Original Article

Arsenite exposure potentiates apoptosis-inducing effects of tumor necrosis factor- α through reactive oxygen species

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ABSTRACT — Tumor necrosis factor- α (TNF- α) is a proinflammatory cytokine released by immune cells during inflammation process. Sodium arsenite (NaAsO₂) is an environmental toxic metal. The effects of excess NaAsO₂ on TNF- α response and its intracellular signaling are not well understood. We hypothesized that NaAsO₂ exposure might affect cellular response to TNF- α . Using HeLa cell model, we found that the combination of NaAsO₂ and TNF- α clearly decreased cell viability and mitochondrial membrane potential, but increased percentage of early and late apoptotic cells and cleaved-poly (ADP-ribose) polymerase (PARP). Moreover, the combination prolonged the phosphorylation of mitogen-activated protein kinase (MAPK) members, including c-Jun-N-terminal kinase (JNK), p38, and extracellular signal related kinases (ERK), and increased intracellular reactive oxygen species (ROS), in comparison to treatment of NaAsO₂ or TNF- α alone. We further investigated the role of ROS and MAPK signaling on this event by inhibiting ROS production and MAPK. An antioxidant *N*-acetylcysteine pretreatment diminished the apoptosis-inducing effect of NaAsO₂ and TNF- α combination and also inhibited MAPK signaling. Using specific inhibitor of p38 (SB203580) and siRNA-p38 surprisingly increased cell apoptosis and this effect was not observed by JNK and ERK inhibition. This study suggests that p38 may possibly be a survival mediator in response to environmental toxicant-related inflammation. In conclusion, NaAsO₂ exposure might amplify inflammation-related tissue injury by potentiating the apoptosis-inducing effect of TNF- α through ROS-dependent mechanism.

Key words: Sodium arsenite (NaAsO₂), Tumor necrosis factor- α (TNF- α), Apoptosis, MAPK signaling pathway, Reactive oxygen species

INTRODUCTION

When bodily injury occurs, the injured area begins healing processes, such as inflammation. It has long been known that tissue injury can cause inflammation (Rocha e Silva, 1978). Inflammation is a defensive response to several stimuli, including bacterial infectious agents, chemicals, immune reactions and foreign agents. After injury, the body naturally releases tumor necrosis factor- α (TNF- α) protein to allow white blood cells get into the injured area to clear the infection. TNF- α is produced by activated macrophages and promotes the death of cells in the inflammatory response (Rock and

Kono, 2008; Wallach *et al.*, 2014). This process is a temporary response in the damaged area. If the body is unable to remove TNF- α protein, it can cause white blood cell accumulation in the damaged area, resulting in autoimmune disorders, whereby the immune system destroys normal tissues (Ware, 2013).

Apoptosis is a programmed cell death which is a reaction to extreme injury (Yang *et al.*, 2015; Ouyang *et al.*, 2012; Dagenais *et al.*, 2014). TNF- α , a major pro-inflammatory mediator, induces apoptosis in several TNF- α -sensitive target cell lines. TNF- α -induced cell death exhibits ultrastructural changes and internucleosome cleavage of DNA, which are characteristics of apoptotic cells

in normal endothelial cells (Robaye *et al.*, 1991). TNF- α -induced apoptosis involves two independent apoptotic signaling cascades, the extrinsic and intrinsic pathways (Elmore, 2007). In the extrinsic pathway, TNF- α binds to its two receptors, TNFR1 (TNF receptor type 1) and TNFR2 (TNF receptor type 2), which are found in most tissue and immune cells, respectively (Wajant *et al.*, 2003). The study of TNF- α signaling is mostly from TNFR1. In programmed cell death, TNF- α /TNFR binding causes the conformational change of the receptor and recruits adaptor protein (TRADD) to bind to the death domain, leading to the initiation of death signaling. TRADD then binds to Fas-associated death domain (FADD) and pro-caspase-8 to form a death complex (DISC) and caspase-8 is activated by autoproteolytic process, which subsequently cleaves the effector caspase (caspase-3), leading to cell death (Goeddel, 1996). On the other hand, the intrinsic pathway or mitochondrial pathway involves stress or reactive oxygen species (ROS) production (Maeda and Fadeel, 2014; Suematsu *et al.*, 2003). TNF- α associates with ROS modulator1 (Romo1) and Bcl-xl, located in the mitochondria to reduce mitochondrial membrane potential, resulting in ROS production and cell death (Kim *et al.*, 2010; Maeda and Fadeel, 2014). During inflammation, the intracellular TNF- α signaling regulates the activation of NF- κ B and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), mitogen-activated protein kinases (p38 and ERK), translocating them into the nucleus and enhancing the activity of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) that control cellular responses, including proliferation, differentiation, cell survival and cell death (Chang and Karin, 2001; Takada and Aggarwal, 2004).

