Sodium arsenite and dimethylarsenic acid induces apoptosis in OC3 oral cavity cancer cells

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Abstract. Although arsenic is an environmental toxicant, arsenic trioxide (ATO) is used to treat acute promyelocytic leukemia (APL) with anticancer effects. Studies have demonstrated oral cancer is in the top 10 cancers in Taiwan. High rate of oral cancers is linked to various behaviors, such as excessive alcohol consumption and tobacco use. Similarly, betel chewing is a strong risk factor in oral cancer. In the present study, oral squamous carcinoma OC3 cells were investigated with the treatments of sodium arsenite ($NaAsO_2$) and dimethylarsenic acid (DMA), respectively, to examine if arsenic compounds have anti-cancer efforts. It was found that 1 μM $NaAsO_2$ and 1 mM DMA for 24 h induced rounded contours with membrane blebbing phenomena in OC3 cells, revealing cell apoptotic characteristics. In addition, $NaAsO_2$ (10-100 μM) and DMA (1-100 mM) significantly decreased OC3 cell survival. In cell cycle regulation detected by flow cytometry, $NaAsO_2$ and DMA significantly augmented percentage of subG_1 and G_2/M phases in OC3 cells, respectively. Annexin V/PI double staining assay was further used to confirm $NaAsO_2$ and DMA did induce OC3 cell apoptosis. In mechanism investigation, western blotting assay was applied and the results showed that $NaAsO_2$ and DMA significantly induced phosphorylation of JNK, ERK1/2 and p38 and then the cleavages of caspase-8, -9, -3 and poly ADP-ribose polymerase (PARP) in OC3 cells, dynamically. In conclusion, $NaAsO_2$ and DMA activated MAPK pathways and then apoptotic pathways to induce OC3 oral cancer cell apoptosis.

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Introduction

Arsenic (As) is a natural element in earth crust with nonmetallic and metallic properties (1,2), arsenic combines with sulfur, chlorine and oxygen to form inorganic compounds; while it will combine with hydrogen and carbon to form organic compounds in animals and plants (1,2). It is well known that organic arsenic is less toxic than inorganic arsenic and pentavalent and zero-valent arsenic is less toxic than trivalent arsenite (3,4). Arsenic trioxide (ATO), an ingredient of Chinese medicine, has also been discovered upon its antitumor potency first in patients with acute promyelocytic leukemia (APL) (5). Several studies have reported that ATO can induce apoptosis in tumor cells, including APL (6), lung cancer (7) and multiple myeloma (8). Arsenic hexoxide, another inorganic arsenic compound, is similarly found to possess anticancer effects against MCF-7 human breast cancer cells (9). In addition, Darinaparsin (S-dimethylarsino-glutathione), an organic arsenic compound, is safer than other organic arsenic compounds in hematologic and solid tumor treatment in preclinical models (10). Hence, not only inorganic arsenic compounds but organic ones possess capability resisting *in vitro* and *in vivo* tumor progression.

There are a number of mechanisms underlying cell death, which occurs when cells sense environmental stresses or intracellular signals (11). Cell death is categorized into autophagy, apoptosis or necrosis according to morphological changes (12). The first two types belong to programmed cell

death (13). Defective apoptosis is considered as major relevant factor in cancer development and that tumor cells avoid apoptosis serves an important role in drug resistance to therapeutic regimens (14).

The features of apoptosis include cell shrinkage, plasma membrane blebbing, DNA fragmentation and chromatin condensation (15). Apoptosis can be characterized into intrinsic and extrinsic pathways according to the stimuli and the related aspartate-specific cysteine protease (caspase) cascade (16). The intrinsic pathway depends on the decrease of mitochondrial membrane potential (MMP), which releases cytochrome c into the cytosol from the mitochondrial intermembrane space when facing cellular stress, such as DNA damage, UV exposure, or hypoxia (17). Then, cytochrome c will bind to apoptotic protease activating factor 1 (Apaf-1) forming a complex named 'apoptosome', which functions to recruit procaspase-9 (18). The active caspase-9 then cleaves procaspase-3 and the cleaved caspase-3 enters cytosol to stimulate the cleavage of poly ADP-ribose polymerase (PARP) for the induction of apoptosis (17,19). By contrast, the extrinsic pathway is initiated through death receptors, such as Fas/CD95, DR3, TNFR1, DR4 and DR5, when these receptors are associated with certain ligands (16). After binding, activated receptor would recruit related signal molecules to interact with death domains, leading to caspase-8 cleavage (16,20). Activated caspase-8 will then stimulate protease cascade to cleave caspase-3 and PARP within cells, resulting in apoptosis (17).

MAPKs are essential signaling pathways maintaining cell survival responding to various stresses (21). Conventional MAPKs include three members: ERK, p38 and JNK (22), which regulate cell mitosis, survival, gene expression, proliferation, differentiation and apoptosis (23). According to different cell types and stimuli, MAPKs can promote cells to survive and/or to undergo cell death (20,21,24). In fact, ATO can induce p38 and JNK phosphorylation, which result in human cervical cancer cell death through mitochondrial apoptotic cascade death (25).

Head and neck squamous cell carcinoma (HNSCC) develops from mucosa of upper aerodigestive tract (26) and usually occurs in males and it is more often among individuals over aged >50 (27). It is strongly associated with lifestyle and environmental risk causes, including betel chewing, alcohol consumption, tobacco smoking, UV light exposure and infection with human papillomavirus (28,29). HNSCC can be treated with chemotherapy, radiation therapy, surgery, gene therapy and immunotherapy (30). However, these treatments are not good enough. In fact, oral cancer has been in the top 10 commonest cancers for years in Taiwan, and the age-standardized incidence rate of head and neck cancers has increased by 5.4% per year among males and 3.1% among females from 1980-2014 (31,32). Thus, other alternative drugs with improved efficacy and lower side effects for therapeutic remedy on HNSCC will be needed. However, whether arsenic compounds could serve as therapeutic agents for oral cavity cancers and the relative regulating mechanisms remains to be elucidated.

Oral squamous cell carcinoma contributes 95% of HNSCC, which is highly aggressive and malignant. Recently, studies have shown the MAPK signaling pathway is stimulated in >50% of human oral cancer cases (33). Thus, an investigation on the MAPK signaling pathway with its potential therapeutic mechanisms for oral squamous cell carcinoma could be useful. To investigate anticancer effect of arsenic compounds with underlying mechanisms associated with possible therapeutic remedy treating oral cavity cancer, OC3 cells (a Taiwan native oral cancer cell line with a long-term areca betel chewer who did not smoke) (34) were used in the present study. OC3 cells were treated with $NaAsO_2$ and DMA to examine if arsenic compounds would influence cell viability, cell cycle and related signal pathways to induce apoptosis with the anti-cancer capability. It was found that both $NaAsO_2$ and DMA could activate MAPK and caspase pathways to cause cell cycle dysregulation, which lead to OC3 cell apoptosis. These findings may provide therapeutic information for oral cancer in Taiwan.

Materials and methods

Chemicals. Disodium hydrogen phosphate (Na_2HPO_4), sodium bicarbonate ($NaHCO_3$) and potassium dihydrogen phosphate (KH_2PO_4) were bought from Honeywell Specialty Chemicals Seelze GmbH. FBS and Trypsin-EDTA were obtained from AG Scientific. The Annexin V-FITC apoptosis detection kit was bought from Strong Biotech Corporation. DMSO, hydrochloric acid, Tween-20, SDS, sodium hydroxide DMA, $NaAsO_2$, staurosporine, high-glucose DMEM, PI, RNase A, MTT and penicillin-streptomycin were purchased from MilliporeSigma. A Micro BCA protein assay kit was purchased from Thermo Fisher Scientific, Inc. HEPES, potassium chloride, sodium chloride and Tris base were procured from J.T. Baker. Antibodies against β-actin (cat. no. 58169; 1:5,000), JNK (cat. no. 9252), phosphorylated (p)-JNK (cat. no. 9251), ERK1/2 (cat. no. 9102), p-ERK1/2 (cat. no. 9101), p38 (cat. no. 9212), p-p38 (cat. no. 9215), cleaved caspase-8 (cat. no. 9429), cleaved PARP (cat. no. 9542), cleaved caspase-9 (cat. no. 9509) and cleaved caspase-3 (cat. no. 9661) were attained from Cell Signaling Technology, Inc. Donkey anti-rabbit IgG (cat. no. NEF81200-1EA) conjugated to HRP was bought from PerkinElmer, Inc. ECL detection kit was obtained from MilliporeSigma.

Cell culture. OC3 cell line, a Taiwan native oral cancer cell line from a long-term areca betel chewer who did not smoke (34), was a gift from Professor Kuo-Wei Chang (National Yang-Ming University). OC3 cells were kept in DMEM/F12 and 2-fold volume of Keratinocyte-SFM medium (Thermo Fisher Scientific, Inc.). All media were complemented with 25 mM HEPES, 24 mM $NaHCO_3$, 10,000 U streptomycin, 10,000 U penicillin and 10% fetal bovine serum at pH 7.4. Cells were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37˚C.

