The Hormetic Effect of Arsenic Trioxide on Rat Pulpal Cells: An In Vitro Preliminary Study

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Abstract

Objectives Despite the agreement that there is no longer any indication for arsenic use in modern endodontics, some concerns are surfacing about the minute amount of arsenic trioxide (As₂O₃) released from Portland cement-based materials. The present study investigated the effect of different concentrations of As₂O₃ on rat pulpal cells and the efficacy of N-acetylcysteine (NAC) in preventing As₂O₃-mediated toxicity.

Materials and Methods Cytotoxicities of 50, 10, or 5 µm As₂O₃ and the effect of cells co-treatment with 50 µm As₂O₃ and 5,000 µm NAC or 500 µm NAC were tested at 24 hours or 3 days. Cell viability was assessed by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and cellular morphological changes were observed under phase contrast microscope.

Statistical Analysis Two-way analysis of variance with Tukey’s post-hoc test was used to evaluate differences between the groups (α = 0.05).

Results At both exposure times, 50 µm As₂O₃ resulted in lower optical density (OD) values when compared with 10 or 5 µm As₂O₃. At 24 hours, 10 µm As₂O₃ resulted in a higher OD value compared with the control; however, at 3 days the difference was statistically insignificant. At each exposure time, the OD value of 5 µm As₂O₃ group was comparable to the control and 10 µm As₂O₃ group. There were no significant differences between 50 µm As₂O₃ group and 500 µm NAC+50 µm As₂O₃ group; however, these two groups had lower OD values when compared with 5,000 µm NAC+50 µm As₂O₃ group at 24 hours and 3 days. The latter group showed significantly lower

Keywords ► arsenic ► heavy metal ► hormesis ► N-acetylcysteine ► pulp cells ► toxicity

DOI https://doi.org/10.1055/s-0040-1718637
ISSN 1305-7456.

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Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India
Introduction

Arsenic is a natural element of the earth's crust and a class I human carcinogen that is readily absorbed from the gastrointestinal tract. The acute signs of arsenic toxicity are manifested as nausea, vomiting, abdominal pain, encephalopathy, and neuropathy, while chronic exposure results in numerous types of cancer including skin, bladder, lung, and liver cancers. Inorganic arsenic compounds occur in trivalent (As\(^{3+}\)) and pentavalent (As\(^{5+}\)) forms with the former being the most toxic type in the form of arsenic trioxide (As\(_2\)O\(_3\)). Currently, concerns are shifting to the health effects of low doses of arsenic and this has put the question of how very low arsenic exposure may affect health under scrutiny. The environmental protection agency states that any exposure to a carcinogen, no matter how small, increases cancer risk to some degree. This had led to phasing out of arsenic commercial use in agriculture and lowering the standards of its commercial use in agriculture and lowering the standards of its amount in drinking water.

Historically, arsenic played an important role in endodontics for pulp devitalization and the treatment of sensitive teeth. Due to severe vital tissues damage caused by arsenic and the improvement in local anesthesia techniques, this practice has declined dramatically. Evidently, there is no longer an indication for arsenic use in today's dental practice, and its continued use is viewed as unjustified practice that must be condemned and prohibited. Notwithstanding the unique properties of NAC in protecting cells from damage, thus heightening glutathione level inside the cells. Due to the hormetic effect induced by low As\(_2\)O\(_3\) concentrations should be interpreted with caution. NAC did not prevent As\(_2\)O\(_3\)-mediated toxicity; however, it demonstrated potential for ameliorating this toxicity.

Conclusions

As\(_2\)O\(_3\) displayed a hormetic effect on pulpal cells; however, the proliferative effect induced by low As\(_2\)O\(_3\) concentrations should be interpreted with caution. NAC did not prevent As\(_2\)O\(_3\)-mediated toxicity; however, it demonstrated potential for ameliorating this toxicity.
Materials and Methods

Cytotoxicity Test and Cell Morphology

The clonal cell line (RPC-C2A) established from dental pulp of rat incisors was used in the present study. The culture medium consisted of Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (Biowest; Instant Sterile Fetal Bovine Serum, Rue de la Caille, Nuaille, France) and antibiotic solution (60 µg/mL of kanamycin). Cultures were supplied with fresh medium every other day, and incubated in a humidified atmosphere of 95% air and 5% CO2, and maintained at 37°C. Confluent cells were detached with a mixture of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid.

