

# *In vitro* Cytotoxic and Genotoxic Effects of Arsenic Trioxide on Human Keratinocytes

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**Abstract**—Although, arsenic trioxide has been the subject of toxicological research, *in vitro* cytotoxicity and genotoxicity studies using relevant cell models and uniform methodology are not well elucidated. Hence, the aim of the present study was to evaluate the cytotoxicity and genotoxicity induced by arsenic trioxide in human keratinocytes (HaCaT) using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and alkaline single cell gel electrophoresis (Comet) assays, respectively. Human keratinocytes were treated with different doses of arsenic trioxide for 4 h prior to cytogenetic assessment. Data obtained from the MTT assay indicated that arsenic trioxide significantly reduced the viability of HaCaT cells in a dose-dependent manner, showing an  $IC_{50}$  value of  $34.18 \pm 0.6 \mu M$ . Data generated from the comet assay also indicated a significant dose-dependent increase in DNA damage in HaCaT cells associated with arsenic trioxide exposure. We observed a significant increase in comet tail length and tail moment, showing an evidence of arsenic trioxide -induced genotoxic damage in HaCaT cells. This study confirms that the comet assay is a sensitive and effective method to detect DNA damage caused by arsenic.

**Keywords**—Arsenic trioxide, cytotoxicity, genotoxicity, HaCaT.

## I. INTRODUCTION

ALTHOUGH arsenic trioxide is a well-known toxicant, it has been in medical use for a long time. Arsenic containing compounds have been used for at least a century in the treatment of syphilis, yaws, amoebic dysentery, and trypanosomiasis [1]. Arsenic trioxide has also been reported to have activity *in vitro* against myeloma cell lines and primary myeloma cells [2]. Moreover, it has been shown to inhibit cell viability and proliferation, as well as to induce programmed cell death in a panel of lymphoma cell lines [3].

Nonetheless, exposure to arsenic has been associated with anemia, peripheral neuropathy, liver and kidney damage, irritation of the skin and mucous membranes, vascular disease, and several types of cancer, such as skin, liver, lung, bladder, and kidney neoplasms [4]. In chronic arsenic exposure, many other clinical symptoms including headache, fatigue, confusion, polyneuritis with distal weakness, exfoliative dermatitis, leucopenia, hyperkeratosis, vomiting, and hyperpigmentation have been reported [4]. Scientific reports from epidemiological studies have also pointed out an association between exposure to inorganic arsenic and increased risk of adverse developmental effects such as congenital malformations, low birth weight, and spontaneous

abortion [5].

*In vitro* experiments with many inorganic and organic arsenicals have shown that they are powerful clastogens in many cell types [6]. Both *in vitro* and *in vivo* genotoxic studies have indicated that arsenic trioxide induces DNA damage in human lymphocytes and in mice leucocytes based on the comet assay [7].

In the present study, we use the human keratinocytes (HaCaT) as a test model to evaluate arsenic trioxide induced cytotoxicity and genotoxicity using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and alkaline single cell gel electrophoresis (Comet) assays, respectively; based on its recent consideration as a salvage therapy in the treatment of acute promyelocytic leukemia.

## II. MATERIALS AND METHODS

### A. Chemicals and Test Media

As<sub>2</sub>O<sub>3</sub> was obtained from Merck (Darmstadt, Germany). MTT assay kit was obtained from Sigma Chemical Company (St. Louis, MO).

### B. Tissue Culture

Cells were grown in petri dishes at a concentration of  $7 \times 10^4$  cells/cm<sup>2</sup> in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C.

