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In vitro Genotoxic Effect of Arsenical Compounds in HepG2 Liver Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FW and EM designed the study, wrote the protocol. Author GB wrote the first draft of the manuscript, managed the literature searches, analyses of the study and managed the experimental process. Authors GB and JA read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to determine the genotoxic effect in HepG2 cells of the four arsenic compounds by measuring DNA damage, depletion of cellular glutathione (GSH) as a measure of oxidative stress as well as antioxidant agent and apoptotic and necrotic events.
Study Design: Arsenic is an environmental chemical of toxicological concern today since it has been shown to be a human genotoxin and carcinogen. This project investigated four forms of inorganic arsenic: arsenate (As^{+5}), sodium arsenite (As^{+3}), arsenic trioxide (As_2O_3) and dimethyl arsenic acid (DMA^{+5}); a major metabolite of arsenate.
Methodology: HepG2 cells ($1.5x10^5$/ml) treated with the four arsenics (10 µM) for 24hr. Harvested

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cells were analysed for GSH concentration by reverse phase HPLC with fluorescence detection. Cells were investigated for DNA damage using the Comet assay. Cells were analysed by flow cytometry to detect apoptosis and necrosis, all running in parallel.

Results: The DNA damage for cells dosed with DMA or As_2O_3 was not significantly different from control. However, significant DNA damage was seen for cells treated with arsenite and arsenate ($p<0.001$, $p<0.05$ respectively). Reduced glutathione was increased by arsenic compounds compared to control. However, this was only statistically significant for arsenite ($p<0.001$) and arsenate ($p<0.05$). Whereas, results not indicated apoptosis and necrosis in viable adherent cells although this was not statistically different.

Conclusions: All four arsenicals appeared to increase GSH content and DNA damage compared to control especially both arsenite and arsenate significantly are different. Further experiments are required to assess the mechanism(s) of genotoxicity of the arsenicals in GSH depletion.

Keywords: Arsenic compounds; HepG2 cells; reduced glutathione (GSH); DNA damage; comet assay.

1. INTRODUCTION

Arsenic presents ubiquitously in the environment and the accumulation of this element in ground water and plants poses a health risk to both humans and animals [1]. This metalloid exists in different oxidative states, the most known inorganic arsenic forms are arsenate and arsenite. In addition, arsenate and arsenite are present in water and seafood [2]. Exposure to inorganic arsenic cause black-foot disease (BFD), a unique peripheral vascular disease. The disease frequently ends up with dry gangrene and spontaneous amputation of affected extremities, with an underlying pathological change of severe systemic atherosclerosis [3].

Metabolism of inorganic arsenic involves 2 steps of chemical reactions: reduction and oxidative methylation [4]. Arsenate is reduced to arsenite before it can be further metabolized. Arsenite is then oxidatively methylated to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) by S-adenosyl methionine [5].

There is uncertainty in the literature regarding the mechanisms underlying the toxicity of arsenics, they may include oxidative stress with depletion of intracellular glutathione (GSH) and apoptotic events. However, Reactive oxygen species produced (ROS) particularly hydroxyl radicals, play an important causal role in the genotoxicity of arsenic compounds [6]. The oxidative damage resulting from free radical attack on DNA has been previously linked to mutagenesis, carcinogenesis and aging. Mitochondrial DNA is known to be particularly susceptible to oxidative damage due to its close proximity to the reactive oxygen species producing systems and lack of protective histone proteins. Levels of oxidative damage have been found to be three times higher in mitochondrial DNA than in nuclear DNA [7].

Cells contain several antioxidant systems to limit the damage caused by increased ROS, and GSH is an antioxidant that protects cells from oxidative stress-induced apoptosis. One mechanism of arsenic toxicity appears to be a result of the ability of arsenite to bind to protein thiols causing the thiols to be unavailable for antioxidant activity. As the protective effect of GSH is consistent with the removal of free radicals following exposure to oxidative damaging agents such as arsenic compounds, it can be predicted that at increasing arsenic concentrations resulting in decreased GSH levels, the cells will become prone to increasing DNA damage and apoptosis [8]. Oxidative stress produced by toxic compounds depletes cellular GSH, and when GSH falls below 10% of the normal level, cellular defence against oxidative stress producing compounds is considerably impaired and cell death can result [9].