Morphology observation. OC3 cells ($6x10^5$) were cultured in 6 cm Petri dish with 2 ml medium. After reaching 70-80% confluence, cells were treated for 24 h with different dosages of sodium arsenite (0, 0.1, 1, 10, 25, 50 and 100 μM) or dimethylarsenic acid (0, 0.1, 1, 2, 5, 10, 25, 50 and 100 mM), respectively. The changes of cell morphology were inspected

with 10 random fields in each treatment using light microscopy at 40x magnification (Olympus CK40; Olympus Corporation) and images captured using an Olympus DP20 digital camera (Olympus Corporation).

MTT viability assay. OC3 cells were cultured at $1x10^4$ cells in 100 μl medium per well. After reaching 70-80% confluence, cells were treated for 24 h with different dosages of sodium arsenite (0, 0.1, 1, 10, 25, 50 and 100 μM) or dimethylarsenic acid (0, 0.1, 1, 2, 5, 10, 25, 50 and 100 mM), respectively. MTT at 0.5 mg/ml final concentration was then added and incubated at 37˚C for another 4 h. The medium was removed and 50 μl DMSO was put into all wells dissolving the crystals through plate shaking for 20 min in the dark. Absorbance values among treatments were then examined at λ=570 nm using VersaMax ELISA reader (Molecular Devices, LLC.).

Cell cycle investigation. To test if sodium arsenite and dimethylarsenic acid could stimulate apoptosis in OC3 cells, cell cycle redistribution was studied using flow cytometry with propidium iodide staining. OC3 cells ($6x10^5$) were cultured and, after 70-80% confluence, treated with different dosages of sodium arsenite (0, 0.1, 1, 10, 25, 50 and 100 μM) and different dosages of dimethylarsenic acid (0, 0.1, 1, 10, 25, 50 and 100 mM) for 24 h, respectively. Cells were then collected with trypsin and centrifuged at 400 x g and 4˚C for 12 min, washed by isoton II and fixed with 70% ethanol at -20˚C for 2 h. After fixation, OC3 cells were rinsed again by isoton II and harvested through centrifugation at 400 x g for 12 min at 4˚C. OC3 cell pellets were suspended again by isoton II and then mixed with 40 μg/ml propidium iodide plus 100 μg/ml RNase for 30 min at 25˚C. A flow cytometer (FACScan; BD Biosciences) was used with excitation set at λ=488 nm to determine stained cell distributions. DNA diploid will be observed in G_1 phase while DNA synthesis progress exists in G_2/M phase in normal cells. Less DNA content and hypodiploid, is detected in subG_1 phase cells, considered as cell apoptosis (35).

Annexin V/PI double staining assay. Treated OC3 cells were collected with trypsin and then rinsed with 2 ml medium. After 160 x g centrifugation at 4˚C for 10 min, cell pellets were suspended again by cold isoton II and centrifuged at 400 x g for 12 min at 4˚C. Cell pellets were subsequently mixed with staining solution (100 μl) and reacted for 15 min at 25˚C. Stained OC3 cells were determined by FACScan flow cytometer (BD Biosciences) with excitation at λ=488 nm using >600 nm band pass filter for PI detection and 515 nm band pass filter for FITC detection, respectively. Plots displayed four quadrants with staining annexin V/PI double-positive (late apoptosis) cells, PI-positive (necrosis) cells, annexin V-positive (early apoptosis) cells and negative (viable) cells, respectively (36). It should be noted that control and staurosporine results in Fig. 5A and Fig. 6A are same. The reason is that control, staurosporine, sodium arsenite and dimethylarsenic acid treatments were conducted simultaneously in experiments. To clearly show the results for sodium arsenite and dimethylarsenic acid treatments, respectively, Figs. 5 and 6 were therefore created using same control and staurosporine results for comparison.

Western blotting. Cells at $6x10^5$ were cultured with 60 mm dish. After 70-80% confluence, different dosages of sodium arsenite (0, 10 and 25 μM) or dimethylarsenic acid (0, 10 and 25 mM) were added to OC3 cells for 3 to 24 h, respectively. Cell culture medium was then moved to a 15 ml tube and collected by centrifugation (1,500 x g; 10 min at 4˚C). Cells were broken down by 100 μl lysis solution plus proteinase inhibitor. Pellets were suspended again by 10 μl lysis solution to blend into cell lysates and conducted with centrifugation (12,000 x g; 12 min at 4˚C). Supernatants were then collected and kept at -80˚C. Protein concentrations of cell lysates were determined by Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Inc.) (19). Cell lysates (20-30 μg) were resolved in SDS-PAGE gel (12%) with running buffer at 25˚C and transferred to PVDF membranes, electrophoretically, at 4˚C. Membranes was blocked in 2-4% skimmed milk at room temperature for 1 h and incubated with primary antibodies [cleaved caspase-3 (cat. no. 9661; 1:1,000), cleaved caspase-9 (cat. no. 9509; 1:1,000), cleaved PARP (cat. no. 9542; 1:1,000), cleaved caspase-8 (cat. no. 9429; 1:1,000), ERK1/2 (cat. no. 9102; 1:4,000), phospho-ERK1/2 (cat. no. 9101; 1:4,000), p38 (cat. no. 9212; 1:4,000), phospho-p38 (cat. no. 9215; 1:1,000), JNK (cat. no. 9252; 1:1,000), phospho-JNK (cat. no. 9251; 1:4,000)] at 4˚C overnight. After washing with TBS Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at 25˚C (Donkey anti-rabbit IgG; cat. no. NEF81200-1EA; 1:2000), membranes were then visualized by enhanced chemiluminescence detection kit using UVP EC3 (BioImaging Systems). Quantification for each band was achieved using ImageJ version 1.50 software (National Institutes of Health).

Statistical analysis. Data were expressed as mean ± SEM from ≥3 separate experiments. Statistical significance between treatment and control groups was examined by one-way ANOVA and then Tukey's test with GraphPad Prism 9 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Arsenic compounds induce morphological changes in OC3 cells. OC3 cells were treated for 24 h in plain medium, sodium arsenite (0, 0.1, 1, 10, 25, 50 and 100 μM; Fig. 1A-G), or dimethylarsenic acid (0, 0.1, 1, 10, 25, 50 and 100 mM; Fig. 1H-M), respectively. Cell morphological alterations were then observed under light microscopy. In sodium arsenite experiment, OC3 cells showed spindle shape in control treatment (Fig. 1A). Cells appeared rounded and floated in medium as the dosages increased and attached cells significantly decreased from 25 to 100 μM groups in OC3 cells (Fig. 1E to G), respectively. In dimethylarsenic acid experiment, cells started to float in OC3 cells treated by 0.1 mM dimethylarsenic acid (Fig. 1H). As concentration of dimethylarsenic acid increased, cells shrank with membrane blebbing, implying that cells underwent apoptosis (Fig. 1I to M). Fig. 1N is a x4 enlargement of cell membrane blebbing in Fig. 1F, which clearly demonstrates sodium arsenite could induce OC3 cell apoptosis.

