Sodium arsenite and arsenic trioxide differently affect the oxidative stress, genotoxicity and apoptosis in A549 cells: An implication for the paradoxical mechanism

Xuejun Jiang, Chengzhi Chen, Wei Zhao, Zunzhen Zhang

Department of Environmental Health, West China School of Public Health, Sichuan University, Chengdu 610041, PR China

ABSTRACT

Although arsenic toxicity greatly depends on its chemical forms, few studies have taken into account the paradoxical phenomenon which is manifested by that sodium arsenite (NaAsO₂) acts as a potent carcinogen but arsenic trioxide (As₂O₃) serves as an effective therapeutic agent. In this study, we compared the in vitro effects of NaAsO₂ and As₂O₃ on cell viability, colony formation, cell cycle progression, apoptosis, genotoxicity and oxidative stress in human lung adenocarcinoma A549 cells. Our results demonstrated that both NaAsO₂ and As₂O₃ caused oxidative stress, genotoxicity, cytotoxicity, cell cycle arrest as well as apoptosis, while As₂O₃ induced higher production of reactive oxygen species (ROS) with a more remarkable decrease in superoxide dismutase (SOD) activities and intracellular levels of glutathione (GSH) than NaAsO₂. Moreover, the degree of DNA damage, chromosomal breakage, cell cycle arrest and apoptosis in As₂O₃-treated cells were more severe than those in NaAsO₂-treated cells. These findings suggest that differential effects and mechanisms of NaAsO₂ and As₂O₃ may responsible for the paradoxical effects of arsenic on the carcinogenesis and anticancer function.

1. Introduction

Arsenic is a significant environmental public health concern worldwide due to its wide distribution and increased hazards for humans and animals (Chen et al., 2009; Wang et al., 2012a). Studies have shown that chronic exposure to arsenic is associated with a variety of adverse effects, such as dermal toxicity, neurodegenerative disorder, cardiovascular disease and multiple cancers (Rahman et al., 2009). Arsenic toxicity greatly depends on the chemical form and physical state of the compound involved. The organic form is generally considered to be less toxic than that of inorganic state, and the trivalent arsenite is more toxic than pentavalent arsenate (Kochhar et al., 1996). Among inorganic arsenicals, the two main forms of trivalent arsenic tested most frequently in experiments are sodium arsenite (NaAsO₂) and arsenic trioxide (As₂O₃) (Cui et al., 2008). However, it is noteworthy that previous studies of NaAsO₂ focused primarily on its carcinogenic effects (Hughes, 2002), while As₂O₃ was received much attention for...
its anticancer function (Miller et al., 2002). So far, the exact molecular mechanisms of NaAsO2 induced carcinogenicity and the therapeutic efficacy mediated by As2O3 are still not well documented and need to be elucidated.

NaAsO2 is a well established human carcinogen and its carcinogenicity has been evaluated by International Agency for Research on Cancer (IARC) for the first time in 1973 (Hughes et al., 2011; IARC, 1973). Epidemiological investigations demonstrate that long-term exposure to NaAsO2 via the consumption of contaminated water leads to different types of cancer in skin, lung, liver, kidney and bladder (Smith et al., 1992). This conclusion has also been further supported by the results of experiments in vitro, showing that chronic exposure to NaAsO2 caused malignant transformation of various human cells (Achanzar et al., 2002; Wang et al., 2012b), although arsenic was also reported not carcinogenic in rodent models (Wang et al., 2002). Nevertheless, it is still unclear how NaAsO2 results in different cancers and what are the mechanisms responsible for the carcinogenesis. Multiple models of NaAsO2-induced carcinogenesis have been proposed, including abnormal gene amplification, induction of apoptosis and DNA damage, inhibition of DNA repair, as well as alteration of DNA methylation and cell proliferation (Miller et al., 2002; Rossman and Klein, 2011), but none of these mechanisms has been widely accepted as the major etiologic event for NaAsO2 carcinogenicity. Oxidative stress is a relatively new and widely recognized theory of NaAsO2 carcinogenesis (Kitchin and Conolly, 2010). An increasing number of studies suggest that NaAsO2 causes mutations and carcinogenicity through the generation of reactive oxygen species (ROS), and this notion is also supported by the observation that NaAsO2 can induce a dose-dependent increase of ROS in cultured human cells, thus leading to DNA damage, chromosomal breakage and cell transformation (Ruiz-Ramos et al., 2009; Zhang et al., 2011). More importantly, the intracellular ROS level depends on the concentration of NaAsO2 and this in turn decides the biological effects of cells. Low concentrations of NaAsO2 (0.5–5 μM) induce excess ROS production in cells, and thereby leading to genomic instability by damaging DNA and causing cell cycle arrest (Ruiz-Ramos et al., 2009). In contrast, exposure to relatively high concentrations of NaAsO2 (about 30–100 μM) mainly results in apoptosis via ROS generation in various cell types (Chou et al., 2008; Watcharasit et al., 2008).