Arsenic compounds inactivate numerous types of enzymes, mostly involved in DNA replication, repair and cellular energy pathways. These enzymes have greater affinity to substituted phosphate in high energy compounds as ATP results in the production of useless energy [10-12]. So, the genotoxicity of arsenic does not communicate directly to DNA, however, indirectly it affects DNA by production of reactive oxygen species (ROS) or deregulation of DNA repair enzymes [13,14].

Several studies have been carried out to determine the genotoxic potential of arsenic compounds [15]. *In vitro* and *in vivo* studies have

been shown that inorganic arsenic increases the frequency of micronuclei, chromosome aberrations and sister chromatid exchanges in both animals and humans [16].

Comet assay has been considered a rapid, simple and sensitive technique for measuring DNA damage. Comet assay has been shown to be a very sensitive method for detecting the genetic damage induced by different genotoxic agents [17].

Comet assay can be assessed genotoxicity of various industrial chemicals, pharmaceuticals and pollutants [18], as well as this assay can detect DNA damage in different types of cells such as eosinophils and ovarian cells [19-22]. It has also been used to detect DNA damage in blood of workers occupationally exposed to inorganic arsenic [23].

Apoptosis is a normal physiological phenomenon put forward by Kerr et al. [24]. It plays an important role in embryonic development, maintenance of tissue homeostasis and pathology, and therefore has become a hotspot of activity in biomedical fields. Flow cytometry is a method for quantitating components or structural features of cells primarily by optical means. Although it makes measurements on one cell at a time, it can process thousands of cells in a few seconds. Since different cell types can be distinguished by quantitating structural features, flow cytometry can be used to count cells of different types in a mixture.

Most of the previous studies on the genotoxicity of arsenic compounds have been carried out with sodium arsenite and with the two most frequent methylated forms, monomethylarsonic acid (MMA) and dimethylarsenic acid (DMA).

In our study we consider that the selected compounds of inorganic arsenic forms to view the *in vitro* DNA damage using comet assay and depletion of cellular glutathione (GSH) as removal of free radicals following exposure to oxidative damaging agents using HPLC as well as to investigate the effect of arsenic compounds on apoptotic and necrotic events using flow cytometry.

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% foetal 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%, arsenic trioxide 99.995% trace metals basis, and dimethylarsinic acid (DMA) ≥98.0%. All chemicals were purchased from Sigma Chemicals Co., Dorset, UK.

2.2 Cell Culture

HepG2 cells were obtained from the European Cell Culture Collection (ECCC, UK). HepG2 cells are human Caucasian hepatocyte carcinoma cells taken from a primary hepatoblastoma (liver biopsy) from an 11 year old male from Argentina in 1979 [25,26]. The HepG2 cells were maintained in suspension culture in Dulbecco's Modified Eagl's Medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM pyruvate, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 2.5 U/ml amphotericin B. The cultures were incubated at 37℃ in a 5% CO_2 incubator at 95% humidity.

2.3 Arsenic Treatment

Multi-well plates were seeded with HepG2 cells approximately ($1.5x10^5$) in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum. The cell monolayers were washed and then arsenate, arsenite, arsenic trioxide and dimethylarsenic acid (10 µM) were added in 1 ml Dulbecco's Modified Eagle's Medium for 24 hr.

2.4 Comet Assay

The size of DNA fragments and the number of fragments are the main principles to determine comet formation. The negatively charged broken DNA molecule freely migrates in an electric field towards the anode. Tice et al. explained comet assay, for the measurement of DNA damage in *in vitro* culture of human cells [27].

Comet assay was carried out under alkaline conditions, basically as described by Singh et al. [28]. Partly frosted microscope slides were pre-

