EVALUATION OF THE PROTECTIVE EFFECT OF GALLIC ACID AGAINST ARSENIC-INDUCED GENOTOXICITY IN HEPG2 CELL LINE

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ABSTRACT

Objective: Arsenic has cytotoxic as well as mutagenic effect in human health due to its indirect effect on oxidative stress on the cells. We aimed to find out the effect of gallic acid (GA), a well-known natural antioxidant in ameliorating in heavy metal toxicity.

Methods: MTT assay was performed to determine the cytotoxicity of sodium arsenite (NaAsO$_2$) on HepG2 cells with the cytoprotectant GA at varying concentrations for exposure durations of 6 h, 12 h, and 24 h. Similarly, the alkaline version of the comet assay was performed to investigate the genotoxicity and assessment of oxidative stress of the cells using flow cytometry.

Results: Cells treated with NaAsO$_2$ at various doses spanning a broad range of concentrations (5–500 μM) showed a dose- and time-dependent decrease in cellular viability as observed. However, the effect of the proposed protectant, GA showed an increase in cellular viability in a concentration-dependent manner.

Conclusion: We assessed the cytotoxicity and genotoxicity induced by NaAsO$_2$, to provide insight into the role of GA on arsenic-induced toxicity in liver cells and to shed light on its possible ameliorative effect at low concentrations in a time-dependent manner.

Keywords: Arsenic toxicity, Gallic acid, MTT assay, Comet assay, Flow cytometry.

INTRODUCTION

Arsenic (As) is a semi-metallic compound that occurs naturally in the earth’s crust. It is primarily found in environmental sources such as soil, rocks, water, and air and occurs in combination with other elements. Its inorganic forms, however, are highly toxic. Arsenic is one of the major chemical contaminants as stated by the World Health Organization (WHO) [1]. The primary source of arsenic toxicity is found to be water sources [2]. The occurrence of increased levels of arsenic is found in groundwater sources as compared to surface water sources such as rivers and lakes. This could occur due to contaminated groundwater caused by the accumulation of arsenic compounds on the bedrock, which further enters water sources through erosion [3]. High levels of arsenic in groundwater are found to be prevalent worldwide. Arsenic exposure through drinking water has been reported in many countries of the world [4]. The provisional guideline value for the high risk of arsenic in drinking water is 10 μg/mL, as stated by the WHO. However, in India, its permissible levels are 0.05 mg/L [5]. Depending on the type of exposure to arsenic compounds, acute and chronic effects are typically observed. The acute exposure to arsenic in the form of larger doses is rare and can result in immediate clinical manifestations such as vomiting, diarrhea, abdominal pain, and muscle cramping [6]. Chronic effects (>50 μg/mL) of arsenic, however, manifest in the form of liver diseases and can further contribute toward the development of bladder, skin, lungs, and liver cancer [7]. Associations between the exposure of arsenite during pregnancy and its effect on infant health and mortality have been correlated to studies related to impaired cognitive development [8].

Toxicity to arsenic compounds such as arsenate (As IV) impairs cellular interactions and energy production through molecular mimicry, whereas arsenite (As III) can exhibit cytotoxic effects by binding directly to sulfur bonds and cause the additional generation of reactive oxygen species (ROS) [9]. Genotoxic effects of arsenic include the formation of strand breaks in the deoxyribonucleic acid (DNA). Meta-arsenite is also shown to bind to the repeat regions of the telomere and causes erosion of the repeats and inhibition of cell proliferation [10]. Oxidative stress as mentioned is found to be one of the prime causes of arsenic-induced toxicity. This has led to the search for effective antioxidants that can counter the stress induced by highly reactive xenobiotic agents [11]. Pharmacologically active compounds or dietary sources are ideal for the protection or treatment against metal-induced toxicity [12]. One such compound is gallic acid (GA) which is a polyphenolic compound that exhibits high antioxidant properties compared to physiologically active and synthetic antioxidants [13]. GA is said to possess strong scavenging activity against reactive free radicals and leads to an increase in intracellular antioxidant capacity. In this study, arsenic-induced toxicity in a tissue-specific in vitro model of hepatocytes-HepG2 cells was studied due to the efficient methylation potential of arsenic compounds in the liver. The arsenic compound used in this study was sodium arsenite (NaAsO$_2$) to evaluate its cytotoxic and genotoxic potential and also explored the possible amelioration of arsenic toxicity with the help of GA, to test for any protective potential in terms of cytotoxicity, genotoxicity, and oxidative stress.

