

# Arsenic compounds activate the MAPK and caspase pathways to induce apoptosis in OEC-M1 gingival epidermal carcinoma

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**Abstract.** Arsenic is a well-documented environmental toxicant that can induce neurotoxicity and peripheral vascular diseases. In fact, arsenic trioxide has been used to treat various cancer types. Oral cancer has been in the top ten common cancers for decades in Taiwan, and the incidence rate is continuously increasing. The majority of oral cancers are associated with excessive tobacco, alcohol consumption and betel chewing. To the best of our knowledge, no study has revealed the effect of arsenic compounds on oral cancers. Thus, the present study used OEC-M1 oral squamous carcinoma cells treated with sodium arsenite (NaAsO<sub>2</sub>) and dimethylarsenic acid (DMA) to determine whether both arsenic compounds could exert anticancer effects on oral cancer. The results demonstrated that NaAsO<sub>2</sub> and DMA induced rounding up and membrane blebbing in OEC-M1 cells, which are morphological characteristics of apoptosis. Annexin V/PI double staining analysis further confirmed that both arsenic compounds induced apoptosis of OEC-M1 cells. In addition, NaAsO<sub>2</sub> and DMA significantly decreased the survival rate and increased the percentage of OEC-M1 cells in the subG1 and G2/M phases (P<0.05). Furthermore, both arsenic compounds significantly activated the cleavage of

caspase-8, -9, -3 and PARP, and the phosphorylation of JNK, ERK1/2 and p38 in OEC-M1 cells (P<0.05). Collectively, the findings of the present study indicated that NaAsO<sub>2</sub> and DMA stimulate extrinsic and intrinsic apoptotic pathways through the activation of the MAPK pathways to induce apoptosis of OEC-M1 cells, suggesting that NaAsO<sub>2</sub> and DMA may be used as novel anticancer drugs for oral cancers.

## Introduction

The most frequent malignant tumor of the head and neck region is squamous cell carcinoma, which typically develops in males (1,2). Squamous cell carcinoma is strongly associated with certain environmental and lifestyle risk factors, including tobacco smoking, alcohol consumption, UV light exposure and infection with high-risk types of human papillomavirus (3). In addition, betel chewing in Southeast Asia is known to be a strong risk factor for developing oral cavity cancers (4). Squamous cell carcinoma can be treated through surgery, radiation therapy and chemotherapy, as well as new investigative treatments, such as immunotherapy and gene therapy (5). Oral cancer has been in the top ten common cancers for decades in Taiwan, with a continuing annual increase in its incidence rate (6).

Arsenic is a naturally occurring element distributed throughout the earth's crust, which exhibits both metallic and non-metallic properties (7). In the environment, arsenic is combined with oxygen, chlorine and sulfur to form inorganic arsenic compounds; while in animals and plants it combines with carbon and hydrogen to form organic arsenic compounds. In general, inorganic arsenic is more toxic than organic arsenic (8). Arsenic has long been applied as a pesticide for agricultural use due to its poisonous effects. In addition, arsenic trioxide (ATO) has been discovered to exert antitumor effects in patients with acute promyelocytic leukemia (APL) for clinical trial (9). Several studies have reported that ATO induces apoptosis of malignant cells, including APL (10), multiple myeloma (11), lung cancer (12), liver cancer (13), cholangiocarcinoma (14), testicular cancer (15) and ovarian cancer (16) cells with *in vitro* and *in vivo* studies. Another inorganic arsenic

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compound, arsenic hexoxide, has also been reported to exert anticancer effects on MCF-7 human breast cancer cells (17) and colon cancer (18). Furthermore, an organic arsenic derivative (OAD), darinaparsin, is potentially safer and more effective for the treatment of hematological and solid tumors according to preclinical models (19). Dipropyl-S-glycerol arsenic, a novel OAD, also exhibits antiproliferative activity by inducing apoptosis of human acute myeloid leukemia cell lines (20). Therefore, inorganic and organic arsenic compounds possess the ability to suppress tumor progression *in vitro* and *in vivo*. To the best of our knowledge, no study has yet determined whether arsenic compounds exert any effects on normal gingival fibroblasts or any anticancer effects on oral tumors.

The unifying characteristics of apoptosis are largely morphological, including cell shrinkage, blebbing of the plasma membrane, chromatin condensation and DNA fragmentation (21,22). Apoptosis can be distinguished into the extrinsic and intrinsic pathways based on the initial stimuli and regulatory caspase (aspartate-specific cysteine protease) cascade. The extrinsic pathway, also termed the death receptor pathway, is activated by death receptors when bound by the appropriate ligand. Upon binding, a trimerized receptor recruits the signal transducing molecules through interaction with death domains, which leads to the cleavage of pro-caspase-8. Activated caspase-8 initiates a protease cascade that cleaves targets within a cell and results in apoptotic cell death (23). Conversely, the intrinsic pathway is dependent on the decrease of mitochondrial membrane potential. When cellular stress occurs, cytochrome *c* is released from the mitochondrial intermembrane space into the cytosol. Subsequently, it binds to an adapter protein, apoptotic protease activating factor 1, which acts to recruit pro-caspase-9. Active caspase-9 subsequently cleaves pro-caspase-3, which is then released into the cytosol to affect the proteolytic degradation of its target substrates (23). Both apoptotic pathways can activate downstream effector caspases and lead to poly(ADP-ribose) polymerase (PARP) cleavage, which inhibits the cellular function of DNA repair (24,25). In fact, previous studies have demonstrated that ATO can induce apoptosis with downregulation of Bcl-2 expression and activation of a caspase cascade (24,25).

Mitogen-activated protein kinases (MAPKs), which are crucial for the maintenance of cell proliferation, differentiation, mitosis, cell survival, gene expression and apoptosis, consist of three family members: c-Jun NH<sub>2</sub>-terminal kinase (JNK)1, 2 and 3; extracellular signal-regulated kinase (ERK1 and 2); and p38 MAPKs (p38-MAPK  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) (26,27). MAPKs are serine/threonine/tyrosine-specific protein kinases involved in directing cellular responses to diverse stimuli (26,27). Depending on the cell type and stimulus, MAPKs promote cell survival or trigger cell death. JNK and ERK activities have been reported to sensitize ovarian carcinoma cells to cisplatin-induced apoptosis (28). In addition, phosphorylation of JNK and p38 is increased in response to ATO treatment, resulting in mitochondrial apoptotic cell death in human cervical cancer cells (29).

On account of the anticancer ability of arsenic compounds and emerging demands for more effective therapeutic remedies to treat oral cavity cancer, OEC-M1 cells, derived from a surgical specimen of buccal mucosa squamous carcinoma from a Taiwanese as a unique indigenous oral cancer

cell line (30,31), were used in the present study. Cells treated with sodium arsenite (NaAsO<sub>2</sub>) and dimethylarsenic acid [(CH<sub>3</sub>)<sub>2</sub>AsO<sub>2</sub>H; DMA] were investigated to determine whether the arsenic compounds could affect cell viability, regulate cell cycle progression, modulate signaling pathways, and then induce apoptosis with anticancer capabilities.

## Materials and methods

**Chemicals.** NaAsO<sub>2</sub> was purchased from Fluka; Sigma-Aldrich. DMA, high glucose Dulbecco's modified Eagle's medium, penicillin-streptomycin, staurosporine, RNase A, propidium iodide, 30% acrylamide/Bis-acrylamide solution, methylthiazole tetrazolium (MTT) and monoclonal antibody against  $\beta$ -actin were purchased from Sigma-Aldrich; Merck KGaA. Fetal bovine serum and trypsin-EDTA were purchased from AG Scientific, Inc. RPMI-1640 medium, Dulbecco's modified Eagle's medium/F12 and Keratinocyte-SFM medium were purchased from Gibco; Thermo Fisher Scientific, Inc. Sodium chloride, potassium chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Tris base were purchased from JT Baker. Disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>), potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) and sodium bicarbonate (NaHCO<sub>3</sub>) were purchased from Riedel-de Haen. Hydrochloric acid, sodium hydroxide, sodium dodecyl sulfate (SDS), Tween-20 and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA. Donkey anti-rabbit IgG conjugated to horseradish peroxidase and donkey anti-mouse IgG conjugated to horseradish peroxidase were purchased from PerkinElmer, Inc. Annexin V-FITC apoptosis detection kit was purchased from Strong Biotech. Micro BCA protein assay kit was purchased from Thermo Fisher Scientific, Inc. Antibodies against PARP, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, phosphorylated (p)-JNK, JNK, p-ERK1/2, ERK1/2, p-p38 and p38 were purchased from Cell Signaling Technology, Inc. Enhanced chemiluminescence (ECL) detection kit was purchased from EMD Millipore.

**Cell culture.** OEC-M1 is a cell line derived from a surgical specimen of buccal mucosa squamous carcinoma from a Taiwanese, a unique oral cancer indigenous in Taiwan, and was a generous gift from Professor Kuo-Wei Chang (National Yang-Ming University, Taipei, Taiwan) (30,31). OEC-M1 cells were maintained in RPMI-1640 medium supplemented with 24 mM NaHCO<sub>3</sub>, 25 mM HEPES, 10,000 U penicillin and 10,000  $\mu$ g streptomycin (both from Sigma-Aldrich; Merck KGaA) and 10% heat-inactivated fetal bovine serum, pH 7.4, incubated in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> at 37°C (32).