### C. Cytotoxicity/MTT Assay

This is a colorimetric assay that measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT reagent enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored, formazan product. The amount of color produced is directly proportional to the number of viable cells [8]. HaCaT cells were maintained in DMEM containing 4 mmol/L L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/streptomycin, and incubated at 37°C in humidified 5% CO<sub>2</sub> incubator. To 542  $\mu$ l aliquots in six replicates of the cell suspension ( $1 \times 10^6$ /mL) seeded to 96-well polystyrene tissue culture plates, 50  $\mu$ L aliquots of stock solutions were added to each well using distilled water as solvent to make-up final arsenic trioxide doses of 0.625, 1.25, 2.5, 5, 10, 20 and 40  $\mu$ M. Control cells received 150  $\mu$ L of distilled water. All chemical exposures were carried in 96-well tissue culture plates for the purpose of chemical dilutions. Cells were placed in a humidified 5% CO<sub>2</sub> incubator for 24 h at 37°C. After

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incubation, 20  $\mu$ L aliquots of MTT solution (5 mg/mL in PBS) were added to each well and re-incubated for 4 h at 37°C following by low centrifugation at 800 rpm for 5 min. Then, the 200  $\mu$ L of supernatant culture medium were carefully aspirated and 50  $\mu$ L aliquots of trypsin were added to each well to dissolve the formazan crystals, followed by incubation for 6 min at 37°C. The culture plate was placed on a Biotek micro-plate reader and the absorbance was measured at 550 nm. All assays were performed in six replicates for each arsenic trioxide dose.

#### D. Cell Treatment for Comet/Genotoxicity Assay

Cells were counted (10,000 cells/well) and resuspended in the growth medium with 10% FBS. Aliquots of 100  $\mu$ L of the cell suspension were placed in each well of 96-well plates, treated with 100  $\mu$ L aliquot of either media or arsenic trioxide (6.25, 12.5, 25, 50, 100, 200  $\mu$ M) and incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C for 24 h. After incubation, the cells were centrifuged, washed with PBS free calcium and magnesium, and re-suspended in 100  $\mu$ L PBS. In a 2 mL tube, 50  $\mu$ L of the cells suspension and 500  $\mu$ L of melted LMAgarose were mixed and 75  $\mu$ L pipetted onto a pre-warmed comet slide. The side of the pipette tip was used to spread completely agarose/cells over the sample area. The slides were placed flat in the dark at 4°C for 6 min to allow the mixture to solidify, and then immersed in pre-chilled lysis solution at 4°C for 1 h. Slides were removed from lysis solution, tapped, and immersed in alkaline solution for 40 min at room temperature in the dark. Slides were washed twice for 5 min with tris-borate-EDTA (TBE). Slides were electrophoresed at low voltage (300 mA, 25 V, 4°C) for 30 min. After electrophoresis, the slides were placed in 70% ethanol for 5 min, removed, tapped, and air dried overnight. Slides were stained with DAPI designed for the Comet assay, and allowed to air dry at room temperature for 4 h. Slides were viewed using a phase contrast microscope and images were obtained using a digital camera. Three hundred cells were randomly selected from three replicated comet slides per arsenic trioxide dose at 20x magnification under the microscope. A mean of 20% of tail intensity is reflecting an effect of genotoxicity.

#### E. Statistical Analysis

Cell viability percentages were calculated from the optimal density readings at 550 nm, using the Xenometrix computer software program. Results were presented as means  $\pm$  SD. Statistical analysis was done using one way analysis of variance (ANOVA) for multiple samples and Student's t-test for comparing paired sample sets. Statistical significances were considered at  $p$ -values less than 0.05.

In the case of comet slides, a total of 300 comets were scored per arsenic trioxide dose. One hundred comets were randomly selected from three replicated slides by visual scoring at 20x magnification under a phase contrast microscope, according to the degree of DNA damage and comet tail length.

Data were analyzed using a non parametric analysis of

variance procedure in the PASW statistics 18 software. Significant differences ( $p < 0.05$ ) between means from all treated groups and the control negative group were determined by Mann-Whitney test.

### III. RESULTS

The results of the cytotoxicity of arsenic trioxide to HaCaT cells are presented in (Fig. 1). Data obtained from this assay indicated a strong dose response relationship with regard to the cytotoxic property of arsenic trioxide. As indicated in this figure, there was a gradual decrease in the viability of HaCaT cells, with increasing doses of arsenic trioxide.

