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ORIGINAL RESEARCH

Cytotoxicity patterns of arsenic trioxide exposure on HaCaT keratinocytes

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Background: Arsenic is a ubiquitous environmental toxicant, and abnormalities of the skin are the most common outcomes of long-term, low-dose, chronic arsenic exposure. If the balance between keratinocyte proliferation, differentiation, and death is perturbed, pathologic changes of the epidermis may result, including psoriasis, atopic dermatitis, and certain forms of ichthyosis. Therefore, research investigations using in vitro human epidermal cells could help elucidate cellular and molecular processes in keratinocytes affected by arsenic. Data from such investigations could also provide the basis for developing cosmetic intervention for skin diseases caused by arsenic.

Methods: The viability of HaCaT keratinocyte cultures with or without prior exposure to low-dose arsenic trioxide was compared for varying concentrations of arsenic trioxide over a time course of 14 days because in untreated control cultures, approximately 2 weeks is required to complete cell differentiation. Long-term cultures were established by culturing HaCaT cells on collagen IV, and cells were subsequently exposed to 0 parts per million (ppm), 1 ppm, 5 ppm, 7.5 ppm, 10 ppm, and 15 ppm of arsenic trioxide. The percentages of viable cells as well as DNA damage after exposure were determined on Day 2, Day 5, Day 8, and Day 14.

Results: Using both statistical and visual analytics approaches for data analysis, we have observed a biphasic response at a 5 ppm dose with cell viability peaking on Day 8 in both chronic and acute exposures. Further, a low dose of 1 ppm arsenic trioxide enhanced HaCaT keratinocyte proliferation, whereas doses above 7.5 ppm inhibited growth.

Conclusion: The time course profiling of arsenic trioxide cytotoxicity using long-term HaCaT keratinocyte cultures presents an approach to modeling the human epidermal cellular responses to varying doses of arsenic trioxide treatment or exposure. A low dose of arsenic trioxide appears to aid cell growth but concomitantly disrupts the DNA transcription process.

Keywords: arsenic trioxide, chronic exposure, DNA damage, HaCaT, keratinocyte, visual analytics

Introduction

Arsenic is a ubiquitous environmental toxicant, and abnormalities of the skin are the most common outcomes of long-term, low-dose, chronic arsenic exposure.[1–3] The common dermatological symptoms of arsenicosis are hyperpigmentation, hyperkeratosis, keratosis on the soles, keratosis on the palms, melanosis, hypopigmentation on the trunk and dorsal surface extremities,[4] and keratosis of the genitalia and eyelids.[2] The major routes of exposure are through inhalation and ingestion through food, drinking water, and medication.[5,6] Skin malignancies linked to arsenic include squamous cell carcinomas, basal cell carcinomas, Merkel cell carcinoma, intraepidermal carcinomas (Bowen disease),[7,8] and multiple cutaneous malignancies.[9,10]

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The maturation and differentiation of keratinocytes from the stem cells in the basal layer of the epidermis to the corneocytes or squamous cells at the outermost layer of the skin follow an organized system and take approximately 14 days.[11] The stratum corneum, which contains corneocytes and extracellular lipids secreted by differentiated keratinocytes, plays a vital immunologic role in the body, acting as a barrier against the entry of chemicals and microbes from the environment and protecting the body from dehydration.[12] The epithelial keratinocytes are the first cells to come into contact with arsenic through ingestion, inhalation, or direct contact, thus explaining why they are the most affected cells.

If the balance between keratinocyte proliferation, differentiation, and death is perturbed, pathologic changes of the epidermis may result, including psoriasis, atopic dermatitis, and certain forms of ichthyosis.[13] Therefore, research investigations using in vitro human epidermal cells could help elucidate cellular and molecular processes in keratinocytes affected by arsenic. Data from such investigations could also provide the basis for developing a cosmetic intervention for skin diseases caused by arsenic.