coated with a smear of 1% low melting-point agarose (LMPA) and allowed to dry for 1 hr at 37℃. The slides were then placed on a bed of ice to pre-cool them before adding an agarose sandwich. An aliquot of 170 µl of 1% LMPA was placed onto the slide and immediately covered with a coverslip. Whilst this was setting, 170 µl of the suspended lymphocytes were mixed 1:1 with 2% LMPA. The coverslip was then removed from the initial agarose layer. An aliquot of 170 µl of the agarose/lymphocyte mix was placed on top of the first layer and again covered immediately with a coverslip. Once set, the coverslip was removed and a final layer of 170 µl 1% LMPA was applied to the existing gels to form an agarose sandwich, with the cells in the middle layer. The final coverslip was removed once the gel had set. For each lymphocyte sample, two slides were prepared. At this point the lights were switched off and the remainder of the assay was performed using indirect light. A pyrex tray containing 500 ml of ice-cold lysis buffer was placed onto a bed of ice and the slides were gently lowered into it. After one hr the slides were removed and washed by placing them in 500 ml ice-cold PBS (again in a pyrex tray on a bed of ice) and allowing them to sit for 15 min. This removed the salt from the lysis buffer and prepared the slides for the next step. A horizontal electrophoresis tank was surrounded by ice and filled with 2 L of ice-cold lysis buffer. Then the slides were gently lowered into a horizontal electrophoresis tank filled with 2 L of cold lysis buffer and surrounded by ice. The slides were sat for 30 min to allow the DNA to unwind in the alkali buffer. Electrophoresis was then performed at 22 V (500 mA) for 30 min. Next, the slides were removed and rinsed by sitting them in 500 ml of ice-cold neutralising buffer for 10 min and then in 500 ml of ice-cold PBS for 15 min. SYBR Gold dye was added to each slide to stain the DNA. The slide was visualised using either a BioRad MRC 600 confocal microscope or a Leica TCS SP2 UV confocal microscope Twenty-five images per slide were captured. Images were analysed using the kinetic Komet version 5.5 software.

2.5 Cell Viability

To determine cell viability, 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). For the experiments cell viability was 85-90%.

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, arsenite, arsenic trioxide and dimethylarsenic acid in 1 ml medium. After the incubation, this solution was removed, 100 µL of 3 mM monobromobimane in (50 mM N-ethylmorpholine) and then 10 µL 100% trichloroacetic acid (TCA) was added to stop the reaction. Glutathione standards were prepared fresh on the day. 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 analysed by integrating the peak area. A Kontron system with a 420 pump, 425 gradient former, 360 auto sampler and SFM25 fluorescence detector was used [29]. The system was controlled by a Kontron Data System 450, software version 3.30 and the column used was a 150 mm x 4.6 mm Hypersil 3ODS with a waters C18 guard column and a flow rate of 1.3 ml/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 [30]. The cells were seeded to and grow to confluence of ($1X10^6$ cells per well). Medium was removed and cells were dosed with the treated material in 1 ml medium. After the incubation time, this solution was removed, and contents of each well placed in a separate culture tubes. 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 mMNaCL, 25 mM $CaCl_2$). Then100 µl of the diluted cell suspension

(1 X 10^5 cells) was transferred to a culture tube that contained 5 µl 7-ADD (7-amino-actinomycin D) of 200 µg/ml and 5 µl of 100 µg/ml Phycoerythrin (PE) both DNA staining. Cells were fully vortexed and incubated for 15 min in the dark at room temperature. 400 µl of binding buffer was added and analysed by flow cytometry (FACS) within 1hr. At least 10,000 event per sample were collected. Staining with PE is typically used in conjunction with 7-Amino-Actinomycin (7-AAD) to identify early apoptotic cells (7-AAD -ve, PE -ve), whereas cells that are considered viable (undamaged) are (7-AAD -ve, PE -ve); cells that are in early apoptosis are (7-AAD -ve, PE -ve); and cells that are in late apoptosis or already dead (necrotic) are, (7-AAD +ve, PE +ve).

2.8 Statistical Evaluation

Data are expressed as Mean values ± Standard error of means *(*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 6).

3. RESULTS AND DISCUSSION

Comparison of the DNA damage in control (0.5±0.1) with cells dosed with arsenate, arsenite, arsenic tiroxide and DMA showed 85% cell viability, a significantly higher damage for arsenite (1.7±0.2, $p<0.05$), and (1.5±0.2, $p<0.01$) arsenate but not arsenic trioxide (1.0±0.1*)* and DMA (1.2±0.2), Fig. 1. Cells viability was 86%.

