

In Vitro Mitigation of Arsenic-Induced Toxicity by Reduced Glutathione in Rat Pulp Cells

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ABSTRACT

Objective: Despite the controversial results regarding the amount of arsenic (As) in mineral trioxide aggregate (MTA) and MTA-like cements, it is prudent to assess the effect of this heavy metal on pulpal cells and search for methods to attenuate its toxicity. This study investigated the toxic effect of As on pulpal-like cells and evaluated the influence of reduced glutathione (GSH) on As-induced toxicity.

Methods: The cytotoxicity of 50 μM As, 50 μM As+50 μM GSH, 50 μM As+500 μM GSH or 50 μM As+5000 μM GSH on rat pulpal cells (RPC-C2A) was evaluated at 24 hours and 72 hours. Cell culture in fresh medium without experimental solution served as the control. Cell viability was measured by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the optical density was measured with microplate reader. The morphology of the cultured cells was observed under phase contrast microscope. Cytotoxicity data were analyzed by two-way ANOVA and Tukey post hoc tests ($P < 0.05$).

Results: There were statistically significant differences in cell viability amongst the tested groups ($P < 0.05$). As elicited remarkable toxic effect on pulpal cells, while 5000 μM GSH protected the cells from As-induced damage at 24-hour exposure time. The cultured control cells were polygonal-shaped; however, As-treated cells exhibited contracted and spherical morphology with increased intercellular spaces indicative of cellular death and decreased proliferation.

Conclusion: As negatively affected the viability of pulpal cells; however, controlled concentration of GSH had a short-term protective effect against As-induced toxicity. Future research is warranted on the clinical use of GSH with MTA and MTA-like cements to minimize initial inflammation resulting from As release during the setting of the aforementioned cements thus enhancing the success of procedures where these cements are placed in direct contact with vital pulp tissues.

Keywords: Arsenic, cytotoxicity, glutathione, heavy metal, pulp cells

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HIGHLIGHTS

- Arsenic has remarkable toxic effect on rat pulpal cells.
- Reduced glutathione at low concentration enhances the toxic effect of arsenic.
- Higher concentration of reduced glutathione has a short-term protective effect against arsenic-induced toxicity.

INTRODUCTION

Mineral trioxide aggregate (MTA) is a popular cement; mainly used in endodontic treatment for root perforation repair, retrograde root filling, and closure of apices in immature teeth. It is also advocated for use as a pulp-capping material due to its biocompatibility and sealing ability (1). MTA is mainly composed of tricalcium silicate, tricalcium aluminate and tetracalcium aluminoferrite which come

from its Portland cement component, in addition to bismuth oxide to give the cement its radiopacity (2, 3). Portland cement, the main component of MTA (4), is a hydraulic material. It is used in building industries and is the fundamental component of mortar and concrete. It has been receiving a lot of attention in dentistry because of its availability and low cost (5, 6), and this has been translated into a surge in the number of commercial products that are claimed to be suitable substitutes for MTA. Portland cement comes from a process in which chalk, limestone, and clay are clinkered at 1400-1600°C and then ground with gypsum, producing Portland cement. Howev-

er, the aforementioned process may include impurities in the form of heavy metals (7). Discrepancies in heavy metal content rely on the manufacturing process and the site of extraction of the minerals (4, 7, 8).

The presence of heavy metal is of concern because these cements are used in situations where they are in contact with vital pulp, periodontal and peri-radicular tissues. The main heavy metal that gained considerable attention is arsenic (As) (7). As is a metalloid, unequivocally known for its toxicity and carcinogenicity with reported acute and chronic effects (9). The most common form of As is the inorganic type which comes either as trivalent (III) or pentavalent (V) (10). Various studies have confirmed the presence of As in MTA and MTA-like cements (2-4, 7, 11-16). The chemical analysis of dental cements concerning As amount showed equivocal results.

Several MTA and Portland cement products were reported to contain levels of As higher (4, 7, 12, 13) than the safe limit specified by the ISO 9917-1 which is 2 mg/kg (17). Minotti et al. evaluated four different products of which three showed As levels higher than the safe limit with the highest level found in a gray Portland cement which contained 18.46 mg/kg (4). All products tested in Schembri et al. study contained significant amount of As in a range between 31.30 mg/kg (MTA ProRoot) and 42.64 mg/kg (gray Portland cement) (7). The exceedingly higher levels of As in gray Portland cement were also confirmed by Monteiro Bramante et al. (12) and Chang et al. (13) at 34.27 mg/kg and 25.01 mg/kg, respectively. However, other researchers concluded that As amount in calcium silicate-based cements was either slightly higher, below the safety limit, or even negligible (2, 3, 11, 14-16). These differences might be attributed to different methodologies used to detect As level and different lot numbers or extraction sites of the used materials. Thus, significant controversy remains regarding As content of these cements. However, lowering potential As-induced toxicity on pulpal cells is prudent, as the clinical implication of As release from these materials is still not clearly understood.