The HaCaT cell line is an immortalized human skin epithelial cell line that exhibits normal keratinocyte differentiation, which makes it a useful in vitro model system for determining regulatory mechanisms during human epidermal cell differentiation.[14] Inorganic arsenic is cytotoxic and genotoxic to keratinocytes and thus able to perturb normal human epidermal cell growth and differentiation.[15–18] Arsenic trioxide is an inorganic arsenic effectively used in the treatment of skin-related diseases such as psoriasis and syphilis-associated skin rashes.[19] Topical application of arsenic trioxide has been proposed as an effective and safe radiosensitizer for palliative radiotherapy for skin-infiltrating lesions of breast cancer.[20] Prolonged use of arsenic trioxide for the treatment of acute promyelocytic leukemia causes side effects including skin dryness, itching, or erythematous changes, and acute toxicity could lead to acute fluid retention, skin rash, kidney failure, and sudden death.[21]

The objective of the research was to compare the cytotoxic effect of various concentrations of arsenic trioxide on HaCaT keratinocytes with or without prior exposure to low-dose arsenic trioxide for ten passages. In this experiment, the chronic cell state was established by exposing HaCaT keratinocytes to a low dose (0.5 parts per million [ppm]) of arsenic over 10 passages, simulating how people in endemic areas are naturally exposed through drinking water. Long-term cultures were established by culturing HaCaT cells on collagen IV and were subsequently exposed to various concentrations (0 ppm, 1 ppm, 5 ppm, 7.5 ppm, 10 ppm, and 15 ppm) of arsenic trioxide. The DNA damage after exposure to various concentrations of arsenic trioxide was determined. Also determined were the percentages of viable cells on Day 2, Day 5, Day 8, and Day 14 cultures. The time course time points were selected based on Rea et al[22] in which the effect of arsenate on normal human epidermal keratinocytes were determined over a time course of approximately 2 weeks. Untreated control cultures maximally express differentiation markers in 14 days.[22] The dose (0.5 ppm) of arsenic selected for establishing chronic HaCaT culture was consistent with the range of arsenic levels in drinking water in endemic areas of Bangladesh, India, and Taiwan, which is from 0.05 ppm to 3.4 ppm.[23–25]

Material and methods

Chemical and reagents

Arsenic trioxide (99.9% purity, Fisher Scientific, Suwanee, GA), fetal bovine serum, Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered solution (PBS 1x) (American Type Culture Collection [ATCC], Manassas, VA), and streptomycin/penicillin antibiotics (Invitrogen, Carlsbad, CA) were purchased for culturing the HaCaT cell line. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) reagent was obtained from ATCC and collagen IV from Sigma Chemical Company (St Louis, MO).

Cell culture

HaCaT keratinocytes were kindly provided by Dr Van Wilson (Microbial and Molecular Pathogenesis, College of Medicine, Texas A&M Health Science Center, College Station, TX). Approximately 1.5×10^5 HaCaT cells were cultured in 7.5 mL of complete DMEM in T-25 culture flasks and incubated in a humidified atmosphere with 5% CO_2 at 37°C. The culture medium was replaced every 96 hours. The cells were split after growing to 90% confluence by aspirating the culture medium and washing the cell monolayer three times with sterile PBS. The cell monolayer was treated with 1 mL 0.25% trypsin-EDTA per plate and incubated briefly at 37°C. The cells were then viewed under the microscope to ensure that cells were completely detached and were resuspended in a complete DMEM medium. An aliquot of the HaCaT cell suspension was stained with 4% trypan blue for 1 to 2 minutes and counted with a hemocytometer. The cells used for chronic exposure were considered chronic after undergoing ten passages (cells trypsinized at 90% confluence and subcultured in another cultured flask). In this research investigation, acute cells are defined as the cells that were exposed to arsenic trioxide for the first time, whereas chronic cells are cells that have been exposed

continuously to up to ten passages with a very low-dose 0.5 ppm of arsenic trioxide.

Collagen coating

Collagen coating was performed using Kleinman's method:[26] 75 μL of collagen IV (30 ppm) was transferred to prechilled wells of 96-well tissue culture plates. The solution was evenly spread to completely cover the flat bottom of the wells. The plates were incubated at 37°C for 2 hours and washed three times with sterile PBS.

