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In-vitro cytotoxic and genotoxic effects of arsenic trioxide on human leukemia (HL-60) cells using the MTT and alkaline single cell gel electrophoresis (Comet) assays

Clement G. Yedjou and **Paul B. Tchounwou***
Molecular Toxicology Research Laboratory, NIH-Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, 1400 Lynch Street, Box 18540, Jackson, Mississippi, USA

Abstract

Although arsenic trioxide (ATO) 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 ATO in a human leukemia (HL-60) cell line using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and alkaline single cell gel electrophoresis (Comet) assays, respectively. HL-60 cells were treated with different doses of ATO for 24 h prior to cytogenetic assessment. Data obtained from the MTT assay indicated that ATO significantly ($p < 0.05$) reduced the viability of HL-60 cells in a dose-dependent manner, showing a LD_{50} value of 6.4 ± 0.6 μg/mL. Data generated from the comet assay also indicated a significant dose-dependent increase in DNA damage in HL-60 cells associated with ATO exposure. We observed a significant increase ($p < 0.05$) in comet tail-length, tail arm and tail moment, as well as in percentages of DNA cleavage at all doses tested, showing an evidence of ATO-induced genotoxic damage in HL-60 cells. This study confirms that the comet assay is a sensitive and effective method to detect DNA damage caused by heavy metals like arsenic. Taken together, our findings suggest that ATO exposure significantly ($p < 0.05$) reduces cellular viability and induces DNA damage in HL-60 cells as assessed by MTT and alkaline single cell gel electrophoresis assays, respectively.

Keywords

Arsenic trioxide; Cytotoxixity; Genotoxicity; HL-60 cells; Promyelocytic leukemia

1. Introduction

Although arsenic trioxide (ATO) 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, 2]. Recently, ATO (Trisenox) has been used as an anticancer agent in the treatment of acute promyelocytic leukemia, and its therapeutic action has been associated with its ability to induce apoptosis with down regulation of Bcl-2 expression and modulation of the PML-RARα/PML proteins in leukemic cells both *in vivo* and *in vitro* [3]. This is indicative of the potential mechanism of action of ATO at low doses, and therefore, its use as a salvage therapy for relapsed and refractory acute promyelocytic leukemia [4]. ATO has also been reported to have activity *in vitro* against myeloma cell lines and primary myeloma cells [4]. Moreover, it has been

*Correspondence to: Dr. Paul B. Tchounwou, paul.b.tchounwou@jsums.edu.

shown to inhibit cell viability and proliferation, as well as to induce programmed cell death in a panel of lymphoma cell lines [5].

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 [6–9]. Symptoms of chronic arsenic intoxication include: headache, fatigue, confusion, polyneuritis with distal weakness, exfoliative dermatitis, leucopenia, hyperkeratosis, vomiting, and hyperpigmentation [9]. There are also several epidemiological studies reporting an association between exposure to inorganic arsenic and increased risk of adverse developmental effects such as congenital malformations, low birth weight, and spontaneous abortion [10, 11].

One of the major mechanisms by which arsenic exerts its toxic effect is through impairment of cellular respiration by the inhibition of various mitochondrial enzymes, and the uncoupling of oxidative phosphorylation. In addition, arsenic toxicity results from its ability to interact with sulphydryl groups of proteins and enzymes, and to substitute phosphorous group in a variety of biochemical reactions [12]. Schwerdtle and his colleagues have reported that ATO binds strongly to sulfhydryl groups and dithiols, and modifications in the glutathione redox system can lead to inhibited DNA repair, mutation in key genetic sites, or increased cell proliferation which can result in subsequent mutation by inhibiting the DNA repair apparatus [13].

