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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 (As_2O_3) released from Portland cement-based materials. The present study investigated the effect of different concentrations of As_2O_3 on rat pulpal cells and the efficacy of *N*-acetylcysteine (NAC) in preventing As_2O_3-mediated toxicity.

Materials and Methods Cytotoxicities of 50, 10, or 5 μm As_2O_3 and the effect of cells co-treatment with 50 μm As_2O_3 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 ($\alpha = 0.05$).

Results At both exposure times, 50 μm As_2O_3 resulted in lower optical density (OD) values when compared with 10 or 5 μm As_2O_3. At 24 hours, 10 μm As_2O_3 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 As_2O_3 group was comparable to the control and 10 μm As_2O_3 group. There were no significant differences between 50 μm As_2O_3 group and 500 μm NAC+50 μm As_2O_3 group; however, these two groups had lower OD values when compared with 5,000 μm NAC+50 μm As_2O_3 group at 24 hours and 3 days. The latter group showed significantly lower

Keywords
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- pulp cells
- toxicity

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OD value in comparison with the control at 24 hours and 3 days. Control cells were polygonal-shaped while 50 µm As_2O_3-treated cells exhibited contracted and spherical morphology with increased intercellular spaces. At 24 hours, 10 µm and 5 µm As_2O_3-treated cells were slightly hypertrophic. Cells co-treated with NAC and As_2O_3 showed increased intercellular spaces and lower cellular density compared with the control.

Conclusions As_2O_3 displayed a hormetic effect on pulpal cells; however, the proliferative effect induced by low As_2O_3 concentrations should be interpreted with caution. NAC did not prevent As_2O_3-mediated toxicity; however, it demonstrated potential for ameliorating this toxicity.

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.[1,2] Inorganic arsenic compounds occur in trivalent (As^{III}) and pentavalent (As^{V}) forms with the former being the most toxic type in the form of arsenic trioxide (As_2O_3).[1] 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 amount in drinking water.[3]

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 evidence, it is still being available to some clinicians in developing countries.[4,5] The current concerns in regard to arsenic are coming from unexpected sources as discussed below.

Nowadays, mineral trioxide aggregate (MTA) is mainly used in endodontic procedures such as perforation repair, vital pulp therapies, and retrograde root canal treatment.[6] It is regarded as a biocompatible material with notable clinical success. MTA is a Portland cement (PC)-based material with at least 75%, composed of tricalcium silicate, tricalcium aluminate, and tetracalcium aluminoferrite, in addition to bismuth oxide and dihydrate calcium sulfate.[7,8] PC, the nucleus of modern construction industry, is usually manufactured by mixing and heating limestone and sand to produce clinker followed by grinding with gypsum.[9,10] PC is being studied as a viable alternative to MTA due to its availability, low cost, and similar properties to commercially available contemporary endodontic cements.[11] However, the raw materials and the manufacturing process of PC might result in the inclusion of contaminants which might come in the form of heavy metals. One of the heavy metals that got most of the attention is arsenic, and this has generated concerns in regards to the application of this cement on vital pulpal tissues.[12] Minotti et al reported that gray PC contained 18.46 mg/kg arsenic,[13] while in another report this amount was 42.64 mg/kg.[12] These high levels of arsenic are consistent with the findings of Monteiro Bramante et al and Chang et al who demonstrated the presence of arsenic in gray PC at 34.27 mg/kg and 25.01 mg/kg levels, respectively.[14,15] A lower amount (5.4 mg/kg) was detected by Dorileo et al,[16] however, this level is higher when compared with the safety limit of arsenic specified by the ISO.[17] De-Deus et al found negligible amount of arsenic in PC and Duarte et al stated that the amount of arsenic released in water from two types of gray PC were considered very low and nontoxic.[18,19] It is noteworthy to mention here that the release is usually higher in simulated body fluid when compared with water.[12] Different cement manufacturing processes and sites of extraction of the raw materials and/or the use of different methodologies for arsenic detection are the sources of much controversy in regard to the amount of this metalloid.[12,15] Hence, it is of utmost importance to have a preliminary investigation on the toxic effect of different concentrations of arsenic on pulpal cells and to explore ways to prevent or minimize arsenic-mediated toxicity, to develop in-depth studies to pave the way for enhancing the bio-compatibility of dental materials that come in contact with vital pulpal tissues. *N*-acetylcysteine (NAC) is a thiol containing substance that has the ability to cross cell membrane and is readily hydrolyzed to cysteine thus heightening glutathione level inside the cells. Due to the unique properties of NAC in protecting cells from damage, its use has been increasing in several branches of medicine.[20] Previous studies have demonstrated the effectiveness of NAC to ameliorate cellular damage caused by arsenic.[21,22]