**Morphological observation.** OEC-M1 cells were seeded at a concentration of  $6 \times 10^5$  cells in a 6-cm Petri dish with 2 ml culture medium. After reaching 70-80% confluence, cells were treated without or with various concentrations of NaAsO<sub>2</sub> (0.1, 1, 10, 25, 50 and 100  $\mu$ M), or various concentrations of DMA (0.1, 1, 2, 5, 10, 25, 50 and 100 mM) for 24 h. Cell morphology was then observed under an Olympus CK40 light microscope and recorded by an Olympus DP20 digital camera (Olympus Corporation).

**MTT viability test.** OEC-M1 cells were seeded at a concentration of  $1 \times 10^4$  cells/well with 100  $\mu$ l culture medium. After reaching 70-80% confluence, cells were treated without or with various concentrations of NaAsO<sub>2</sub> (0.1, 1, 10, 25, 50 and 100  $\mu$ M) or various concentrations of DMA (0.1, 1, 10, 25, 50 and 100 mM) for 24 h. MTT was added to a final concentration of 0.5 mg/ml and incubated at 37°C for 4 h. The medium was discarded and 50  $\mu$ l DMSO was added to each well to dissolve the crystals by shaking the plate for 20 min in the dark (33,34). The absorbance values in each treatment group were determined at a wavelength of 570 nm by VersaMax ELISA reader (Molecular Devices, LLC).

**Cell cycle analysis.** To further confirm whether NaAsO<sub>2</sub> and DMA could induce apoptosis among these oral cancer cell lines, the redistribution of the cell cycle was examined by flow cytometric analysis through propidium iodide (PI) staining. OEC-M1 cells were seeded at a concentration of  $6 \times 10^5$  cells. After reaching 70-80% confluence, cells were treated without or with various concentrations of NaAsO<sub>2</sub> (0.1, 1, 10, 25, 50 and 100  $\mu$ M), or various concentrations of DMA (0.1, 1, 10, 25, 50 and 100 mM) for 24 h. Treated cells were harvested by trypsin and centrifuged (400 x g for 12 min at 4°C), and then washed by isoton II and fixed in 70% ethanol at -20°C for  $\geq 2$  h. After fixation, cells were washed with isoton II again and collected by centrifugation (400 x g for 12 min at 4°C). Cell pellets were resuspended with isoton II and mixed with 100  $\mu$ g/ml RNase and 40  $\mu$ g/ml PI for 30 min (15). The stained cells were analyzed using a flow cytometer (FACScan; BD Biosciences) with excitation set at a wavelength of 488 nm. The DNA contents of normal cells in the G1 phase are diploid, while that in the G2/M phase increases after DNA synthesis progression. Cells in the subG1 phase have less DNA contents in cell cycle distribution, which is called hypodiploid and considered as an outcome of cell apoptosis (34,35). The percentages of sub-G1, S and G2/M phase cells were analyzed using FACStation v6.1x and Modfit LT v3.3 software (BD Biosciences).

**Annexin V/PI double staining assay.** Treated cells were harvested by trypsin and washed with 2 ml culture medium. Following centrifugation at 160 x g for 10 min at 4°C, the pellets were resuspended with cold isoton II and centrifuged (400 x g for 12 min at 4°C) again. The pellets were subsequently mixed with 100  $\mu$ l staining solution for 15 min according to the user's manual of the Annexin V-FITC apoptosis detection kit (Strong Biotech). The stained cells were analyzed at a wavelength of 488 nm excitation using a 515-nm band pass filter for FITC detection and  $>600$ -nm band pass filter for PI detection by a FACScan flow cytometer (BD Biosciences). The plots were divided into four quadrants, which represent negative staining (viable) cells, Annexin V-positive (early apoptosis) cells, PI-positive (necrosis) cells, and Annexin V/PI double-positive (late apoptosis) cells. In addition, cells were also treated with staurosporine which was considered as a positive control. The percentage of cells in the 4 quadrants was analyzed using FACStation v6.1x software.

**Protein extraction and immunoblotting analysis.** Cells ( $6 \times 10^5$ ) were seeded in a 60-mm dish. After reaching 70-80% confluence, cells were treated without or with various concentrations

of NaAsO<sub>2</sub> (10 and 25  $\mu$ M), or various concentrations of DMA (10 and 25 mM) for 3, 6, 12 and 24 h. Medium was then transferred to a 15-ml tube, and centrifuged at 1,500 x g for 10 min at 4°C. Attached cells were lysed using 100  $\mu$ l lysis buffer with proteinase inhibitor (cat. no. P8340; Sigma-Aldrich; Merck KGaA). The pellets were resuspended with 10  $\mu$ l lysis buffer, blended into cell lysates, and centrifuged at 12,000 x g for 12 min at 4°C. The supernatants were collected and stored at -80°C. The protein concentrations of cell lysates were determined by the Lowry assay (35,36).

For immunoblotting analysis, cell lysates (30  $\mu$ g) were resolved on 12% SDS-PAGE gel with standard running buffer at room temperature, and electrophoretically transferred to polyvinylidene difluoride membranes at 4°C. The membranes were blocked in 4% milk for 1 h at room temperature and incubated with primary antibodies [cleaved caspase-8 (product no. 9429; 1:1,000), cleaved caspase-9 (product no. 9509; 1:1,000), cleaved caspase-3 (product no. 9661; 1:1,000), cleaved PARP (product no. 9542; 1:1,000), phospho-JNK (product no. 9251; 1/4,000), JNK (product no. 9252; 1:1,000), phospho-ERK1/2 (product no. 9101; 1:4,000), ERK1/2 (product no. 9102; 1:4,000), phospho-p38 (product no. 9215; 1:1,000), p38 (product no. 9212; 1:4,000)] overnight at 4°C. Following washing with TBS Tween-20 and incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature [Donkey anti-rabbit IgG; cat. no. NEF81200-1EA; 1:2,000], the membranes were visualized using the ECL detection kit and UVP EC3 BioImaging Systems (UVP; Analytik Jena) (37). The quantification of each band was performed using the ImageJ version 1.50 software (National Institutes of Health).

**Statistical analysis.** The data are expressed as the mean  $\pm$  standard error of the mean (SEM) of at least three independent experiments. The statistical significance of differences between the control and treatment groups was determined by one-way ANOVA, followed by Tukey's post hoc test comparisons. Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of arsenic compounds on morphological changes in OEC-M1 cells.** OEC-M1 cells were treated with medium, NaAsO<sub>2</sub> (trivalent arsenic) (0.1, 1, 10, 25, 50 and 100  $\mu$ M) or DMA (penta-valent arsenic) (0.1, 1, 10, 25, 50 and 100 mM) for 24 h, and the morphological changes were examined. In the experiment with NaAsO<sub>2</sub>, OEC-M1 cells exhibited a spindle-like shape in the control group (Fig. 1A), and became rounded up and suspended in the medium as the concentration increased, with the number of attached cells considerably decreasing in the 25-100  $\mu$ M treatment groups (Fig. 1E-G). In the DMA experiment, cells started to float in the groups of OEC-M1 cells treated with 0.1-1 mM DMA (Fig. 1H and I). As the concentration of DMA increased, cells shrank with membrane blebbing, indicating that they were undergoing apoptosis (Fig. 1H-M). Floating cells tended to aggregate in a high concentration of DMA treatment compared with NaAsO<sub>2</sub> treatment. These results indicated that both NaAsO<sub>2</sub>

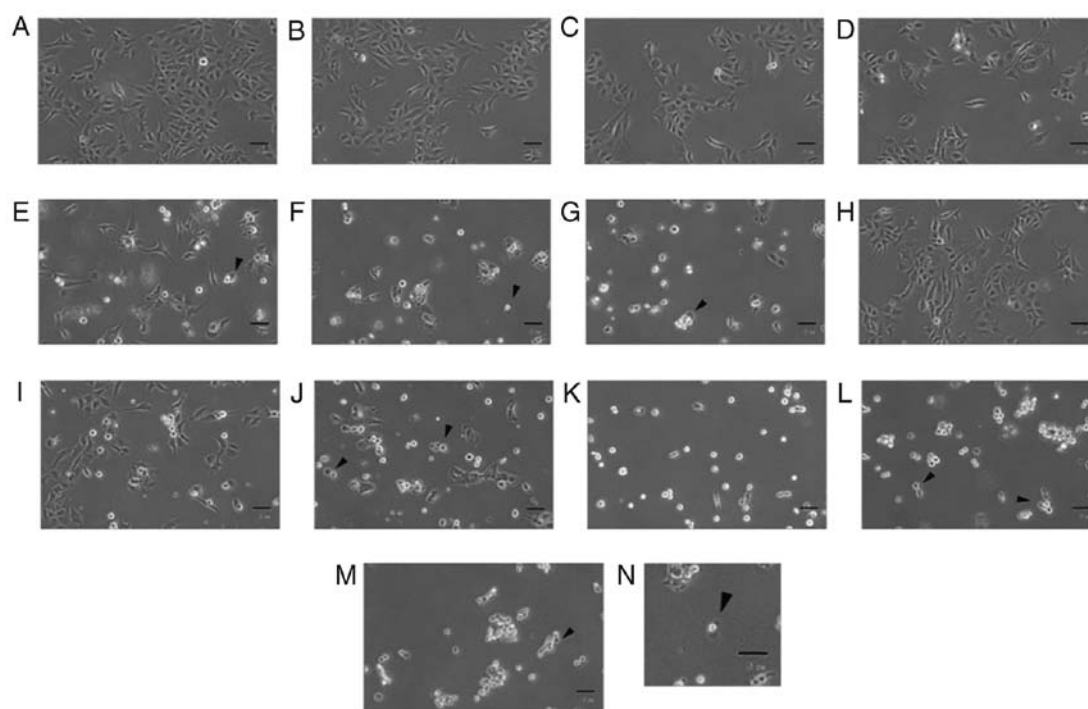


Figure 1. Effects of arsenic compounds on morphological changes in OEC-M1 cells. Cells were treated with (A) plain medium, (B-G) 0.1, 1, 10, 25, 50 and 100  $\mu$ M sodium arsenite, respectively, or (H-M) 0.1, 1, 10, 25, 50 and 100 mM dimethylarsenic acid in OEC-M1 cells for 24 h, respectively. Morphological changes were observed under light microscopy (scale bar, 50  $\mu$ m). Arrowheads indicate membrane-blebbing cells. (N) Apoptotic cell (scale bar, 10  $\mu$ m). The arrowhead indicates an apoptotic cell with membrane blebbing). Experiments were performed three times with similar results.