Few studies suggest that the mechanism of genotoxicity of arsenic compounds may be due to the ability of arsenite to inhibit DNA replicating or repair or to the ability of arsenate to act as phosphate analog enzyme [11]. Arsenic-induced oxidative stress can provoke a broad spectrum of effects on the DNA including DNA single- and double-strand breaks, abasic sites, DNA-protein cross-links and DNA base modifications [14]. Among the DNA base modifications, 7, 8-dihydro-8-oxoguanine (8-hydroguanine) has received special attention because it is mutagenic by causing GC to TA transversions [15]. The scavenging of reactive oxygen species by superoxide dismutase, catalase, glutathione peroxidase and dimethyl sulfoxide (DMSO) can counteract the deletion of mutations in human chromosomes as well as sister chromatid exchanges and thus exercise a collateral damaging effect on the DNA [14, 16].

Research has shown that ATO can be mutagenic in mammalian cells in culture. Reversion assays with *Salmonella typhimurium* fail to detect mutations that are induced by arsenic compounds [17]. Tests for genotoxicity have indicated that arsenic compounds inhibit DNA repair, induce chromosomal aberrations, and sister chromatid exchanges [11, 18]. *In vitro* experiments with many inorganic and organic arsenicals have shown that they are powerful clastogens in many cell types [19, 20]. Both *in vitro* and *in vivo* genotoxic studies have indicated that ATO induces DNA damage in human lymphocytes and in mice leucocytes based on the comet assay [21].

In a recent *in-vivo* study, we also demonstrated that ATO is genotoxic and able to induce structural chromosomal aberrations and micronuclei formation in bone marrow cells of Sprague-Dawley rats [18]. Studies in our laboratory have demonstrated that ATO, *in vitro*, is cytotoxic to human liver carcinoma ($HepG_2$) cells, and is able to transcriptionally activate a significant number of stress genes and related proteins in human liver carcinoma ($HepG_2$) cells [22, 23]. In the present study, we use the HL-60 human leukemia cell line as a test model to evaluate ATO-induced cytototoxicity 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.

2. Materials and methods

2.1. Chemicals and test media

Arsenic trioxide (As_2O_3), CASRN 1327-53-3, MW 197.84, with an active ingredient of 100% (w/v) arsenic in 10% nitric acid was purchased from Fisher Scientific in Houston, Texas. Growth medium RMPI 1640 containing 1 mmol/L L-glutamine was purchased from Gibco BRL products (Grand Island, NY). Ninety-six well plates were purchased from Costar (Cambridge, MA). Fetal bovine serum (FBS), antibiotics (penicillin G and streptomycin), phosphate buffered saline (PBS), and MTT assay kit were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Tissue culture

The HL-60 promyelocytic leukemia cell line was purchased from the American Type Culture Collection –ATCC (Manassas, VA). It is a promyelocytic cell line derived from peripheral blood cells from a 36 year-old Caucasian female with acute promyelocytic leukemia (APL). The HL-60 cells grow as a suspension culture. The predominant cell population consists of neutrophilic promyelocytes [24, 25].

In the laboratory, cells were stored in the liquid nitrogen until use. They were thawed by gentle agitation of their containers (vials) for 2 minutes in a water bath at 37°C. After thawing, the content of each vial of cells was transferred to a 25 cm^2 tissue culture flask, diluted with up to 10 mL of RMPI 1640 containing 1 mmol/L L-glutamine (GIBCO/BRL, Gaithersburg, MD) and supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% (w/v) penicillin/streptomycin. The 25 cm^2 culture flasks containing 2×10^6 viable cells were observed under the inverted microscope, followed by incubation in a humidified 5 % CO_2 incubator at 37 °C. Three times a week, they were diluted and maintained under same conditions at a density of 5×10^5/mL and harvested in the exponential phase of growth. The cell viability was assessed by the trypan blue exclusion test (Life Technologies) and manually counted using a hemocytometer.

2.3. Cytotoxicity/MTT assay

Principle of the Assay—This is a colorimetric assay that measures the reduction of 3-(4, 5-dimethylthiasol-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 cells are then solubilised with an organic solvent (DMSO or isopropanol) and the released, solubilised fromazan product is measured by spectro-photometry. The amount of color produced is directly proportional to the number of viable cells [26, 27].

