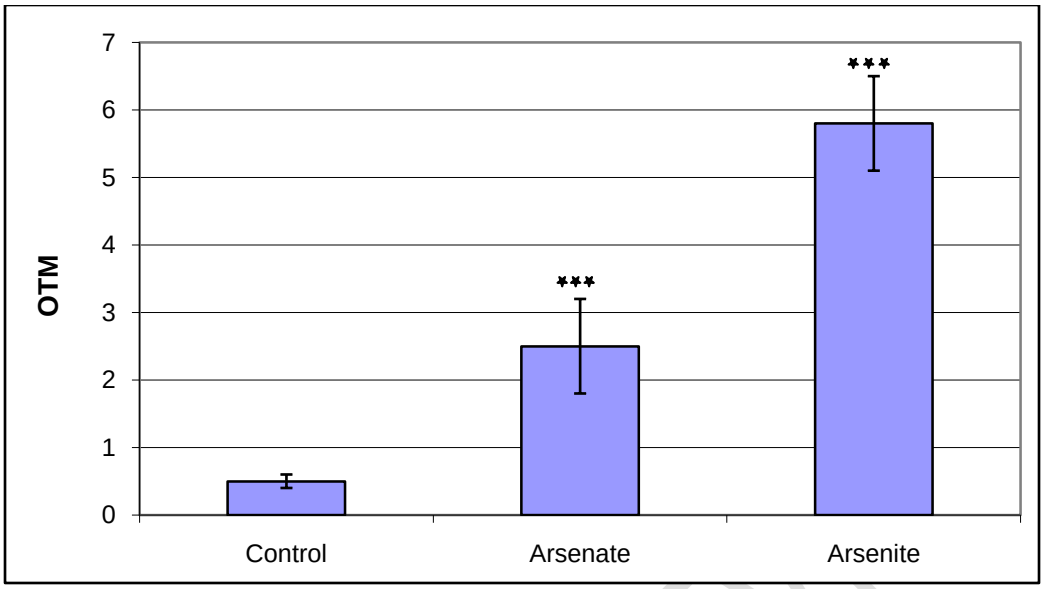

Figure 1: DNA damage in HaCat cells treated with (10 µM) arsenate and arsenite for 24h using Comet assay. OTM were recorded for 50 cells per each arsenic compound.