Cytotoxicity assay

Arsenic trioxide (arsenic trioxide dissolved in dilute nitric acid, 1 mL = 1 mg AS; Reference Standard Solution, 1000 ppm +/−1%/certified, 99.9% purity, Fisher Scientific) dilutions were prepared using a complete DMEM medium as the diluent. Eight different 96-well plates were used for the different time points: four different 96-well plates were labeled Day 2+AS, Day 5+AS, Day 8+AS, and Day 14+AS for the chronic cells treatment group; and an additional four different 96-well plates were labeled Day 2–AS, Day 5–AS, Day 8–AS, and Day 14–AS for the acute cells, cells without previous arsenic exposure (+AS = with arsenic and −AS = without previous arsenic exposure). Column 1 of the precollagen-coated 96-well plates was used for media alone, column 2 for media and cells (cells grown in culture medium without arsenic, which served as passage-matched control group), and columns 3–12 for the various concentrations of arsenic trioxide. Each of the concentrations was performed in triplicate. A total of 200 μL complete medium only was added to the first column, then 100 μL of HaCaT cell suspension in complete medium containing approximately 20,000 cells/well was added to column 3–12 wells of the precollagen-coated 96-well plates, 100 μL of medium was added to column 2, and 100 μL of arsenic trioxide concentrations was added to columns 3–12, ensuring that the final concentrations were 0, 1.0, 5.0, 7.5, 10, and 15 ppm, respectively. They were incubated in a humidified incubator at 37°C in a 5% CO_2 for a period of 2 days, 5 days, 8 days, and 15 days. Arsenic has a very short half-life of approximately 5 days;[27] consequently, medium and arsenic were replaced every 4 days to maintain arsenic concentration level and nutrient for the cells, thus removing any confounding factor that may come from depleted nutrients and cell metabolism waste.

At each of the time points, the culture plate was removed from the incubator and 10 μL of the yellow MTT reagent was added to each of the wells and incubated at 37°C for 4 hours until purple formazan precipitate was visible. Then 100 μL of the detergent reagent was added to each of the wells and kept in the dark at room temperature for 2 hours. The absorbance was measured at 570 nm wave length using a microtiter plate reader (Fluoroskan II microplate reader, Helsinki, Finland). LD_{10} and LD_{50} were determined from the dose-response curve. The experiment was repeated using chronic HaCaT cells (HaCaT that have been exposed to low-dose 0.5 ppm arsenic trioxide for up to ten passages).

Single gel electrophoresis (Comet Assay)

Single gel electrophoresis was performed to determine DNA damage in HaCaT cells when exposed to arsenic trioxide using a Comet Assay kit (Trevigen Inc, Gaithersburg, MD). The manufacturer's protocol was followed to conduct the experiment. About 1.5×10^5 HaCaT cells were cultured in 7.5 mL of complete DMEM in a T-25 culture flask and incubated in a humidified atmosphere with 5% CO_2 at 37°C. After growing to 80% confluence, the DMEM was aspirated and replaced with different concentrations of arsenic trioxide diluted in complete DMEM (0, 1, 5, 7.5, 10, and 15 ppm) and incubated for 48 hours. The untreated HaCaT Cells (0 ppm) served as the DNA damage control. After arsenic trioxide treatments, the medium was removed and the cells were washed three times with prechilled PBS and trypsinized with 1 mL of 0.25% trypsin-EDTA and then transferred to a centrifuge tube and counted. The cells were centrifuged at 3000 rpm for 5 minutes and washed once in ice-cold PBS.