The chemical dose required to cause 50% reduction in cell viability was computed to be  $34.18 \pm 0.6$   $\mu$ M. HaCaT cells exposed to arsenic trioxide doses of 5, 10, 20 and 40  $\mu$ M showed significant mortalities compared to control cells (Fig. 1).

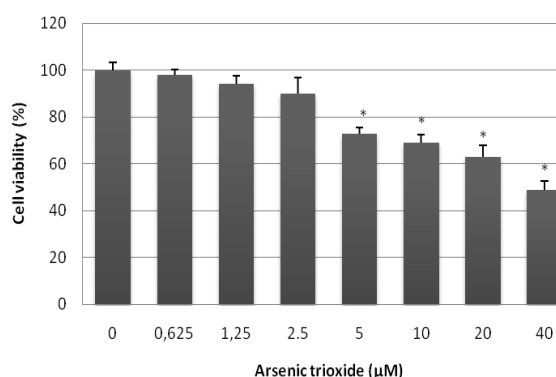


Fig. 1 Toxicity of arsenic trioxide to HaCaT cells. Cell viability was determined based on the MTT assay: Each point represents a mean  $\pm$  SD of 3 experiments with 6 replicates per dose. \*Significantly different ( $p < 0.05$ ) from the control group

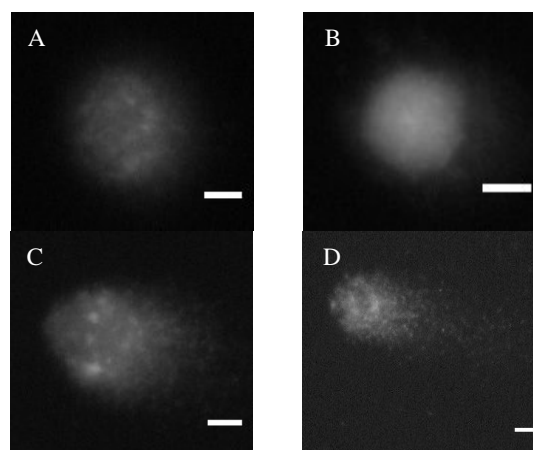


Fig. 2 Representative comet assay images of control and arsenic trioxide-treated HaCaT cells. (A) Control negative group (tail intensity = 1.92); (B) AS<sub>1</sub>-AS<sub>6</sub> groups (tail intensity = 19.49); (C) AS<sub>7</sub> group (tail intensity = 39.20); (D) Control positive group (tail intensity = 56.77). A mean of 20% of tail intensity is reflecting an effect of genotoxicity. Scale bar for all photograph sizes 10  $\mu$ m.

Representative comet assay images of control negative, control positive, and arsenic trioxide-treated HaCaT cells are presented in (Fig. 2). These results indicated that the distribution of cells by comet classes was highly dependent of the dose of arsenic trioxide. There was a gradual increase in the percentage of damaged cells with increasing doses of arsenic trioxide (Fig. 2). We observed also a significant increase in comet tail length and tail moment at doses 12.5, 25, 50, 100, 200  $\mu\text{M}$ , showing an evidence of arsenic trioxide-induced genotoxic damage in HaCaT cells (Fig. 3).