The viability of cells was 87%. As shown in Fig. 2 there was an increase in the reduced GSH level in arsenic trioxide (22.0±1.0 nmolGSH/mg protein), DMA (19.4±0.8 nmolGSH/mg protein) and significantly difference with arsenite and arsenate (40.4±4.3 nmolGSH/mg protein $p<0.001$ and 23.4±4.3 nmolGSH/mg protein $p<0.05$) respectively compared to the control (15.4±3.0 nmolGSH/mg protein).

The percentage of cell viability was 90% and for apoptotic cells (7-AAD+ve, PE-ve) was similar in control HepG2s and cells treated with arsenate, arsenite, arsenic trioxide or DMA at concentrations of 10 µM for 24 hr. Similarly, the percentage of necrotic (7-AAD+ve, PE+ve) cells was similar in control HepG2s and cells treated with arsenate, arsenite, arsenic trioxide or DMA at concentrations of 10 µM for 24 hr (Fig. 3).

In our study, arsenic induced genotoxocity was measured by comet assay in cell line (HepG2 cells). 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

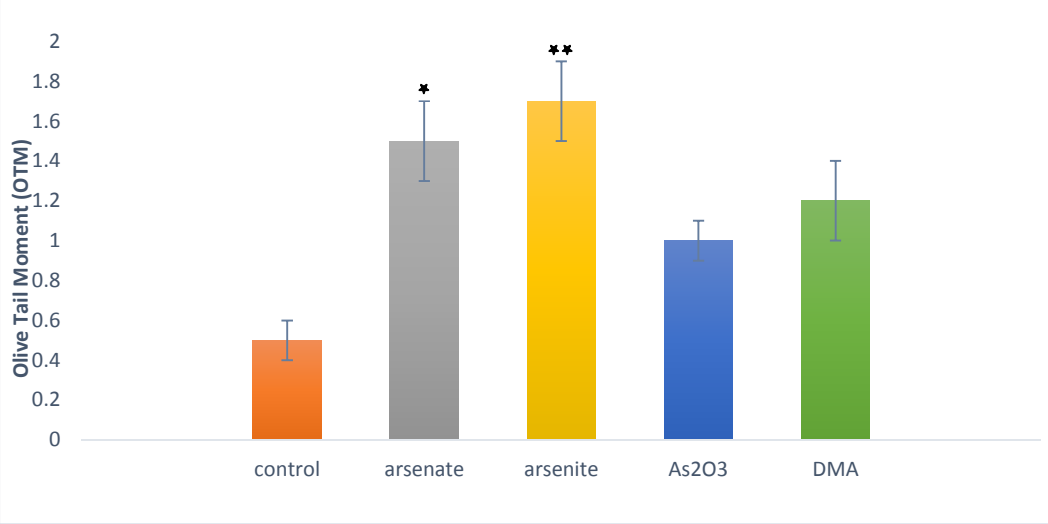

Fig. 1. DNA damage in HepG2 cells treated *in vitro* by arsenate, arsenite, arsenic trioxide and DMA (10 µM) for 24 hr

Olive tail moment (OTM) were recorded for 50 cells per each arsenic compound.
** P < 0.05; ** P< 0.001 compared to control.*
Mean ± S.E.M = Mean values ± Standard error of means of six experiments