The pellet was resuspended in ice-cold PBS at 1×10^5 cells/mL. The cells were mixed with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v), and 50 μL was immediately pipetted onto precoated CometSlide™ (Trevigen Inc, Gaithersburg, MD). The slides were placed flat in a refrigerator at 4°C in the dark for 30 minutes. The slides were immersed in a prechilled lysis solution and left on ice for 60 minutes. Excess buffers were drained from the slides and then immersed in a freshly prepared alkaline solution, pH > 13 (0.6 g of NaOH pellets, 250 μL of 200 mM EDTA, and 49.75 mL of dH2O) for 60 minutes at room temperature. Slides were washed twice for 5 minutes with 1X TBE (Tris-Borate-EDTA neutral buffer) electrophoresis buffer. The slides were aligned equidistant from electrodes in a horizontal gel apparatus and 1X TBE was added and electrophoresed at 1 V/cm (22 V) for 10 minutes. Excess TBE was tapped out and the slides were immersed twice in distilled water for 10 minutes and in 70% ethanol for 5 minutes. Excess 70% ethanol was removed and the slides were air dried overnight and stained with 100 μL of diluted SYBR Green and placed in the refrigerator for 5 minutes, removed, and tapped to remove SYBR Green.

Slides were allowed to air dry at room temperature in the dark. The slides were examined using an Olympus Epifluorescence Microscope, and the LAI's Automated Comet Assay Analysis System (LACAAS) (Loates Associates, Inc, Westminster, MD) was used to determine the extent of DNA damage by measuring the percent DNA in the tail (the integrated tail intensity × 100/the total integrated cell intensity for a normalized measure of the percent of total cell DNA found in the tail),[28] comet moment (% DNA in tail x tail length), and tail length (the distance of DNA migration from the body of the nuclear core).[28] Seventy comets were randomly selected from each slide for statistical analysis, and photographs were taken to show the changes in DNA morphology due to exposure to arsenic trioxide. Experiments were conducted in triplicate.

Statistical analysis

The absorbance values obtained per treatment from the MTT assay were converted to percentages of cell viability. Statistical analysis for differences in mean levels of arsenic trioxide was done using the Student's *t*-test for comparing two sample sets at $P < 0.05$. The data generated from the Comet assay were analyzed by using the two-sample *F*-test for variance of the treated and untreated (control) HaCaT cells and the significance level was $P < 0.05$.

Visual analytics

Visual analytics is increasingly used to analyze multidimensional biological datasets of all sizes and presenting them in an interactive visual display with one of the purposes being to identify patterns.[29–31] For example, paired analysis permits analysis within and between datasets. Tools available in the Tableau Public Software (http://www.tableausoftware.com/public) were used to visualize the cytotoxicity data produced from the experimental results.

Results

Long-term cultures were established by culturing HaCaT cells on collagen IV. MTT cytotoxicity assay was used to evaluate chronic toxicity of arsenic trioxide on keratinocytes. An LD_{10} value was obtained with a 1 ppm dose, and a dose of 0.5 ppm arsenic trioxide was used to establish the chronic HaCaT cells for up to ten passages. Two visual analytic representations of the cell viability data are presented in Figures 1 and 2. Figure 1 presents a time course for each dose, whereas Figure 2 presents a view comparing the exposure types for each time point and concentration. In addition, Figures 3 and 4 presents the cytotoxicity data as a bar chart with error bars shown. Overall, in both acute and chronic exposure, the HaCaT cell viability was dose dependent. Thus, an increase in dose resulted in decreased cell viability. The 1 ppm dose in the acute treatment cells resulted in 92.4% viability on Day 2, and cells continued to grow in the time course with viability rates of 93.4%, 107%, and 115.9% for Days 5, 8, and 14, respectively (Figures 1–3). The 5 ppm concentration resulted in viability rates of 30.8%, 36%, 46.7%, and 32.7% for Day 2, Day 5, Day 8, and Day 14 time points respectively.

Treatment doses of 7.5 ppm, 10 ppm, and 15 ppm inhibited growth of both acute and chronic cells (Figures 1 and 2). In the chronic treatment cells, the 1 ppm dose gave viability rates of 92.1%, 98.5%, 114.5%, and 120% for Days 2, 5, 8, and 14, respectively, whereas the 5 ppm gave viability rates of 42.9%, 45.9%, 67.1%, and 56.7% for Days 2, 5, 8, and 14, respectively (Figures 1, 2, and 4). The chronic cell had higher viability values that could resist the cytotoxic effect of the high doses of 7.5 ppm, 10 ppm, and 15 ppm. There was no significant difference at $P < 0.05$ when the chronic HaCaT cells were compared with the acute HaCaT cells at the various time points except for the 5 ppm dose using the Student's *t*-test. Additionally, there was no significant difference when the controls were compared with the 1 ppm dose in both acute and chronic cells at $P < 0.05$. However, there are significant differences at $P < 0.05$ on Day 2 between the controls and other treatment groups: 5 ppm, 7.5 ppm, 10 ppm, and 15 ppm in both acute and chronic cells.