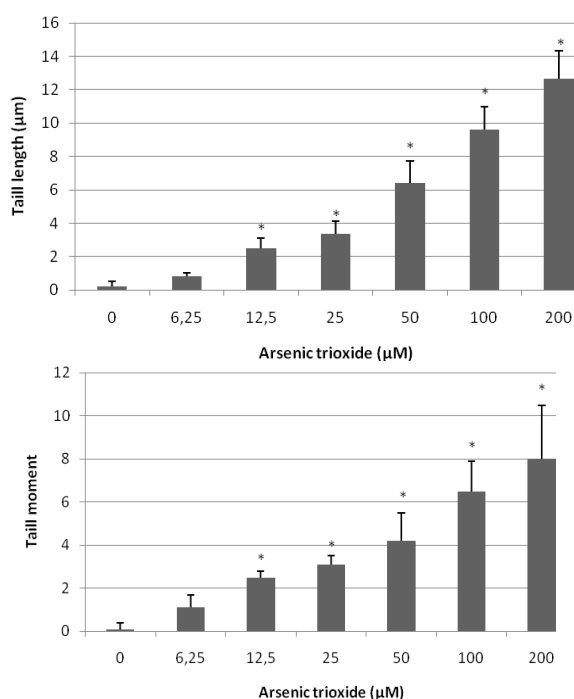


Fig. 3 Comet assay of HaCaT cells showing the tail length and tail moment as a function of arsenic trioxide doses: Each point represents mean  $\pm$  SD of three independent experiments; \* significantly different ( $p < 0.05$ ) from the control group

#### IV. DISCUSSION

Cytotoxicity can be defined as the cell killing property of a chemical compound independent from the mechanism of death. In this research, we examined the cytotoxic effect of arsenic trioxide on the HaCaT cells. Findings from our study clearly demonstrated that arsenic trioxide is highly cytotoxic to HaCaT cells, showing a 4 h-IC<sub>50</sub> of  $34.18 \pm 0.6 \mu\text{M}$ .

Several studies have addressed the cytotoxicity of arsenic to various cells. One of such studies has shown that exposure to arsenic trioxide within the dose range of 0.5–1  $\mu\text{M/L}$  induces apoptosis in monocytic (NB4) cells [9]. Cytotoxicity studies with two multiple myeloma (MM)-derived cell lines, RPMI 8226 and U266, have reported that exposure to 1.0  $\mu\text{M/L}$  arsenic trioxide inhibits cell proliferation resulting in a weak degree of apoptosis induction, while an exposure of 2.0  $\mu\text{M/L}$  strongly induces cell apoptosis. This investigation pointed out that arsenic trioxide exerts apoptosis-inducing and growth inhibiting effects on MM-derived cells [10]. Findings from

other studies suggest that low doses of arsenic are dramatically effective in acute promyelocytic leukemia and show considerable promise in preclinical models of other tumor types [11].

Comet assay, also known as single cell gel electrophoresis (SCGE), is a microgel electrophoresis technique, which detects DNA damage and repair in individual cells [12]. In the comet assay, a damaged cell takes on the appearance of a comet, with head and tail regions. The head corresponds to the amount of DNA that still remains in the region of the nuclear matrix, whereas the tail visualizes the fragments of DNA migrating from the nucleus [13]. By means of the comet assay, the present study has elucidated some of the molecular changes in HaCaT cells exposed to arsenic trioxide. Similar to those reported in previous literature, our results demonstrate that arsenic trioxide induces DNA damage in a dose-dependent manner. Our data indicate that the amount of DNA liberated from the nuclei of HaCaT cells is highly dependent on the arsenic trioxide dose.

Other toxic end points including tail length and tail moment also follow the same pattern. Our findings are consistent with those of previous literature reporting an elevated level of DNA damage in cervical epithelial cells, and peripheral blood leucocytes of cervix cancer patients and patients with pre-cancerous lesions categorized into mild and severe dysplasia [14].

The extent of DNA damage increased proportionately with increasing doses of arsenic trioxide. The migration of the DNA from the head region into the tail region reflects the number of single-strand breaks in the DNA. No data was found in the literature regarding the genotoxicity of arsenic trioxide to HaCaT cells. Here, we report for the first time the genotoxic effect of arsenic trioxide to these cells using the alkaline single cell gel electrophoresis assay.

#### V. CONCLUSION

Arsenic trioxide exposure above 5  $\mu\text{M}$  exerts a potent cytotoxic effect on human keratinocytes (HaCaT) by inhibiting cell proliferation and inducing cell death. Using the MTT assay, we found a strong dose-response relationship between the degree of cytotoxicity and arsenic trioxide exposure level. Data generated from comet assay indicate that arsenic trioxide also has a high genotoxic potential. We found that exposure to this compound significantly induces a substantial amount of DNA damage in HaCaT cells. Taken together, our research demonstrates arsenic trioxide is highly cytotoxic and genotoxic to HaCaT cells. These findings provide new insights in the understanding of the use of arsenic trioxide as a salvage therapy in the treatment of acute promyelocytic leukemia.