To the best of our knowledge and the most recent available literature, there is scarce information about the effect of different concentrations of As_2O_3 on pulpal cells and the role of NAC to attenuate this toxicity. Thus, the aims of the present study were to evaluate the effect of different concentrations of As_2O_3 on the viability and morphology of pulpal cells and assess the efficacy of NAC co-administration in preventing As_2O_3-mediated 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.[23] 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% CO_2 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 As_2O_3 (FUJIFILM; Wako Pure Chemical, Osaka, Japan); (b) 10 µM As_2O_3; and (c) 5 µM As_2O_3. To each well of 24-well culture plates, 5×10^4 cells were placed and incubated for 24 hours in a 5% CO_2 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% CO_2 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 (OD_{570}) of the formazan solution, which is directly 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 (1X70; 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 As_2O_3; (b) 5,000 µM NAC (Sigma Aldrich Co.; St. Louis, MO) + 50 µM As_2O_3; and (b) 500 µM NAC + 50 µM As_2O_3.

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 As_2O_3 concentrations on pulpal-like cells at 24 hours or 3 days of exposure are depicted in ►**Fig. 1**. At both exposure times, 50 µM As_2O_3 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 As_2O_3 showed a significantly higher OD value when compared with the control group ($p < 0.05$). 5 µM As_2O_3 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 As_2O_3 and 5 µM As_2O_3 showed no significant difference compared with the control ($p > 0.05$).

The effect of NAC on As_2O_3-meditated toxicity is shown in ►**Fig. 2**. At each exposure time, there was no significant difference between 50 µm As_2O_3 group and 500 µm NAC+50 µm As_2O_3 group. The former groups had lower OD values when compared with 5,000 µm NAC+50 µm As_2O_3 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. 3**. The cultured RPC-C2A control cells exhibited polygonal-shaped fibroblast-like morphology (►**Fig. 3A** and **3E**). The exposure of cells to 50 µm As_2O_3 for 24 hours or 3 days resulted in spherical retractions and increases in intercellular spaces (►**Fig. 3B** and **3F**). Slight

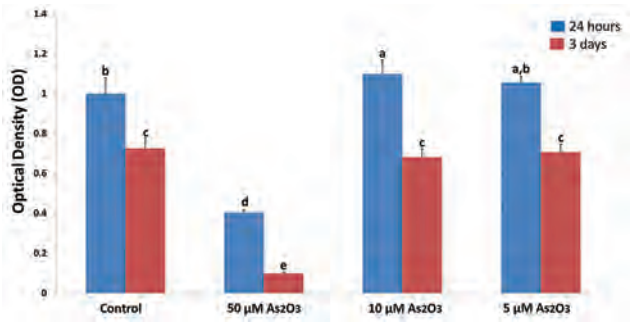

Fig. 1 Cytotoxicity of culture medium containing 50, 10 or 5 µM As_2O_3 on rat dental pulp cells after an exposure time of 24 hours or 3 days. Cell viability was determined using MTT assay (n = 6/group). Groups identified by different lowercase letters indicate statistically significant differences ($p > 0.05$). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