Figure 2 illustrates a time course cell viability pattern, showing acute versus chronic exposure to arsenic trioxide. Chronic HaCaT cells were more tolerant to the 5 ppm dose compared with the acute HaCaT cells. A low dose of 1 ppm was observed to improve cell viability, whereas high doses above 5 ppm were toxic to both chronic and acute HaCaT cells.

Comet Assay was performed in HaCaT cells exposed to different concentrations of arsenic trioxide to determine the dose that has the highest genotoxic effect on HaCaT cells. Comet Assay was done using TBE, a neutral buffer that produces elongated comet shape, stained with SYBR® Green and images generated and measured automatically on a Loats System. The parameters measured in the 70 comets randomly selected per sample were the moment, comet tail length, and DNA damage. There was an increase in the length and rate of DNA migration in the HaCaT cells treated with arsenic trioxide when compared with the untreated control HaCaT cell.

The Comet Assay result shows a dose-dependent response to arsenic trioxide exposure as shown in Figure 4. The percentage

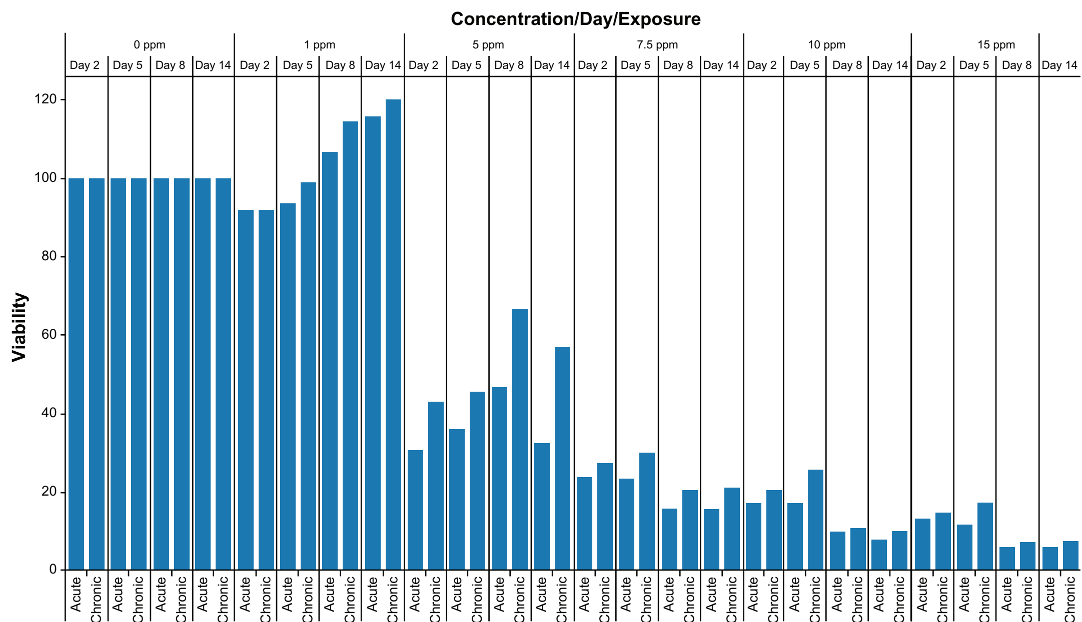

Figure 1 Dose- and time-dependent response to the cytotoxic effect of arsenic trioxide on HaCaT keratinocytes. Interactive visual analytics resource for image available at http://public.tableausoftware.com/views/hacat_arsenic/viability_timecourse.