***P<0.001 compared to control.

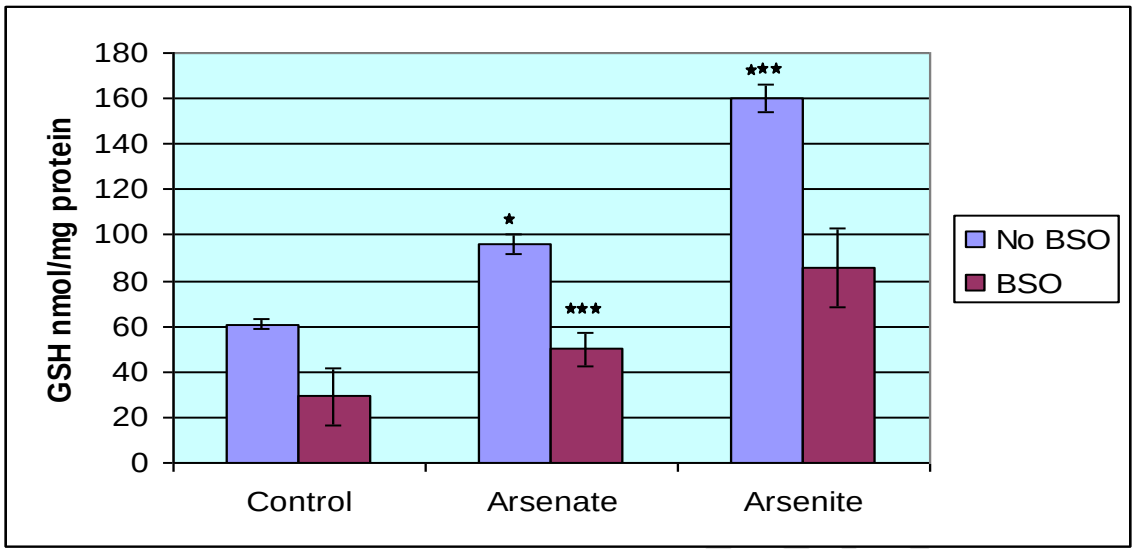

Figure 2: Effects of arsenate and arsenite (10µM) on reduced GSH levels in HaCat cells at 24h using HPLC-fluorescence detection. Results are expressed as nmolGSH/mg protein.

*$p<0.05$ compared to the control.

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

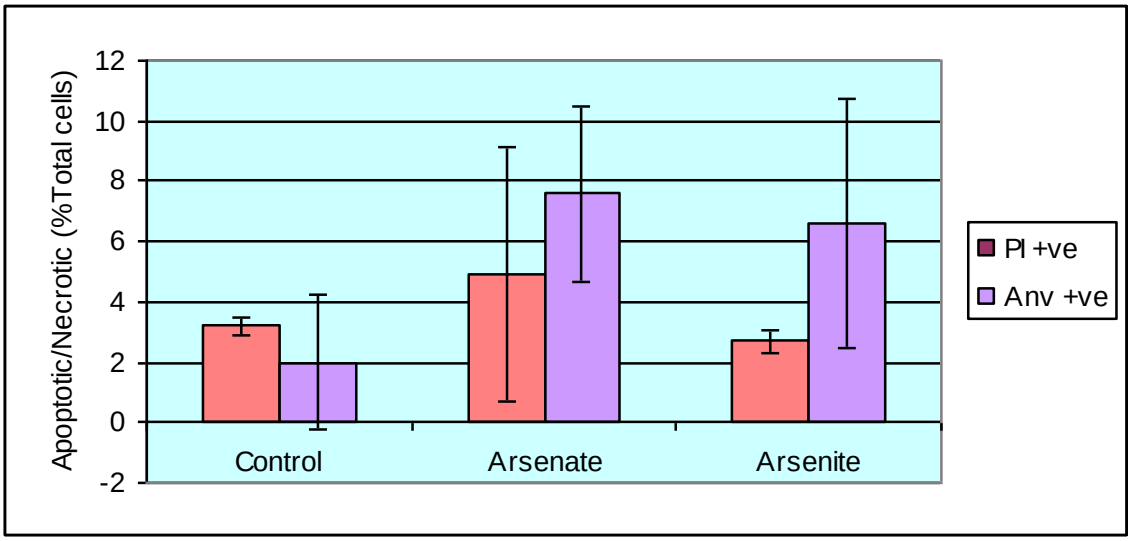

Figure 3: Apoptosis and necrosis in HaCat cells induced by arsenate and arsenite (10 µM) at 24 hr**.**

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.

	DNA damage (OTM)	GSH (nmol/mg protein)		Apoptotic/necrotic events (% total cells)		
		No BSO	BSO	An V −ve & PI −ve	An V +ve & PI −ve	An V +ve & PI +ve
Control	0.5 ± 0.1	61.1 ± 2.3	29.2 ± 12.4	94.6 ± 3.1	2.0 ± 2.8	3.2 ± 0.3
Arsenate	2.5 ± 0.7***	96.0 ± 4.7*	85.6 ± 17***	87.5 ± 6.7	7.6 ± 2.9	4.9 ± 4.2
Arsenite	5.8 ± 0.7***	160.3 ± 5.9***	50.0 ± 7.2	90.6 ± 4.2	6.6 ± 4.1	2.7 ± 0.4

Values are the mean ± SEM.
* $p < 0.05$ One-Way ANOVA compared to control.
*** $p < 0.001$ One-Way ANOVA compared to control.