Another form of trivalent arsenic compound, As2O3 has been used therapeutically as a part of traditional Chinese medicine that can be dated from more than two thousand years ago (Hughes et al., 2011), and recently, it is introduced into treatment of acute promyelocytic leukemia (APL) with mild side-effects (Powell et al., 2010). The therapeutic potential of As2O3 is not limited to APL cells, but also observed in some solid tumor cells, such as lung cancer, neuroblastoma, head and neck cancer (Dilda and Hogg, 2007). A series of studies have been conducted to reveal the mechanisms of anticancer effects of As2O3, showing that As2O3 exerts its therapeutic function mainly by induction of apoptosis, activation or inhibition of a variety of cellular signal transduction and stimulation of cell differentiation (Yen et al., 2012; Yih et al., 2012). Through the process of apoptosis, As2O3 normally eliminates damaged or unwanted cells from organisms, causes the cellular dysfunction in the malignant cells, thus providing benefits in cancer therapy (Miller et al., 2002). Moreover, the clinically achievable concentrations of As2O3 was also found to increase the production of intracellular ROS (Haga et al., 2005). In the context that both As2O3 and NaAsO2 can trigger ROS generation and apoptosis in various cells, why their biological effects in some extent are different? The paradoxical effects attract much attention and are under intensive study.

To date, few studies have taken into account to distinguish the exact molecular basis of NaAsO2 carcinogenicity and the anticancer efficacy of As2O3 in an experiment. In the present study, cells were treated with NaAsO2 or As2O3 under the same condition, and then, MTT assay, colonic formation assay, micronucleus assay and comet assay were used to determine the cytotoxicity and genotoxicity of NaAsO2 and As2O3. The Annexin V-FITC/PI staining assay, TdT-mediated dUTP nick end labeling (TUNEL) assay and cell cycle analysis were employed to test the apoptosis and cell cycle arrest induced by the arsenic compounds. In addition, several parameters, including ROS, glutathione (GSH) and superoxide dismutase (SOD), were used to evaluate the levels of oxidative stress caused by NaAsO2 and As2O3.

2. Materials and methods

2.1. Reagents

Ethidium bromide (EB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange, normal and low melting point agarose were all from Amresco Inc. (Solon, OH, USA). Glutathione (GSH), 5,5′-dithio bis-(2-nitrobenzoic acid) (DTNB), sodium azide (NaN3), propidium iodide (PI), ribonuclease A (RNase A) were from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and trypsin were obtained from Gibco Life Technologies (Grand Island, NY, USA).

As2O3 was purchased from YiDa Pharmaceutical Co. Ltd. (Harbin Medical University, Heilongjiang, China), and the working concentrations of As2O3 were freshly prepared by diluting the stocks (5 × 10^-3 M) with DMEM. NaAsO2 powder, obtained from Fluka Chemical Co. (Buchs, Switzerland), was dissolved in distilled-deionized water to make a 10^-6 M stock solution. Working solutions of NaAsO2 were prepared by DMEM.

2.2. Cell culture

Human lung adenocarcinoma A549 cell line was obtained from Gene Therapy Cancer Drug Engineering Research Center, Chengdu Huasun Group Inc. Ltd. (Chengdu, China). Cells were maintained in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37 °C in a 5% CO2 atmosphere.

2.3. Cytotoxicity assay

2.3.1. MTT assay

Short-term cytotoxicity was determined by MTT assay as described previously (Twenteman and Luscombe, 1987). Briefly, cells were seeded at a density of 10,000 cells/well in
96-well microplates. After an overnight incubation, cells were exposed to NaAsO₂ or As₂O₃ at a series of concentrations for 24 h. Then the cells were incubated with fresh DMEM containing 0.5 mg/ml MTT for an additional 4 h at 37 °C in the dark. Subsequently, the formazan precipitate was dissolved in 100 μl dimethyl sulfoxide (DMSO), and the absorbance value of each well was measured by a spectrophotometer at 570 nm (Bio-Rad, Hercules, CA, USA). Cell viability (%) was expressed as a percentage of absorbance relative to the untreated cells. In addition, each experiment was conducted in 8 replicates for each dosage studied, and DMSO was used as blank control.

2.3.2. Colony forming assay
In order to assess the long-term effects of NaAsO₂ and As₂O₃ on the cell proliferation, the colony forming assay was performed as described previously (Cordes and van Beuningen, 2004). In brief, cells were seeded in 24-well plates at a density of 200 cells/well. On the next day, the cells were treated with increasing concentrations of NaAsO₂ or As₂O₃ (5–25 μM) in triplicates for 24 h, and untreated cells served as the control. Then cells were washed twice with phosphate buffer saline (PBS, 0.1 M of sodium phosphate and 0.15 M of sodium chloride, pH 7.2), and reincubated in drug-free medium for an additional 12 days. During the culture, the medium was changed every 3 days. At the end of incubation, colonies were fixed with 100% methanol and stained with 10% Giemsa. Colonies greater than 50 cells were counted, and the percentage of colonies for each treatment group was calculated by adjusting the control group to 100%.

2.4. Assays for oxidative stress

2.4.1. Measurement of intracellular reactive oxygen species (ROS)
Intracellular ROS generation was determined by a ROS assay kit (Applygen Technologies Inc., Beijing, China) based on the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method (Tobi et al., 2000). Briefly, cells were plated at a density of 10⁶ cells/well on 6-well plates with glass coverslips (24 mm × 24 mm). Cells were exposed to the desired concentrations of NaAsO₂ or As₂O₃ for 24 h. One hour before the end of exposure, 1 μl DCFH-DA solution was added to the medium at a final concentration of 10 μM. After treatments, coverslips with cultured cells were washed with PBS and observed under fluorescence microscope (Leica, Wetzlar, Germany) with excitation at 488 nm and emission at 530 nm. To evaluate the fluorescent density for a single cell, the parameter of average optical density (AOD) was measured and calculated by dividing the sum of integral optical density (IOD) by the sum area (sum IOD/sum area). Values of sum IOD and sum area were obtained using Comet Assay Software Project (CASP).