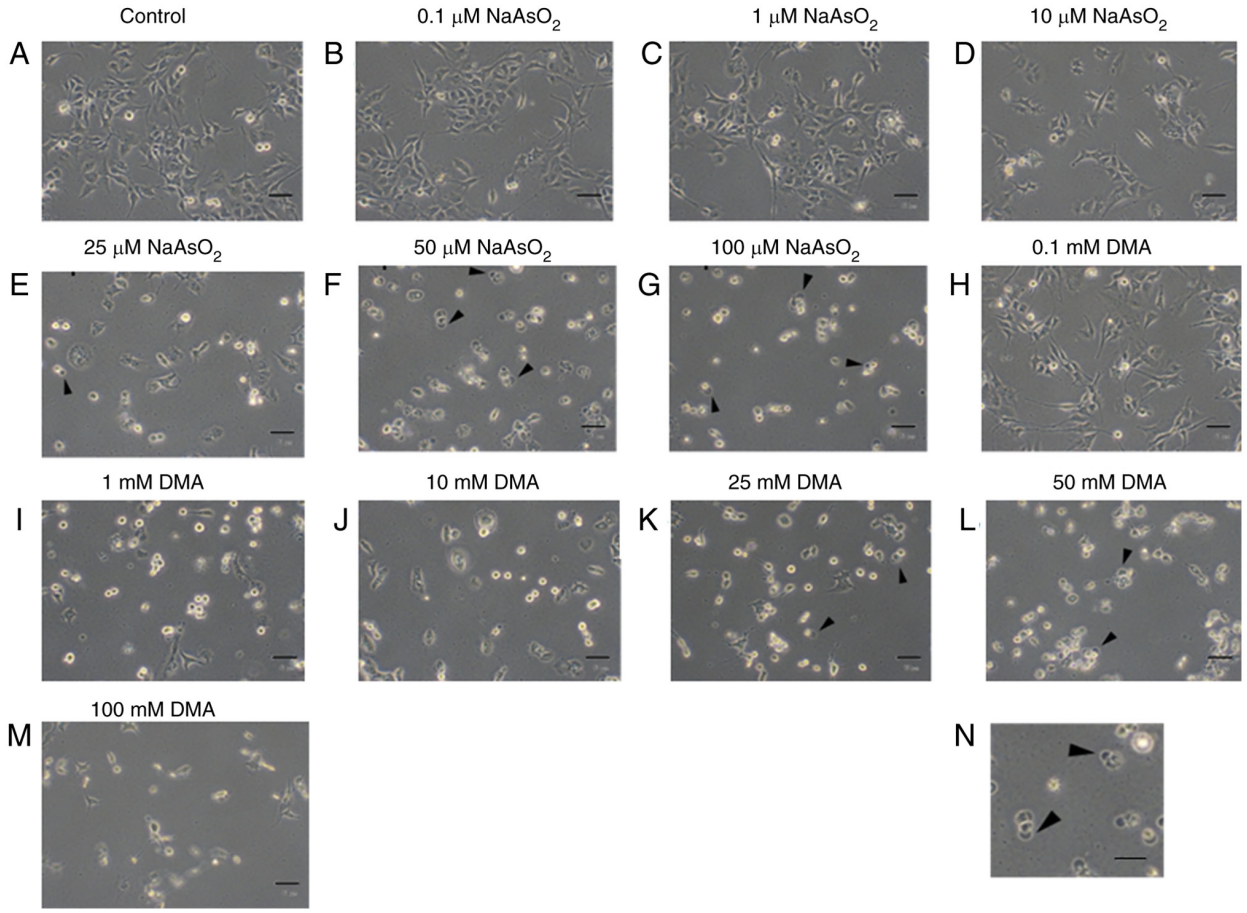

Figure 1. Arsenic compounds induce morphological changes in OC3 cells. OC3 cells were incubated with (A) plain medium control, (B) 0.1 μM, (C) 1 μM, (D) 10 μM, (E) 25 μM, (F) 50 μM and (G) 100 μM sodium arsenite, or (H) 0.1 mM, (I) 1 mM, (J) 10 mM, (K) 25 mM, (L) 50 mM and (M) 100 mM dimethylarsenic acid for 24 h, respectively, repeated three times. Changes of cell morphology were observed under phase contrast microscopy (scale bar, 50 μm). Cell membrane blebbings are arrowed. (N) the x4 enlarged image of cell membrane blebbing in (F) (scale bar, 200 μm). DMA, dimethylarsenic acid.

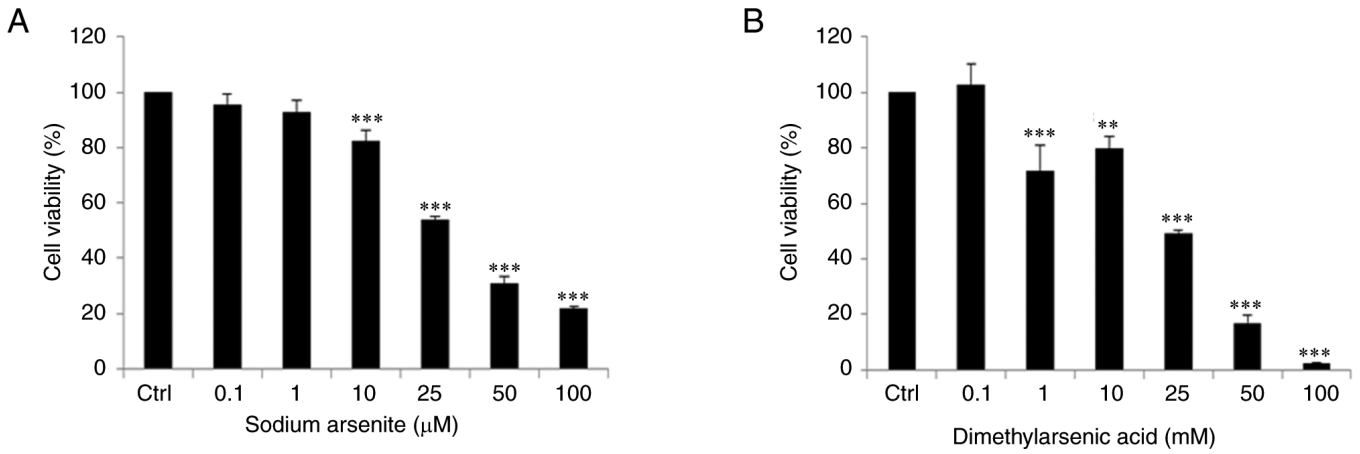

Figure 2. Arsenic compounds reduce cell viability in OC3 cells. Cells were incubated with (A) 0, 0.1, 1, 10, 25, 50 and 100 μM sodium arsenite and with (B) 0, 0.1, 1, 10, 25, 50 and 100 mM dimethylarsenic acid for 24 h, respectively. The cell viability was determined through MTT assay. Data are illustrated by percentages of cell proliferation rate compared with control group. Results are mean ± standard error of the mean of three independent experiments. **P<0.01 and ***P<0.001 vs. control. Ctrl, control.

These data suggested both dimethylarsenic acid and sodium arsenite could induce the abnormal morphological changes associated with apoptotic cell death in OC3 oral cancer cells with dose-dependent relationships.

Arsenic compounds reduces cell viability in OC3 cells. Survival rates in OC3 cells following treatments of arsenic compounds were further determined by MTT assay. OC3 cells were treated for 24 h with sodium arsenite (0, 0.1, 1, 10,

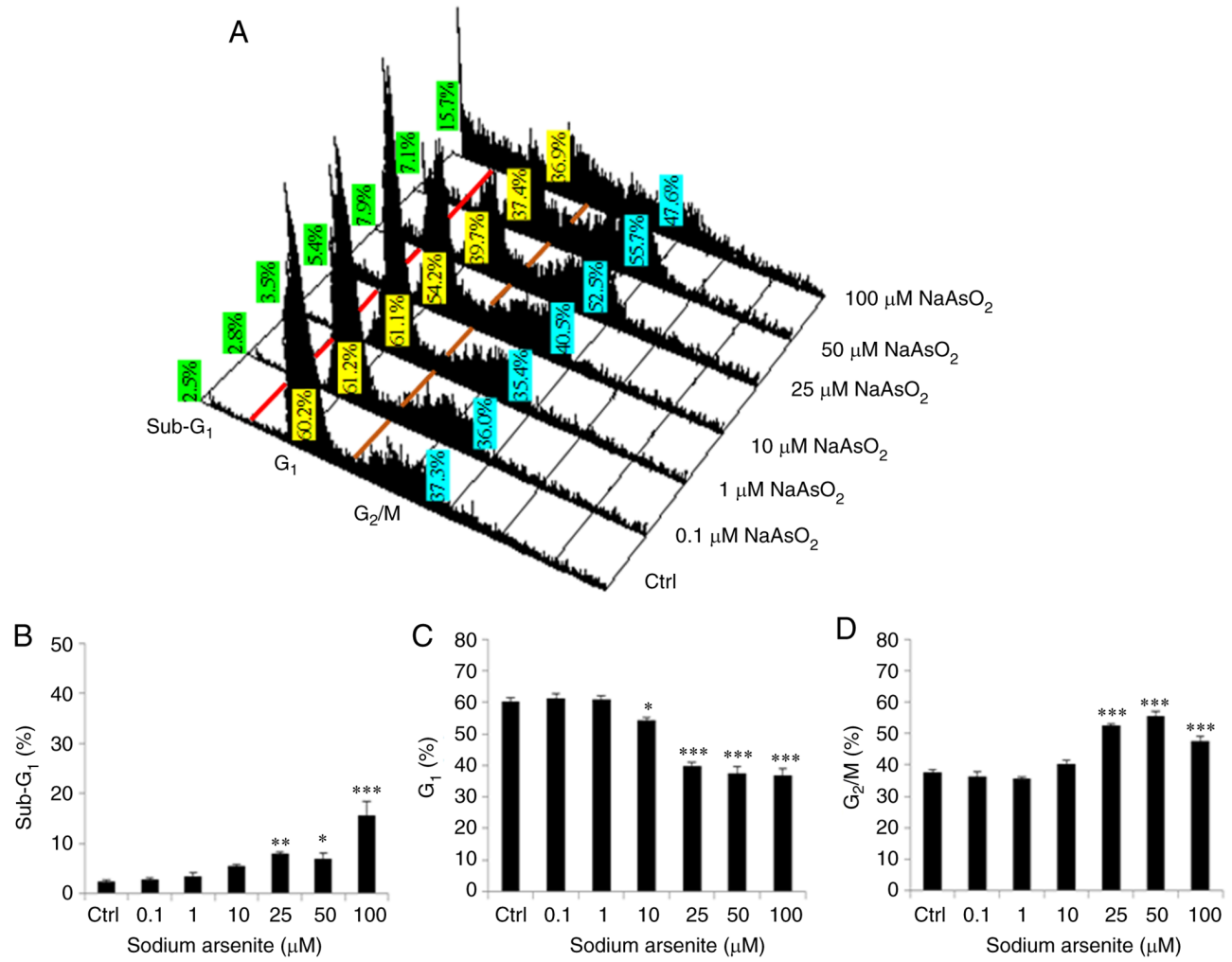

Figure 3. Sodium arsenite modulates redistribution of cell cycle in OC3 cells. OC3 cells were treated with sodium arsenite at 0, 0.1, 1, 10, 25, 50 and 100 μM for 24 h and cells were then fixed, stained with propidium iodide and evaluated through flow cytometry assay. (A) Red and brown lines are plotted to illustrate the changes of sub-G_1 (left to red line), G0/G_1 (between red and brown lines) and G_2/M phases (right to brown line) in the different treatment groups. Values with green background illustrate percentages of sub G_1 cells (left to red line) in different treatments, which is also shown in (B) Values with yellow background illustrate percentages of G0/G_1 cells (between red and brown lines) in different treatments, which is also shown in (C) Values with blue background illustrate percentages of G_2/M cells (right to brown line) in different treatments, which is also shown in (D) There is less DNA content in sub-G_1 phase cells than normal cells, which indicates apoptosis. Results are demonstrated as mean ± standard error of the mean of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control. Ctrl, control.