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).

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 (1992) 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, (2002) 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 CI 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., (1998) 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).

In 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.

References

1. Smith Allan H., Smith Meera H.,"*Arsenic drinking water regulations in developing countries with extensive exposure.*" Toxicology, 2004. (1-3): 39-44.
2. Hughes, M. F. "*Arsenic toxicity and potential mechanism of action*." Toxicology Letters, 2002. **133**: 1-16.
3. Pradosh R., A. S. "*Metabolism and toxicity of arsenic: A human carcinogen*." Current Science, 2002. **82**(1): 83-45.
4. Takahiko Y., Hiroshi Y., Gui F. S. "*Chronic health effects in people exposed to arsenic via the drinking water: dose–response relationships in review*." Toxcology and Applied Pharmacology, 2004. **198**: 243-251.
5. El-Ghawi, U.M., *The level of trace elements in Tripoli city groundwater.* Instrumentation Science and Technology 2005. **33**: p. 609-617.
6. Balazs N., Zolatan. G. "*Reduction of arsenate to arsenite in hepatic cytosol*." Toxicological Sciences, 2002. **70**: 4-12.
7. Thomas J. D., Lin. J., Waters S. B., Xing W., Adair B. M., Drobne Z., Devesa V., Styblo M. "*Arsenic (+3 oxidation state) methyltransferase and methylation of arsenicals.*" Experimental Biology and Medicine, 2007. **232**(3): 3-13.
8. Aposhian V., Zakkharyan. R., Avram Mihaela D., Kopplin Micheal J. Wollenberg Michael L. "*Oxidation and detoxification of Trivalent arsenic species*." Toxicology and Applied Pharmacology, 2003. **193**: 1-8.
9. Rradabaugh T., Aposhian H. "*Enzymatic reduction of arsenic compounds in mammalian systems: reduction of arsenate to arsenite by human liver arsenate reductase*." Chemical research in Toxicology, 2002. **13**: p26-30.
10. Vahter M., Concha G. "*Role of Metabolism in Arsenic Toxicity*." Pharmacology and Toxicology, 2001. **89**: p1-5.
11. Lever, J. H. "*Paget's disease of bone in Lancashire and arsenic pesticide in cotton mill waste water: a speculative hypothesis*." Bone, 2002. **31**(3): 434-436.
12. Huang R., L.C., "*Cellular uptake of trivalent arsenite and pentavalent arsenate in KB cells cultured in phosphate-free medium*". Toxicology and Applied pharmacology, 1996. **136**: p. 243-249.
13. Scott N., H.K., Mackenzie N., and Carter D. "*Reactions of arsenic (III) and arsenic(V) species with glutathione*". Chemical Research in Toxicology, 1993. **6**: p. 102-106.
14. Hsin-Su Yu, W.-T.L., Chee-Yin Chai "*Arsenic carcinogenesis in the skin*". Journal of Biochemical Science, 2006. **13**(5): p. 657-666.
15. Ghazalla M. Benhusein, E.M., Suher Aburawi and Faith M. Williams "*Genotoxic effect induced by hydrogen peroxide in human hepatoma cells using comet assay*". Libyan Journal of Medicine, 2010. **5**(1): p. 1-5.