2.4.2. Measurement of intracellular glutathione (GSH)
The total intracellular GSH content was detected by the method of Beutler et al. (1963) with modifications. In brief, 10⁶ cells/well were incubated with the indicated doses of NaAsO₂ or As₂O₃ for 24 h. Cells were then harvested by trypsinization, washed with PBS, and suspended in 0.1 ml of lysis buffer [0.05 mM EDTA, 1% Triton-X100 (v/v), pH 8.0] for 1 h at 4 °C. Subsequently, the suspension was centrifuged at 12,000 rpm for 2 min, and the supernatant was mixed with 1.9 ml of freshly made disodium hydrogen phosphate (0.32 M) solution and 0.5 ml of 0.004% DTNB (w/v). Absorbance of the yellow color developed from the GSH-DTNB conjugates was read in spectrometer (WTJ 7200, Unico Inc., Shanghai, China) at the wavelength of 420 nm. In addition, the GSH contents were normalized by the supernatant protein concentration determined by the Bradford method.

2.4.3. Measurement of cellular superoxide dismutase (SOD) activity
Total SOD activity was determined by a commercial Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China, Cat. No. A001-1), in which, the activity of SOD was defined by its ability to inhibit the oxidation reaction of hydroxylamine hydrochloride to nitrite with superoxide. Briefly, 10⁶ cells were exposed to desired doses of NaAsO₂ or As₂O₃ for 24 h in 6-well plates. Cells were collected by trypsinization, followed by lysis and centrifugation at 3000 rpm for 5 min, and the supernatant was analyzed according to the manufacturer’s protocol. The absorbance was measured at 550 nm using a spectrometer (WTJ 7200, Unico Inc., Shanghai, China). Fifty percent inhibition was defined as one unit of enzyme activity and normalized by the protein concentration.

2.5. Evaluation of genotoxicity

2.5.1. Alkaline comet assay
To measure the DNA damage induced by both NaAsO₂ and As₂O₃, the alkaline comet assay was conducted according to the method of Singh et al. (1988) with some modifications. Briefly, 10⁵ cells were treated with NaAsO₂ or As₂O₃ for 24 h, and untreated cells served as control. At the end of the exposure, cells were harvested and the cell viability was checked immediately by trypan blue exclusion technique. The following procedures were conducted only when cell viability was greater than 70%. Cells in agarose were then lysed, followed by unwinding and electrophoresis. Slides were stained with 25 μl ethidium bromide and observed at 200× magnification with a fluorescence microscopy (Leica, Wetzlar, Germany). Comet rate and olive tail moment (OTM) were utilized as qualitative and quantitative parameters to estimate the level of DNA damage, respectively. For each concentration, three slides were visually analyzed, and 200 randomly chosen cells were scored from each slide to calculate comet rate as follow: Comet rate (%)=(the total number of cells with tails in each group/the total number of counted cells) × 100%. Meanwhile, 50 comets of each treatment were randomly selected to determine OTM using Comet Assay Software Project (CASP).

2.5.2. Micronucleus assay
Micronucleus assay was conducted to measure the chromosomal breakage caused by both NaAsO₂ and As₂O₃, as described by Fenech (2000). Cells were plated in 6-well culture plates at a density of 5 × 10⁵ cells/well and cultured overnight. On the next day, cells were exposed to NaAsO₂ or As₂O₃ at various concentrations for 24 h, and the cells treated with 0.5 μg/ml mitomycin C (MMC) were used as a positive control. After treatment, cells were washed with PBS and collected by trypsinization. Subsequently, cells were resuspended in
potassium chloride solutions (0.075 M), fixed with methanol-glacial acetic acid (3:1, v/v), and centrifugated at 1000 rpm for 5 min. This fixation process was repeated three times. In the end, cells were resuspended in 0.1 ml methanol/acetic acid (99:1, v/v) and spread onto pre-cold slides. The slides were then stained with 40 μg/ml acridine orange and observed with a fluorescence microscope (Leica, Wetzlar, Germany). One thousand cells on each slide were randomly selected to count the number of micronuclei. The frequency of micronuclei (%) was expressed as total number of micronuclei per 1000 counted cells.

2.6. Apoptosis detection

2.6.1. TdT-mediated dUTP nick end labeling (TUNEL) assay

As a biochemical hallmark of apoptosis, internucleosomal DNA fragmentation was measured by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method using the one step TUNEL Apoptosis Assay Kit (KeyGen Biotechnology, Nanjing, China, Cat. No. KGA7073). In brief, cells were seeded on 6-well plates with glass coverslips (24 mm × 24 mm), and then subjected to a series of desired doses of NaAsO2 or As2O3 for 24 h. Subsequently, coverslips were washed with PBS, fixed by 4% (w/v) parafomaldehyde and permeabilized with 1% (v/v) Triton X-100. After incubation in the reaction mixture for 1 h, the stained cells adhering the coverslips were observed by a fluorescence microscopy (Leica, Wetzlar, Germany) at 100× magnification.

Image-Pro® Plus 6.0 software (Media Cybernetics Inc., USA) was applied to analyze the fluorescence intensity.