Non-protein thiols, low molecular weight thiol-compounds containing a sulfhydryl group (-SH) in their structure, are classified as antioxidants that function through different mechanisms such as metal chelators and reactive oxygen species (ROS) quenchers (18). One of the most important non-protein thiols is glutathione that exists in two forms; reduced glutathione (GSH) and oxidized glutathione (GSSH) with the former being the active form that consists of cysteine, glutamic acid and glycine. Moreover, GSH is the most abundant intracellular thiol-disulfide redox buffer in mammalian cells that has the major contribution to the intracellular non-protein thiols (19). GSH appears to play a major role in detoxification of As toxicity and its metabolism, consequently, causing a depletion in GSH levels (20).

To the best of knowledge, no previous study has evaluated the effect of As, specifically arsenic trioxide (As₂O₃), on pulpal cells, nor was there a report on the effect of GSH on As-induced toxicity. Hence, the objectives of the present study were to examine the effect of As on the viability of pulpal cells and evaluate the efficacy of GSH on preventing As-induced pulpal cell damage. The null hypotheses were that: (i) As has no adverse effect

on pulpal-like cells, and (ii) GSH does not have the ability to offset As-induced damage on pulpal-like cells.

MATERIALS AND METHODS

Cytotoxicity test and cell morphology

The study was approved by the Ethics Committee of Tokyo Medical and Dental University. 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, 5% CO₂ and maintained at 37°C. Confluent cells were detached with a mixture of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid.

Four types of experimental solutions were prepared using cell culture medium for the cytotoxicity testing: (a) 50 µM As (As₂O₃, FUJIFILM, WakoPure Chemical, Osaka, Japan); (b) a mixture of 50 µM As and 50 µM GSH (L-Glutathione reduced, Sigma Aldrich Co., St. Louis, MO); (c) a mixture of 50 µM As and 500 µM GSH; and (d) a mixture of 50 µM As and 5000 µM GSH. To each well of 24-well culture plates, 5 × 10⁴ 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 72 hours. 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. New culture medium (180 µl) 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). Twenty microliters of MTT solution were added to each well of the plate and incubated for 3 hours at 37°C. In presence of living cells with functional mitochondria, MTT is reduced to insoluble purple formazan crystals. After the incubation, dimethylsulfoxide (200 µl) was added to dissolve the reduced formazan crystals. The optical density (OD₅₇₀) 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).

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences version 16.0 (SPSS 16.0, SPSS Inc, Chicago, IL, USA). Two-way ANOVA was used to compare the mean OD values of the groups by using the tested groups and exposure times as 2 factors, and Tukey's test was performed for post hoc comparisons. The significance level was set at 0.05.

RESULTS

Cytotoxicity test and cell morphology

The effects on pulpal-like cells after 24 hours and 72 hours of exposure to solutions containing As with or without GSH are presented in Figure 1. Two-way ANOVA showed that the factors "tested groups" and "exposure times" were significant ($P < 0.001$) and the interaction between these 2 factors was also significant ($P < 0.001$). At both exposure times, 50 μM As, 50 μM As+50 μM GSH and 50 μM As+500 μM GSH groups caused dramatic decreases in the OD value that were statistically significantly different when compared to the control group ($P < 0.001$). At 24-hour exposure time, 50 μM As+500 μM GSH group did not show statistical significant difference when compared to 50 μM As or 50 μM As+50 μM GSH group. An interesting observation was the statistically significantly lower OD value of 50 μM As+50 μM GSH group when compared to 50 μM As at 24-hour exposure time. At 24-hour exposure time, 50 μM As+5000 μM

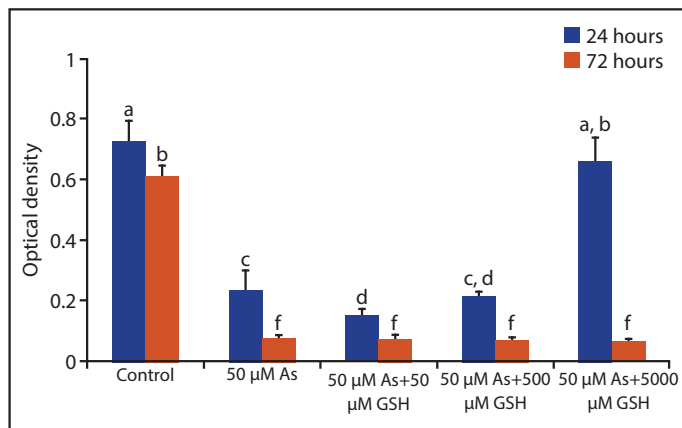


Figure 1. Cytotoxicity of culture medium containing the test solutions on rat dental pulp cells after 24 hours and 72 hours of exposure time. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay ($n=6$ each group). The same lowercase letter indicates no significant difference ($P > 0.05$)