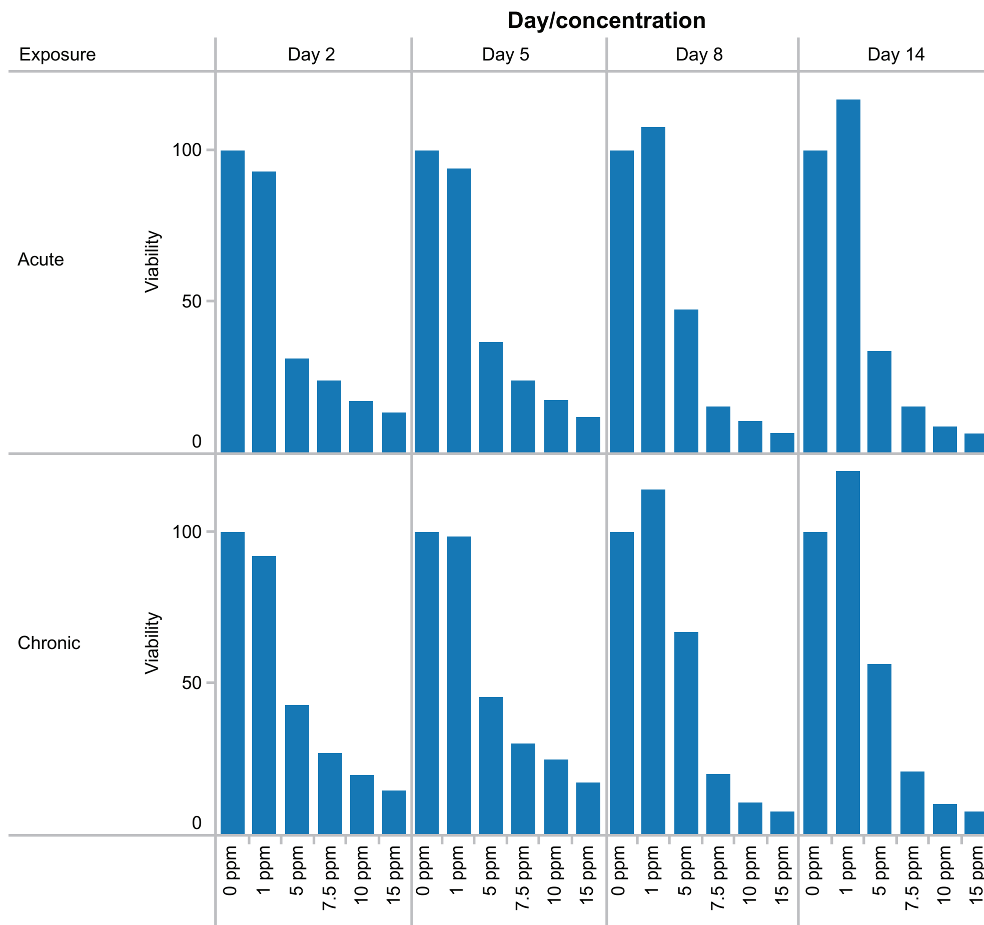

Figure 2 Time course cell viability pattern showing acute versus chronic exposure to arsenic trioxide. Both the acute and chronic HaCaT cells tolerated the 1 part per million (ppm) dose, but the chronic cells had higher viability with the doses ≤5 ppm. Interactive visual analytics resource for image available at http://public.tableausoftware.com/views/hacat_arsenic/viability_figure.

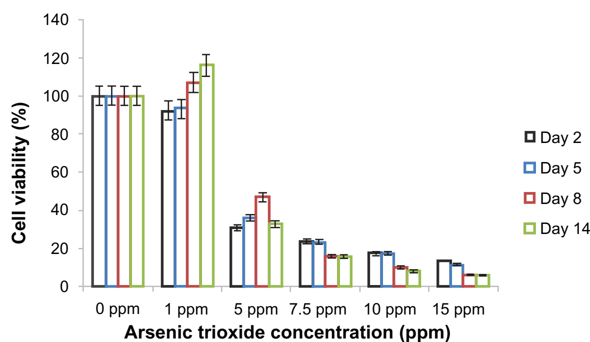

Figure 3 Dose- and time-dependent response of HaCaT keratinocytes without prior exposure to low dose (0.5 parts per million [ppm]) of arsenic trioxide over ten passages.