25, 50 and 100 μM) or dimethylarsenic acid (0, 0.1, 1, 10, 25, 50 and 100 mM), respectively and results showed that both arsenic compounds significantly reduced OC3 cell viability with dose-dependent phenomena (P<0.05). The survival rate significantly decreased by sodium arsenite from 10 to 100 μM (Fig. 2A) and by dimethylarsenic acid from 1 to 100 mM (Fig. 2B) in OC3 cells, respectively (P<0.05).

The concentration of dimethylarsenic acid reducing cell viabilities to 50% in OC3 cells was ~1,000 times higher compared with the concentration of sodium arsenite. Therefore, sodium arsenite showed stronger cytotoxicity than dimethylarsenic acid in OC3 cells.

Arsenic compounds modulates redistribution of cell cycle in OC3 cells. Studies have shown the subG$_1$ phase is a sign of DNA fragmentation for apoptosis and G_2/M phase arrest could lead cells to undergo apoptosis (20,37). To test whether sodium arsenite and dimethylarsenic acid could induce cell death through apoptosis, OC3 cells were challenged with arsenic compounds and DNA contents were detected through propidium iodide staining with flow cytometry analysis. OC3 cells were treated with different concentrations of sodium arsenite (0, 0.1, 1, 10, 25, 50 and 100 μM) and dimethylarsenic acid (0, 0.1, 1, 10, 25, 50 and 100 mM) for 24 h to examine possible impacts on cell cycle regulation (Figs. 3 and 4), respectively.

In the present study, significant increase of subG$_1$ phase cell number was observed following 24 h treatment of sodium arsenite at 25 to 100 μM in OC3 cells (Fig. 3A and B; P<0.05). The number of G_1 phase cells was decreased following 24 h treatment of sodium arsenite from 10 to 100 μM in OC3 cells (Fig. 3A and C; P<0.05). In addition, 25 to 100 μM sodium arsenite significantly induced OC3 cell G_2/M phase arrest (Fig. 3A and D; P<0.05).

In addition, dimethylarsenic acid at 1, 50 and 100 mM significantly increased subG$_1$ phase cell numbers (Fig. 4A and B; P<0.05); 1-100 mM significantly reduced G_1 phase cell numbers (Fig. 4A and C; P<0.05); and 1 to 25 mM

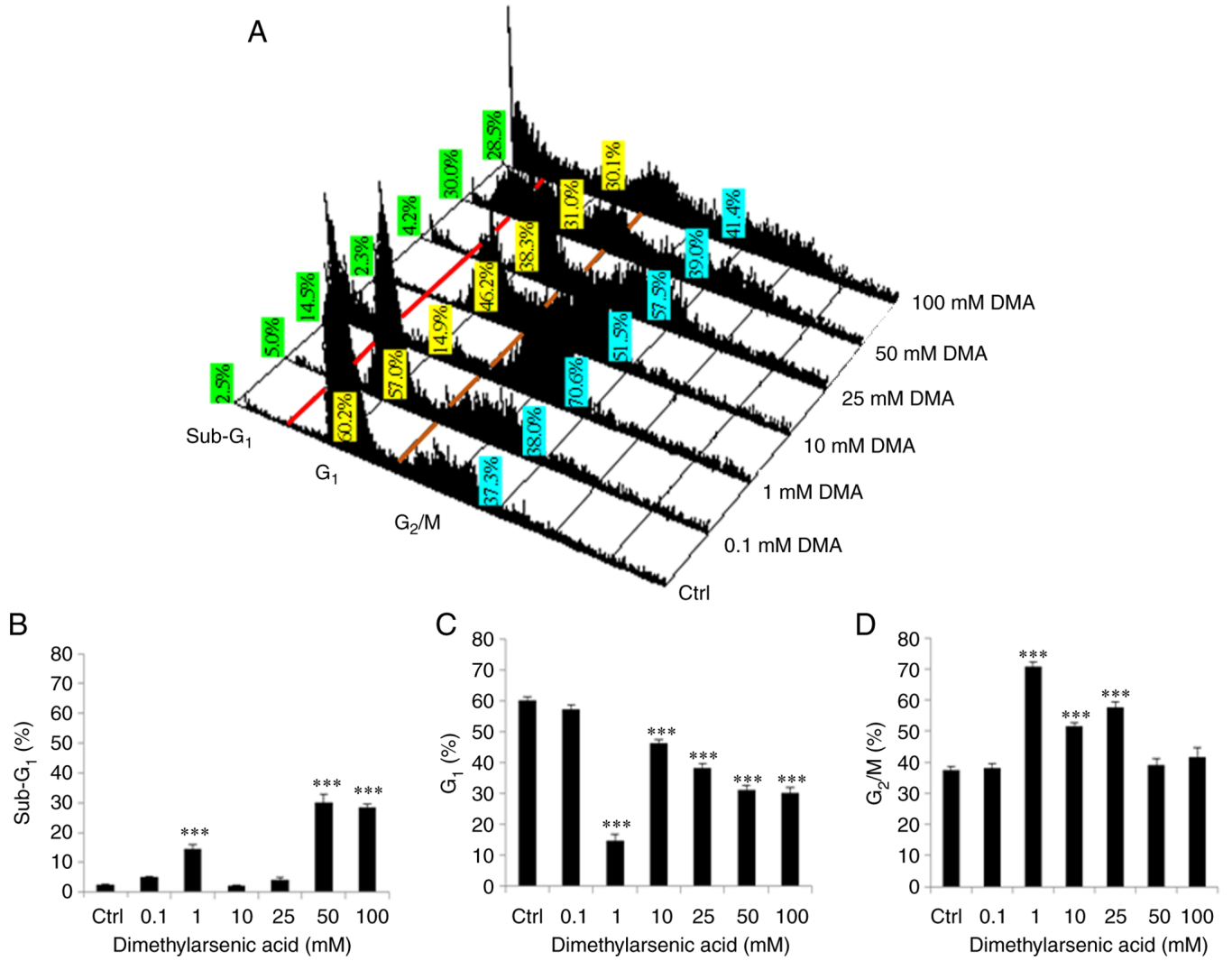

Figure 4. Dimethylarsenic acid modulates redistribution of cell cycle in OC3 cells. OC3 cells were treated with dimethylarsenic acid at 0, 0.1, 1, 10, 25, 50 and 100 mM for 24 h and cells were then fixed, stained with propidium iodide and evaluated through flow cytometry assay. (A) Red and brown lines are plotted to illustrate the changes of sub-G_1 (left to red line), G0/G_1 (between red and brown lines) and G_2/M phases (right to brown line) in the different treatment groups. Values with green background illustrate percentages of sub G_1 cells (left to red line) in different treatments, which is also shown in (B) Values with yellow background illustrate percentages of G0/G_1 cells (between red and brown lines) in different treatments, which is also shown in (C) Values with blue background illustrate percentages of G_2/M cells (right to brown line) in different treatments, which is also shown in (D) There is less DNA content in subG_1 phase cells than normal cells, which indicates apoptosis. Results are demonstrated as mean ± standard error of the mean of three independent experiments. ***P<0.001 vs. control. Ctrl, control.

significantly increased G_2/M phase cell number, respectively (Fig. 4A and D; P<0.05).

It should be noted that dimethylarsenic acid at 1, 50 and 100 mM, but not 25 mM, could increase subG_1 phase cell number in OC3 cells (Fig. 4A and B). Moreover, dimethylarsenic acid at high concentration could increase G_2/M phase cell number in OC3 cells (Fig. 4A and D). The data illustrated that sodium arsenite and dimethylarsenic acid could modulate subG_1, G_1 and G_2/M phase numbers in OC3 cells.

Arsenic compounds induce cell apoptosis in OC3 cells. To confirm if apoptosis was induced with arsenic compounds in OC3 cells, annexin V and propidium iodide double staining assay with flow cytometry was used. Percentages of double-positive (late apoptotic), annexin V single-positive (early apoptotic), PI single-positive (necrotic) and double-negative (viable) cells are shown in a four quadrants to determine cell apoptosis (36). The results showed that 24 h treatments with sodium arsenite (50 and 100 μM; Fig. 5A-C) and dimethylarsenic acid (10-100 mM; Fig. 6A-C) for significantly induced OC3 cell apoptosis (P<0.05).