16. Lee T.C., and .H.I.C. "*Modulation of cellular antioxidant defense activities by sodium arsenite in human fibroblasts*". Archives of Toxicology 1995. **69**(7): p. 498-504.
17. Barchowsky A., K.L.R., Dudek E.J., Swartz H.M., James P.E, "*Stimulation of reactive oxygen, but not reactive nitrogen species, in vascular endothelial cells exposed to low levels of arsenite - possible role in protection against cytotoxicity*"*?* Free Radical Biology and Medicine, 1999. **27**(11): p. 1405-1412.
18. Wang D. H., W.H.L., Zhao H. S., Hao C Y., Min Z. H, Liu J. M, "*Arsenic trioxide overcomes apoptosis inhibition in K562/ADM cells by regulating vital components in apoptotic pathway*". Pharmocological Research 2005. **52**: p. 376-385.
19. Wang D. H., W.H.L., Zhao H. S., Hao C Y., Min Z. H, Liu J. M, "*Arsenic trioxide overcomes apoptosis inhibition in K562/ADM cells by regulating vital components in apoptotic pathway*". Pharmocological Research 2005. **52**: p. 376-385.
20. Meister, A., *"Glutathione-ascorbic acid antioxidant system in animals."*. Journal of Biololgy and Chemistry., 1993. **269**: p. 9397-9400.
21. Huang H, C.F.H., D. R. Wu, C. M. Jinn and K. Y. Jan "*Glutathione as a cellular defence against arsenite toxicity in cultured Chinese hamster ovary cells."*. Toxicology, 1993. **79**: p. 195-204.
22. Vahter, M., *Mechanism of arsenic biotransformation."*. Toxicology and_Applied Pharmacology, 2002. **181-182**: p. 212-217.
23. Seamus J. Martin, Chris P. M. Reutelingsperger, Anne J. McGahon, James A. Rader, Rob C.A.A van Schie, Drake M. Laface, Douglas R. Green "*Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by over expression of Bcl-2 and Abl*." Journal of Experimental Medicine, 1995. **182**: 1545-1556.
24. Wei-Ting Liao, K.-L. C., Chai-LiYu, Gow-Shing Chen, Louis W. Chang, Hsin-SuYu "*Arsenic Induces Human Keratinocyte Apoptosis by the FAS/FAS Ligand Pathway, Which Correlates with Alterations in Nuclear Factor-κB and Activator Protein-1 Activity*." Journal of Investigative Dermatology, 2004. **122**(1): 125-129.
25. Elena V. Komissarova, S.K.S., Toby G. Rossman *"Dead or dying: the importance of time in cytotoxicity assays using arsenite as an example."* Toxicology and Applied Pharmacology, 2005, **202**(1): 99-107.
26. Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H., Xu, T., "*PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development*". Development, 1999. **126**(23): p. 5365-5372.
27. Zhao S., T.T., Kawakami K., Shi C. Kawamoto K "*Effect of As_2O_3 on cell cycle progression and cyclins D1 and B1 expression in two glioblastoma cell lines differing in p53 status."* International Journal of Oncology 2002. **21**: p. 49-55.
28. P Boukamp, R.T.P., D Breitkreutz, J Hornung, A Markham, N E Fusenig, "*Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line*". Journal of Cell Biology, 1988. **106**: p. 761-771.
29. Ghazalla Benhusein, E.M., Jamal Elmezogi Yousef Taher, Adel Abushofa, Faith Williams, *A Comparative Study of Susceptibility of HepG2 and HaCat cells to DNA Damage.* WJRR, 2016b. **3**(2): p. 59-62.