GSH group showed an OD value that was statistically significantly higher when compared to all other experimental solutions; however, there was no significant difference among the former group and the control at 24 hours. At 72-hour exposure time, there were no significant differences among all experimental solutions; meanwhile, the control group had a statistically significantly higher OD value when compared to all experimental solutions. At 72 hours, each experimental group had a significantly lower OD value when compared to its counterpart group at 24-hour exposure time.

Morphologically, cultured RPC-C2A cells were polygonal-shaped and showed fibroblast-like characteristic (Fig. 2a). Cells treated with 50 μM As for 24 hours or 72 hours became rounded with cellular retraction and increases in intercellular spaces (Fig. 2a and f). Cells treated with 50 μM As+50 μM GSH or 50 μM As+500 μM GSH showed disrupted cellular morphology similar to the group treated with 50 μM As which is an indication of the ineffectiveness of GSH at these two concentrations to protect the cells from As-induced damage at both exposure times (Fig. 2c, d, h and i). Cells treated with 50 μM As+5000 μM GSH retained their normal morphology at 24-hour exposure time; however, decreased cellular density is noted (Fig. 2e). At 72-hour, cells treated with 50 μM As+5000 μM GSH exhibited contracted, spherical morphology and increases in intercellular spaces (Fig. 2j), which are indicators of cellular death and decreased proliferation.

DISCUSSION

This study assessed the effect of As on the viability of pulpal-like cells and the ability of GSH to mitigate As-induced toxicity. The results of this study showed that As had drastic negative effect on the used cells, and a short-term prevention of this toxicity was achieved by the use of GSH at a certain concentration. Thus, these results lead to partial rejection of the null hypotheses.

MTA and MTA-like products are becoming the materials of choice for direct pulp capping and other vital pulp therapies.

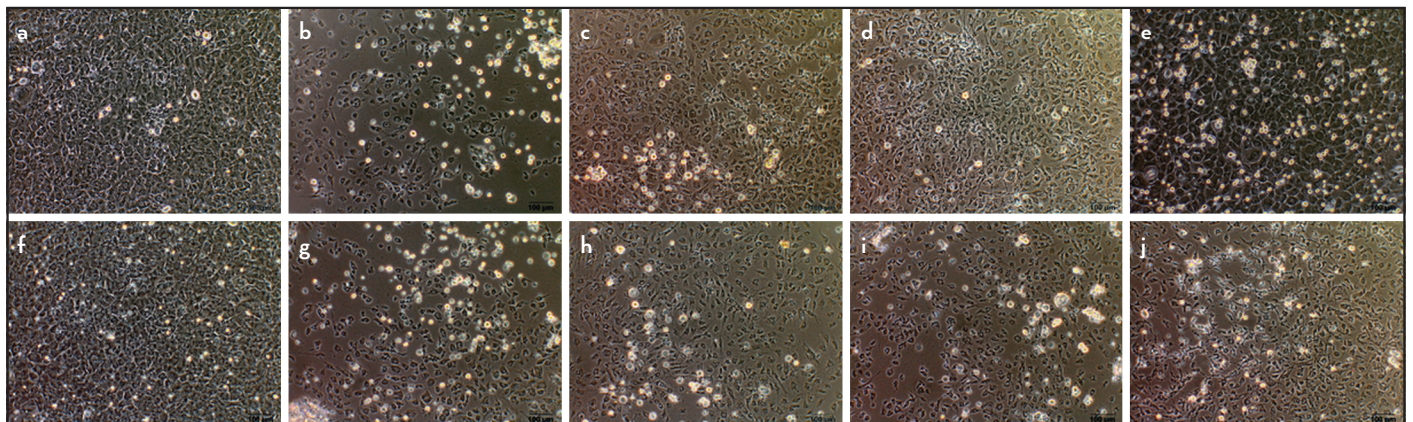


Figure 2. Morphologic changes of RPC-C2A cells after 24 hours of exposure to test solutions (a-e). (a) Control: polygonal-shaped cells (b-d). Cells treated with 50 μM As, 50 μM As+50 μM GSH, or 50 μM As+500 μM GSH, respectively. Contracted, spherical morphology and increases in intercellular spaces were observed (e). Normal polygonal morphology of the cells was retained with 50 μM As+5000 μM GSH treatment; however, decreased cellular density is noted. Morphologic changes of RPC-C2A cells after 72 hours of exposure to test solutions (f-j). (f) Control: polygonal-shaped cells (g-j). Cells treated with 50 μM As, 50 μM As+50 μM GSH, or 50 μM As+500 μM GSH, or 50 μM As+5000 μM GSH, respectively. Contracted and spherical morphology and increases in intercellular spaces were observed which are indicators of cellular damage