Arsenic (As) is an environmental pollutant and well-known human carcinogen (Rossman, 2003; Schoen *et al.*, 2004; Shi *et al.*, 2004) which is involved in the inflammatory responses and DNA damage. The study from Dutta *et al.* (2015) revealed a high level of arsenic in groundwater and arsenic was found in the nails of rural women of West Bengal. Examination of the body fluid from the exposed women found the presence of pro-inflammatory cytokines (interleukins (ILs)-IL-6, IL-8 and IL-12) in plasma, inflammatory markers (MMP-2 and MMP-9) in sputum and inflammatory mediator (TNF- α) in leucocytes and airway cells. Low levels of arsenic exposure evoked pro-inflammatory cytokines to promote oxidative DNA damage. Arsenite (AsO₂⁻) is an oxidative form of arsenic which usually appears in sodium salt form. Exposure of sodium arsenite (NaAsO₂) at single dose administration and subchronic exposure in drinking water, increased hyperalgesia and inflammatory

response in rats (Aguirre-Bañuelos *et al.*, 2008). This indicates that As and AsO₂⁻ play a critical role in tissue injury and cell death (Porter *et al.*, 1999). With regard to the role of NaAsO₂ and TNF- α in inflammation, NaAsO₂ was shown to participate in the inflammation process by contributing with TNF- α affecting the expression of vascular cell adhesion molecular-1 (VCAM-1) protein in human umbilical vein endothelial cells (HUVECs) (Tsou *et al.*, 2005). Expressions of pro-inflammatory cytokines such as TNF- α , IL-6, IL-8 and IL-10 were increased in response to NaAsO₂ under hypoxic condition in human bronchial epithelial cells (Xu *et al.*, 2013).

However, the excess of NaAsO₂ during TNF- α overproduction during the injury regarding cell death and its intracellular mechanism are still poorly understood. We therefore hypothesized that the excess of NaAsO₂ affects the overproduction of TNF- α -induced cell death program and changes the intracellular signaling pathways, including MAPK pathway in tissue injury. We used HeLa cells as a human cellular model as it is known that TNF- α can activate intracellular signaling pathways and cell death program in this cell system.

MATERIALS AND METHODS

Cell culture

HeLa (Henrietta Lacks) cells were cultured in DMEM-high glucose (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin mixture (Gibco, Thermo Fisher Scientific, Gaithersburg, MD, USA) and cells were maintained at 37°C in 5% CO₂. HeLa cells were prepared at 80% confluency in 6-cm cell culture dishes and 96-well plates before being exposed in all experiments.

Reagents and antibodies

All antibodies, including anti-phospho-specific MAPK family antibodies kit and antibodies against apoptosis family kit, were purchased from Cell Signaling Technology (Danvers, MA, USA). Recombinant human TNF- α was obtained from R&D Systems (Minneapolis, MN, USA). NaAsO₂ was purchased from Ajax Finechem (Taren Point, NSW, AU) and MAPK inhibitors, including SB203580, SP600125 and U0126, were purchased from Calbiochem (Darmstadt, Germany). Small interfering RNA against p38, JNK and ERK were purchased from Cell Signaling Technology.

MTT Assay

Cells were exposed to NaAsO₂, alone and in combination, with TNF- α in serum-free DMEM media in dose-de-

pendent manner for 24 hr. Cell viability was determined by MTT method. Briefly, freshly prepared 10% MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) solution in serum-free media was added to treated cells and incubated in 37°C for 3 hr. After removing the MTT solution, purple formazan was solubilized with DMSO and the intensity was measured by microplate reader (Molecular Devices, Silicon Valley, CA, USA) at excitation wavelength (Ex) of 550 nm and emission wavelength (Em) of 650 nm.

Western blot analysis

After exposure, whole-cell lysates were prepared in lysis buffer (25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol) containing 1% protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were resolved by 7.5%, 10%, or 12.5% SDS-PAGE and transferred onto an Immobilon-P nylon membrane (Millipore Corporation, Bedford, MA, USA). The membrane was blocked with Rapid Block solution (Amresco, Solon, OH, USA) for nonspecific binding site blocking and probed with primary antibodies in Antibody Signal Enhancer solution (Amresco). The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit (Cell Signaling Technology) and visualized with the ECL system (GE Healthcare, Little Chalfont, UK). For reprobing, the membranes were stripped with Gentle Review TM Buffer solution (Amresco). The pre-stained protein marker (Invitrogen Corporation, Thermo Fisher Scientific) was used to refer to molecular size of developed proteins.