2.6.2. Annexin V-FITC/PI staining assay

To evaluate the surface exposed phosphatidylserine (PS) on cells, which is an early indication of apoptosis, Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotechnology, Nanjing, China, Cat. No. KGA108) was utilized. In this kit, Annexin V and propidium iodide (PI) were used to distinguish the apoptotic and necrotic cells. According to the manufacture’s protocol, the exponentially proliferating cells were exposed to the designed doses of NaAsO2 or As2O3 in 6-well plates at a density of 10^5 cells/well for 24 h. Cells were washed in cold PBS and trypsinized with 0.25% (w/v) trypsinase. Thereafter, cells were centrifuged at 1000 rpm for 5 min at 4 °C, resuspended in 400 μl binding buffer containing 5 μl of FITC-conjugated Annexin V and 5 μl of PI. After incubation in the dark at 37 °C for 30 min, the cells were measured by a flow cytometer (Beckman Coulter, FC500, USA) and the data were analyzed by the software of Windows Multiple Document Interface for Flow Cytometry, version 2.8 (The Scripps Research Institute, San Diego, CA, USA).

2.7. Cell cycle analysis

Cell cycle distribution was detected by flow cytometry using PI staining as described by Fried et al. (Fried et al., 1976). Except for the negative control, cells were cultured in the presence of NaAsO2 or As2O3 at the designed concentrations for 24 h. Then, cells were harvested and washed in cold PBS followed by fixation in 75% ethanol (v/v) at 4 °C overnight. On the next day, cells were rinsed and centrifuged, and then incubated with PI staining solution [recipe: 50 μg/ml PI, 20 μg/ml Ribonuclease A (RNase A), 0.2% Triton X-100 (v/v) in PBS] for 30 min. Samples (20,000 cells/sample) were read on a flow cytometer (Beckman Coulter, FC500, USA) and analyzed using the software of Multicycle for windows (Phoenix Flow Systems, San Diego, CA, USA).

2.8. Statistical analysis

All assays were carried out at least three independent times. Data were expressed as mean±standard deviation, and analyzed using one-way analysis of variance (ANOVA) and independent-sample Student t-test. Post hoc pairwise comparison was commonly utilized by the least significant difference (LSD) t-test when ANOVA was significant. To analyze the significant difference among groups in micronucleus assay, Poisson distribution was conducted. P < 0.05 was considered statistically significant and all statistical analyses were performed by the SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of NaAsO2 and As2O3 on cell viability and colony formation

To evaluate both the short-term and long-term cytotoxic effects of NaAsO2 and As2O3 in A549 cells, MTT assay and colony forming assay were employed. In MTT assay, at low concentrations (0.125–2.5 μM), there was no observed cytotoxic effect in both As2O3 and NaAsO2 treatment groups (P > 0.05). However, a significant enhancement of cytotoxicity was observed after exposure of cells to 20 μM NaAsO2 or 5 μM As2O3 (P < 0.05), and the cytotoxicity increased gradually with increasing concentrations of NaAsO2 (20–100 μM) and As2O3 (5–100 μM). Moreover, at 10–100 μM, the cytotoxic effect of As2O3 was more obvious than that of NaAsO2 (P > 0.05), but at 0.125–2.5 μM, the difference in cell viability between the two groups did not achieve statistical significance (P > 0.05) (Fig. 1). In colony forming assay, cells were allowed to grow for 12 days after a 24-h exposure to the designed concentrations (0–25 μM) of NaAsO2 or As2O3. Our results illustrated that both agents caused long-term cytotoxic effects (Fig. 2). In As2O3 treatment group, the colonies (% of control) decreased remarkably with increasing concentrations (P < 0.05). In group treated by NaAsO2, the significant decrease in colonies was not observed at low concentrations (5, 10 μM, P > 0.05), but found at high concentrations (15–25 μM, P < 0.05). Furthermore, As2O3 appeared to be more potent than NaAsO2 in the inhibition of colony formation at all tested concentrations (P < 0.05). In short, our results demonstrate that As2O3 is more cytotoxic than NaAsO2 in A549 cells.

3.2. Effects of NaAsO2 and As2O3 on cell cycle distribution

Cell cycle analysis was conducted to compare the effects of NaAsO2 and As2O3 on cell cycle progression. As shown in Table 1, treatment of NaAsO2 (10–25 μM) caused a significant
increase in the percentage of cells in the G2/M phase (P<0.05) and an obvious decrease in G0/G1 phase (P<0.05), while no remarkable change of cell profile was found after treatment with 5 μM NaAsO2 (Fig. 3A). In As2O3-treated group, the percentage of cells in the G2/M phase was increased with increasing concentrations (0–15 μM), and the fraction began to decrease in a dose-dependent manner up to 25 μM (Fig. 3B). At all tested concentrations, the percentage of cells in G2/M phase of As2O3 treatment group was always higher than that of the control (P<0.05). The fraction of As2O3 treatment group was higher than that of the NaAsO2 treatment group at concentrations of 5–15 μM, but lower at 25 μM (P<0.05). These results indicate that both NaAsO2 and As2O3 induce cell cycle arrest at G2/M phase, and As2O3 is more effective than NaAsO2 for the arrest of A549 cells in G2/M phase.