Method—Human leukemia HL-60 cells were maintained in RMPI 1640 containing 1 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_2 incubator. To 180 μL aliquots in six replicates of the cell suspension (5×10^5/mL) seeded to 96-well polystyrene tissue culture plates, 20 μL aliquots of stock solutions were added to each well using distilled water as solvent to make-up final ATO doses of 0.04, 0.08, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, and 20 μg/mL. Control cells received 20 μL 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_2 incubator for 24 h at 37°C. After incubation, 20 μL 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 μL of supernatant culture medium were carefully aspirated and 200 μL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals, followed by incubation for 10 min to dissolve air bubbles. 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 ATO dose. Statistical analysis was done to determine the means ± SDs of cell viability. Cell viability rate was calculated as the percentage of MTT absorption as follows: % survival = (mean experimental absorbance/mean control absorbance×100).

2.4. Cell treatment for Comet/genotoxicity assay

Cells were counted (10,000 cells/well) and re-suspended in the growth medium with 10% FBS. Aliquots of 100 μL of the cell suspension were placed in each well of 96-well plates, treated with 100μL aliquot of either media or ATO (2.5, 5, and 10 μg/mL) and incubated in a humidified 5% CO_2 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 μL PBS. In a 2 mL tube, 50 μL of the cells suspension and 500 μL of melted LMAgarose were mixed and 75 μL pipetted onto a pre-warmed cometslide. 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 10 min to allow the mixture to solidify, and then immersed in pre-chilled lysis solution at 4°C for 40 min. 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 20 min. After electrophoresis, the slides were placed in 70% ethanol for 5 min, removed, tapped, and air dried overnight. Slides were stained with Silver stain or SYBR Green stain designed for the Comet assay, and allowed to air dry at room temperature for 6 h.

Silver-stained cometslides were viewed using a phase contrast microscope and images were obtained using a digital camera. Three hundred cells were randomly selected from three replicated cometslides per ATO dose at 20x magnification under the microscope. The comets were classified visually into five categories according to the appearance resulting from the relative proportion of DNA in the tail. The extent of DNA damage was expressed in arbitrary units: Class 0 - control cell with no DNA damage and no tail (intact nuclei); Class 1 - comet cell with short tail (tail length less than the head diameter); Class 2 - comet cell with tail length longer than the head diameter and with low DNA damage; Class 3 - comet cell with tail length longer than the head diameter and with high DNA damage; and Class 4 - comet cell with tail length longer than the head diameter, high DNA damage, and insignificant head [16].

SYBR Green-stained cometslides were viewed with an Olympus fluorescence microscope and analyzed using LAI's Comet Assay Analysis System software (Loates Associates, Inc. Westminster, MD).

3. Statistical analysis

3.1. Cytotoxicity assay data

Cell viability percentages were calculated using the Xenometrix computer software program based of the optimal density readings at 550 nm [28]. Results were presented as means ± SDs. 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. The percentages of cell viability

were presented graphically in the form of histograms, using Microsoft Excel computer program.

3.2. Comet/genotoxicity assay data

In the case of Silver stained cometslides, a total of 300 comets were scored per ATO 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 as described in the Materials and Methods.

In the case of SYBR Green stained cometslides, a total of 150 comets were scored per ATO dose. Seventy five comets were randomly selected from three replicated slides using LAI's Comet Assay Analysis System software (Loates Associates, Inc. Westminster, MD). The data from three experiments were pooled and the means ± SDs were calculated.

The least significant difference (LSD) test was used to determine statistical differences between the control cells and ATO-treated HL-60 cells. All p-values <0.05 were considered to be significant. The percentage of DNA cleavage, comet tail length, tail arm and tail moment of control and ATO-treated HL-60 cells were presented graphically in the form of histograms, using Microsoft Excel computer program.