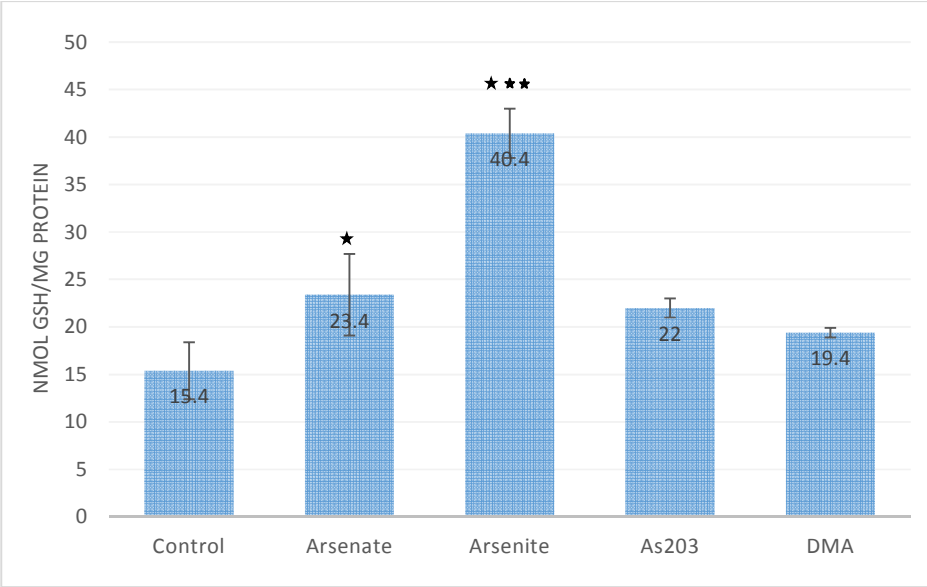

Fig. 2. Effects of sodium arsenate, sodium arsenite, arsenic trioxide and DMA (10 µM) on reduced GSH levels in HepG2 cells at 24 hrs using HPLC-fluorescence detection

Results are expressed as nmol GSH /mg protein, (mean±SEM).
** P < 0.05 compared to the control.*
** * * P < 0.001 compared to the contr*

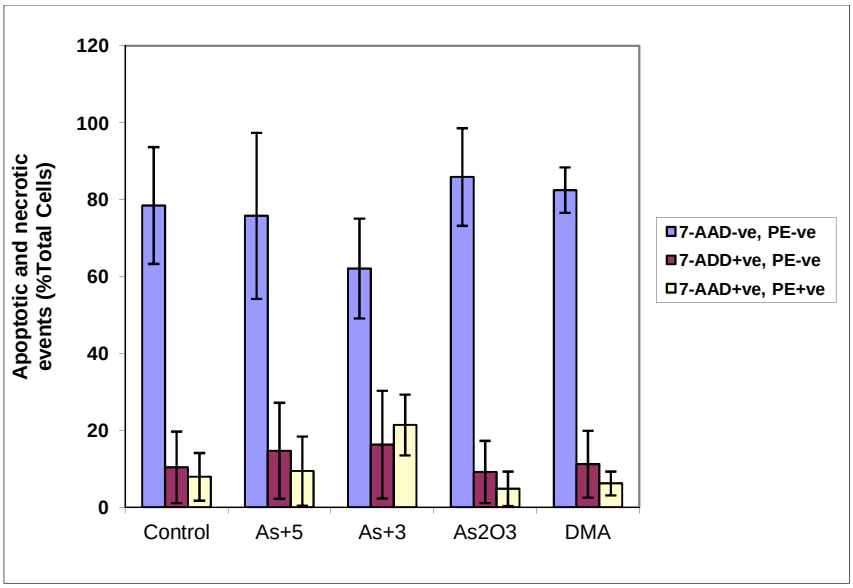

Fig. 3. Apoptotic and necrotic events in adherent HepG2 cells treated with arsenate, arsenite, As_2O_3 and DMA at a concentration of 10 µM for 24 hr. Undamaged (7-AAD-ve, PE-ve), apoptotic (7-AAD+ve, PE-ve) and necrotic (7-AAD+ve, PE +ve) cells were detected by flow cytometry

Results are expressed as % of total cells (mean±SEM).
P=ns compared to control

reported are DNA damage, oxidative stress, chromosome aberrations, apoptosis, DNA methylation, signal transduction and cell proliferation [31]. Generation of reactive oxygen species is one of the major pathways for arsenic mediated genotoxicity [32].

Our results obtained the DNA damage of arsenics using the Comet assay indicates that, both arsenate and arsenite, but not DMA or arsenic trioxide induced DNA damage in HepG2 cells. However, mean comet tail-length induced by arsenate was less than arsenite.

One mechanism of arsenic toxicity appears to be a result of the ability of arsenite to bind to protein thiols causing the thiols to be unavailable for antioxidant activity. As the protective effect of GSH is consistent with the removal of free radicals following exposure to oxidative damaging agents such as arsenic compounds, at increasing arsenic concentrations resulting in decreased GSH levels, the cells will become prone to increasing DNA damage and apoptosis [33]. It was predicted that in our studies arsenic compounds in low concentrations at 10 µM would increase GSH levels. In fact, the profile of cellular GSH depletion was complex with toxic and subtoxic doses of arsenic compounds which is tending to decreas GSH level while 10 µM concentration of arsenics for 24 hr increased GSH levels. Arsenite was significantly increased GSH level therefore; arsenite appeared to have the greatest effect on GSH levels, consistent with its potential for greater toxicity [34].