3.3. Effects of NaAsO2 and As2O3 on apoptosis

In this study, we conducted both TUNEL and Annexin V-FITC/PI staining assays to examine whether NaAsO2 and As2O3 differentially affected apoptosis in A549 cells. The results of TUNEL staining assay were summarized in Fig. 4A. Compared with the control, treatments of cells with NaAsO2 or As2O3 significantly induced apoptosis at all tested concentrations.

<table>
<thead>
<tr>
<th>Dose (μM)</th>
<th>NaAsO2 (%)</th>
<th>As2O3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G0/G1</td>
<td>S</td>
</tr>
<tr>
<td>0</td>
<td>53.1 ± 6.1</td>
<td>11.8 ± 2.2</td>
</tr>
<tr>
<td>5</td>
<td>51.8 ± 5.5</td>
<td>10.5 ± 2.0</td>
</tr>
<tr>
<td>10</td>
<td>41.7 ± 5.3</td>
<td>14.5 ± 2.1</td>
</tr>
<tr>
<td>15</td>
<td>28.9 ± 4.3</td>
<td>18.7 ± 3.2</td>
</tr>
<tr>
<td>20</td>
<td>29.5 ± 4.6</td>
<td>3.1 ± 1.2</td>
</tr>
<tr>
<td>25</td>
<td>28.2 ± 3.5</td>
<td>3.5 ± 1.5</td>
</tr>
</tbody>
</table>

Data were representative of three independent experiments (mean ± S.D.).

* P<0.05, significant difference between the treatment group and the control.

** P<0.05, significant difference between NaAsO2 and As2O3 treatment group at the same concentration.
Fig. 3 – Effects of NaAsO₂ and As₂O₃ on cell cycle distribution. Cells were treated with 0–25 μM of NaAsO₂ or As₂O₃ for 24 h. Cell cycle distribution was detected by flow cytometry using PI staining. For each sample, 20,000 cells were collected and analyzed. Data were obtained from three independent experiments. In A and B, the stacked bar chart was used to represent the percentage of NaAsO₂-treated cells or As₂O₃-treated cells in each phase.

Moreover, As₂O₃ was more potent than NaAsO₂ in increasing the AOD at various concentrations ranging from 5 to 25 μM (P < 0.05). In order to further assess the early apoptosis induced by NaAsO₂ or As₂O₃, the annexin V-FITC/PI staining assay was performed. Our results showed that percentage of apoptotic cells was significantly increased in the two treatment groups compared with the untreated group, and the apoptotic rates of the two treatment groups were both elevated in a dose-dependent manner (P < 0.05, shown in Fig. 4B). However, the significant differences in apoptotic rate between NaAsO₂ and As₂O₃ treatment group were observed only at 20 and 25 μM (P < 0.05). Together, these results suggest that both As₂O₃ and NaAsO₂ induce cell apoptosis in a dose-dependent manner, and As₂O₃ is more effective than NaAsO₂ to trigger apoptosis in A549 cells.

3.4. Effects of NaAsO₂ and As₂O₃ on genotoxicity

Comet assay is a rapid and sensitive technique to measure multiple classes of DNA damage in individual cells. In this study, alkaline comet assay was used to compare the DNA damage between NaAsO₂ and As₂O₃ treatment group. To rule out the potential false positive effects of dead cells on comet assay, trypan blue exclusion experiment was carried out, and our results showed that cell viabilities of all treatment groups in this experiment were above 70% (data not shown), which were in agreement with the results of MTT assay. Compared
Fig. 5 – DNA damage of cells following exposure to NaAsO₂ and As₂O₃. (A) The average of comet rate was calculated in the three independent comet assays. In each experiment, 200 cells/slide were scored for calculating the comet rate. (B) Fifty comet cells of each slide were used to measure the Oliver tail moment (OTM) by Comet Assay Software Project (CASP). One-way ANOVA analysis and LSD-t test were employed to identify statistically significant difference. Data were reported as mean ± S.D. Asterisk (*) denotes a significant difference between NaAsO₂ or As₂O₃ treatment group and the control, while pond (#) represents a significant difference between NaAsO₂- and As₂O₃-treated group under the same concentration. *P < 0.05 was considered as significantly different.

with the control, treatment of cells with NaAsO₂ (15–25 μM) or As₂O₃ (10–25 μM) for 24 h resulted in a significant increase in comet rate (P < 0.05, Fig. 5A), while OTM was enhanced after exposure of cells to 10–25 μM of NaAsO₂ or As₂O₃ (P < 0.05, Fig. 5B). Furthermore, post hoc pairwise comparisons revealed that at high doses (15–25 μM), the comet rate and OTM of cells in As₂O₃ group were both higher than those of NaAsO₂-treated cells (P < 0.05).

Micronucleus test in vitro has been extensively utilized to detect chromosomal damage. In the present study, we employed micronucleus assay to investigate the chromosomal breakage and aberrations induced by NaAsO₂ and As₂O₃. As shown in Fig. 6, at 10–25 μM, both treatment groups exhibited a higher frequency of micronuclei than that of untreated group (P < 0.05), and with increasing concentrations of NaAsO₂ or As₂O₃ (10–25 μM), the frequencies of micronuclei increased (NaAsO₂: 32.85–67.21‰; As₂O₃: 39.05–103.94‰). Moreover, at 15–25 μM, cells exposed to As₂O₃ showed a higher frequency of micronuclei than that of NaAsO₂-treated cells (P < 0.05). Our results further indicate that As₂O₃ induces DNA damage and chromosome breakage with higher potency than NaAsO₂.