4. Results

4.1. Cytotoxicity/MTT assay

The results of the cytotoxicity of ATO to human leukemia (HL-60) cells are presented in (Fig 1). Data obtained from this assay indicated a strong dose-response relationship with regard to the cytotoxic property of ATO. As indicated in this figure, there was a gradual decrease in the viability of HL-60 cells, with increasing doses of ATO. Upon 24 h of exposure, the mean percentages of cell viability were 100 ± 13; 109 ± 10; 107 ± 13; 76± 11; 66 ± 18; 53 ± 6; 54 ± 3; 53 ± 2; 48 ± 5; 16 ± 3 and 3 ± 3 in 0, 0.04, 0.08, 0.16, 0.31, 0.62, 1.25, 2.5, 5, 10, and 20 μg/mL of ATO, respectively. The chemical dose required to cause 50% reduction in cell viability was computed to be 6.4 ± 0.6 μg/mL. These results showed that ATO induced a slight increase in cell viability between 0μg/mL (control) and 0.08 μg/mL, followed by a gradual decrease between 0.16 and 20 μg/mL. We previously reported a similar chemical hormesis effect from a study of the cytotoxicity of ATO to human liver carcinoma cells [23]. HL-60 cells exposed to ATO doses of 0.31, 0.62, 1.25, 2.5, 5, 10, and 20 μg/mL showed significant mortalities ($p < 0.05$) compared to control cells, according to ANOVA Dunnett's test (Fig 1).

4.2. Comet assay using silver stain

Representative comet assay images of control and ATO-treated HL-60 cells using the silver staining protocol are presented in (Fig 2). These results indicated that the distribution of cells by comet classes was highly dependent of the dose of ATO. For example, in the control (0 μg/mL ATO), we found about 62%, 25% and 13% of cells in Class 0, Class 1, and Class 2, respectively, and 0% in Classes 3 and 4. In contrary, about 70%, 47%, and 2% of cells were found in Class 4, Class 3, and Class 1, respectively following exposure to the 10 μg/mL dose. In general, there was a gradual increase in the percentage of damaged cells with increasing doses of ATO (Fig 3).

4.3. Comet assay using SYBR green stain

Representative comet assay images of control and ATO-treated HL-60 cells using SYBR green stain are presented in (Fig 4). As in the case with silver staining, these results showed a significant ($p < 0.05$) increase of DNA damage in ATO-treated HL-60 cells compared to

the control cells. Data generated from this experiment indicated a gradual increase in the comet tail length, tail moment, tail arm, and percentage of DNA cleavage of HL-60 cells, with increasing doses of ATO (Fig 4). After 24 h of exposure, the mean percentages of DNA cleavage of exposure were 1.72 ± 1.9%, 3.25 ± 2.3%, 12.02 ± 8.3%, and 18.87 ± 6.8% in 0, 2.5, 5, and 10 μg/mL of ATO, respectively. Similarly, the mean values tail length were computed to be 1 ± 2, 3 ± 2, 17 ± 12, and 24 ± 14 μM in 0, 2.5, 5, and 10 μg/mL, respectively; showing a clear evidence that ATO causes DNA damage in a dose-dependent manner (Fig. 5).

5. Discussion

5.1. Cytotoxicity/MTT assay

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 ATO on the HL-60 cells. Findings from our study clearly demonstrated that ATO is highly cytotoxic to human leukemia (HL-60) cells, showing a 24 h-LD_{50} of 6.4 ±0.6 μg/mL. Recently, we also reported that ATO is cytotoxic to human liver carcinoma ($HepG_2$) cells, showing a 48 h-LD_{50} of 8.55 ±0.58 μg/ml [20]. These results indicate that HL-60 cells are more sensitive to ATO toxicity than $HepG_2$ cells.