30. Singh, N.P., McCoy, M. T., Tice, R. R., Schneider, E. L *"A simple technique for quantification of low levels of DNA damage in individual cells."* Experimental Cell Research, 1988. **175**: 184-19.
31. Ghazalla M. Benhusein, E.M., Faith Williams, "*Detection of DNA damage and reliability of Comet assay parameters using HaCat cells*". Tripolitana Medical Journal, 2012. **1**(1): p. 31-35.
32. Barbara Graham-Evans, Hary H.P.Cohly, Hongtao Yu and Paul B Tchounwou "*Arsenic-Induced Genotoxic and cytotoxic Effects in Human Keratinocytes, Melanocytes and Dendritic Cells*". Environmental Research and Public Health 2004. **1**(2): p. 83-89.
33. Rossman, T.G., "*Mechanism of arsenic carcinogenesis: an integrated approach*". Mutation Research 2003. **533** p. 37-65.
34. Pilger A., Rudeger H.W., "*8-hydroxy-2-deoxyguanosine as a marker of oxidative DNA damage related to occupational and environmental exposures*". International Archives of Occupational and Environmental Health 2006. **80**: p. 1-5.
35. Kitchin KT, W.K., "*Evidence against the nuclear in situ binding of arsenicals-oxidative stress theory of arsenic carcinogenesis*". Toxicol Appl Pharmacol 2008. **232**: p. 252-257.
36. Ghazalla Benhusein, E.M., Jamal Almezogi, Faith Williams, "*In vitro Genotoxic Effect of Arsenical Compounds in HepG2 Liver Cells*". British Journal of Pharmaceutical Research, 2016a. **5**(13): p. 1-10.
37. Sun X., L. Li B., Li X., Wang Y., Xu Y., Jin Y., Piao F., Sun G. "*Effects of sodium arsenite on catalase activity, gene and protein expression in HaCat cells.*" Toxicology in Vitro**,** 2006. **20**: p1139-1144.
38. Starcevic S. L., Diotte N. M., Zukowski K.L., Cameron M.J., Novak R. F. N. M., Zukowski K.L., Cameron M.J., Novak R. F. *"Oxidative DNA Damage and Repair in a Cell Lineage Model of Human Proliferative Breast Disease (PBD)."* Toxicological Sciences, 2003. **75**: p.74-81.
39. Wei Ding, L.G.H., Ke Jian Liu, "*Inorganic arsenic compounds cause oxidative damage to DNA and protein by inducing ROS and RNS generation in human keratinocytes*". Mol Cell Biochem, 2005. **279**(1-2): p. 105-12.
40. Schwerdtle T, W.I., Mackiw I, Hartwig A, "*Induction of oxidative DNA damage by arsenite and its trivalent and pentavalent methylated metabolites in cultured human cells and isolated DNA*". Carcinogenesis 2003. **24** (5): p. 967-974.
41. Li D, M.K., Takeshita T, Lu Y, "*Arsenic induces DNA damage via reactive oxygen species in human cells*". Environ Health Prev Med, 2001. **6**: p. 27-32.
42. Scott N., H.K., Mackenzie N., and Carter D *Reactions of arsenic (III) and arsenic(V) species with glutathione.* Chemical Research in Toxicology, 1993. **6**: p. 102-106.
43. Deneke S. M. "*Induction of cysteine transport in bovine pulmonary artery endothelial cells by sodium arsenite*." Biochimica et Biophysica Acta**,** 1992. **1109**: p127– 131.

44. Schuliga M., Chouchane S., Snow E. "*Upregulation of glutathione-related genes and enzyme activities in cultured human cells by sublethal concentrations of inorganic arsenic*." Toxicological Sciences, 2002. **70**: p183-192.
45. Engel, R. R., Hopenhayn-Rich, C., Receveur, O., Smith, A. H."*Vascular effects of chronic arsenic exposure."* Epidemiologic Reviews, 1994. **16**: p184–208.
46. Dong-Hyun Lee, Timothy R. O and Gred P. "Oxidative DNA damage induced by copper and hydrogen peroxide promotes CG-TT tandem mutations at methylated CpG dinucleotides in nucleotide excision repair-deficient cells." Nucleic Acids Research, 2002. **30**: 3566-3573.
47. Zhong-Ying Shen, Wen-Ying Sheng, Ming-Hua Chen, Jian Shen and Yi Zeng. "*Reactive oxygen species and antioxidants in apoptosis of esophageal cancer cells induced by As_2O_3*." International Journal of Molecular Medicine, 2003. 11: p479-484.
48. Yen-Chou Chien, Shoei-Yn lin -Shiau, and Jen-Kun Lin. *"Involvement of reactive oxygen species and caspase3 activation in arsenite-induced apoptosis*." Journal of Cellular Physiology, 1998. **177**: p324-333.