In the first experiment, four types of solutions were prepared using cell culture medium for the cytotoxicity testing: (a) 50 µM As2O3 (FUJIFILM; Wako Pure Chemical, Osaka, Japan); (b) 10 µM As2O3; and (c) 5 µM As2O3. To each well of 24-well culture plates, 5×10^4 cells were placed and incubated for 24 hours in a 5% CO2 incubator at 37°C. Six wells were allocated for each test solution. An aliquot of 300 µL of each experimental solution was added to each well and incubated in a 5% CO2 incubator at 37°C for either 24 hours or 3 days. Cell culture in fresh medium without experimental solution served as the control. After the incubation times, cell culture medium was discarded and cells were washed with 200 µL of phosphate buffer solution to avoid any interaction between the experimental solutions and the colorimetric assay. A 180 µL of new culture medium was added to each well and cell viability was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics GmbH, Germany). MTT solution was added to each well of the plate and incubated for 3 hours at 37°C. In the presence of living cells with functional mitochondria MTT is reduced to insoluble purple formazan crystals. After the incubation, dimethyl sulfoxide was added to dissolve the reduced formazan crystals. The optical density (OD570) of the formazan solution, which is proportional to the number of viable cells present in the solution, was measured with a microplate reader. A blank well was regularly used for data subtraction by placing the same volume of culture medium with MTT into culture wells.

The morphology of the cultured cells was observed using phase contrast microscope (IX70; Olympus, Tokyo, Japan).

The procedure of the second experiment was the same as the first, except for some differences in the used experimental solutions which included: (a) 50 µM As2O3; (b) 5,000 µM NAC (Sigma Aldrich Co.; St. Louis, MO) + 50 µM As2O3; and (b) 500 µM NAC + 5 µM As2O3.

Data were analyzed using the Statistical Package for Social Sciences version 16.0 (SPSS 16.0; SPSS Inc, Chicago, Illinois, United States) by applying two-way analysis of variance (ANOVA) and Tukey’s post hoc test using the experimental solution and exposure time as two factors. The preset significance level of α was 0.05.

Results

Cytotoxicity Test and Cell Morphology

The effects of different As2O3 concentrations on pulpal-like cells at 24 hours or 3 days of exposure are depicted in Fig. 1. At both exposure times, 50 µM As2O3 caused a marked decrease in the OD value when compared with the control and the other experimental groups (p < 0.05). At 24 hours exposure, 10 µM As2O3 showed a significantly higher OD value when compared with the control group (p < 0.05). 5 µM As2O3 showed a slightly higher OD value when compared with the control, however, it did not reach to the level of statistical significance (p > 0.05). There was no significant difference between the two lowest concentrations at each exposure time (p > 0.05). At 3 days, 10 µM As2O3 and 5 µM As2O3 showed no significant difference compared with the control (p > 0.05).

The effect of NAC on As2O3-mediated toxicity is shown in Fig. 2. At each exposure time, there was no significant difference between 50 µM As2O3 group and 500 µM NAC+50 µM As2O3 group. The former groups had lower OD values when compared with 5,000 µM NAC+50 µM As2O3 group at 24 hours and 3 days. All experimental groups showed significantly lower OD values when compared with the control at 24 hours and 3 days.