Several studies have addressed the cytotoxicity of arsenic to various cells. One of such studies has shown that exposure to ATO within the dose range of 0.5 to 1 μM/L induces apoptosis in monocytic (NB_4) cells [29]. Cytotoxicity studies with two multiple myeloma (MM)-derived cell lines, RPMI 8226 and U266, have reported that exposure to 1.0 μM/L ATO inhibits cell proliferation resulting in a weak degree of apoptosis induction, while an exposure of 2.0 μM/L strongly induces cell apoptosis. These investigations pointed out that ATO exerts apoptosis-inducing and growth-inhibiting effects on MM-derived cells [30]. 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 [31].

5.2. Comet/genotoxicity assay

Comet assay, also known as single cell gel electrophoresis (SCGE), is a microgel electrophoresis technique which detects DNA damage and repair in individual cells [32]. 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 [33]. By means of the comet assay, the present study has elucidated some of the molecular changes in HL-60 cells exposed to ATO. Similar to those reported in previous literature, our results demonstrate that ATO induces DNA damage in a dose-dependent manner.

In the SYBR green-staining protocol, a variety of geometric and densitometric parameters are provided by the image analysis software, which allows an estimation of the amount of DNA in the head and tail regions and the extent of migration into the tail region. Our data show individually isolated nuclei in cells comprising heads and tails, forming comet images (Figs 2&4). Also, these data indicate that the amount of DNA liberated from the nuclei of HL-60 cells is highly dependent on the ATO dose. The mean values of tail length were computed to be 1 ± 2, 3 ± 2, 17 ± 12, and 24 ± 14 μM in 0, 2.5, 5, and 10 μg/mL, respectively; indicating that ATO is a potent inducer of DNA damage. Other toxic end points including tail moment, tail area, and percentage of DNA damage 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 [34].

In this research, we estimated DNA damage in HL-60 cells using both silver, and SYBR green staining protocols. We did not find any significant differences in comet images using the two staining techniques. However, the silver-staining protocol only provided a qualitative analysis whereas SYBR green-staining provided both qualitative and quantitative assessments. Both protocols confirm that the comet assay is a sensitive and effective method for detecting DNA damage caused by heavy metals such as arsenic. The genotoxic effect by ATO was significantly higher at the 5 and 10 μg/mL dose levels (mean tail lengths of 17 ± 12 and 24 ± 14 μM, respectively) compared to the control (1 ± 2 μM). In general, the extent of DNA damage increased proportionately with increasing doses of ATO. 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 ATO to human leukemia (HL-60) cells. Here, we report for the first time the genotoxic effect of ATO to these cells using the alkaline single cell gel electophoresis assay.

6. Conclusions

Arsenic trioxide (ATO) exerts a potent cytotoxic effect on human leukemia (HL-60) cells 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 ATO exposure level; yielding a 24 h-LD_{50} of 6.4 ± 0.6 μg/mL. Such cytotoxic effects have been observed in other cell lines and animal models, as well as in clinical studies. Data generated from comet assay indicate that ATO also has a high genotoxic potential. We found that exposure to this compound significantly induces the amount of DNA damage in HL-60 cells. Taken together, our research demonstrates ATO is highly cytotoxic and genotoxic to HL-60 cells. These findings provide new insights in the understanding of the use of ATO as a salvage therapy in the treatment of acute promyelocytic leukemia.