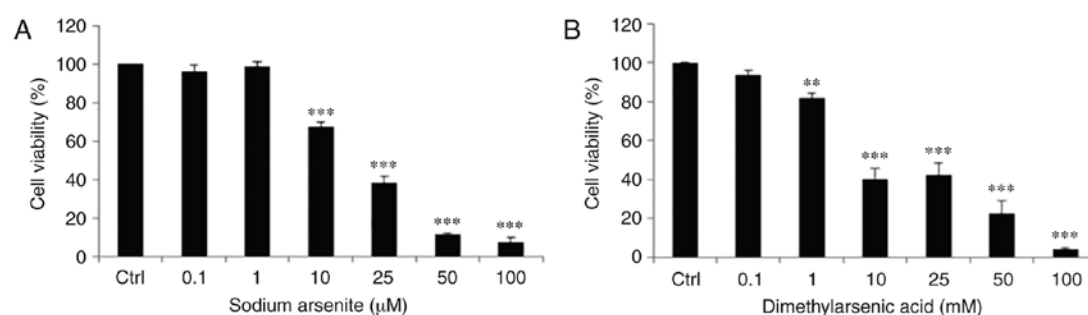


Figure 2. Effects of arsenic compounds on cell viability in OEC-M1 cells. Cells were treated with (A) 0, 0.1, 1, 10, 25, 50 and 100  $\mu$ M NaAsO<sub>2</sub>, or (B) 0, 0.1, 1, 10, 25, 50 and 100 mM DMA for 24 h. Cell viability was quantified by MTT assay. Results are expressed as percentages of cell growth relative to the control groups as 100%. The data are expressed as mean  $\pm$  SEM of at least three separate experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  represent statistical differences compared to the Ctrl. NaAsO<sub>2</sub>, sodium arsenite; DMA, dimethylarsenic acid; SEM, standard error of the mean; Ctrl, control.

and DMA could induce the morphological changes associated with apoptosis in OEC-M1 oral cancer cells.

**Effects of arsenic compounds on the viability of OEC-M1 cells.** The survival rate of OEC-M1 cells treated with arsenic compounds was further examined by MTT cell viability assay. Cells were treated with medium, NaAsO<sub>2</sub> (0.1, 1, 10, 25, 50 and 100  $\mu$ M) or DMA (0.1, 1, 10, 25, 50 and 100 mM) for 24 h. The OEC-M1 cell survival rate was significantly decreased by NaAsO<sub>2</sub> at doses of 10-100  $\mu$ M (Fig. 2A) and by DMA from 1-100 mM (Fig. 2B) in dose-dependent manners ( $P < 0.05$ ). The concentration of DMA required to reduce cell viability to 50% in OEC-M1 cells was ~1,000-times higher compared with that of NaAsO<sub>2</sub>.

**Effects of arsenic compounds on cell cycle distribution of OEC-M1 cells.** To assess whether NaAsO<sub>2</sub> and DMA could

induce OEC-M1 oral cancer cell death via apoptosis, cells were treated with arsenic compounds and then evaluated for their DNA contents by PI staining via flow cytometric analysis. Thus, OEC-M1 cells were treated with medium, NaAsO<sub>2</sub> (0.1, 1, 10, 25, 50 and 100  $\mu$ M) or DMA (0.1, 1, 10, 25, 50 and 100 mM) for 24 h, and the changes in the number of cells in the subG1, G1 and G2/M phases related to cell cycle regulation were investigated.

NaAsO<sub>2</sub> at 50 and 100  $\mu$ M significantly increased the number of cells in the subG1 phase in OEC-M1 cells (Fig. 3A and B) ( $P < 0.05$ ). In addition, the number of cells in the G1 phase was decreased by 25-100  $\mu$ M NaAsO<sub>2</sub> in OEC-M1 cells (Fig. 3A and C) ( $P < 0.05$ ). Furthermore, 25-100  $\mu$ M NaAsO<sub>2</sub> significantly increased the number of OEC-M1 cells in the G2/M phase (Fig. 3A and D) ( $P < 0.05$ ). DMA at 1, 10, 50 and 100 mM significantly increased

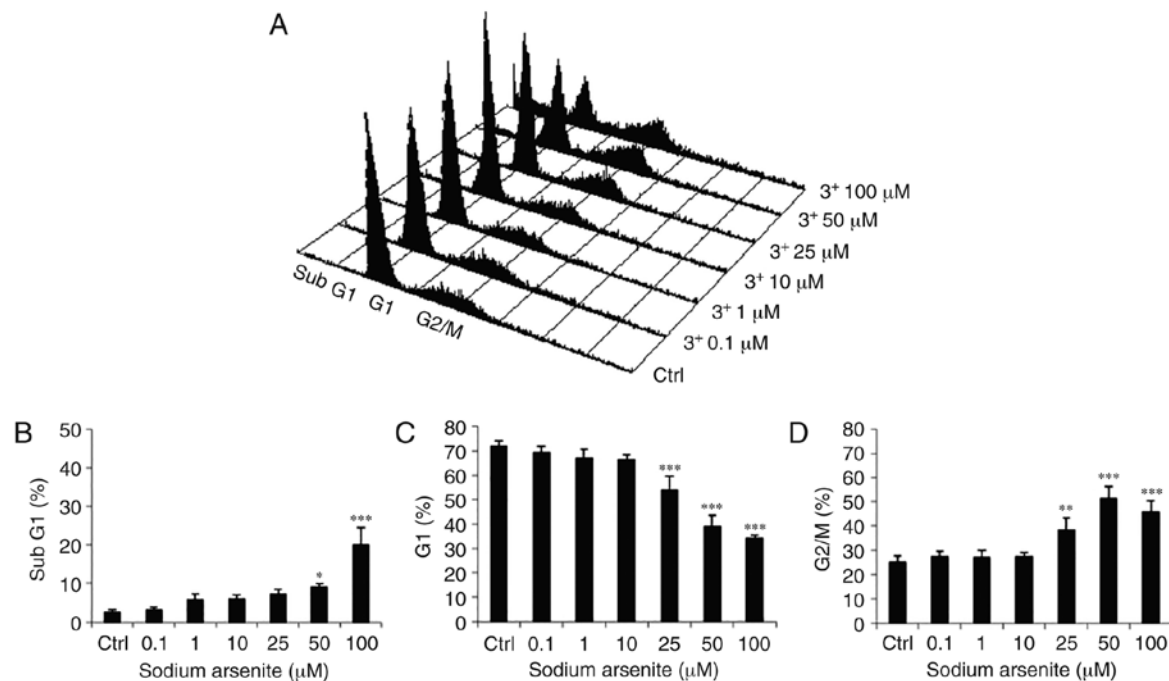


Figure 3. Effects of NaAsO<sub>2</sub> on cell cycle redistribution in OEC-M1 cells. (A) Various concentrations of NaAsO<sub>2</sub> (0, 0.1, 1, 10, 25, 50 and 100 μM) were used to treat OEC-M1 for 24 h. Cells were fixed, stained with PI, and analyzed by flow cytometry. Cells in subG1 phase contain less DNA content than normal cells, indicating apoptosis. The percentage of (B) sub G1, (C) G1 and (D) G2/M phase cells are presented, respectively. The data are expressed as the mean ± SEM of at least three separate experiments. \*P<0.05, \*\*P<0.01, and \*\*\*P<0.001 represent statistical differences compared to the Ctrl group. NaAsO<sub>2</sub>, sodium arsenite; PI, propidium iodide; SEM, standard error of the mean; Ctrl, control.