Representative images of cell morphology obtained from the first experiment are shown in Fig. 3A and 3B. The cultured RPC-C2A control cells exhibited polygonal-shaped fibroblast-like morphology. The exposure of cells to 50 µM As2O3 for 24 hours or 3 days resulted in spherical retractions and increases in intercellular spaces (Fig. 3B and 3D). Slight

Fig. 1 Cytotoxicity of culture medium containing 50, 10 or 5 µM As2O3 on rat dental pulp cells after an exposure time of 24 hours or 3 days. Cell viability was determined using MTT assay (n = 6/group). Groups identified by different lowercase letters indicate statistically significant differences (p < 0.05). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Fig. 2 Cytotoxicity of culture medium containing 50 µM As2O3, 5,000 µM NAC+50 µM As2O3, or 500 µM NAC+50 µM As2O3 on rat dental pulp cells after an exposure time of 24 hours or 3 days. Cell viability was determined using MTT assay (n = 6/group). Groups identified by different lowercase letters indicate statistically significant differences (p < 0.05). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine.
increases in cell size at 24 hours were observed in the groups treated with 10 or 5 µm As$_2$O$_3$, which might be an indication of a hypertrophic response (►Fig. 3C and 3D). After 3 days, the latter two groups showed fibroblast-like cells similar to the control group (►Fig. 3G and 3H).

►Fig. 3 Rat pulpal cells morphological changes seen under phase contrast microscope after 24 hours of exposure to control or experimental solutions (A–D). (A) Control cells: polygonal-shaped. (B–D) Cells treated with 50, 10, or 5 µm As$_2$O$_3$, respectively. (C, D) Normal polygonal morphology with slightly hypertrophic response. Morphologic changes of the cells after 3 days of exposure to control or experimental solutions (E–H). (E) Control cells: polygonal-shaped. (F–H) Cells treated with 50, 10, or 5 µm As$_2$O$_3$, respectively. (F) Contracted and spherical morphology and increases in intercellular spaces. (G, H) Normal polygonal morphology.

►Fig. 4 Rat pulpal cells morphological changes seen under phase contrast microscope after 24 hours (A–D) or 3 days (E–H) of exposure to control or experimental solutions. (A, E) Control cells: polygonal-shaped. (B–D) Cells treated with 50 µM As$_2$O$_3$, 5,000 µm NAC+50 µM As$_2$O$_3$, or 500 µM NAC+50 µM As$_2$O$_3$, respectively after 24 hours. (F–H) Cells treated with 50 µM As$_2$O$_3$, 5,000 µm NAC+50 µM As$_2$O$_3$, or 500 µM NAC+50 µM As$_2$O$_3$ respectively after 3 days. Lower cellular density and increased intercellular spaces are observed in all experimental groups at each exposure time; however, 5,000 µm NAC+50 µM As$_2$O$_3$-treated group (C, G) showed higher cellular density compared with the other experimental groups. NAC, N-acetylcysteine.

Discussion
Based on the results obtained in this study, As$_2$O$_3$ showed a biphasic dose response on pulpal cells. This type of response is termed hormesis which is characterized by what appears like a beneficial effect at low doses and a harmful effect at high doses. NAC did not prevent As$_2$O$_3$-mediated toxicity as shown in the results of the viability test and cell morphology observation; however, these results showed a potential of this antioxidant at a certain concentration to minimize the negative impact of As$_2$O$_3$ on pulpal cells.