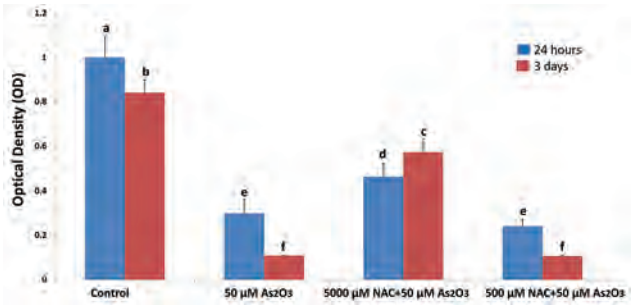

Fig. 2 Cytotoxicity of culture medium containing 50 µM As_2O_3, 5,000 µm NAC+50 µM As_2O_3, or 500 µM NAC+50 µm As_2O_3 on rat dental pulp cells after an exposure time of 24 hours or 3 days. Cell viability was determined using MTT assay (n = 6/group). Groups identified by different lowercase letters indicate statistically significant differences ($p > 0.05$). MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NAC, *N*-acetylcysteine.

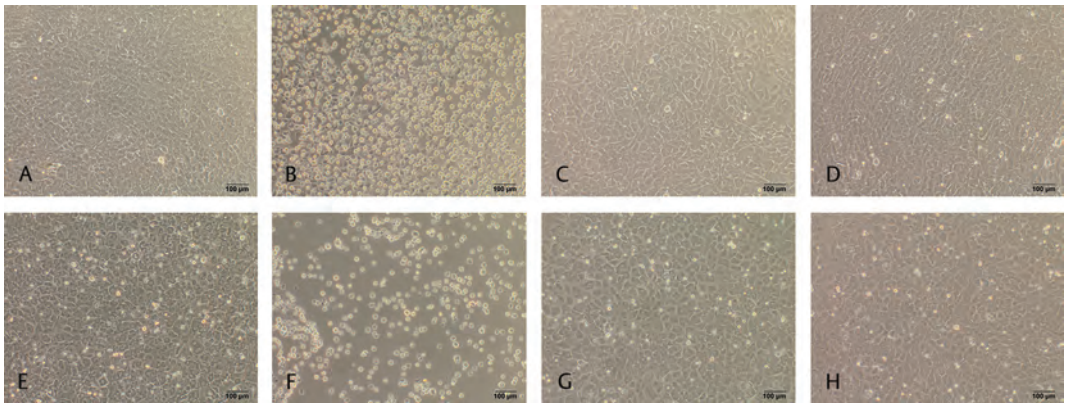

Fig. 3 Rat pulpal cells morphological changes seen under phase contrast microscope after 24 hours of exposure to control or experimental solutions (**A–D**). (**A**) Control cells: polygonal-shaped. (**B–D**) Cells treated with 50, 10, or 5 µm As_2O_3, respectively. (**B**) Contracted and spherical morphology and increases in intercellular spaces. (**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_2O_3, respectively. (**F**) Contracted and spherical morphology and increases in intercellular spaces. (**G, H**) Normal polygonal morphology.