Our findings agree with these of Takafumi, [35] who found that arsenite, DMA and other arsenic compounds produced an increase in GSH levels following exposure to 5 µM subtoxic concentrations in Chinese hamster V79 cells. The mechanism was likely to be an early reduction in glutathione levels resulting in an increase in glutathione synthesis, as well as Schuliga et al. [36], reported that inorganic arsenic induced reactive oxygen species (ROS) in mammalian cells which lead to significant upregulation in the level of cellular glutathione. Both human AGO6 keratinocytes cells and W1-38 fibroblast cells were treated with 0.1-10 µM arsenite for 24 hr. The baseline levels of GSH in both W1-38 fibroblasts and AGO6 keratinocytes was significant increased by sublethal concentrations of arsenic compound. These results are in agreement with our data and indicate the cytotoxic action of arsenite at concentrations of 10 µM and above. Arsenite and As_2O_3 disrupted the balance of GSH homeostasis, and $NaAsO_2$- and As_2O_3-induced oxidative damage was closely associated with the imbalance in GSH synthesis, recycling and utilization [37].

There is conflict in literatures regarding the relative toxicity of arsenicals. Moore et al. [38] reported that the organic arsenicals are thus much less potent as mutagenic agents than the inorganic arsenicals, also arsenic compounds appear to act by mechanisms that cause chromosomal mutations. Vahter et al. [39] investigated that inorganic arsenic is methylated to methylarsonic acid (MMA) and dimethylarsinic acid (DMA), which are less toxic and more readily excreted in urine than the inorganic arsenic, these findings are in agreement with our results. But Hughes, [40] showing the higher acute toxicity of monomethylarsonous acid (MMA^{III}) than arsenite *in vitro* and *in vivo*, the carcinogenic.

Both arsenite and As_2O_3 caused oxidative stress, genotoxicity, cytotoxicity, cell cycle arrest as well as apoptosis, while As_2O_3 induced higher production of reactive oxygen species (ROS) with a more remarkable decrease in superoxide dismutase (SOD) activities and intracellular levels of glutathione (GSH) than arsenite. Moreover, the degree of DNA damage, chromosomal breakage, cell cycle arrest and apoptosis in As_2O_3-treated cells were more severe than those in arsenite treated cells which is not in agreement with our results [41].

The high release of reactive oxygen species leads to decrease of glutathione level, collapse of mitochondrial membrane potential, release of cytochrome c, activation of Caspase-3 and apoptosis, whereas all of the phenomena can be eliminated by the addition of antioxidants. Therefore, arsenicals compounds can induce the oxidative stress-mediated intrinsic apoptosis in HL-60 cells [42].

Previous research demonstrated that arsenic trioxide induces mitochondrial pathway of apoptosis in HL-60 cells. This apoptotic signaling is modulated via oxidative stress, DNA damage, and change in mitochondrial membrane potential, translocation and upregulation of apoptotic proteins leading to apoptosis [43]. There was a significant increase in the rate of apoptosis at 80 and 160 µM arsenite concentrations in polymorphonuclear leukocytes [44]. This study demonstrates that arsenite not induce statistically different apoptosis through low asenite concentration.

4. CONCLUSION

Our current work concluded that both arsenate and arsenite but not arsenic trioxide 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 ROS formation by increase secretion of glutathione for cells protection.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