Arsenic compounds induces extrinsic and intrinsic caspases pathways in OC3 cells. To further investigate whether arsenic compounds could induce cell death involved in extrinsic (death receptor) or intrinsic (mitochondrial) apoptotic pathways, expressions of cleaved caspase-9 and caspase-8 with the downstream targets of caspase-3 plus PARP cleavages were determined through western blotting. The results showed that 24 h treatment with 25 μM sodium arsenite could considerably stimulate cleaved caspase-8, -9, -3 and PARP expressions in OC3 cells (Fig. 7A to E; P<0.05). Treatment for 24 h with 10 μM sodium arsenite could also significantly stimulate the expressions of cleaved caspase-9, -3 and PARP in OC3 cells

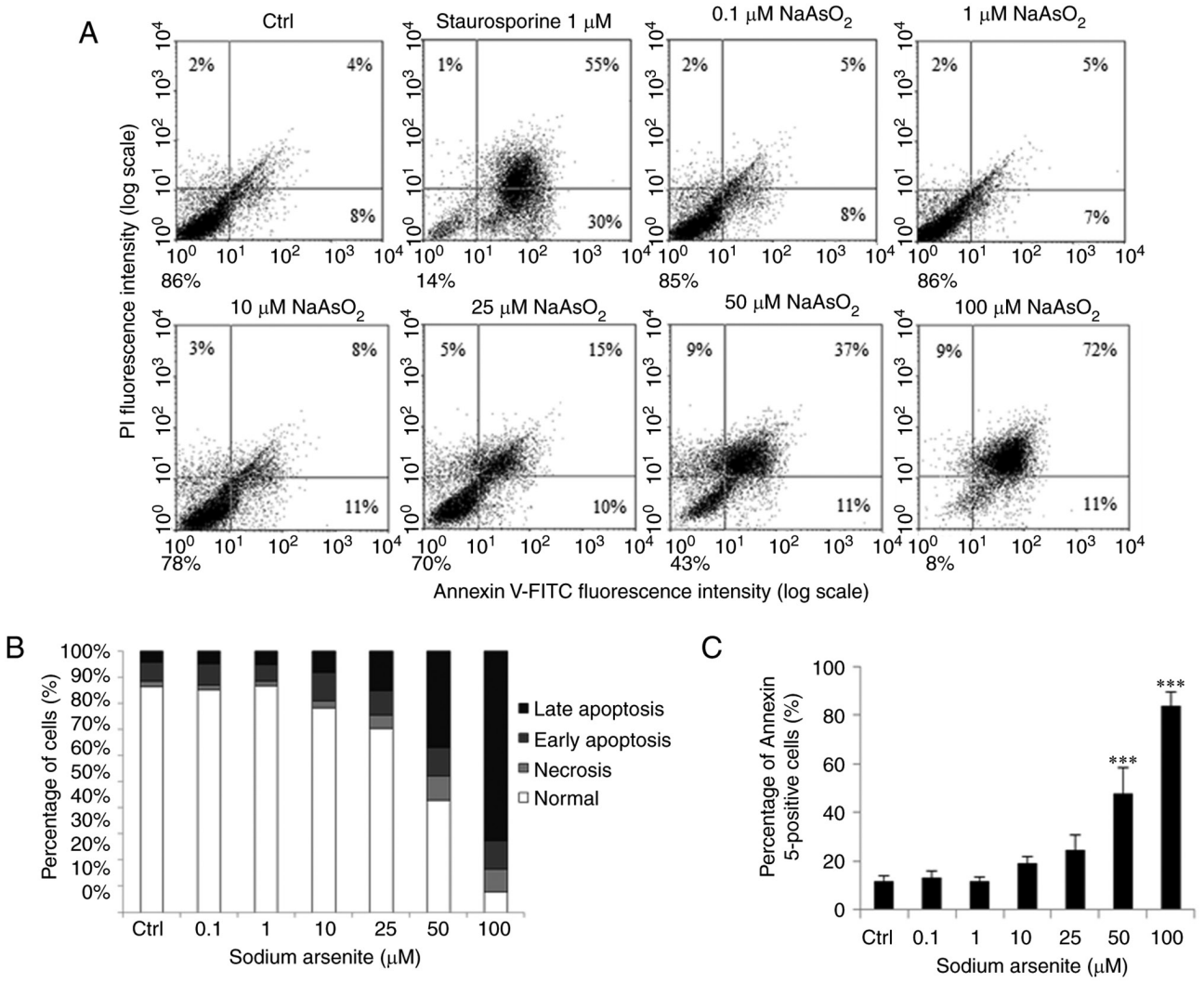

Figure 5. Sodium arsenite induces cell apoptosis in OC3 cells. OC3 cells were incubated with $NaAsO_2$ at 0, 0.1, 1, 10, 25, 5,0 and 100 μM for 24 h. Staurosporine was used to treat cells for positive control. (A) Annexin V/PI double staining assay was used to determine cell apoptotic status. (B) Percentages of double-positive (late apoptotic), annexin V single-positive (early apoptotic), PI single-positive (necrotic) and double-negative (viable) cells. (C) Double-positive (late apoptotic) and annexin V single-positive (early apoptotic) cells were analyzed. Results are demonstrated as mean ± standard error of the mean of three independent experiments. ***P<0.001 vs. control. Ctrl, control; PI, propidium iodide.

(Fig. 7A to E; P<0.05). In addition, treatment for 12 h with 25 μM sodium arsenite could significantly induce cleaved caspase-3 expression (Fig. 7A and D; P<0.05). Treatments for 12 and 24 h with 1 mM dimethylarsenic acid significantly activated expressions of cleaved caspase-8, -9 and -3, resulting in PARP cleavage at 24 h treatment in OC3 cells (Fig. 8A to E; P<0.05).

Arsenic compounds induce MAPK pathways in OC3 cells. Studies have shown MAPK signaling pathways are involved to regulate cell survival, differentiation, gene expression, proliferation, mitosis and/or apoptosis (21-24). To test if MAPK pathways were associated with apoptosis induced by arsenic compounds in OC3 cells, JNK, ERK1/2 and p38 phosphorylation was analyzed with western blotting. Results showed that treatments for 6 and 24 h with 25 μM sodium arsenite significantly increased JNK phosphorylation in OC3 cells (Fig. 9A and B; P<0.05), while treatments for 3, 6, 12 and 24 h with 25 μM sodium arsenite significantly increased ERK1/2 phosphorylation in OC3 cells (Fig. 9A and C; P<0.05). Phosphorylated p38 was significantly induced after 24 h treatment of 25 μM sodium arsenite (Fig. 9A and D; P<0.05). Treatments for 3 and 6 h with 10 mM dimethylarsenic acid significantly increased JNK phosphorylation (Fig. 10A and B; P<0.05), while treatments for 3, 6, 12 and 24 h with 10 mM dimethylarsenic acid significantly increased ERK1/2 phosphorylation (Fig. 10A and C; P<0.05). Phosphorylated p38 was significantly increased with 12 and 24 h treatments of 10 mM dimethylarsenic acid (Fig. 10A and D; P<0.05).

Discussion

ATO possesses anticancer ability in APL patients (6). Studies have also shown that arsenic compounds have been used to efficiently treat numerous types of cancers, such as colon, breast, pancreatic, prostate and neuroblastoma cancers, by inducing cell apoptosis (9,38-41). However, whether arsenic compounds could serve as therapeutic agents for oral cavity cancers and the relative regulating mechanisms remain to be elucidated.