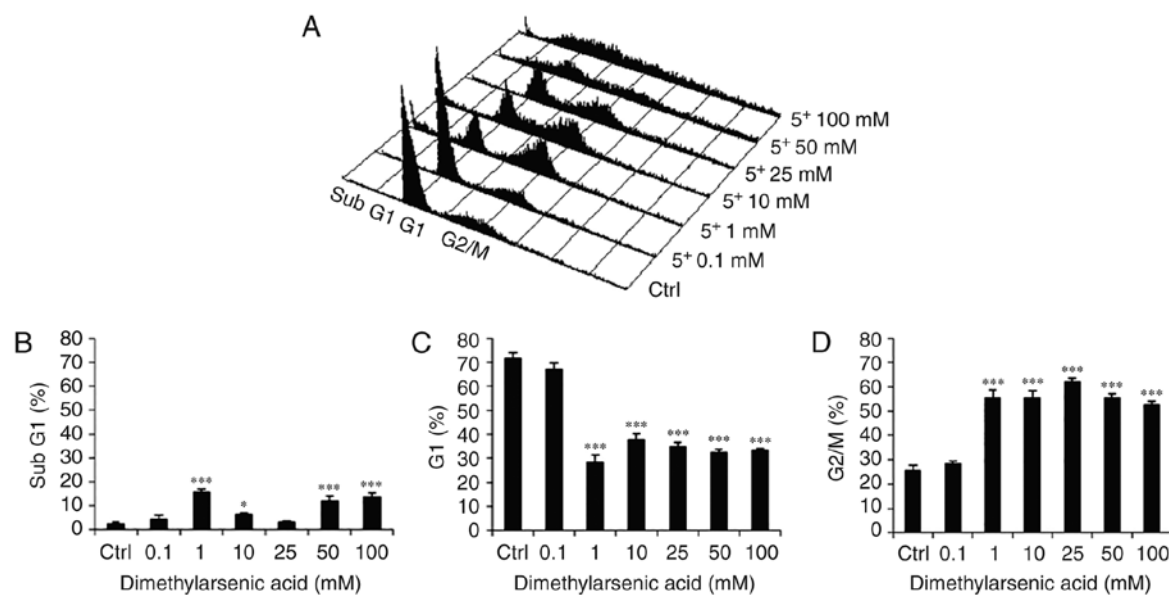


Figure 4. Effects of DMA on cell cycle redistribution in OEC-M1 cells. (A) Various concentrations of DMA (0, 0.1, 1, 10, 25, 50 and 100 mM) were used to treat OEC-M1 for 24 h. Cells were fixed, stained with PI, and analyzed by flow cytometry. Cells in subG1 phase contain less DNA content than normal cells, indicating apoptosis. The percentage of (B) sub G1, (C) G1 and (D) G2/M phase cells are presented, respectively. The data are expressed as the mean ± SEM of at least three separate experiments. \*P<0.05 and \*\*\*P<0.001 represent statistical differences compared to the Ctrl group. DMA, dimethylarsenic acid; SEM, standard error of the mean; PI, propidium iodide; Ctrl, control.

the number of cells in the subG1 phase (Fig. 4A and B); 1-100 mM significantly reduced the number of cells in the G1 phase (Fig. 4A and C) (P<0.05); and 1-100 mM significantly increased the number of cells in the G2/M phase (Fig. 4A and D) (P<0.05). These results demonstrated that both NaAsO<sub>2</sub> and DMA could regulate the distribution of

OEC-M1 cells in the subG1, G1 and G2/M phases of the cell cycle to induce cell apoptosis.

*Arsenic compounds induce apoptosis in OEC-M1 cells.* To further confirm whether apoptosis is induced by arsenic compounds in OEC-M1 cells, double staining with Annexin V

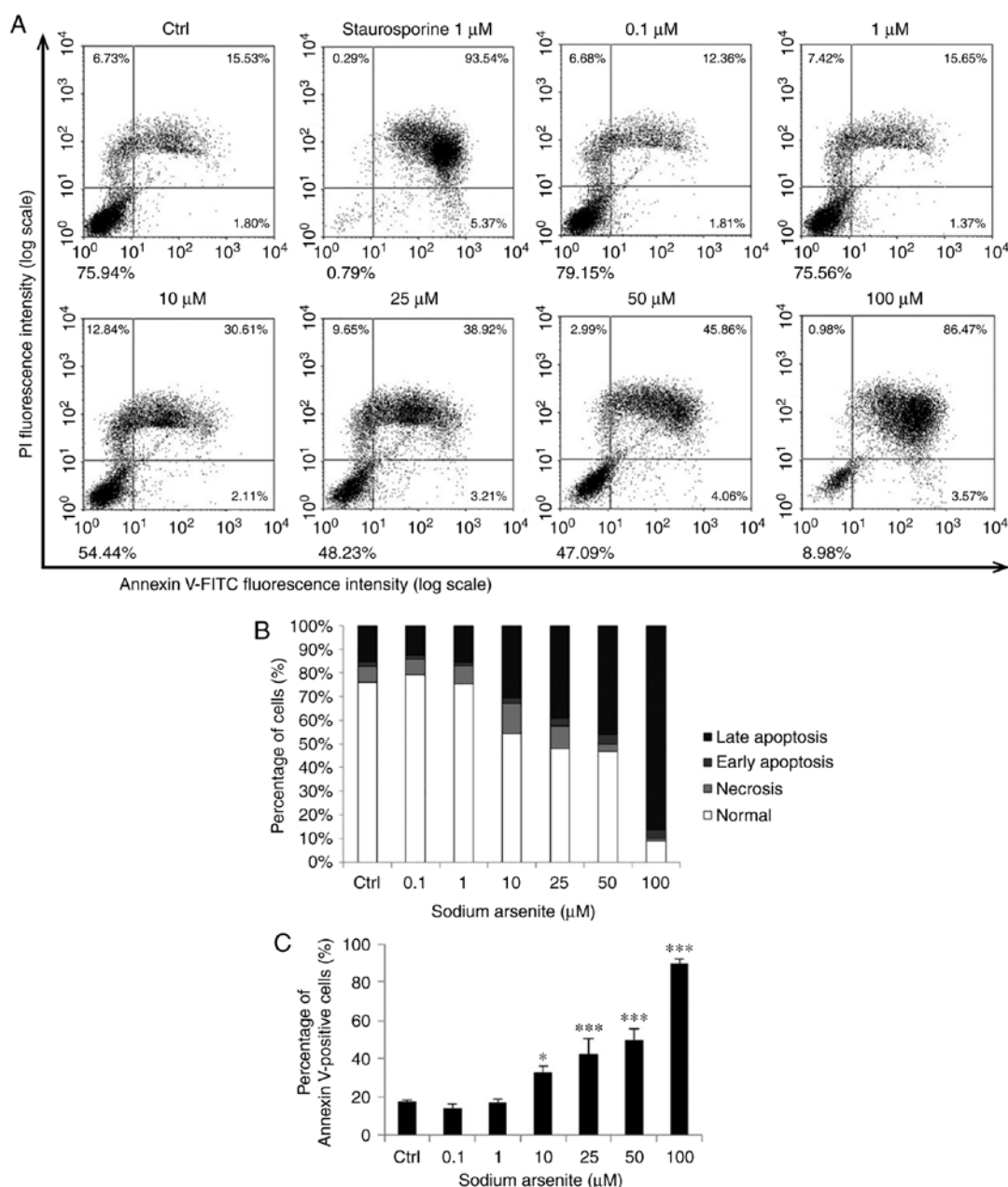


Figure 5. NaAsO<sub>2</sub> induces OEC-M1 cell apoptosis. Various concentrations of NaAsO<sub>2</sub> (0, 0.1, 1, 10, 25, 50 and 100  $\mu$ M) were used to treat OEC-M1 for 24 h. (A) The apoptotic status was determined by Annexin V/PI double staining assay, and the staurosporine-treated cells were considered as a positive control. (B) The percentages of double-negative (viable), PI single-positive (necrotic), Annexin V single-positive (early apoptotic), and double-positive (late apoptotic) cells are presented in. (C) Annexin V-positive cells were analyzed and are presented. The data are expressed as the mean  $\pm$  SEM of at least three separate experiments. \* $P < 0.05$  and \*\*\* $P < 0.001$  represent statistical differences compared to the Ctrl group. NaAsO<sub>2</sub> sodium arsenite; SEM, standard error of the mean; PI, propidium iodide; Ctrl, control.

and PI via flow cytometer was performed. The percentages of double-negative (viable), PI-positive (necrotic), Annexin V-positive (early apoptotic) and double-positive (late apoptotic) cells can be revealed in four quadrants by the double staining assay to assess cell apoptosis (38). Treatment with NaAsO<sub>2</sub> (10–100  $\mu$ M) (Fig. 5A) and DMA (1–100 mM) (Fig. 6A) for 24 h significantly induced cell apoptosis, and the number of Annexin V-positive cells significantly increased as the concentrations of sodium NaAsO<sub>2</sub> (Fig. 5B and C) and DMA increased (Fig. 6B and C) ( $P < 0.05$ ). These results demonstrated that both NaAsO<sub>2</sub> and DMA promoted apoptosis of OEC-M1 cells.