Despite the claimed biocompatibility of these cements, the presence of heavy metals, As in particular, in the powder part is considered as one of the drawbacks associated with their use in procedures where they come in direct contact with vital pulpal tissue. Despite the controversial results about the amount of As in these products, it is important to study the effect of As on pulpal cells taking into account that the main type of As detected in these cements is As_2O_3 (14, 16) which is the most toxic form of inorganic As (10), thus it was the type of As chosen to be evaluated in the present study where it was found to adversely affect the viability of pulpal-like cells and change their morphology from being polygonal to become rounded and smaller with increased intercellular spaces. The molecular mechanisms of As toxicity are not completely understood and different biological systems have developed various strategies of sustaining As-induced damage (21). However, it is thought that GSH depletion is one of the major mechanisms that occurs through the induction of ROS generation, formation of As-GSH complexes; and/or inhibition of glutathione reductase, therefore, retarding the regeneration of GSH (22, 23). Additionally, As metabolism entails the production of a variety of arsenicals with different properties and toxicities (24), which requires oxidation of further amount of GSH. It is postulated that the extracellular surfaces of cells are more prone to As-induced damage due to the naturally lower extracellular concentrations of GSH compared to its intracellular levels (25) and the sensitivity of the cells to As toxicity is correlated with amount of GSH in these cells (26).

Due to the immense involvement of GSH in the metabolism of As, the former was chosen in this study in an attempt to increase the levels of GSH in pulpal-like cells to enhance their resistance and ameliorate As-induced toxicity. In this study, 50 μ M and 500 μ M GSH were ineffective in preventing As-induced cellular damage at 24 hours or 72 hours of exposure and this was substantiated by the obtained cell morphology in these two groups that indicated cellular death. At 5000 μ M GSH concentration, an attenuation of As-induced damage was observed at 24 hours, which unfortunately was lost at 72 hours. One of the most important As detoxification mechanisms is the direct interaction between GSH and As by forming As-triglutathione [As(GSH)₃] complexes which is attributed to the high affinity of As to cysteine residue of GSH. Cánovas et al. reported that the formation of these complexes might not be sufficient for As detoxification as they are not stable (21). The half-life of As(GSH)₃ ranges from few minutes to few hours depending on the pH and GSH concentration (27). Acidic conditions were reported to stabilize these As(GSH)₃ complexes (21). This might be an explanation to the short-lasting protective effect of 5000 μ M GSH in the present study. In 2011, Minamikawa et al. directly cultured rat-pulpal cells on MTA with or without n-acetylcysteine, a precursor of GSH, and they reported higher number of viable cells and better cell attachment and spreading behavior of the cells that had n-acetylcysteine in the culture medium. The intracellular levels of GSH in cells treated with NAC in presence of MTA were higher than the levels in cells treated with MTA alone. In their paper, they attributed the inhibitory effect of MTA on cell growth to aluminum ions released from MTA (28).

A noteworthy finding to mention is the enhanced toxic effect of As in the presence of 50 μ M GSH at 24-hour exposure time. There is evidence that GSH might behave as pro-oxidant through its interaction with ROS or its iron-mediated oxidative metabolism thus generating thiyl radicals which result in increased cellular damage (29).

Rat pulpal cells were used in this study; however, the effect of As on human pulpal cells is anticipated too as As was shown to be more efficient at producing ROS in human cells when compared to animal cells (30). It is important to mention here that despite some worries about heavy metal contents of MTA and MTA-like cements, these materials have been used in various clinical applications for several years with success. However, understanding the metabolism of As by pulpal cells is essential for developing biocompatible naturally sourced cements and improving the biocompatibility of the currently used cements.

CONCLUSION

As negatively affected the viability of pulpal cells; however, controlled concentration of GSH had a short-term protective effect against As-induced toxicity. The results obtained in this study merit further experiments to evaluate the effect of As on human cells and the possible incorporation of GSH in MTA-like cements to further enhance their biocompatibility thus bringing additional improvement for the outcome of vital pulp therapies.

Disclosures

Conflict of interest: The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Ethics Committee Approval: This study was conducted according to the protocol approved by the Human Research Ethics Committee, Tokyo Medical and Dental University, Japan.

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