#### REFERENCES

- [1] NRC, "Arsenic in drinking water." U.S. National Research Council, National Academy Press. Washington, DC, 1999.
- [2] P. Rousselot, S. Labaume, JP. Marolleau, J. Larghero, MK. Noguera, JC. Brouet, and JP. Ermand, "Arsenic trioxide and melarsoprol induce apoptosis in plasma cell lines and in plasma cells from myeloma patients." *Cancer Res*, vol. 59, pp. 1041–1048, 1999.

- [3] K. Zhang, K. Ohnishi, Shigeno, S. Fujisawa, K. Naito, S. Nakamura, K. Takeshita, A. Akeshita, and R. Ohno, "The induction of apoptosis and cell cycle arrest by arsenic trioxide in lymphoid neoplasms." *Leukemia* vol. 12, pp.1383–1391, 1998.
- [4] IARC, "Monographs on the Evaluation of Carcinogenic Risks of Chemicals to Humans." Supplement F. Overall Evaluation of Carcinogenicity. International Agency for Research on Cancer. World Health Organization. Lyon, France, pp 29–57, 1987.
- [5] A. Aschengran, S. Zierler, and A. Cohen, "Quality of community drinking water and the occurrence of spontaneous abortion." *Arch Environ Health* vol. 44, pp. 283–290, 1989.
- [6] JC. Barrett, PW. Lamb, TC. Wang, and TC. Lee, "Mechanisms of arsenic-induced cell transformation." *Biol Trace Elem Res* vol. 21, pp. 421–429, 1989.
- [7] B. Saleha Banu, K. Danadevi, J. Kaiser, YR. Ahuja, K. Visweswara Rao, and M. Ishap, "In vivo genotoxic effect of arsenic trioxide in mice using comet assay." *Toxicol* vol. 162, pp. 171–177, 2001.
- [8] PB. Tchounwou, CG. Yedjou, D. Foxx, A. Ishaque, and E. Shen, "Lead-induced cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells." *Mol Cell Biochem* vol. 255, pp. 161–170, 2004.
- [9] Y. Jing, J. Dai, RM. Chalmers-Redman, WG. Totto, and S. Waxman, "Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide dependent pathway." *Blood* vol. 94, pp. 102–2111, 1999.
- [10] P. Jai, G. Chen, X. Huang, X. Cai, J. Yang, L. Wang, Y. Zhou, Y. Shen, L. Zhou, Y. Yu, S. Chen, X. Zhang, and Z. Wang, "Arsenic trioxide induces multiple myeloma cell apoptosis via disruption of mitochondrial transmembrane potentials and activation of caspase-3." *Chin Med J* vol. 114, pp. 19–24, 1999.
- [11] SL. Soignet, SR. Frankel, D. Douer, MS. Tallman, H. Kantarjian, E. Calleja, RM. Stone, M. Kalaycio, DA. Scheinberg, P. Steinherz, EL. Sievers, S. Coutre, S. Dahlberg, R. Ellison, and RP. Warrell, "United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia." *J Clin Oncol* vol. 19, pp. 3852–3860, 2001.
- [12] O. Ostling, KI. Johanson, "Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells." *Biochem Biophys Res Commun* vol. 123, pp. 291–298, 1984.
- [13] E. Rojas, MC. Lopez, and M. Valverde, "Single gel electrophoresis assay: methodology and application." *J Chromatogr* vol. 722, pp. 225–254, 1999.
- [14] M. Jaiswal, G. Anuradha, N. Rajeswari, KN. Raj, N. Balakrishna, KV. Rao, IS. Prasand, SN. Jain, and YR. Ahuja, "Comet assay on cervical epithelial cells and leucocytes of patients with precancerous and cancerous lesions of the cervix." *Med Sci Res* vol. 22, pp. 879–881, 1994.