*Involvement of the extrinsic and intrinsic pathways of caspases in arsenic-induced OEC-M1 cell apoptosis.* To further investigate whether arsenic compounds can induce OEC-M1 cell apoptosis via the death receptor (extrinsic) and/or mitochondrial (intrinsic) apoptotic pathways, the expression levels of cleaved caspase-8 and caspase-9, and the downstream targets of cleaved caspase-3 and PARP were examined by western blotting (Fig. 7). The selected concentrations of NaAsO<sub>2</sub> (10 and 25  $\mu$ M) and DMA (1 and 10 mM) used to determine the involvement of the extrinsic and intrinsic pathways in the present experiments were based on the doses that could reduce cell viability from ~40 to 60% covering 50% (Fig. 2) and

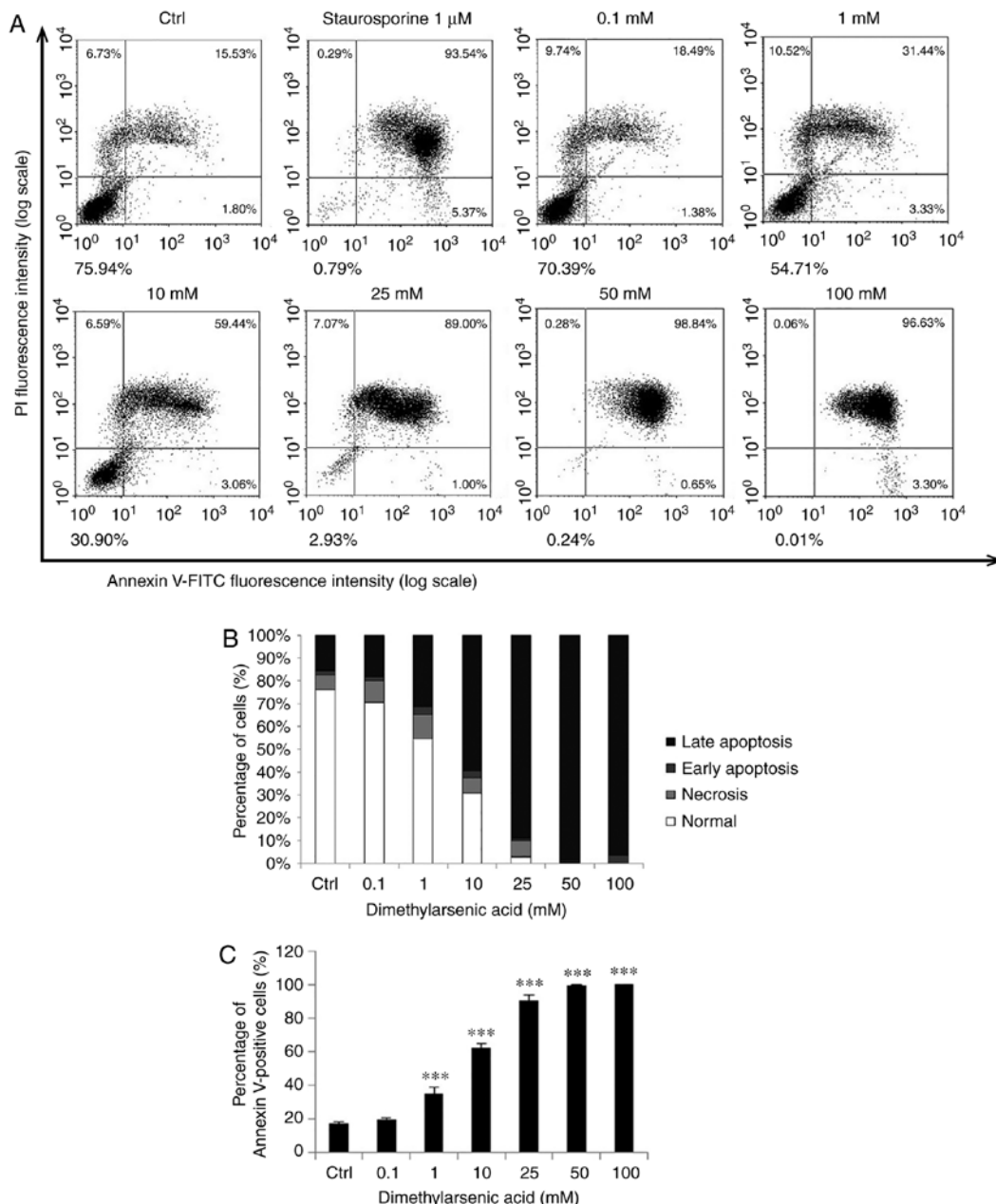


Figure 6. DMA induces OEC-M1 cell apoptosis. Various concentrations of DMA (0, 0.1, 1, 10, 25, 50 and 100 mM) were used to treat OEC-M1 for 24 h. (A) The apoptotic status was determined by Annexin V/PI double staining assay, and the staurosporine-treated cells were considered as a positive control. (B) The percentages of double-negative (viable), PI single-positive (necrotic), Annexin V single-positive (early apoptotic), and double-positive (late apoptotic) cells are presented. (C) Annexin V-positive cells were analyzed and are presented. The data are expressed as the mean  $\pm$  SEM of at least three separate experiments. \*\*\* $P < 0.001$  represent statistical differences compared to the Ctrl group. DMA, dimethylarsenic acid; SEM, standard error of the mean; PI, propidium iodide; Ctrl, control.

induce cell apoptosis from ~30 to 45% for NaAsO<sub>2</sub> (Fig. 5) and from ~35 to 60% for DMA, respectively (Fig. 6).

NaAsO<sub>2</sub> at 10 and 25  $\mu$ M for 12 and 24 h significantly activated caspase-8 (Fig. 7A and B) and PARP (Fig. 7A and E), while caspase-9 was significantly activated by 10 and 25  $\mu$ M NaAsO<sub>2</sub> for 12 h, and 25  $\mu$ M NaAsO<sub>2</sub> for 24 h (Fig. 7A and C) ( $P < 0.05$ ). Caspase-3 was significantly activated by treatment with 25  $\mu$ M NaAsO<sub>2</sub> for 12 and 24 h (Fig. 7A and D) ( $P < 0.05$ ). DMA at 1 mM for 24 h could significantly induce the cleavage of caspase-8, -9, -3 and PARP (Fig. 8A-E) ( $P < 0.05$ ). The expression of cleaved PARP was also significantly increased by 10 mM DMA for 24 h (Fig. 8A and E) ( $P < 0.05$ ). In addition,

10 mM DMA for 12 h could induce cleavage of caspase-3 and PARP (Fig. 8A, D and E) ( $P < 0.05$ ). These results indicated that long-term exposure of both sodium compounds could activate caspase-8 and caspase-9, followed by caspase-3 and PARP, to induce both the extrinsic and intrinsic apoptotic pathways in OEC-M1 cells.

*Involvement of the MAPK pathways in arsenic-induced apoptosis of OEC-M1 cells.* Previous studies have revealed that MAPK pathways may be involved in regulating cell proliferation, differentiation, mitosis, cell survival, gene expression and apoptosis (26,27). To evaluate whether MAPK pathways