DNA damage results were 0.27%, 3.45%, 3.22%, 4.0%, 7.5%, and 11% for 0 ppm, 1 ppm, 5 ppm, 7.5 ppm, 10 ppm, and 15 ppm, respectively. There was a significant difference in DNA damage between the control and the treatment doses at ($P < 0.05$) using two-sample F-test for variance. A similar pattern was observed for comet moment and comet tail length results. Images representing the comets observed are shown in Figure 5 and values in Table 1. In Figure 5, panel A represents a comet from the untreated cell control, moment 0.0, % DNA 0.08, length 0.0; panel B represents 1 ppm, moment 0.04, % DNA 2.1, length 5; and panel C represents 15 pp, moment 8.18, % DNA 28.62, length 73.

Discussion

We have established chronic cultures of HaCaT cells treated with 0.5 ppm arsenic trioxide up to passage ten and subsequently exposed the chronic HaCaT cells and untreated control HaCaT cells to higher doses of arsenic trioxide. Visual analytics software was used to construct interactive visualizations of the acute and chronic exposures at the various time points (Figures 1 and 2) and to demonstrate the genotoxic effect of arsenic on HaCaT cells (Figures 5 and 6). The visual analytics resources in this report, unlike the standard static figures, will allow for user-defined queries beyond those reported here. The visualization could help generate hypothesis from novel insights on the patterns of arsenic cytotoxicity as the cells mature and differentiate from basal cell to squamous cells.

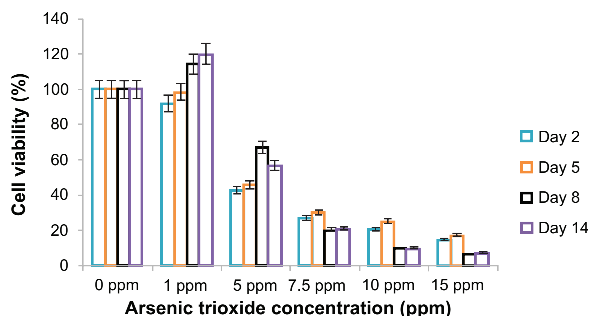

Figure 4 Dose- and time-dependent response of HaCaT keratinocytes with prior exposure to low dose (0.5 parts per million [ppm]) of arsenic trioxide over ten passages.

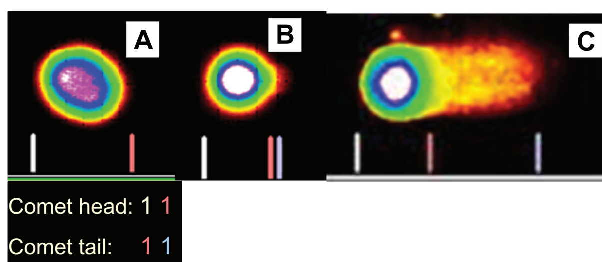

Figure 5 Representative comets from exposure of HaCaT keratinocytes to concentrations of arsenic trioxide. Panel A represents a comet from the untreated cell (control), Panel B represents 1 ppm, and Panel C represents 15 ppm treated cells.

Our results confirmed that arsenic trioxide is cytotoxic to HaCaT cells.[17] We observed a biphasic response at a 5 ppm dose with cell viability peaking on Day 8 in both chronic cells and acutely exposed cells. It is striking that a low dose of 1 ppm arsenic trioxide enhanced HaCaT keratinocyte proliferation and doses above 7.5 ppm inhibited growth. This observation concurs with previous reports,[17,32] but the time course profiling of arsenic trioxide cytotoxicity using long-term HaCaT cell cultures provides an approach to modeling the human epidermal cellular response to varying doses of arsenic trioxide exposure.