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REFERENCES

1. Allan H. Smith, Meera MH. Smith. Arsenic drinking water regulations in developing countries with extensive exposure. Toxicology. 2004;(1-3):39-44.
2. Cullen William R, Reimer Kenneth J. Arsenic speciation in the environment. Chemical Reviews. 1989;89(4):713-764.
3. Aposhian HV, Aposhian MM. Arsenic toxicology: Five questions. Chemical Research in Toxicology. 2006;19:1-15.
4. Thomas J, Stayblo M, Lin S. The cellular metabolism and systemic toxicity of arsenic. Toxicology and Applied Pharmacology. 2001;176:127-144.
5. Vahter M, Concha G. Role of metabolism in arsenic toxicology. Pharmacology and Toxicology. 2001;89:1-5.
6. Guillamet E, Creus A. Ponti J, Abbiooni E, Fortaner S, Marcos R. *In vitro* DNA damage by arsenic compounds in a human lymphoblastoid cell line (TK6) assessed by the alkaline comet assay. Mutagenesis. 2004;19(2):129-135.
7. Camougrand N, Rigoulet M. Ageing and oxidative stress: Studies of some genes involved in both ageing and in response to oxidative stress. Respiration Physiology. 2001;128:393-401.
8. Wu XX, Ogawa O, Kakehi Y. Enhancement of arsenic trioxide-induced apoptosis in renal cell carcinoma cells by L-buthionine sulfoximine. International Journal of Oncology. 2004;24:1489-1497.
9. Rong Chung Y, Kuang-Hung H, Chein-Jen C, John RF. Arsenic methylation capacity and skin cancer. Cancer Epidemiology, Biomarkers and Prevention. 2000;9(21): 1259-1262.
10. Ratnaike RN. Acute and chronic arsenic toxicity. Postgrad Med J. 2003;79:391-396.
11. Abernathy CO, Liu YP, Longfellow D, Aposhian HV, Beck B, et al. Arsenic: Health effects, mechanisms of actions, and research issues. Environ Health Perspect. 1999;107: 593-597.
12. Duker AA, Carranza EJ, Hale M. Arsenic geochemistry and health. Environ Int. 2005;31:631-641.
13. Kitchin KT, Ahmad S. Oxidative stress as a possible mode of action for arsenic carcinogenesis. Toxicol Lett. 2003;137:3-13.
14. Basu A, Som A, Ghoshal S, Mondal L, Chaubey RC, et al. Assessment of DNA damage in peripheral blood lymphocytes of individuals susceptible to arsenic induced toxicity in West Bengal, India. Toxicol Lett. 2005;159:100-112.
15. Basu A, Mahata J, Gupta S, Giri AK. Genetic toxicology of a paradoxical human carcinogen, arsenic-a review. Mutation Research. 2001;488:171-194.
16. Aposhian V. ZR, Avram MD, Kopplin MJ, Wollenberg ML. Oxidation and detoxification of trivalent arsenic species. Toxicology and Applied Pharmacology. 2003;193:1-8.
17. Vera Garaj-Vrhovac, Nevenka Kopjar. The alkaline comet assay as biomarker in assessment of DNA damage in medical personnel occupationally exposed to ionizing radiation. Mutagenesis. 2003; 18(3):265-271.
18. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, et al. Recommendations for conducting the *in vivo* alkaline comet assay. 4th International Comet Assay Workshop. Mutagenesis. 2003;18:45-51.
19. Yasuhara S, Zhu Y, Matsui T, Tipirneni N, Yasuhara Y, et al. Comparison of comet assay, electron microscopy and flow cytometry for detection of apoptosis. J Histochem Cytochem. 2003;51:873-885.
20. Sinkó I, Mórocz M, Zádori J, Kokavszky K, Raskó I. Effect of cigarette smoking on DNA damage of human cumulus cells analyzed by comet assay. Reprod Toxicol. 2005;20:65-71.