METHODS

Chemicals

NaAsO$_2$, GR was purchased from Romali, American Preparate, India. Dulbecco’s Modified Eagle’s Medium (DMEM) and 0.1% Trypsin were obtained from HiMedia, fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), ethidium bromide (EtBr), Agarose, Triton X-100, and dichlorodihydrofluorescein diacetate (DCFH-DA) dye were purchased from Sigma-Aldrich, Bangalore, India. GA and dimethyl sulfoxide (DMSO) as purchased from SRL Chemicals, Pvt. Ltd., India, and Merck Life Sciences Pvt. Ltd., India, respectively.
Cell culture and maintenance of HepG2 cell lines

HepG2 cell line was acquired from the National Centre for Cell Science, Pune, which was grown in DMEM with 10% FBS as a supplement. Cells maintained in T-25 and T-75 flasks were supplemented with the fresh medium until 70% confluency was obtained and they were subcultured every 2–3 days. Exponentially growing HepG2 cells were used for the following assays.

Preparation of test solutions

NaAsO$_2$ was dissolved in autoclaved Milli-Q water to obtain a stock of 100 mM and constituted with DMEM + 10% FBS. GA was dissolved in the organic solvent, dimethyl sulfoxide, and DMSO (0.02%), and a further stock solution of 100 mM was made up with DMEM supplemented with 10% FBS. Further dilutions of NaAsO$_2$/GA were prepared from the stock solution in culture medium for all the assays.

Assessment of cytotoxicity induced by NaAsO$_2$ by MTT assay

MTT assay was performed to determine the cytotoxicity of NaAsO$_2$ on HepG2 cell lines. The classic protocol used was modified to suit the present experiment [14]. HepG2 cells (1 × 10$^4$) were seeded into each well of a 96-well plate (Greiner, CellStar, India) and allowed to adhere for about 24 h in 5% CO$_2$ incubator. After the attachment of the cells to the plate, the media were then replaced with varying concentrations of NaAsO$_2$ (As III) ranging from 5 to 500 µM and were incubated for different durations, i.e. 12, 24, and 48 h. Following the incubation at different time points, arsenite-containing media were then replaced with 100 µL MTT (5 mg/mL) and incubated for a minimum of 3 h at 37°C. Crystals of formazan formed in purple color were solubilized by the addition of 100 µL of DMSO. The optical density (OD) was then detected at 570 nm with a reference wavelength of 630 nm, using a multiwell plate reader (Tecan, Austria) and measured using the software Magellan. Cell viability was calculated as follows:

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\% \text{ Viability} = \frac{\text{Test OD}}{\text{Control OD}} \times 100
\]

Effect of GA on cellular viability by arsenite-induced toxicity assessed by MTT assay

Parallel to the previous method, 1 × 10$^4$ cells were seeded into each well of a 96-well plate and allowed to adhere for about 24 h in a humidified 5% CO$_2$ incubator. Cells were treated in batches as per the following scheme:

- **Group A:** Treatment of cells with GA alone in concentrations ranging from 0.5 to 30 µM for 6 h, 12 h, and 24 h, respectively.
- **Group B:** Pre-treatment of cells with GA in a range of concentrations (0.5–30 µM) for 6 h, 12 h, and 24 h, respectively. Further treatment with a sublethal dose of arsenite (25 µM) for 48 h.
- **Group C:** Pre-treatment of cells with GA in concentrations ranging from 0.5 to 30 µM for 6 h, 12 h, and 24 h. Further treatment with IC$_{50}$ dose of arsenite (~50 µM) for 48 h.
- **Group D:** Controls – positive controls – mitomycin, negative control – DMSO, and individual doses arsenic (25 µM and 50 µM) were added to the HepG2 cells.