Acknowledgments

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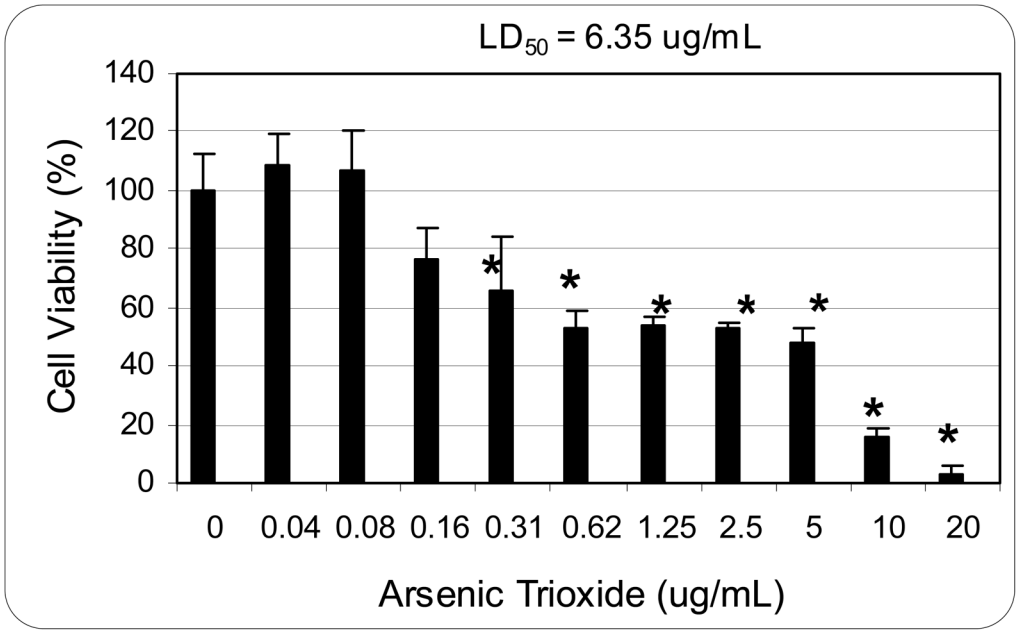

Figure 1.

Toxicity of arsenic trioxide to human leukemia (HL-60) cells. HL-60 cells were cultured with different doses of arsenic trioxide for 24 hours as indicated in the Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean ± SD of 3 experiments with 6 replicates per dose. *Significantly different ($p < 0.05$) from the control, according to the Dunnett's test.

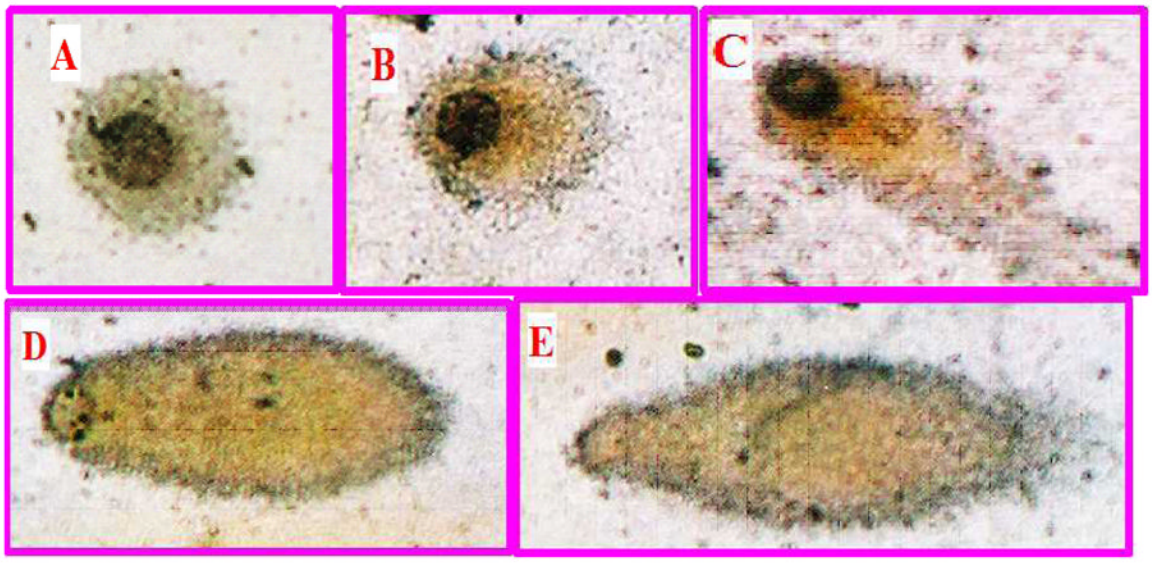

Figure 2.