3.5. Effects of NaAsO₂ and As₂O₃ on oxidative stress

The intracellular ROS levels were determined by a commercial kit using DCFH-DA as a probe. After 24 h incubation, both NaAsO₂ and As₂O₃ treatment greatly increased ROS levels in cells at all treated concentrations compared with the controls (P < 0.05). The AOD in As₂O₃-treated group was elevated more rapidly than that of NaAsO₂ treatment group. Furthermore, at 10–25 μM, treatment of cells with As₂O₃ resulted in higher levels of ROS than NaAsO₂ (P < 0.05) (Fig. 7A).

SOD serves as a key antioxidant enzyme in cells to scavenge superoxide anion radicals. To compare the antioxidant ability of cells in NaAsO₂- and As₂O₃-treated group, the cellular SOD activity was measured. After exposure to NaAsO₂ or As₂O₃ for 24 h, SOD activities of the cells dropped significantly at all tested concentrations except at 5 μM NaAsO₂ (P > 0.05). Moreover, SOD enzyme activity decreased more appreciably in
Fig. 7 – Effects of NaAsO₂ and As₂O₃ on the level of oxidative stress. Cells were treated by 5–25 μM NaAsO₂ or As₂O₃ for 24 h. The parameters of oxidative stress, including ROS, GSH and SOD, were measured, respectively. (A) The intracellular ROS level was determined by using a fluorescent probe (DCF), and the fluorescent intensity was evaluated with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. (B) SOD activity was tested by a commercial SOD kit, and the SOD activity was normalized according to the protein concentration. (C) The content of GSH was detected by reacting with DTNB to form a yellow colored product, and the absorbance was measured by spectrometer at a wavelength of 420 nm. Data were derived from three independent experiments and expressed as mean ± S.D. One-way ANOVA analysis including the least significant difference (LSD-t test) multiple comparisons were performed to evaluate the statistically significant difference. **A** indicates P < 0.05, compared with the control group; **B** denotes P < 0.05, compared with the NaAsO₂ at the corresponding concentration of As₂O₃.

4. Discussion

Arsenic is a common metalloid that occurs naturally in the environment, and exists in organic and inorganic forms (Hughes et al., 2011). Both NaAsO₂ and As₂O₃ are the two major forms of inorganic trivalent arsenic. Importantly, NaAsO₂ is a well-documented carcinogen, while As₂O₃ appears to be not only a poison but also an effective therapeutic tool in the treatment of APL and some solid tumors (Cui et al., 2008). Numerous studies have shown that inorganic trivalent arsenic induces paradoxical effects in the target cells (Dilda and Hogg, 2007; Hughes, 2002), but the further detailed mechanisms underlying the different effects between NaAsO₂ and As₂O₃ are still elusive. In this study, we compared the influence of these two agents on cell proliferation, cell cycle distribution, oxidative stress, genetic damage, and apoptosis, which were directly or indirectly associated with their paradoxical effects, in cells.

In the present study, two different methods were conducted to distinguish different effects between NaAsO₂ and As₂O₃ on the cytotoxicity. MTT assay was utilized to examine the cytotoxic effects of both agents on cell viability under multiple treatments (dosage ranged from 0.125 to 100 μM) at short term exposure (24 h). Also, the colony forming assay was employed to measure the long term effects of NaAsO₂ and As₂O₃ on the cell proliferation. Our results clearly demonstrated that, after NaAsO₂ or As₂O₃ treatment, the cell viabilities were markedly decreased in a dose-dependent manner as compared with the control, and As₂O₃ showed more toxic to the cells than NaAsO₂ at equimolar concentrations. In addition, concentrations of As₂O₃ used in our experiment may be representative of clinically achievable (0.25–5 μM), even one-fold lower (0.125 μM), many folds higher (10–50 μM) or only encountered accidentally concentrations (60, 75 and 100 μM). As required by US Environmental Protection Agency, the safety level in drinking water is less than 10 μg/l (0.133 μM) for NaAsO₂ (Brown and Ross, 2002; EPA, 1988), and thus, the concentrations of NaAsO₂ designed in this study were comparable with that in the environmental exposure.
study covered the levels commonly encountered in the environment for human being. Previous studies have reported that arsenic is cytotoxic at micromolar concentration (Hu et al., 1998), and similar results were observed in our study, demonstrating that the minimum doses of As$_2$O$_3$ and NaAsO$_2$ for induction of cytotoxicity were 5 µM and 20 µM, respectively. However, at clinically relevant concentrations (1–2 µM) (Zhu et al., 1999), As$_2$O$_3$ did not exhibit any cytotoxic effect to A549 cells in MTT assay, which was consistent with the earlier study showing that A549 cells were relative resistant to As$_2$O$_3$ than other solid tumor cells (Han et al., 2008; Zhang et al., 1999), and the sensitivity of As$_2$O$_3$ in different cell lines might be due to the coupling of GSH depletion (Davison et al., 2003).