21. Malm-Erjefalt M, Stevens TR, Persson CGA, Erjefalt JS. Discontinuous percoll gradient centrifugation combined with immunomagnetic separation obviates the need for erythrocyte lysis and yield isolated eosinophils with minimal granule abnormalities. J Immunol Methods. 2004; 288:99-109.
22. Akram Z, Jalali S, Shami SA, Ahmad L, Batool S, et al. Genotoxicity of sodium arsenite and DNA fragmentation in ovarian cells of rat. Toxicol. Lett. 2009;190:81-85.
23. Palus J, Lewinska D, Dziubaltowska E, Stepnik M, Beck J, et al. DNA damage in leukocytes of workers occupationally exposed to arsenic in copper smelters. Environ Mol Mutagen. 2005;46:81-87.
24. Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer. 1972;26:239-257.
25. Aden DF, Fogel A, Plotkin S, Damjanov I, Knowles BB. Controlled synthesis of NbsAg in a differentiated human liver carcinoma-derived cell line. Nature. 1979; 282(5739):615-616.
26. Dehn PF, WCM, Conners DE, Shipkey G, Cumbo TA. Characterization of the human hepatocellular carcinoma (HepG2) cell line as *in vitro* model for cadmium toxicity studies. *In vitro* Cellular & Developmental Biology. 2004;40:172-182.
27. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Muyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: Guideline for *in vitro* and *in vivo* genetic toxicology testing. Environmental and Molecular Mutagenesis. 2000;35:206-221.
28. Singh NP, McCoy MT, Tice RR, Schneider LL. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research. 1988;175:184–191.
29. Isvan Vermes, Clemens Haanen, Helga Steffens-Nakken, Chris Reutelingsperger. A novel assay for apoptosis Flow cytometric detection of phosphatidylserine early apoptotic cells using fluorescein labelled expression on Annexin V. Journal of Immunological Methods. 1995;184:39-51.
30. Rita Paroni, Elena De Vecchi, Giuliana Cighetti, Cinzia Arcelloni, Isabella Fermo, Adalberto Grossi, Pierangelo Bonini. HPLC with o-phthalaldehyde precolumn derivatization to measure total, oxidized, and protein-bound glutathione in blood, plasma and tissue. Otomation and Analytical Techniques. 1995;41/3:448-454.
31. Rossman TG. Mechanism of arsenic carcinogenesis: An integrated approach. Mutation Research. 2003;533(1-2):37-65.
32. Kitchin K. Recent advances in arsenic carcinogenesis: modes of action, animal model systems and methylated arsenic metabolites. Toxicology and Applied Pharmacology 2001;172:249-261.
33. Wu XX, Ogawa O, Kakehi Y. Enhancement of arsenic trioxide-induced apoptosis in renal cell carcinoma cells by L-buthionine sulfoximine. International Journal of Oncology. 2004;24:1489-1497.
34. Kumagai Y, Sumi D. Arsenic: Signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity. Annu Rev Pharmacol Toxicol. 2007;47:243-262.
35. Takafumi O. Arsenic compound-induced increase in glutathione levels in cultured chinese hamster V79 cells and mechanisms associated with changes in γ-glutamlycysteine synthetase activity, cystein uptake and utilization of cysteine. Archives of Toxicology. 1997;71:730-740.
36. Schuliga M, Choichane S, Snow E. Upregulation of glutathione-related genes and enzyme activities in cultured human cells by sublethal concentrations of inorganic arsenic. Toxicological Sciences. 2002;70:183-192.
37. Moore Martha M, Karen Harrington-Brock, Carolyn L. Doerr. Relative genotoxic if arsenic and its methylated metabolites. Mutation Research. 1997;386:279-290.
38. Vahter M. Genetic polymorphism in the biotransformation of inorganic arsenic and its role in toxicity. Toxicology Letters. 2000;112-113:211-217.
39. Hughes MF. Arsenic toxicity and potential mechanism of action. Toxicology Letters. 2002;133:1-16.
40. Xuejun Jiang, Chengzhi Chen, Wei Zhao, Zunzhen Zhang. Sodium arsenite and arsenic trioxide differently affect the oxidative stress, genotoxicity and apoptosis in A549 cells: An implication for the paradoxical mechanism. Environmental Toxicology and Pharmacology. 2013;36: 891-902.
41. Fan XY, Chen XY, Liu YJ, Zhong HM, Jiang FL, Liu Y. Oxidative stress-mediated intrinsic apoptosis in human

promyelocytic leukemia HL-60 cells induced by organic arsenicals. Sci Rep. 2016;19:1-12.

42. Kumar S, Yedjou CG, Tchounwou PB. Arsenic trioxide induces oxidative stress, DNA damage, and mitochondrial pathway of apoptosis in human leukemia (HL-60) cells. J Exp. Clin. Cancer Res. 2014;16; 33:42.

43. Iwama K[1], Nakajo S, Aiuchi T, Nakaya K. Apoptosis induced by arsenic trioxide in leukemia U937 cells is dependent on activation of p38, inactivation of ERK and the Ca2+-dependent production of superoxide. Int J Cancer. 2001; 15;92(4):518-26.

44. Watson RW, Redmond HP, Wang JH, Bouchier-Hayes D. Mechanisms involved in sodium arsenite-induced apoptosis of human neutrophils. J. Leukoc. Biol. 1996; 60(5):625-32.

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