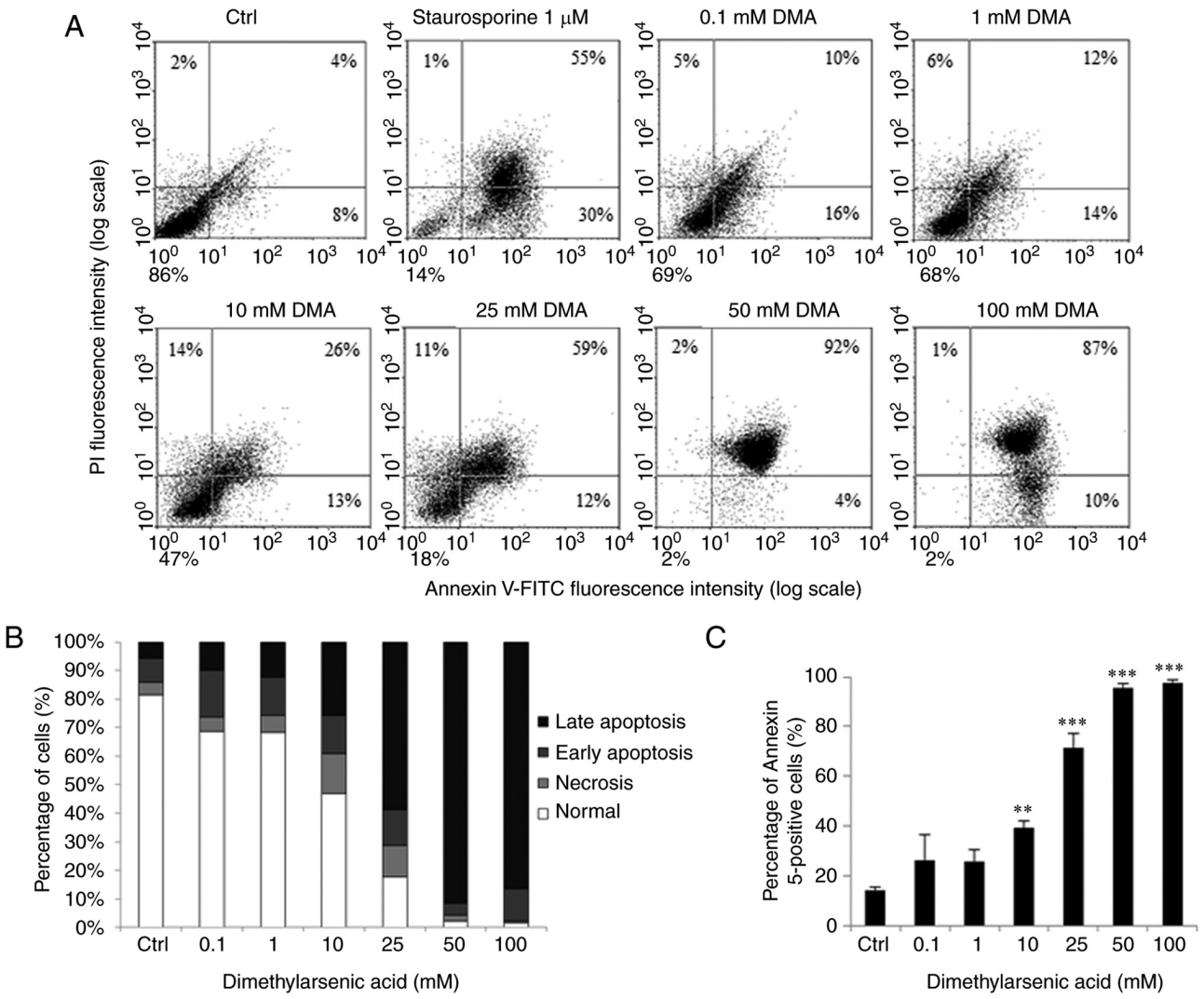

Figure 6. Dimethylarsenic acid induces cell apoptosis in OC3 cells. OC3 cells were incubated with DMA at 0, 0.1, 1, 10, 25, 50 and 100 mM for 24 h. Staurosporine was used to treat cells for positive control. (A) Annexin V/PI double staining assay was used to determine cell apoptotic status. (B) Percentages of double-positive (late apoptotic), annexin V single-positive (early apoptotic), PI single-positive (necrotic) and double-negative (viable) cells. (C) Double-positive (late apoptotic) and annexin V single-positive (early apoptotic) cells. Results are demonstrated as mean ± standard error of the mean of three independent experiments. Control and staurosporine results in (A) are same as in Fig. 5, as Control, staurosporine, sodium arsenite and dimethylarsenic acid treatments were conducted simultaneously. **P<0.01 and ***P<0.001 vs. control. PI, propidium iodide; Ctrl, control.

Oral cancers have long been the refractory solid tumors, most of which consist of squamous cell carcinoma (38-41). The incidence and mortality increases year by year in Taiwan (31,32). Treatments remain poor and new therapeutic strategies are required. In the present study, sodium arsenite and dimethylarsenic acid showed significant effects to induce apoptosis in OC3 cells.

In the present study, sodium arsenite and dimethylarsenic acid decreased OC3 cell viability in dose-dependent manners (Fig. 2), consistent with the morphological change shown in Fig. 1. It should be noted that the concentration of sodium arsenite was lower than that of dimethylarsenic acid to perform similar effects; sodium arsenite at 25 μM for 24 h treatment significantly reduced cell viability to 53%, while dimethylarsenic acid 25 mM for 24 h treatment was needed to reduce OC3 cell viability to 49% in MTT cell viability test. It is known that inorganic arsenic compounds are more toxic than organic arsenic compounds (3,4,42). Inorganic arsenic compounds can be methylated to become less toxic compounds processed by methyltransferases in liver, a detoxification reaction, and the methylation occurs through alternating reductive and oxidative methylation reactions (43). Thus, the findings of the present study were not unprecedented.

Eukaryotic cell cycle proceeds to the next phase when cellular upstream events accomplish checkpoint necessities (44). After suffering cellular injury, cell cycle arrest can occur at certain phase and cells can proceed to programmed cell death as they cannot be properly repaired (45). Arsenite can stimulate microtubule network disruption, which can result in spindle aberrations to induce cell apoptosis (46). Indeed, arsenic-induced cell cycle arrest is a necessary step to activate apoptotic pathways among various tumor cells (47). There is a close relationship between apoptosis induction and G_2/M arrest in ovarian carcinoma responding to DNA damage (48). In the present study, sodium arsenite and dimethylarsenic acid significantly stimulated subG_1 phase cells and induced G_2/M phase arrest, which suggested that sodium arsenite and

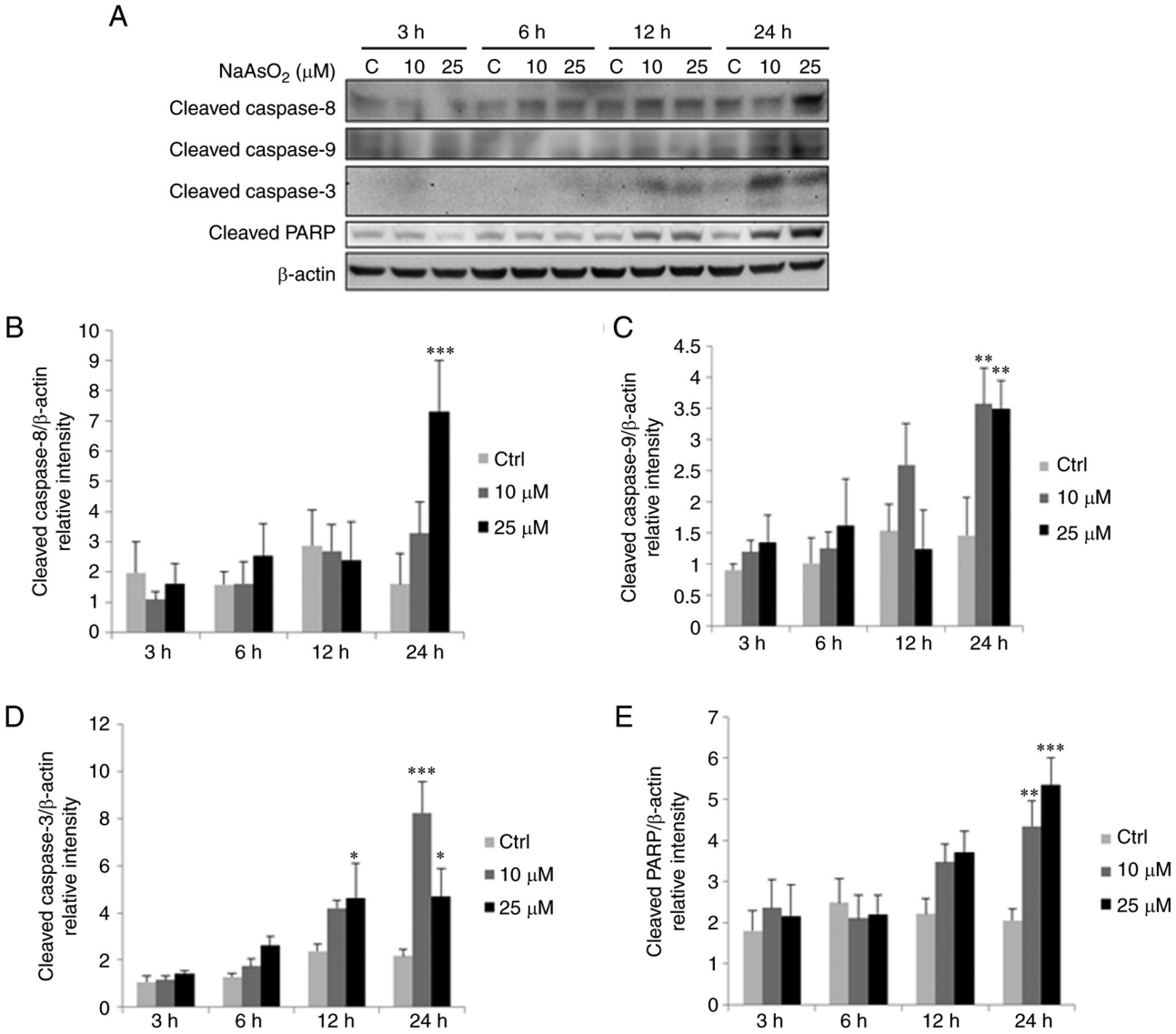

Figure 7. Sodium arsenite upregulates cleaved caspase-9, -8, -3 and PARP protein expressions in OC3 cells. OC3 cells were incubated with 0, 10 and 25 μM $NaAsO_2$ for 3, 6, 12 and 24 h, respectively. (A) Expression of cleaved caspase-9 (35/37 kDa), -8 (43 kDa), -3 (17/19 kDa) and PARP (85~90 kDa) were examine via western blotting. Integrated optical intensities of (B) cleaved caspase-8, (C) -9, (D) -3 and (E) PARP were standardized by β-actin (43 kDa) among lanes. Results are demonstrated as mean ± standard error of the mean of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control. Ctrl, control.

dimethylarsenic acid stimulated apoptosis in OC3 cells. The present study illustrated that arsenic-induced cell apoptosis was associated with abnormal redistribution of cell cycles. Data assessed by annexin V/PI double staining method also confirmed that apoptosis induction with sodium arsenite and dimethylarsenic acid was a dose-dependent phenomenon in OC3 oral cancer cells.

Apoptosis is a critical process for homeostasis in individuals and apoptosis resistance is always hypothesized as a central reason for cancer development (49). Apoptosis originates either by extrinsic or intrinsic death signaling, which activates caspase cascade (16). Arsenic trioxide could stimulate apoptosis in laryngeal cancer through survivin mRNA downregulation, which functions to inhibit caspases activation (50). Arsenic trioxide can also induce apoptosis among various types of cancer by activating caspase-9, -8 and -3 (8). In the present study, cleaved caspase-9, -8, -3 and PARP expressions were significantly induced following treatments of sodium arsenite or dimethylarsenic acid for 12 and/or 24 h in OC3 cells, respectively. Consequently, the data suggested sodium arsenite and dimethylarsenic acid could induce both extrinsic and intrinsic apoptotic pathways to induce OC3 cell apoptosis.