The development of PC-based materials has come a long way; however, some concerns are surfacing with regard to the presence of heavy metals such as arsenic which is receiving the most attention due to its cytotoxic and carcinogenic potential. One of the first steps taken to address the matter of PC-heavy metals content was the introduction of white PC that retains significantly lower amount of arsenic when compared with its gray counterpart. Despite the low amount of arsenic, the deleterious effect caused by continuous release of low levels of arsenic is still unclear because the ISO standards only specify the
limits for the total arsenic content and not for the amount released. The type of arsenic used in the present study was As₂O₃, since it is the main type of inorganic arsenic found in PC-based materials. In the present study, the response elicited with the application of 50 µM As₂O₃ indicates the cytotoxic capability of this metalloid on pulpal cells. Arsenic is known for its ability to induce apoptosis, the mechanism of action is speculated to be through the induction of radical oxygen species (ROS) due to its high affinity to the sulfur-containing thiol groups, sequentially hindering cell signaling pathways and sabotaging the cellular redox system governed by glutathione. In addition to that, arsenic-induced oxidative stress results in the formation of 8-hydroxy-2-deoxyguanosine which is a quintessential DNA adduct and a critical biomarker of carcinogenesis. On the other hand, the application of 10 or 5 µM As₂O₃ resulted in a stimulatory effect at 24 hours exposure time as characterized by slight increases in cell viability compared with control cells. This finding can be viewed as either a health promoting effect or an indication of the carcinogenic potential of arsenic. The enhanced proliferation obtained in the present study corroborates some of the findings of previous reports on lung epithelial cells and keratinocytes; however, the exact mechanism underlying this finding is speculative and not properly understood. Several pathways have been suggested as possible mechanisms, such as P53 protein inhibition and the activation of antiapoptotic molecules, thus contributing to the proliferation of the affected cells or inhibiting the autophagy pathway. Snow et al reported that low arsenic exposure has a protective effect against oxidative stress as it promotes the activities of important intracellular glutathione-related enzymes. Moreover, transiently increased ROS level as a result of exposure to low doses of arsenic is speculated to act as transducers of arsenite effects on lifespan, a process known as hormesis. It is thought that low doses of arsenic trigger an adaptive response that curtails the adverse effects of oxidative stress; however, this response is cell- and tissue-specific. Despite aiding cell growth at low doses, a concomitant disruption of the DNA transcription process was also reported. Some authors proposed that the proliferation-enhancing effect of arsenic is consistent with its role as tumor promoter which leads to uncontrolled proliferation and carcinogenesis. Due to the ability of arsenic in transforming normal stem cells into cancer stem cells, coupled with the fact that pulpal stem cells might have the potential to undergo neoplastic alteration, one must exercise caution and avoid overzealous interpretation of the viability test results obtained in our study. Low levels of As₂O₃ rendered the pulpal cells slightly hypertrophic; recently, Samanta et al demonstrated an in vitro hypertrophic effect of 1 µM arsenic when applied for 24 hours on rat cardiomyocytes. They attributed this finding to decreased activity of adenosine monophosphate-activated protein kinase and forhead box transcription factor along with increased expression of nuclear factor of activated T-cells, cytoplasmic 3. Whether these effects can be translated into in vivo studies remains to be investigated, and thus future studies are essential to provide confirming evidence.

The efficacy of NAC to prevent the toxicity of 50 µM As₂O₃ was tested in this study. We used two different concentrations of NAC, e.g., 5,000 µM and 500 µM, and it was apparent that neither concentration proved effective in preventing As₂O₃-induced toxicity; however, 5,000 µM NAC showed a potential to reduce the cellular damage. The mechanism for its ameliorating effect is through decreasing lipid peroxidation, activating antioxidant enzymes, scavenging ROS, chelating with arsenic and/or increasing the intracellular level of glutathione. Although some studies have reported decreased toxicity of arsenic with the application of NAC, the results have been always equivocal; the administration of NAC in combination with zinc or Monoisoamyl DMSA, a lipophilic chelating agent, was more effective than NAC monotherapy which showed only minimal or no effect in protecting against arsenic toxicity. In another study, NAC exacerbated the toxic effect of arsenic metabolites and this was attributed to the ability of NAC to act as pro-oxidant or to produce further reactive metabolites. At 24-hour exposure time, the co-administration of 500 µM NAC with 50 µM As₂O₃ resulted in a lower OD value compared with 50 µM As₂O₃ alone, but this difference did not reach the level of significance. Some of the differences between the studies evaluating the protective effect of NAC on arsenic-induced toxicity can be attributed to variation in cell lineage, methodology, or concentration and type of reagents.

Conclusions
This study showed the dose-dependent effect of As₂O₃ on pulpal cells and the inability of NAC to prevent As₂O₃-mediated cellular damage, and these findings are consistent with some reports in literature discussed earlier. The effect of arsenic on pulpal cells received very little, if any, consideration and has been poorly studied and understood to this date, thus, we here highlight the need for considering further studies to precisely determine the potential detrimental or protective effect of long-term exposure of pulpal cells to low concentrations of arsenic, and if needed, to explore innovative approaches such as combination therapy to prevent this toxic effect.

Conflict of Interest
None declared.

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