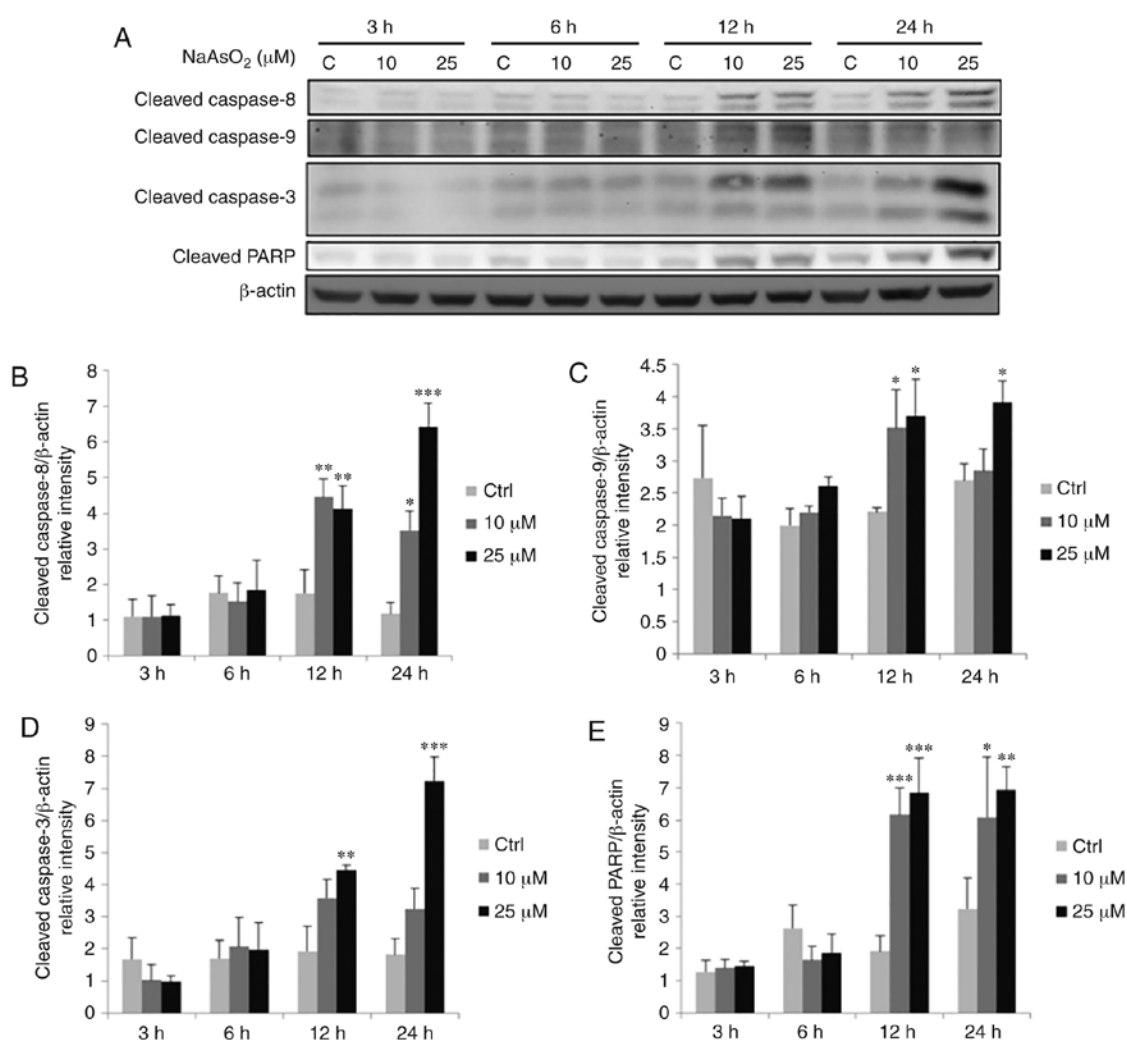


Figure 7. Effects of NaAsO<sub>2</sub> on the expression levels of cleaved caspase-8, -9, -3 and PARP proteins in OEC-M1 cells. Cells were treated without or with 10 and 25 μM NaAsO<sub>2</sub> for 3, 6, 12 and 24 h, respectively. (A) Cleaved caspase-8 (43 kDa), -9 (35/37 kDa), -3 (17/19 kDa) and PARP (~85-90 kDa) were detected by western blotting. The IODs of cleaved (B) caspase-8, (C) caspase-9, (D) caspase-3 and (E) PARP proteins were normalized with β-actin (43 kDa) in each lane. The data are expressed as the mean ± SEM of at least three separate experiments. \*P<0.05, \*\*P<0.01, and \*\*\*P<0.001 represent statistical differences compared to the Ctrl group. NaAsO<sub>2</sub>, sodium arsenite; PARP, poly(ADP-ribose) polymerase; IODs, integrated optical densities; SEM, standard error of the mean; C or Ctrl, control.

are associated with arsenic compound-induced OEC-M1 cell apoptosis, the phosphorylation of JNK, ERK1/2 and p38 was investigated using western blotting.

NaAsO<sub>2</sub> at 10 μM for 24 h and 25 μM for 3, 6 and 24 h could significantly increase the level of p-JNK (Fig. 9A and B), while 25 μM NaAsO<sub>2</sub> for 3, 6, 12 and 24 h significantly increased the expression of p-ERK1/2 (Fig. 9A and C) (P<0.05). The p-p38 level could be significantly increased by 25 μM NaAsO<sub>2</sub> for 6 and 24 h (Fig. 9A and D) (P<0.05). DMA at 10 mM for 3, 6, 12 and 24 h could significantly increase the level of p-JNK (Fig. 10A and B), and 10 mM DMA for 6, 12 and 24 h could significantly increase the level of p-ERK1/2 (Fig. 10A and C) (P<0.05). The p-p38 level was significantly increased after treatment with 10 mM DMA for 24 h (Fig. 10A and D) (P<0.05).

## Discussion

In the present study, NaAsO<sub>2</sub> (inorganic arsenic) and DMA (organic arsenic) both demonstrated the ability to induce

apoptosis of OEC-M1 oral cavity cancer cells. Studies have revealed that the cytoskeleton may be a target of NaAsO<sub>2</sub> and DMA (39,40), and the extent of morphological changes upon modulating the cytoskeleton correlates with drug concentration and incubation time (41). The present results demonstrated that NaAsO<sub>2</sub> resulted in a higher number of shriveled and floating cells with increasing concentration compared with DMA in OEC-M1 cells. In addition, a greater extent of membrane blebbing cells could be observed, and floating cells tended to assemble at 50-100 mM DMA treatments. Compared with cells with abalone-like shape under lower doses of NaAsO<sub>2</sub>, high doses of DMA induced cells to attach to the ground matrix but almost rounded up, while higher doses caused cells to float in clusters. The regulatory mechanisms through which arsenic compounds lead to different cell morphologies are unknown. However, the results indicated that their effect on the cytoskeleton differs with drug types and concentrations.

Inorganic arsenic is significantly more toxic compared with organic arsenic compounds (42). Once entering the food

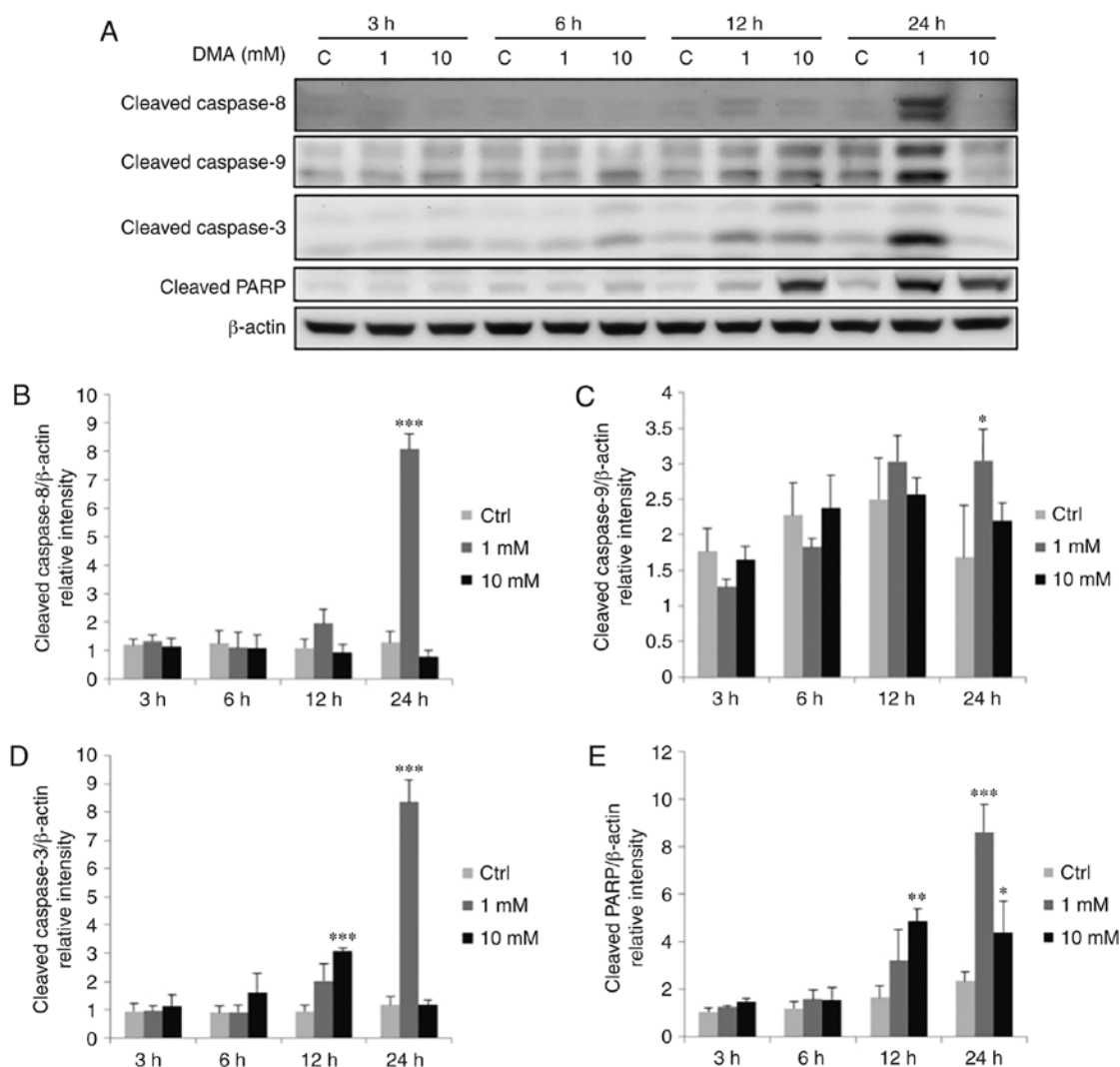


Figure 8. Effects of DMA on the expression levels of cleaved caspase-8, -9, -3 and PARP proteins in OEC-M1 cells. Cells were treated without or with 1 and 10 mM DMA for 3, 6, 12 and 24 h, respectively. (A) Cleaved caspase-8 (43 kDa), -9 (35/37 kDa), -3 (17/19 kDa) and PARP (~85-90 kDa) were detected by western blotting. The IODs of cleaved (B) caspase-8, (C) caspase-9, (D) caspase-3 and (E) PARP proteins were normalized with  $\beta$ -actin (43 kDa) in each lane. The data are expressed as the mean  $\pm$  SEM of at least three separate experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  represent statistical differences compared to the Ctrl group. DMA, dimethylarsenic acid; PARP, poly(ADP-ribose) polymerase; IODs, integrated optical densities; SEM, standard error of the mean; C or Ctrl, control.

chain, inorganic compounds could be metabolized by methyltransferases in the liver of several mammalian species (42). Inorganic arsenic could also be methylated to less toxic compounds, which is considered to be a detoxification reaction. For example, DMA arises from the methylation of arsenic oxide (43). In the present study, NaAsO<sub>2</sub> and DMA decreased OEC-M1 cell viability in a dose-dependent manner, which was consistent with the results of the morphological analysis. The concentration of NaAsO<sub>2</sub> required to perform similar effects was 1,000-fold lower compared with that of DMA, indicating that NaAsO<sub>2</sub> exhibited stronger cytotoxicity than DMA in OEC-M1 oral cancer cells.