Apoptotic cascades are regulated by a number of cellular signal transduction pathways and the MAPK signaling pathway is one associated with cellular stress (15,16,21-24). It has been shown that MAPK signaling pathways can enhance cell survival or promote sensitivity to apoptosis, which depends on different stimuli, cell types and activation latency (23). JNK contributes to cell survival by suppressing apoptosis through BAD phosphorylation (51). By contrast, the apoptosis induced by ATO in APL cells is through JNK activation (52). ERK demonstrates proapoptotic effect induced by various antitumor compounds (53). A previous report illustrates ATO can activate ERK1/2 and JNK1/2 pathways to induce apoptosis in human mesothelioma cells (54).

Table I. Time course of activated apoptotic and MAPK pathways by sodium arsenite and dimethylarsenic acid treatments between FaDu (26), OEC-M1 (44) and OC3 cells. Cells were treated without or with $NaAsO_2$ or DMA for 3, 6, 12 and 24 h. Apoptotic markers and MAPKs were detected by western blotting. The arsenic-induced apoptotic and MAPK pathways were time-dependent.

Cell line	As	Pathway	3 h	6 h	12 h	24 h
FaDu	3+	Apoptosis				V
	3+	MAPK	V		V	V
	5+	Apoptosis			V	V
	5+	MAPK			V	V
OEC-M1	3+	Apoptosis			V	V
	3+	MAPK	V	V	V	V
	5+	Apoptosis			V	V
	5+	MAPK	V	V	V	V
OC3	3+	Apoptosis			V	V
	3+	MAPK	V	V	V	V
	3+	Apoptosis			V	V
	5+	MAPK	V	V	V	V

V, arsenic-induced activation; 3^+, NaAsO2; 5^+, DMA.

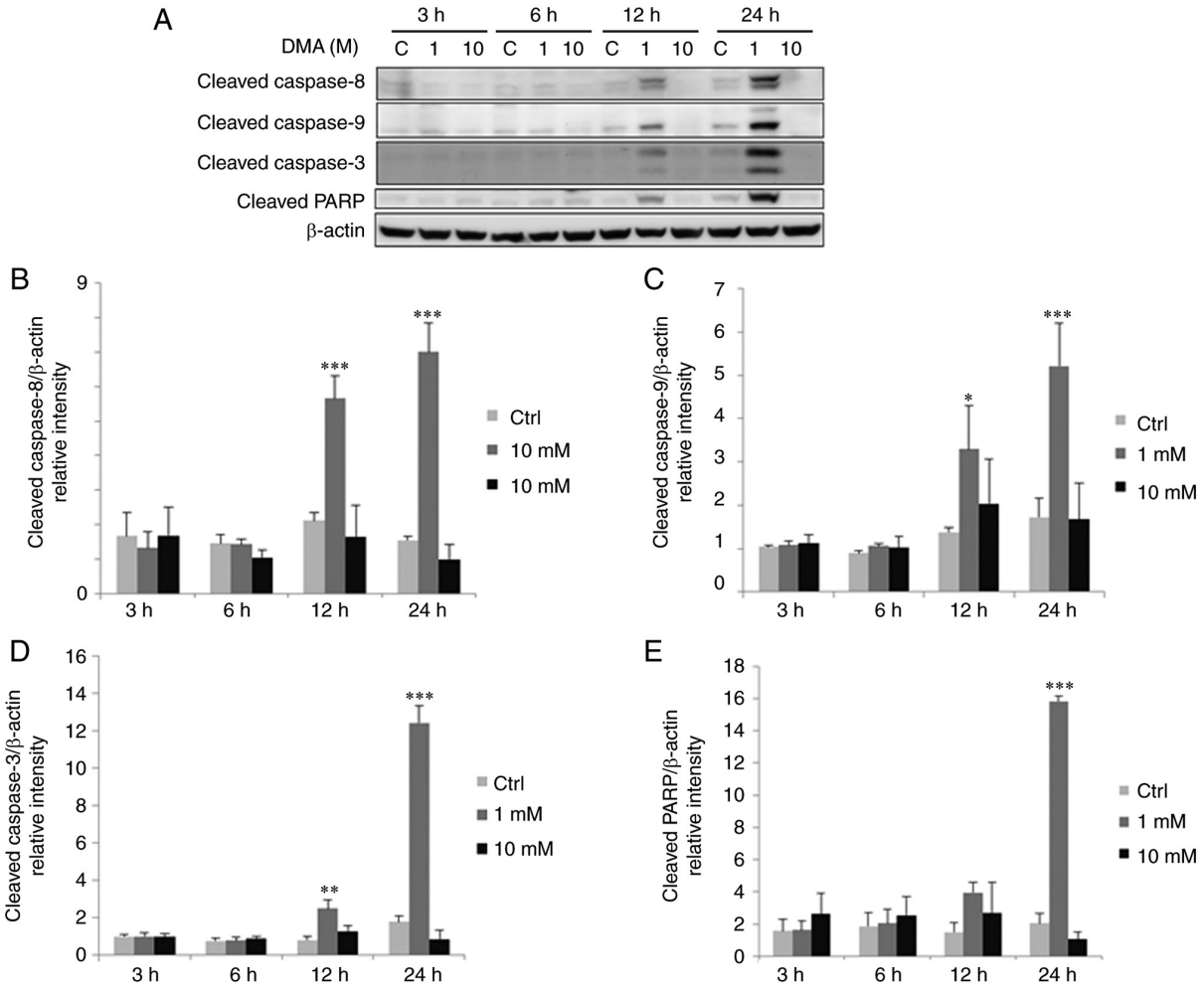

Figure 8. Dimethylarsenic acid upregulates cleaved caspase-9, -8, -3 and PARP protein expressions in OC3 cells. OC3 cells were incubated with 0, 1 and 10 mM DMA for 3, 6, 12 and 24 h. (A) Expression of cleaved caspase-9 (35/37 kDa), -8 (43 kDa), -3 (17/19 kDa) and PARP (85~90 kDa) were examine via western blotting (A) Integrated optical intensities (B) cleaved caspase-8, (C) -9, (D) -3 and (E) PARP were standardized by β-actin (43 kDa) among lanes. Results are demonstrated as mean ± standard error of the mean of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control. DMA, dimethylarsenic acid; C/Ctrl, control.