Combination MTT was performed using a pre-treatment of HepG2 cells with the proposed cytoprotectant, GA at varying concentrations ranging from 0.5 to 30 µM for exposure durations of 6 h, 12 h, and 24 h. Following different pre-treatments, the medium was replaced with arsenite in sets of two concentrations:

1. A sublethal dose of arsenite – 25 µM against GA concentrations ranging from 0.5 to 30 µM
2. IC$_{50}$ dose of arsenite – 50 µM against GA concentrations ranging from 0.5 to 30 µM.

The cells were treated with arsenite for 48 h, following which the medium was replaced with MTT reagent (5 mg/ml) and incubated at 37°C for 3 h. The purple crystals of formazan were solubilized in 100 µL of DMSO. The optical density was then detected at 570 nm with a reference wavelength of 630 nm, using a spectrophotometer (Tecan, Austria) and measured using the software Magellan. The readings were analyzed, and the percentage viability was calculated as described earlier.

Estimation of arsenite-induced genotoxicity by comet assay

The alkaline version of the comet assay was performed to investigate the DNA damage/genotoxicity caused by the toxicant – NaAsO$_2$ referred from the modified protocol [15,16]. Alkaline conditions enabled the detection of both single-strand and double-strand breaks in the genome. HepG2 cells exhibiting exponential growth were seeded into 6 cm culture dishes (Cell Star, Sigma, USA) and treated with a range of arsenite concentrations (10–50 µM). A batch of cells was treated with 200 µM H$_2$O$_2$, as a control, for the standardization of the electrophoresis run time. A combination of the proposed protectant GA as pre-treatment for 6 h followed by arsenic treatment for a duration of 24 h was performed for comet analysis.

Following treatment, cells were harvested by trypsinization and the comet slides were prepared. Briefly, clean glass slides were coated with a thin layer of 1.5% normal melting agarose at 37°C. Approximately 4 × 10$^3$ cells were harvested and centrifuged at 155 × g for 5 min following a phosphate-buffered saline wash. The pellet obtained was resuspended in 200 µL of 0.75% low melting agarose which was mixed well to form the second layer and placed on ice. About 150 µl of 0.75% low melting agarose without cells was used to form the third layer correspondingly. After solidification, agarose-embedded glass slides were immersed in a 24°C lytic working solution (2.5 M NaCl, 100 mM Tris base, 0.2 M NaOH, and 100 mM ethylenediaminetetraacetic acid [EDTA], DMSO, and trypsin, overnight at 4°C).

The slides were then transferred carefully to an alkaline electrophoresis buffer (10 N NaOH and 200 mM EDTA-pH 13) for 30 min and gel electrophoresis was performed at 19 V, 300 mA, further for a range of time points between 18 and 30 min. A neutralization buffer (0.4 M Tris at pH 7.9) was then used to neutralize the slides and was briefly dehydrated with 70–90% alcohol. The glass slides were stained under a reduced light with EthBr (2 mg/ml).

The samples were further visualized at ×40 using a fluorescence microscope (Olympus Microscopes, Japan) at 525 nm excitation filter and the images were captured using a CCD camera using the software CellSens (CellBens B.V., Netherlands). Comet features such as % DNA and olive moment were scored for each sample and analyzed using the Open Comet tool using ImageJ software (Wisconsin, USA).

Assessment of oxidative stress of the cell (ROS production) by flow cytometry

ROS generated by the cell in response to arsenic and the oxidative effect of GA on arsenic-induced toxicity was estimated by the ROS assay protocol outlined by Satish Rao et al. [17] with minor modifications. Exponentially growing cells were seeded at a density of 4–5 × 10$^4$ cells per well in a 6-well cell plate (CellStar, Sigma, USA). Following 24 h of culture initiation, the cells were treated in batches in the following manner:

1. As treatment: Cells were treated with increasing doses of NaAsO$_2$ in a range of concentrations (10–50 µM) for 24 h.
2. Combination of GA + As: Two concentrations of GA were used, 5 µM and 20 µM as a pre-treatment for 6 h and 12 h against a sublethal dose of arsenite, 25 µM, for 24 h.