Representative comet assay images of control and arsenic trioxide-treated HL-60 cells using the silver staining protocol, as mentioned in the Materials and Methods. A: Class 0 - control cell with no DNA damage and no tail (intact nuclei); B: Class 1 - comet cell with short tail (tail length less than the head diameter); C: Class 2 - comet cell with tail length longer than the head diameter and with low DNA damage; D: Class 3 - comet cell with tail length longer than the head diameter and with high DNA damage; and E: class 4 - comet cell with tail length longer than the head diameter and with high DNA damage and insignificant head.

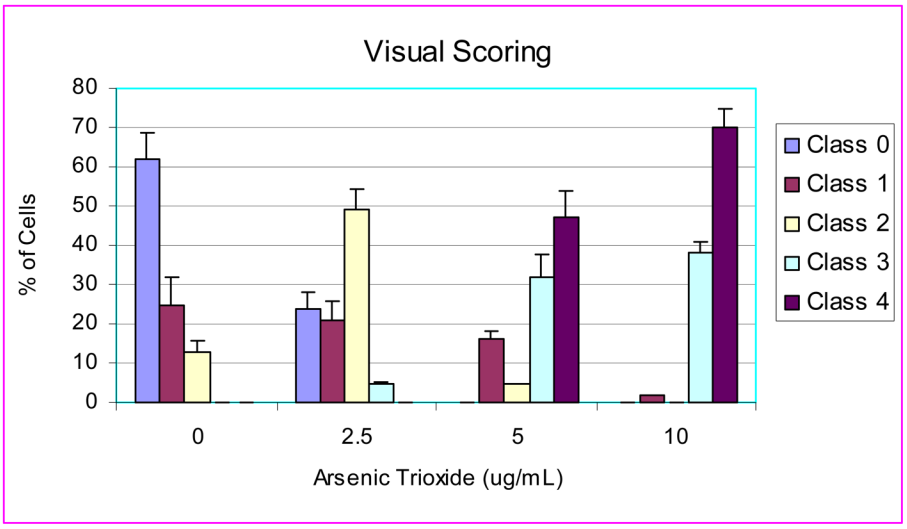

Figure 3.

Silver staining Comet assay data showing the mean percentages of cells in different comet classes as a function of arsenic trioxide doses (0, 2.5, 5, and 10 μg/mL). Each point represents a mean ± SD of 3 experiments. Comets were randomly selected from three replicated slides and grouped into five classes based on the degree of DNA damage and the comet tail length, as described in the Materials and Methods.

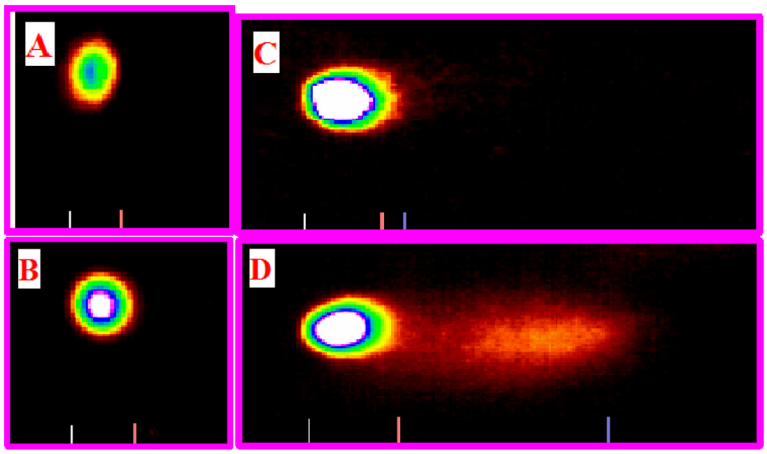

Figure 4.
Representative SYBR Green Comet assay images of untreated (A-control) and arsenic trioxide-treated HL-60 cells at 2.5 μg/mL (B), 5 μg/mL (C), and 10 μg/mL (D). High percentages (>60%) of selected images were observed in specific arsenic trioxide does, as indicated above.