At low concentrations, arsenic trioxide regulated the expression of cell cycling pathway genes such as cyclin-dependent kinase 4 and transcription factor 1,[16] and this may explain the observation with the 1 ppm dose. Additionally, in our previous microarray gene expression investigation,[33] long-term exposure of HaCaT cells to low-dose 0.5 ppm of arsenic trioxide up to 22 passages enhanced the upregulation of insulin growth factor-like family member 1 (IGFL1), which was confirmed with the quantitative reverse transcription polymerase chain reaction method.

Table 1 Summary of Comet Assay data showing percentage DNA damage of HaCaT cells exposed to arsenic trioxide

Concentration (ppm)	% DNA	Length	Moment
0	0.27	2.00	0.26
1	3.45	3.00	0.97
5	3.22	4.50	1.03
7.5	4.00	4.50	5.00
10	7.50	6.50	2.34
15	11.00	10.00	9.00

Note: Data in table can be downloaded at: http://public.tableausoftware.com/views/hacat_arsenic/comet_table.
Abbreviation: ppm, parts per million.

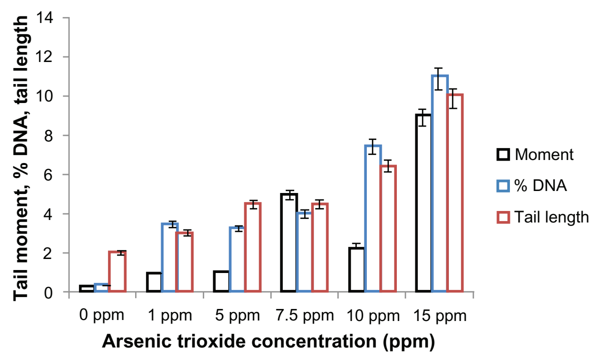

Figure 6 Genotoxic effect of arsenic trioxide on HaCaT keratinocytes. Comet Assay was performed in HaCaT keratinocytes exposed to different concentrations of arsenic trioxide. The % DNA and tail length increased as the concentration of arsenic trioxide increased.
Abbreviation: ppm, parts per million.

IGFL1 similar to other related IGF is involved in cellular energy metabolism, growth and development, and promotion of cell division.[34] IGFL1 is known to be upregulated in skin conditions such as psoriasis that promote the abnormal proliferation and differentiation of epidermal keratinocytes.[35,36] Additionally, the observed increase in cell proliferation at a 1 ppm dose could be attributed to induction of cyclin D1 transcription by low-dose arsenic as reported by Hwang et al.[37] Cyclin D1 stimulates growth by shifting the G1 growth phase into the S/G2 cell cycle compartment. A similar observation had been made with arsenic trioxide on porcine aortic endothelial cells, in which low concentrations stimulated cell proliferation with increases in superoxide and hydrogen peroxide (H_2O_2) accumulation, H_2O_2-dependent tyrosine phosphorylation, and NF-κB-dependent transcription.[38]

There was a significant difference between the control cell and the 1 ppm dose in the Comet Assay ($P < 0.05$). Our results show that arsenic trioxide has differential effects on cell growth and DNA damage depending on its concentration.[3,39] Arsenic dose-dependent effects have been observed on telomerase activity where low doses result in an increase in activity observed as elongated telomere length and promotion of cell proliferation, whereas at high doses of arsenic, telomerase activity is reduced with reduced telomere length and cell death induction.[18] Telomere homeostasis is critical in maintaining chromosome and genomic stability.[40,41] In normal nontransformed normal human epidermal keratinocytes (NHEK) Comet assay, arsenite was reported to induce cell proliferation by increasing redox-related gene expression and decreasing DNA repair.[42] Therefore, low concentrations of arsenic trioxide can significantly enhance HaCaT keratinocyte proliferation.

Conclusion

Long-term exposure of HaCaT cells to low-dose ≤1.0 ppm of arsenic trioxide may enhance cell proliferation, but higher doses are cytotoxic. A low dose of arsenic trioxide appears to aid cell growth but concomitantly disrupts the DNA transcription process.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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