After the respective treatment of the cells, the media were discarded, and the cells were washed with 1 ml of PBS to remove any residual serum which could interfere with the binding of the dye DCFH-DA to the cells. A working solution of 5 µM of DCFH-DA dye was prepared from a stock of 10 mM, dissolved in DMEM (serum-free). Roughly 3 ml of the working dye solution was dispensed into the wells and incubated for 30 min in a humidified CO$_2$ incubator. Following incubation, the dye solution was discarded, and the cells were washed briefly with 1 ml of PBS. The cells were harvested by mild trypsin treatment of 300 µl and centrifuged at 1200 rpm (155 × g) for 1 min. The pellet was washed with PBS and the final pellet obtained was further resuspended.
Comet assay enabled the assessment of the genotoxicity induced by arsenite. Estimation of arsenite-induced genotoxicity by comet assay did not show any significant difference in cell viability (25 µM), following which there was a resultant decrease in cellular viability. GA was found to be at 5 µM treated with a sublethal dose of arsenite (25 µM), following which there was a resultant decrease in cellular viability (Fig. 3a). However, the effect of GA on a higher dose of arsenic did not show any significant difference in cell viability (Fig. 3b).

Effect of GA on cellular viability by arsenite-induced toxicity assessed by MTT assay
The effect of the proposed protectant, GA was also evaluated by the MTT assay in a dose-dependent (5–50 µM) and time-dependent increase in cellular viability, as shown in Fig. 1. The IC50 value of arsenite was estimated to be between 40 and 60 µM for an exposure duration of 24–48 h. Log scale of arsenite concentration was taken for this estimation. The IC50 for the three time points (12, 24, and 48 h) was estimated to be 506 µM, 143 µM, and 47.63 µM, respectively.

Combinatorial treatment of GA and NaAsO2 showed a significant increase in cellular viability compared to the cells treated with no significant differences observed between the control and the treatments. It was observed from the graph that 12 h showed maximum cellular viability and GA was observed to show negligible cytotoxic effects on the cell line by its own (Fig. 2).

Combimolar treatment of GA and NaAsO2 with a pre-treatment of 0.5–30 µM followed by a sublethal dose of arsenite (25 µM) showed a significant increase in cellular viability compared to the cells treated with arsenite alone (Fig. 3a) (p<0.01). The optimum concentration of GA was found to be at 5 µM treated with a sublethal dose of arsenite (25 µM), following which there was a resultant decrease in cellular viability (Fig. 3a). However, the effect of GA on a higher dose of arsenic did not show any significant difference in cell viability (Fig. 3b).

Estimation of arsenite-induced genotoxicity by comet assay
Comet assay enabled the assessment of the genotoxicity induced by NaAsO2 in varying doses for different time points against a positive control, H2O2. The electrophoresis run time and time of exposure of arsenite were standardized to be 20 min and 24 h, respectively. The % head and tail DNA induced by NaAsO2 were also quantified (Fig. 4). Combination treatment of GA and arsenite was assessed by comet assay, wherein 5 µM of GA showed a significant reduction in head and tail DNA % and olive moment (Fig. 5) (p<0.001).

Assessment of the oxidative stress of the cell (ROS production) by flow cytometry
The oxidative state of the cell was evaluated in terms of ROS production by the binding of the fluorescent probe DCFH-DA and its oxidation by ROS to a reduced state that could be quantified with the help of fluorescence detection by fluorescence-activated cell sorting, which can measure the ROS production of cells individually. The treatment of cells with increasing doses of arsenic induced an increase in the ROS production, measured in terms of mean fluorescence intensity (Fig. 6) (p<0.0001). Combinatorial treatment, on the other hand, resulted in a decrease in mean fluorescence intensity in comparison to the treatment with the toxicant by itself (Fig. 7).

DISCUSSION
Arsenic toxicity is one of the major heavy metal toxicities commonly observed due to several environmental and anthropogenic activities. It enters biological systems through the oral route, mainly through the ingestion of arsenic-laden water and in rare cases through surface contact with the skin. Its exposure can cause some adverse effects on human health and is a major contributor to the carcinogenesis of vital organs such as the liver, lungs, bladder, and skin [18]. Its metabolism in biological systems through the one-carbon and transsulfuration pathway leads to oxidative stress in cells, imparting its indirect effect on the cellular and DNA levels [19]. The use of antioxidants such as GA that is naturally derived can be administered through dietary supplements to combat such heavy metal toxicity or serve as a protectant [20].