The eukaryotic cell cycle proceeds to the next phase as the upstream events fulfill the requirements of checkpoints (44). When undergoing cellular damage, cells may become arrest at certain phases and may undergo programmed cell death if the damage is not properly repaired (44). It has been reported that arsenite can promote disruption of the microtubule network, resulting in spindle abnormalities and, thus, mitotic

cell apoptosis (45). In fact, arsenic-induced mitotic arrest may be a necessary step for the activation of apoptotic pathways in numerous human tumor cell lines (46). A previous study has also demonstrated a strong correlation between G2/M arrest and the induction of apoptosis in ovarian carcinoma cell lines in response to DNA damage (47). It is well known that the subG1 phase is considered as a sign of DNA fragmentation, which indicates cell apoptosis (48), and a previous study has also revealed that G2/M-phase arrest can induce cells to undergo apoptosis (49). In the present study, NaAsO<sub>2</sub> and DMA significantly induced G2/M-phase arrest and increased the number of subG1-phase cells, which suggests that both arsenic compounds could induce apoptosis of OEC-M1 cells. Hence, the present data indicated that arsenic-induced apoptosis is associated with abnormal cell cycle redistribution. The Annexin V/PI double-staining assay further verified that the induction of apoptosis by NaAsO<sub>2</sub> and DMA occurred in a dose-dependent manner in OEC-M1 cells. However, the examination of cell cycle protein levels was not conducted in

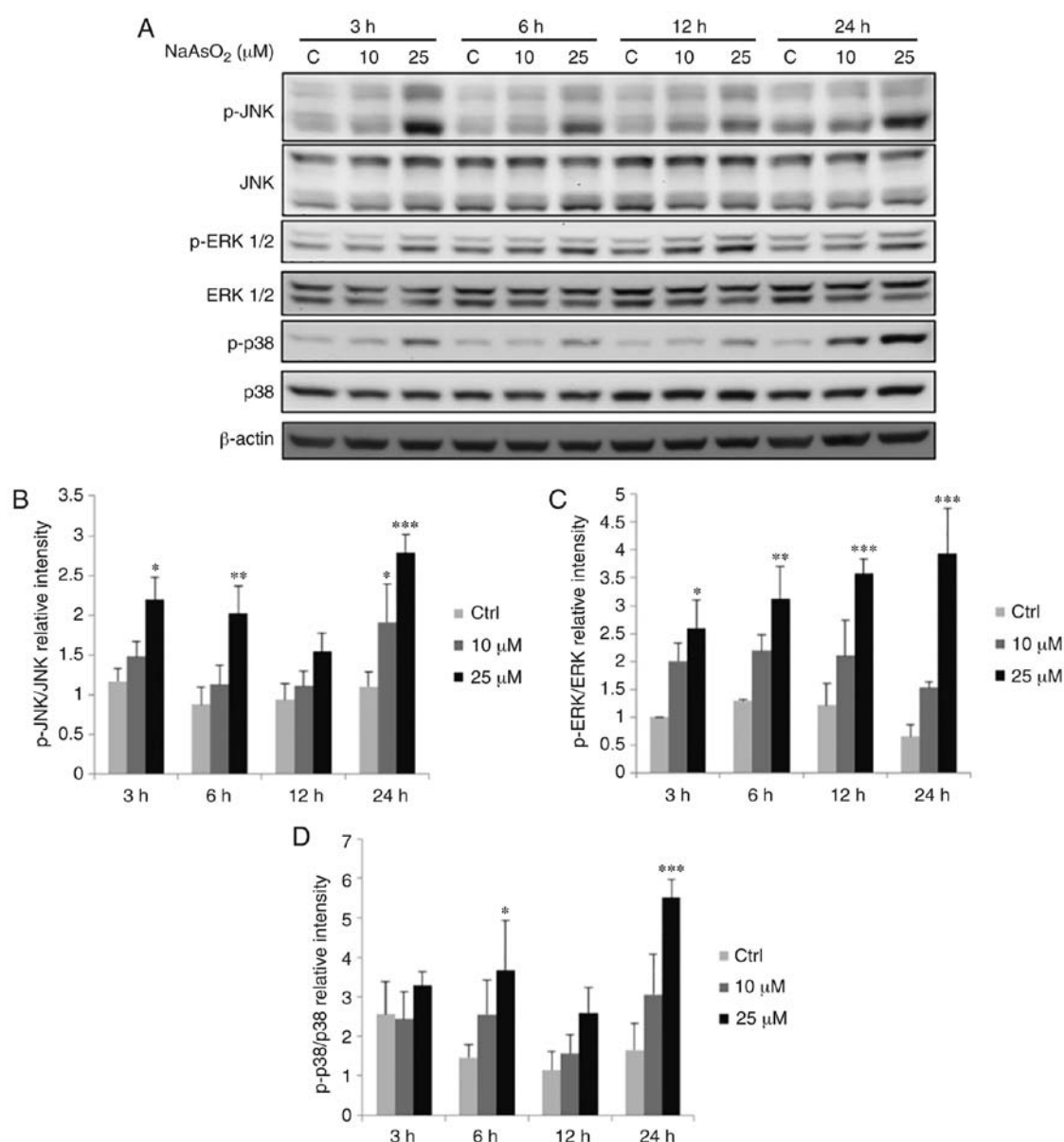


Figure 9. Effects of NaAsO<sub>2</sub> on the phosphorylation of the MAPK signaling pathway in OEC-M1 cells. Cells were treated without or with 10 and 25 μM NaAsO<sub>2</sub> for 3, 6, 12 and 24 h, respectively. (A) p-JNK (46/54 kDa), JNK, p-ERK1/2 (42/44 kDa), ERK1/2, p-p38 (43 kDa) and p38 were detected by western blotting. The IODs of (B) p-JNK, (C) p-ERK and (D) p-p38 proteins were normalized with total forms of themselves in each lane. The data are expressed as the mean ± SEM of at least three separate experiments. \*P<0.05, \*\*P<0.01, and \*\*\*P<0.001 represent statistical differences compared to the Ctrl group. NaAsO<sub>2</sub>, sodium arsenite; MAPK, mitogen-activated protein kinase; p-, phosphorylated; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; IODs, integrated optical densities; SEM, standard error of the mean; C or Ctrl, control.

the present study, and it is worth investigating, in a future study, at cellular and molecular levels how the expression of cell cycle proteins are regulated by NaAsO<sub>2</sub> and DMA to induce OEC-M1 cell apoptosis. Similar to the cell viability assay, once again, NaAsO<sub>2</sub> demonstrated 1,000-fold stronger effects compared with DMA on cell cycle regulation in OEC-M1 oral cancer cells.

Apoptosis is a critical process for maintaining homeostasis (50). Dysregulation of apoptosis is considered to be a key factor in the development of cancer (50). It is well known that apoptosis is initiated by either an extrinsic or an intrinsic death signal, and executed by the activated caspase cascade (51). It is reported that arsenic trioxide can induce the apoptosis of laryngeal cancer cells via downregulation of survivin mRNA,

which inhibits activation of caspases (52). Arsenic trioxide also induces apoptosis in different cancers by activating caspase-8, -9 and -3 (11,53). In the present study, the expression levels of cleaved caspase-8, -9, -3 and the downstream substrate PARP were found to be increased by both NaAsO<sub>2</sub> and DMA in OEC-M1 cells, and NaAsO<sub>2</sub> did exhibit a stronger activating effect compared with DMA. Thus, these results supported that both arsenic compounds induced oral cavity cancer cell apoptosis through extrinsic and intrinsic apoptotic pathways. It should be noted that, in the results, the expression of some caspases was most prominently increased by DAM at 1 mM, but not at 10 mM. It is highly possible that DAM at 1 mM has the maximal effect on the caspase pathway, which then starts to degrade. Thus, DAM at 10 mM could not induce a