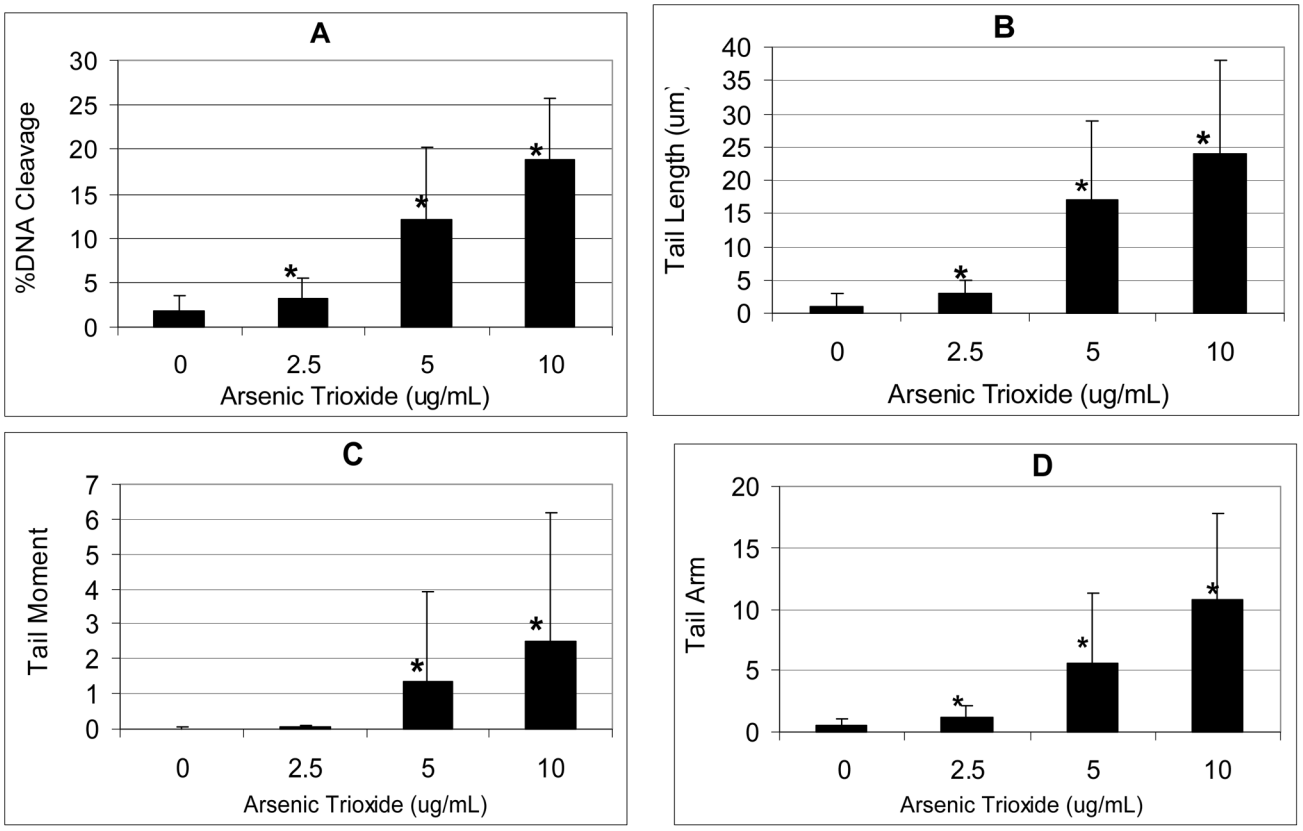

Figure 5.

Comet assay of HL-60 cells showing the percentage of DNA cleavage (A), tail length (B), tail moment (C), and tail arm (D) as a function of arsenic trioxide doses. Each point represents mean ± SD of 3 independent experiments. *Significantly different ($p < 0.05$) from the control, according to the Dunnett's test.