To understand if the different effects of NaAsO$_2$ and As$_2$O$_3$ on cell proliferation result from their different cell cycle arrest and apoptosis, we detected the cell cycle distribution and apoptosis by flow cytometry. Our data demonstrated that both NaAsO$_2$ and As$_2$O$_3$ reduced the cell population in G0/G1 phase and arrested cells in the G2/M phase, which were consistent with the previous reports showing that NaAsO$_2$ and As$_2$O$_3$ increased the percentage of cells in G2/M phase (Ge-ping et al., 2009; Mei et al., 2003). In our study, As$_2$O$_3$ treatment caused G2/M arrest in a dose-dependent manner at concentrations from 5 to 15 µM, but the percentage of cells in this phase decreased with increasing concentration up to 25 µM. Similar phenomenon was observed in NaAsO$_2$ treatment group, the maximum percentage of cells recruited to G2/M phase occurred at 20 µM, and the fraction did not further increase when the concentration was increased up to 25 µM. It is interesting that the percentage of cells in G2/M phase in As$_2$O$_3$ treatment group was higher than that of NaAsO$_2$-treated group at concentrations of 5–15 µM, while there was no significant difference in this fraction between the two groups at 20 µM. More importantly, the fraction of As$_2$O$_3$ treatment group was remarkable lower than that of NaAsO$_2$-treated group at concentration of 25 µM. Given that genomic stability is important for mammalian cells and its maintenance mainly depends on repair replication of damaged DNA, the cell cycle arrest in the G2/M phase after DNA damage is essential to protect genomic stability via giving enough time for the cell to repair damaged DNA before entry into mitosis (Wang et al., 1996a). Furthermore, cell cycle arrest in response to arsenic-induced genetic damage would facilitate to prevent the propagation of potentially detrimental mutation (Hartwig et al., 2002).

To reveal the difference between NaAsO$_2$ and As$_2$O$_3$ in induction of genotoxicity in A549 cells, both micronucleus assay and alkaline comet assay were conducted in this study. As expected, our results showed that treatment of cells with NaAsO$_2$ or As$_2$O$_3$ resulted in a significant increase in both DNA damage and chromosomal breakage, which were consistent with previous studies (Liu et al., 2005; Yi et al., 2007). Moreover, As$_2$O$_3$ exposure produced a higher frequency of micronucleus, comet rate, and OTM in cells than that of NaAsO$_2$ at concentrations above 10 µM. These findings indicate that NaAsO$_2$ and As$_2$O$_3$ differentially affect chromosomal breakage and DNA injury, which may be associated with their different abilities to trigger oxidative stress (Hei and Filipic, 2004) and/or to inhibit DNA replication or repair (Rossman and Klein, 2011). Increasing evidences suggest that cells were arrested to permit repair of mutations before replication to maintain genomic stability after DNA damage (Hoeijmakers, 2001). Once the damage reaches a level that cannot be fixed by the repair mechanisms, cells would undergo one kind of the basic cell death pathways, apoptosis, autophagy or necrosis (Roos and Kaina, 2006). As demonstrated in our study, As$_2$O$_3$ is more genotoxic than NaAsO$_2$ at concentrations from 5 to 15 µM, and also, at these concentrations, the percentage of cells arrested in G2/M phase in As$_2$O$_3$ treatment group is higher than that of NaAsO$_2$-treated group. These results suggest that the different effects of the two agents exerted on cell cycle distribution may reflect their different abilities to induce DNA damage. However, cells may fail to handle As$_2$O$_3$-induced genetic damage by repair systems at the concentrations above 15 µM, manifested by a decrease in the percentage of cells in G2/M phase. Since NaAsO$_2$ is less genotoxic than As$_2$O$_3$, the percentage of cells in G2/M phase was found to be increased after 20 µM NaAsO$_2$ treatment, and remained unchanged with increasing the concentration up to 25 µM.

Apoptosis plays a vital role in the removal of transformed or mutated cells from the body (Cottet, 2009). In the early stage of tumorigenesis, cancerous cells and their precursors try to develop in multiple and highly efficient ways to evade apoptosis so as to survive (Shivapurkar et al., 2003). In fact, the avoidance of apoptosis is not only considered as an important hallmark of cancer cells, but also a pivotal reason of tumor cells for drug resistance in cancer therapy (Hanahan and Weinberg, 2011). Hence, apoptosis-inducing agent could be used as a potential therapeutic tool for cancer. Plenty of evidence has already been accumulated to show that As$_2$O$_3$ exerts its anti-leukemia effect mainly by the induction of apoptosis (Perkins et al., 2000; Powell et al., 2010), while whether NaAsO$_2$ is able to induce apoptosis in tumor cells needs to be validated. In this study, apoptosis was determined by both TUNEL and annexin V-FITC/PI staining. TUNEL staining is used to assess the internucleosomal DNA fragmentation in situ as the result of endonuclease activation (Charriaut-Marlangue and Ben-Ari, 1995). Compare to TUNEL assay, Annexin V-FITC/PI assay can identify apoptosis at an earlier phase based on phosphatidylserine (PS) exposure, which can be detected prior to the measurement of nuclear changes such as DNA strand breaks (van Engeland et al., 1998). In the present study, our data showed that both NaAsO$_2$ and As$_2$O$_3$ significantly induced apoptosis at relatively high concentrations (10–25 µM) in the two assays. Moreover, As$_2$O$_3$ showed a stronger induction of apoptosis than NaAsO$_2$. It is worth noting that our results obtained from TUNEL and Annexin V-FITC/PI assay were in disagreement. In the Annexin V-FITC/PI assay, the degree of apoptosis in As$_2$O$_3$-treated group was more severe than that of NaAsO$_2$ treatment group at high concentrations (20–25 µM), while similar trend was found at concentrations of 5–25 µM by TUNEL assay. These discrepancies may result from the different sensitivity and specificity of the two assays. Annexin V-FITC/PI assay is a relative sensitive and specific method in which annexin V, a phospholipid-binding protein, is combined with PI (a membrane impermeable DNA stain) to easily discriminate the dead and apoptotic cells (Wlodkowic et al., 2012). However, TUNEL assay is used for apoptosis detection based on the ability of terminal deoxynucleotidyl transferase (TdT) to bind to DNA fragmentation. Since DNA
fragmentation occurs in apoptosis, necrosis and autolysis, TUNEL assay would fail to discriminate between apoptotic and dead cells. Although TUNEL assay may be of high sensitivity to determine apoptosis, its specificity is relative low because that the results can be confounded by necrosis and autolysis, thus leading to apoptosis overestimation (Kraupp et al., 1995). Although mechanisms responsible for the action of arsenic-induced apoptosis have not been fully understood, it is well documented that the generation of ROS is implicated in arsenic-induced apoptosis. This notion is also supported by the evidence that both NaAsO2 and As2O3 are shown to induce the mitochondrial-dependent apoptosis and cause excessive accumulation of intracellular ROS (Miller et al., 2002; Wang et al., 1996b; Yen et al., 2012).