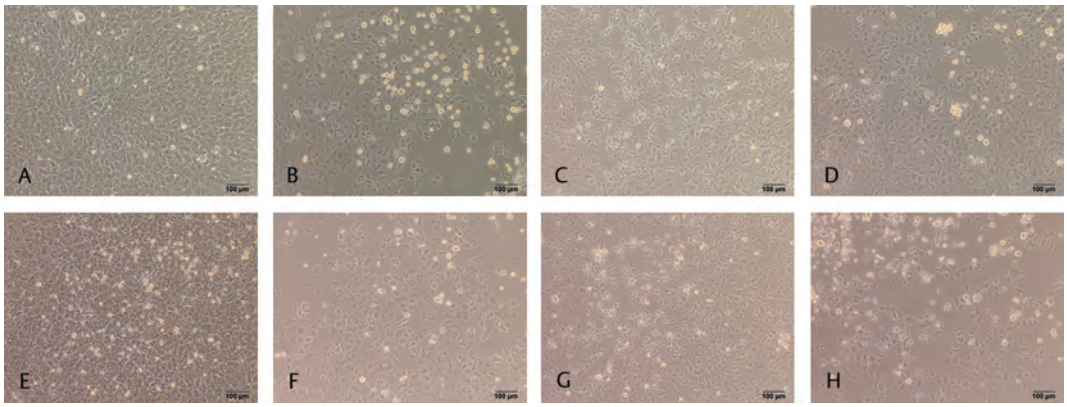

Fig. 4 Rat pulpal cells morphological changes seen under phase contrast microscope after 24 hours (**A–D**) or 3 days (**E–H**) of exposure to control or experimental solutions. (**A, E**) Control cells: polygonal-shaped. (**B–D**) Cells treated with 50 µM As_2O_3, 5,000 µm NAC+50 µM As_2O_3 or 500 µM NAC+50 µm As_2O_3, respectively after 24 hours. (**F–H**) Cells treated with 50 µM As_2O_3, 5,000 µm NAC+50 µM As_2O_3 or 500 µM NAC+50 µm As_2O_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_2O_3-treated group (**C, G**) showed higher cellular density compared with the other experimental groups. NAC, *N*-acetylcysteine.

increases in cell size at 24 hours were observed in the groups treated with 10 or 5 µm As_2O_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. 4** shows representative images of cellular morphological changes obtained from the second experiment. All experimental groups (►**Fig. 4B**, **4C, 4D, 4F, 4G,** and **4H**) exhibited lower cellular density and increased intercellular spaces compared with the control (►**Fig. 4A** and **4E**). However, higher cellular density was observed in the group of cells treated with 5000 µm NAC+50 µm As_2O_3 (►**Fig. 4C** and **4G**) when compared with the other experimental groups (►**Fig. 4B**, **4D, 4F,** and **4H**).

Discussion

Based on the results obtained in this study, As_2O_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.[24] NAC did not prevent As_2O_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_2O_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.[1] 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.[14,15] 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.[12]

The type of arsenic used in the present study was As_2O_3, since it is the main type of inorganic arsenic found in PC-based materials.[18,19] In the present study, the response elicited with the application of 50 µM As_2O_3 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.[25,26] In addition to that, arsenic-induced oxidative stress results in the formation of 8-hydroxy-20-deoxyguanosine which is a quintessential DNA adduct and a critical biomarker of carcinogenesis.[27,28] On the other hand, the application of 10 or 5 µM As_2O_3 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.[29,30] 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.[30,31] 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.[31,32] 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.[33] 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.[29] 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.[33] Despite aiding cell growth at low doses, a concomitant disruption of the DNA transcription process was also reported.[34] Some authors proposed that the proliferation-enhancing effect of arsenic is consistent with its role as tumor promoter[30] which leads to uncontrolled proliferation and carcinogenesis.[35] Due to the ability of arsenic in transforming normal stem cells into cancer stem cells,[35] coupled with the fact that pulpal stem cells might have the potential to undergo neoplastic alteration,[36] one must exercise caution and avoid overzealous interpretation of the viability test results obtained in our study. Low levels of As_2O_3 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 forkhead box transcription factor along with increased expression of nuclear factor of activated T-cells, cytoplasmic 3.[37] 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 As_2O_3 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 As_2O_3-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.[22,38] 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.[21,39] 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.[40] At 24-hour exposure time, the co-administration of 500 µM NAC with 50 µM As_2O_3 resulted in a lower OD value compared with 50 µM As_2O_3 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 As_2O_3 on pulpal cells and the inability of NAC to prevent As_2O_3-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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