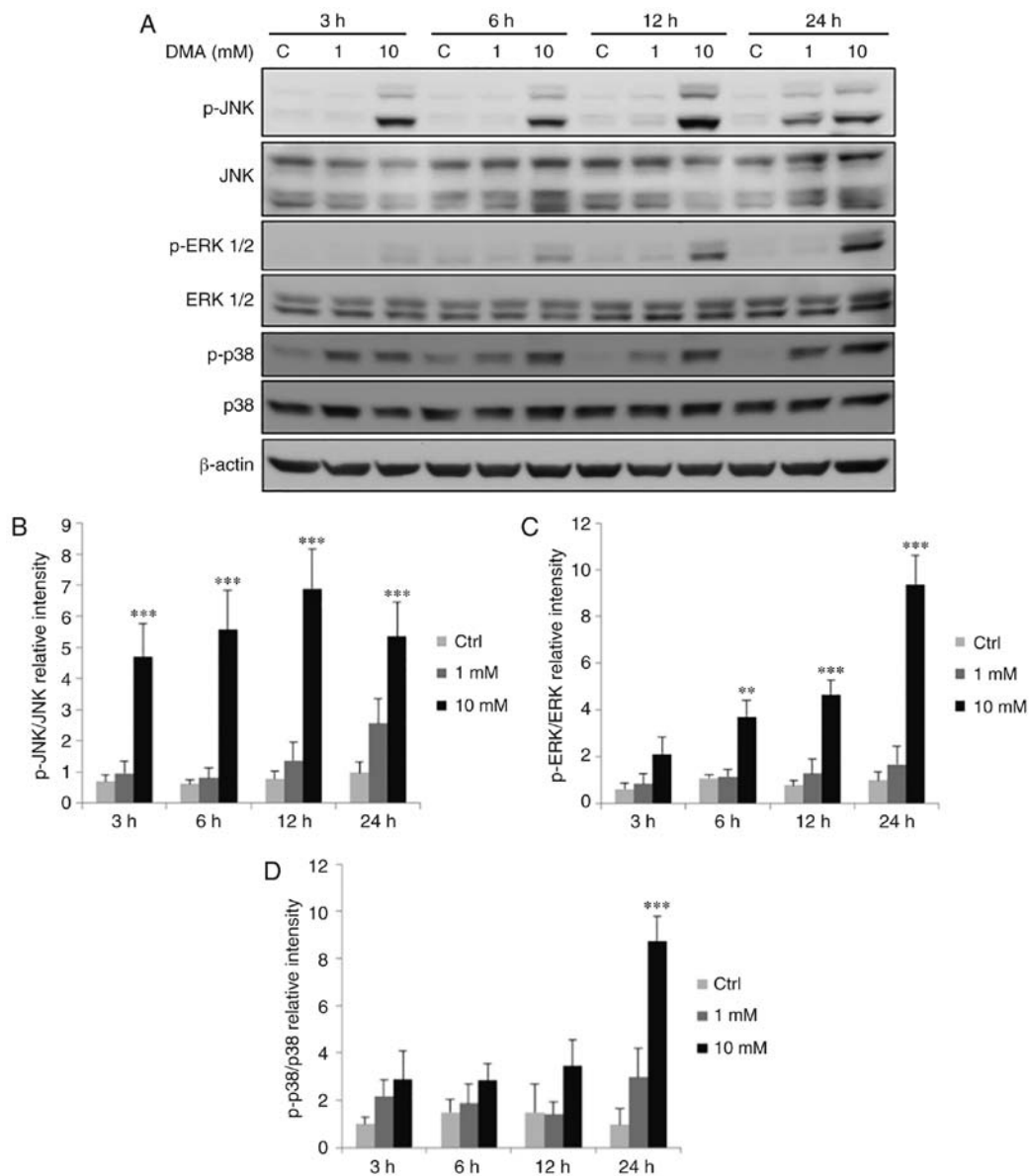


Figure 10. Effects of DMA on the phosphorylation of the MAPK signaling pathway in OEC-M1 cells. Cells were treated without or with 1 and 10 mM DMA for 3, 6, 12 and 24 h, respectively. (A) p-JNK (46/54 kDa), JNK, p-ERK1/2 (42/44 kDa), ERK1/2, p-p38 (43 kDa) and p38 were detected by western blotting. The IODs of (B) p-JNK, (C) p-ERK and (D) p-p38 proteins were normalized with total forms of themselves in each lane. The data are expressed as the mean  $\pm$  SEM of at least three separate experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  represent statistical differences compared to the Ctrl group. DMA, dimethylarsenic acid; MAPK, mitogen-activated protein kinase; p-, phosphorylated; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; IODs, integrated optical densities; SEM, standard error of the mean; C or Ctrl, control.

greater effect on OEC-M1 cell apoptosis. In addition, DAM exclusively induced some caspases at a concentration of 1 mM, indicating that phosphorylation of JNK, ERK and p38, significantly induced by DAM at 10 mM, could not be associated with the activation of caspases. In fact, DAM at 10 mM also induced the cleavage of PARP at 24 h. It is highly possible that the expression of cleaved caspase-8, -9 and -3 had degraded at 24 h after the treatment of DAM. Thus, DAM at 10 mM could stimulate phosphorylation of JNK, ERK and p38, and then further induce the caspase pathway.

In fact, studies have reported that arsenic compounds can activate different signaling pathways to induce cell death related to apoptotic pathways among different cancer cell types, such as myeloma cells, neutrophils and/or leukemia (54-58). Studies have demonstrated that arsenic

compounds could only activate the caspase-8 pathway to induce human melanoma cell apoptosis by enhancing TRAIL-induced apoptosis with the suppression of the NF- $\kappa$ B and STAT3-transcriptional targets (59) and could only stimulate the caspase-9 pathway to induce liver cancer cell apoptosis by downregulating phosphorylated-STAT3 expression with Bcl-2, XIAP, and surviving proteins (60), respectively. Studies have also revealed that arsenic compounds could activate both the caspase-8 and caspase-9 pathways concurrently to induce myeloma and promonocytic leukemia cell apoptosis by decreasing the expression of Bcl-2 families and/or regulating p38-MAPK process (61,62). In the present study, NaAsO<sub>2</sub> and DMA could concurrently induce both the caspase-8 and -9 pathways in OEC-M1 cells, indicating that our observations are not unprecedented. It is well

known that up- and downstream of the caspase-8 and -9 pathways, pathways including Fas/Fas ligand, BCL-2, ER stress, and/or p53, are markedly involved with the finer regulation of cancer cell apoptosis (59-62). Thus, it is worth further investigating how caspase-8 and -9 pathways are regulated by up- and downstream regulators to induce OEC-M1 cell apoptosis.

Apoptotic pathways are associated with numerous regulating mechanisms, one of which is the MAPK signaling pathway responding to cellular stress. MAPK signaling may either promote survival or enhance sensitivity to apoptosis depending on the cell types, stimuli and the latency of the activation of MAPKs (26,27). In the present study, p-JNK, p-ERK1/2 and p-p38 were all increased by both NaAsO<sub>2</sub> and DMA in OEC-M1 cells. These results indicated that both NaAsO<sub>2</sub> and DMA could activate the phosphorylation of JNK, ERK1/2 and/or p38 to induce apoptosis in OEC-M1 cells. Notably, these results demonstrated that activation of caspases and PARP was observed following treatments with arsenic compounds for 12 and 24 h, and the phosphorylation of MAPK occurred 3-6 h earlier compared with the caspase-mediated pathway, indicating that arsenic compounds possibly activated MAPK pathways prior to the caspases and PARP to induce apoptosis in OEC-M1 cells. It should be noted that 1 mM DMA significantly suppressed OEC-M1 cell viability, affected the cell cycle, increased the number of apoptotic cells and activated caspase proteins, but not MAPK proteins. With a closer examination of the western blot results, 1 mM DMA induced an increasing trend of MAPK protein expression although there was no statistical difference. Thus, these observations demonstrated that arsenic compounds activated different MAPK pathways first and then induced apoptosis in OEC-M1 oral cavity cells, which is consistent with the findings of other studies that have revealed that the induction of MAPK pathways and then caspase cascade under stimuli occurs in sequence (63-65).

In the present study, both NaAsO<sub>2</sub> and DMA could induce cell apoptosis through extrinsic and intrinsic apoptotic pathways, exerting potential antitumor effects on OEC-M1 oral cancer cells. Furthermore, both arsenic compounds were able to induce phosphorylation of JNK, ERK and p38 MAPKs to modulate the activation of the caspase cascade to stimulate apoptosis of OEC-M1 cells. However, the precise roles upon the activation of MAPK and caspase pathways by NaAsO<sub>2</sub> and DMA in OEC-M1 cells remain elusive, since specific inhibitors and/or siRNA were not used in the present study to block apoptosis. With the use of specific inhibitors and/or siRNA to suppress OEC-M1 cell apoptosis, the activation of MAPK and caspase pathways by NaAsO<sub>2</sub> and DMA in OEC-M1 cells can then be confirmed, which will conclusively reveal the underlying molecular mechanism. In addition, studies have demonstrated that intravenous arsenic trioxide and its derivatives both alone and/or in combination with other agents have been used successfully for the treatment of APL and myeloid neoplasms, and arsenic trioxide is a standard treatment for APL patients to date (66,67). In fact, oral liquid and pill formulations of arsenic trioxide have recently entered clinical practice for APL patients (66,67). Clinical trials involving arsenic compounds have never been applied to head and neck cancers for their possible

use in clinical practice. With the observations in the present study, NaAsO<sub>2</sub> and DMA could possibly be applied for the therapy of oral cancers with intravenous infusion and/or oral intake as a tablet or pill (66,67). Thus, clinical trials should be performed with arsenic compounds in order to possibly provide a helpful alternative method for the treatment of oral cancers.

It should be noted that the error bars are rather sizable in certain figures, regarding the western blot analysis of caspase and MAPK protein expression activated by NaAsO<sub>2</sub> and DMA in OEC-M1 cells. It is well known that in *in vitro* cell culture experiments, the expression levels of proteins may markedly vary due to the cell conditions related to the cell passages, cell quality, and cell seeding number with the possible changes in experimental room temperature and humidity, incubator temperature and humidity, western blot-conducting skills, and the integrating optical intensities of protein bands with UVP skills. In addition, the original data from 3 independent experiments without replicates for each treatment were used to perform statistical analysis and to draw figures in the present study. Thus, the error bars became rather sizable. Reliably, the statistical analysis could still show there are differences between treatments. Thereafter, in a future study, the same experiment should be repeated more than 3 times with 2 and/or 3 more replicates in each treatment, which could decrease error bar values and increase the fidelity of results. In conclusion, NaAsO<sub>2</sub> and DMA stimulated the extrinsic and intrinsic apoptotic pathways through the activation of MAPK pathways to induce apoptosis in OEC-M1 cells, demonstrating that both arsenic compounds exhibit promising antitumor properties and may be used for clinical therapy with marked potential for oral cancers.

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## Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Authors' contributions

NPF, CLK and CYC designed the experiments, carried out the experiments, interpreted the results, and wrote the manuscript. CYW, ECS and BMH participated in the design and coordination of the study, were involved in the statistical analysis of the obtained results and were responsible for the revision of the manuscript for substantive and methodological correctness. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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