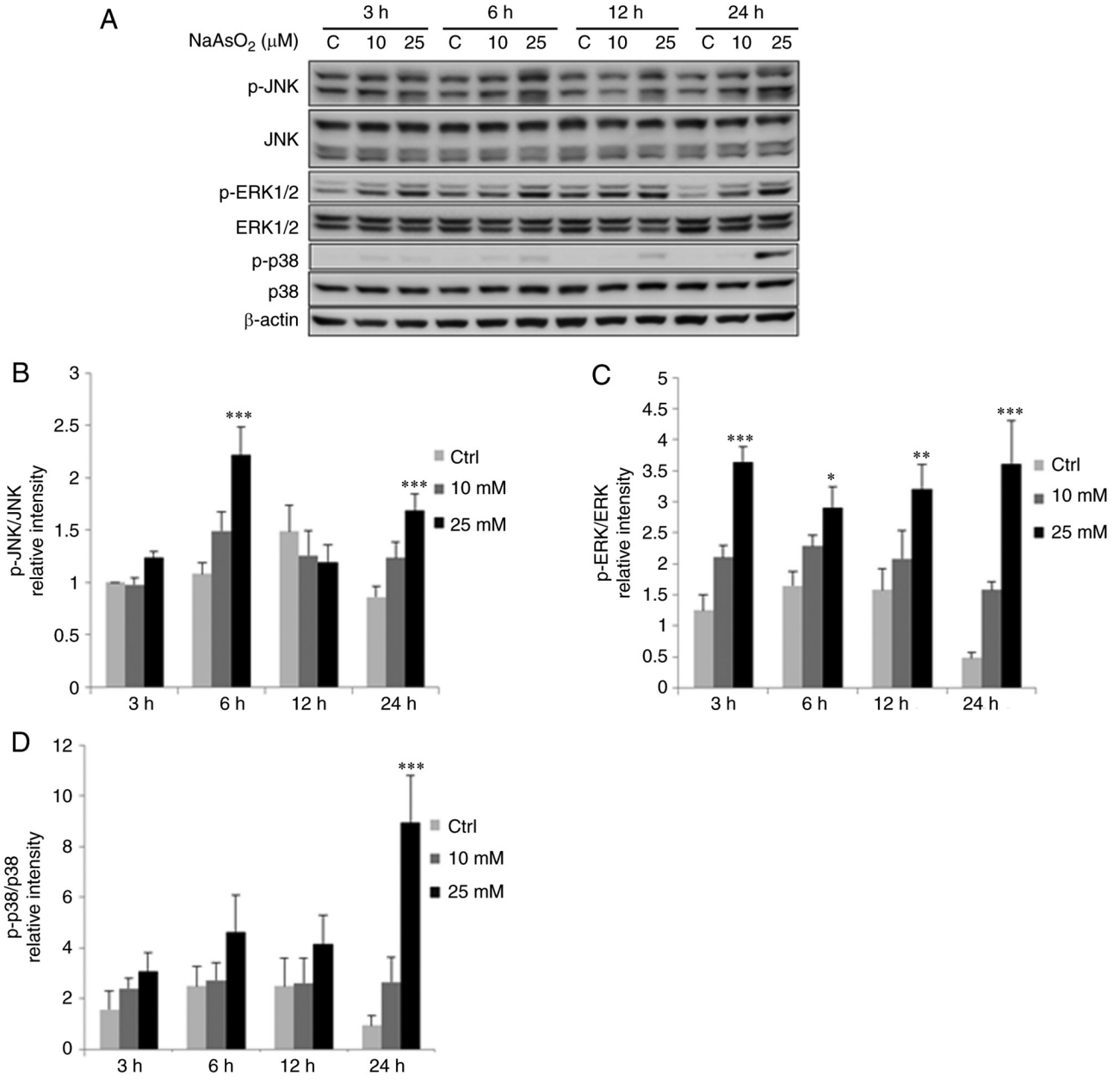

Figure 9. Sodium arsenite modulates phosphorylation of MAPK pathways in OC3 cells. OC3 cells were incubated with 0, 10 and 25 μM $NaAsO_2$ for 3, 6, 12 and 24 h, respectively. (A) JNK (46/54 kDa), p-JNK (46/54 kDa), ERK1/2 (42/44 kDa), p-ERK1/2 (42/44 kDa), p38 (43 kDa) plus p-p38 (43 kDa) were examined via western blotting. Integrated optical intensities of (B) p-JNK, (C) p-ERK and (D) p-p38 proteins were standardized with their total forms in each lane, respectively. Results are demonstrated as mean ± SEM of 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control. p-, phosphorylated; C/Ctrl, control.

Furthermore, involvement of p38 associated with apoptosis is also varied and can suppress caspase-3 and caspase-8 activities in human neutrophils (55). In the present study, ERK1/2, p38 and JNK activities were stimulated by sodium arsenite and dimethylarsenic acid in OC3 cells. Markedly, these results demonstrated that activations of caspases plus PARP were detected at 12 and 24 h treatments by arsenic compounds and the phosphorylation of MAP kinases occurred at 3 to 6 h which is earlier as compared with the caspase-induced pathway, demonstrating both sodium arsenite and dimethylarsenic acid possibly stimulated MAPK pathways before caspase pathways to stimulate OC3 cell apoptosis (Table I). Therefore, these results illustrated sodium arsenite and dimethylarsenic acid activated MAPK pathways to stimulate apoptosis in OC3 oral cavity cells.

Studies have revealed that sodium arsenite and dimethylarsenic acid can modulate cell morphological changes associated with cell apoptosis in dosage- and time-dependent manners in FaDu and OEC-M1 cells, respectively (20,37). The present study showed that sodium arsenite caused more shriveled and floating cells as concentration increased in OC3 cells, compared with FaDu and OEC-M1 cells (20,37). Also, 50 and 100 mM dimethylarsenic acid induced more floating OC3 cells with cell membrane blebbing. In addition, the observations of the present study in OC3 cells is similar to other finding that stimulations of MAPK and caspase pathways by arsenic compound

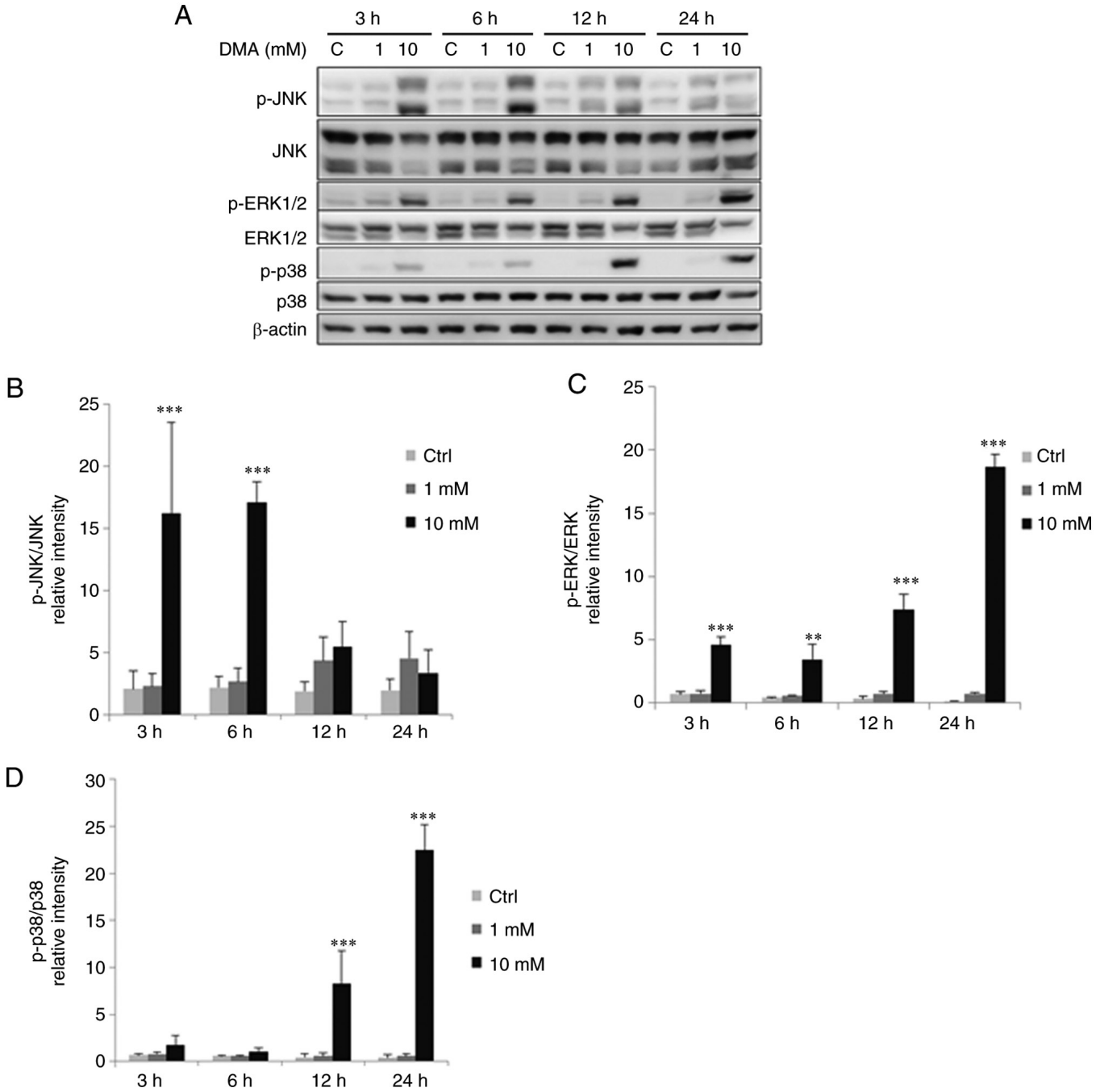

Figure 10. DMA modulates phosphorylation of MAPK pathways in OC3 cells. OC3 cells were incubated with 0, 1 and 10 mM DMA for 3, 6, 12 and 24 h, respectively. (A) JNK (46/54 kDa), p-JNK (46/54 kDa), ERK1/2 (42/44 kDa), p-ERK1/2 (42/44 kDa), p38 (43 kDa) plus p-p38 (43 kDa) were examined via western blotting. Integrated optical intensities of (B) p-JNK, (C) p-ERK and (D) p-p38 proteins were standardized with their total forms in each lane, respectively. Results are demonstrated as mean ± SEM of 3 independent experiments. **P<0.01 and ***P<0.001 vs. control. DMA, dimethylarsenic acid; p-, phosphorylated; C/Ctrl, control.

stimuli are similar as shown in OEC-M1 cells (Table I) (37). However, in FaDu cells, activation of MAP kinases occurs at 3, 12 and 24 h, but caspase activation occurs at 24 h under sodium arsenite treatment (Table I) (20). Notably, activations of MAP kinases and caspase pathway occurred simultaneously at 12 and 24 h treatments with dimethylarsenic acid in FaDu cells (Table I) (20). Hence, sodium arsenite and dimethylarsenic acid activate MAPK and caspase pathways, however, the dynamic phenomena are somewhat dissimilar between different oral cavity cancer cells (Table I). It would have been be more meaningful if non-cancerous oral mucosal cells could be used to study and comparison for the difference with the current data among tumor cell lines and this is a limitation of the present study, which should be investigated in the near future.

Taken together, sodium arsenite and dimethylarsenic acid could stimulate apoptosis via intrinsic and extrinsic apoptotic cascades, showing antitumor properties in OC3 cells. Moreover, sodium arsenite and dimethylarsenic acid could activate MAPKs to further regulate caspase cascade activation and then to induce apoptosis in OC3 oral cancer cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request, which adheres to the FAIR principles (https://www.go-fair. org/fair-principles/), including the fundamental principles of Findability, Accessibility, Interoperability and Reusability.

Authors' contributions

SW, YYL, CC and YPL designed and conducted the experiments, interpreted results and wrote the manuscript. HC and BH participated in the design and coordination of the present study. SW, YYL, CC, HC and BH were involved in the statistical analysis of results and revised manuscript for substantive and methodological correctness. HC and BH confirm the authenticity of all the raw data. 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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