Oxidative damage has been proposed to play a vital role in the toxic effects of arsenic (Flora, 2011). ROS, such as superoxide anion (O2•−), hydrogen peroxide (H2O2) and hydroxyl radical (•OH), are inevitably generated in cells after arsenic exposure. ROS can react with cellular constituents, such as lipids, thiols, protein and DNA, and regulate the process of cell differentiation and apoptosis in various cell lines. Excessive production of ROS by arsenic would disturb the intrinsic oxidation and reduction equilibria of cells and activate or suppress cellular signaling pathways, thereby leading to cell death (Miller et al., 2002). Fortunately, cells develop antioxidant defense system to scavenge the excessive ROS and maintain the balance of redox. The primary defence system is comprised of many enzymes, such as catalase (CAT), SOD, glutathione peroxidase (GSH-Px), as well as the antioxidants like vitamins A, C, E and GSH. Whether ROS would disrupt the antioxidant system of cells or not is complex, and the ultimate outcome mainly depends on the intracellular levels of ROS and the activity of antioxidant system (Sies, 1993). In this study, to further elucidate the potential mechanisms underlying the paradoxical effects of NaAsO2 and As2O3, we assessed the production of intracellular ROS, GSH contents and SOD activities after exposure of cells to the two agents separately for 24 h. Our data showed that both As2O3 and NaAsO2 increased intracellular ROS and reduced GSH contents and SOD activities at concentrations of 10 to 25 μM, and As2O3 was more severe than NaAsO2 in oxidative stress induction. These findings were in agreement with most previous studies, which demonstrated that intracellular ROS levels were elevated and/or the GSH contents were depleted accompanied by the reduction of SOD activity in tumor cells after exposure to As2O3 or NaAsO2 (Han et al., 2008; Ruiz-Ramos et al., 2009). Nevertheless, there were also some results which were inconsistent with our findings. (i) The intracellular ROS levels were remarkably decreased in Calu-6 cells after 72-h exposure to 10 μM As2O3 (Han et al., 2008). (ii) Treatment with 20 μM NaAsO2 for 72 h induced an increase in SOD activity in porcine endothelial cells, but As2O3 did not (Yeh et al., 2003). (iii) As2O3 or NaAsO2 treatment at 1 μM for 24 h increased GSH contents in porcine aortic endothelial cells (Cheng et al., 2008). These discrepancies may result from cell-type specificity, diverse incubation conditions and detection methods. To some extent, these data may also reflect the complexity of toxicity from trivalent inorganic arsenic.

SOD is a ubiquitous enzyme that exerts its antioxidant effects mainly via catalyzing the conversion of O2•− to H2O2. O2•− overproduction by NaAsO2 or As2O3 has been revealed to result in an increase in SOD consumption accompanied by the accumulation of H2O2, subsequently leading to a decrease in SOD activity. GSH is an important endogenous antioxidant, excessive production of O2•−, H2O2 and other free radicals by NaAsO2 or As2O3 can not only directly bind to GSH by virtue of their thiol preference, but also activate a series of antioxidant enzymes, which function as free radical scavengers via utilizing GSH as an electron donor, thus resulting in a rapid decrease in GSH content in observed cells (Flora, 2011). It is worth noting that, as a primary non-protein antioxidant with multiple functions (such as xenobiotics detoxification, bio-reductive reactions and protection), GSH depletion may present a stressful and detrimental status to cells (Shi et al., 2004).

In summary, our results demonstrated both NaAsO2 and As2O3 induced oxidative stress with subsequent DNA damage and chromosomal breakage, thereby leading to cell cycle arrest and apoptosis in A549 cells, and among these effects observed in this study, As2O3 was more effective than NaAsO2. All of the findings indicate that the different effects and mechanisms of NaAsO2 and As2O3 may be very important in understanding the paradoxical effects of arsenic.

Conflicts of interest statement

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Prof. Ping Zhang, State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, for his helpful assistance on the manipulation of flow cytometry and the analysis of the original data in apoptotic assay.

This work was supported by the National Natural Science Foundation of China (Grant number 81172632 to zunzhen zhang).

References