This study assesses the cytotoxic and genotoxic potential of arsenic on HepG2 cell lines taken as an in vitro model. Cytotoxicity induced by NaAsO2 was assessed with the help of a classic cell viability assay that employed the use of MTT reagent. The IC50 of arsenite was estimated to be around 40–60 µM for an exposure duration of 24–48 h which was found to be in concordance with a previous study [21]. The equivalent dose in humans for the range of doses taken is around 5–10 µg/L. This indicates that chronic dosing of arsenic on cell lines led to an increase in cell death. As stated by Watanabe et al. [22], high concentrations of arsenite could lead to the inactivation of methyltransferases that are required in arsenic metabolism and further downstream detoxification processes. Cells treated with increasing doses of GA alone for 6–24 h showed an increase in cellular viability (Fig. 2) which was found to be non-toxic at all concentrations used. On the contrary, in the combinatorial MTT experiment, it was observed that cells which were
Fig. 2: Bar graphs showing the changes in percentage of cells on the treatment with gallic acid (GA) (5–30 µM) for 6 h, 12 h, and 24 h. GA was found to be non-toxic at all concentrations and in increasing the duration of exposure.

Fig. 3: The effect of a pre-treatment of gallic acid in concentrations ranging from 0.5 to 25 µM for 6 h followed by (a) 25 µM and (b) 50 µM NaAsO$_2$ for 24 h. A significant observation was found until about 5 µM in the 24 µM treatment (*p<0.01)

Fig. 4: Bar graphs showing a change in % head and tail DNA and the olive moment when treated with arsenite at 40 µM, compared against a positive control – H$_2$O$_2$ (200 µM) treated for 24 h. Significant differences were observed between treated and control groups in terms of variations in % head and tail DNA and subsequent reduction in an olive tail moment (p<0.0001)

Fig. 5: Comet analysis of different treatment groups along with the combination of gallic acid (5–25 µM) + 25 µM of NaAsO$_2$, showing variations in (a) % head DNA, % tail DNA; (b) olive moment as a measure of genotoxicity. The relative decrease in comet head and a relative decrease in comet tail % DNA was found to be statistically significant at 5 µM GA + 25 µM (**p<0.0001)

Preconditioned with GA followed by treatment with arsenite depicted a significant increase in cellular viability, in comparison to the cells treated with arsenite alone (Fig. 3). Higher doses of arsenite along with increasing concentrations of GA, on the other hand, did not show any significant decrease in cell viability.

Similarly, as shown in other toxicity studies, it is observed that an increase in concentrations of the pure form of GA could lead to further cell death, due to its pro-oxidant activity after a threshold concentration [23, 24].
The study of antioxidants such as GA to mitigate arsenic-induced toxicity has also been recently reported in the previous toxicology studies. A study conducted by Gholamine et al. [26] showed that GA was found to target organs such as the liver and kidney specifically that can aid in its amelioration and alteration in biochemical parameters which reduce its toxic effects. Thus, the antioxidantive potential of GA proves to be substantial against arsenic toxicity. Other studies such as combination therapy, by the utilization of chelating agents and antioxidants, have proven to be effective in toxicity studies [27].

Thus, the combination therapy may serve as a suggested therapeutic for arsenic toxicity, in replacement for the commonly used and less efficient treatment options.

**CONCLUSION**

We assessed the cytotoxicity and genotoxicity induced by NaAsO₂ to provide insight into the role of GA on arsenic-induced toxicity in liver cells and to shed light on its possible ameliorative effect at low concentrations in a time-dependent manner. The antigenotoxic potential was also observed significantly with the use of GA. This naturally occurring compound may prove to be of therapeutic value for the treatment of various liver diseases. However, due to its low threshold for non-cytotoxic effects, further research using specific models that investigate the mechanism and biochemistry of these compounds is required to be done, to explore the definite role of GA in arsenic-induced toxicity.

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**AUTHORS’ CONTRIBUTIONS**

Nidhya Teresa Joseph contributed to conducting experiments, collection of data, and preparation of manuscript. Moka Rajasekhar, conceptualization of the article, guidance, and feedback.

**CONFLICTS OF INTEREST**

We declare that we have no conflicts of interest.

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We assessed the cytotoxicity and genotoxicity induced by NaAsO₂ for arsenic toxicity, in replacement for the commonly used and less efficient treatment options.

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