Detection of reactive oxygen species (ROS)

Dihydroethidium (DHE) is used to monitor superoxide production. The reaction of DHE with superoxide is transformed to a red fluorescent of ethidium form (Owusu-Ansah *et al.*, 2008). Cells were cultured in black 96-well plates at 80% confluency, treated with TNF- α , alone and in combination, with NaAsO₂ in serum-free media in a time-dependent manner, washed with PBS and then stained with freshly prepared 10 μ M DHE (Sigma-Aldrich) solution in HBSS buffer in 37°C incubator for 60 min and protected from light. The intensity of DHE fluorescence by superoxide production was measured at Ex/Em 495/595 nm using microplate fluorescent reader (Molecular Devices).

Mitochondrial membrane potential (MTP) ($\Delta\psi_m$) assay

NIR mitochondrial membrane potential assay kit for microplate (Abcam, Cambridge, UK) was used to detect membrane permeability of mitochondria when cell apoptosis occurs. Cells were prepared in black 96-well plates and cultured to 80% confluency. According to the instructions, treated cells were stained with NIR dye in buffer solution for 45 min at 37°C and protected from light. The red fluorescence intensity, which is increased in normal cells when NIR dye accumulates into mitochondria and decreased in apoptotic cells, is measured using microplate fluorescent reader at Ex/Em 640/680 nm.

Annexin-V and dead cell assay

Muse® annexin V & dead cell kit (Merck Millipore, Billerica, MA, USA) was used to detect cells in various stages of apoptosis, including live cells, early and late apoptosis. Cells were prepared in 6-well plates at 80% confluency and treated with TNF- α , alone and in combination, with NaAsO₂ for 6 and 24 hr. Treated cells were trypsinized and resuspended in media containing 1% FBS. Cell suspensions were stained with Muse annexin V & dead cell reagent for 20 min at room temperature and protected from light. Cells were analyzed by flow cytometric technique using Muse® Cell Analyzer. All experiments were independently repeated 3 times.

Statistical analysis

The bar graphs are reported as the mean $\pm$ standard deviation. Comparison among treatment groups were performed by one-way analysis of variance and Tukey HSD post hoc comparison. Values of $p \leq 0.05$ were considered significant.

RESULTS

The combined effect of NaAsO₂ and TNF- α on cell viability

It is well-known that NaAsO₂ and TNF- α induce cell death in several cell types. In this study, we examined the excess of NaAsO₂ and TNF- α , alone and in combination, on cell cytotoxicity, as shown in Fig. 1. HeLa cells were exposed to various concentrations of NaAsO₂ (0-100 μ M), with and without 20 ng/mL TNF- α , and cell viability was measured by MTT assay. After 24 hr exposure, combination of NaAsO₂ and TNF- α showed significant difference in cell viability compared to NaAsO₂ alone at 50 μ M concentration. However, we did not observe any significant difference in cell viability between NaAsO₂ alone and NaAsO₂ and TNF- α combination at

Fig. 1. The effects of NaAsO₂ and TNF-α on cell viability. HeLa cells were stimulated with NaAsO₂ (0-100 μM), alone and in combination with 20 ng/mL TNF-α for 24 hr and cell viability was detected by MTT assay. The bars show percentage of cell viability compared to untreated cells.

100 μM because most cells were almost completely killed at this concentration.

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Next, we showed the effect of NaAsO₂ and TNF-α combination on the cell characteristics when apoptosis occurs, including phosphatidylserine (PS) translocation, mitochondrial membrane potential changes and caspase signaling pathway, as shown in Fig. 2. The percentage of Annexin-V and 7-ADD cell population, indication of the activation of cell apoptosis was measured after NaAsO₂ and TNF-α exposures for 6 and 24 hr (Fig. 2). The combination of NaAsO₂ and TNF-α increased the early and late cell apoptosis about 30% more than TNF-α and NaAsO₂ alone at 6 hr in Fig. 2A and 2C, and the highest proportion of late apoptosis stage showing Annexin-V and 7-ADD positive was more than 90% after 24 hr exposure (Fig. 2B and 2D). The results indicated that NaAsO₂ affected TNF-α-induced apoptosis at dependent time. Next, the mitochondrial membrane potential changes were shown after NaAsO₂ and TNF-α exposure for 6 hr and 24 hr (Fig. 2E and 2F) and the results demonstrated that the combination of NaAsO₂ and TNF-α significantly reduced mitochondrial membrane potential when compared to TNF-α alone at 6 hr and significantly reduced at 24 hr in all stimuli. In addition, western blot analysis revealed that NaAsO₂ and TNF-α combination increased PARP cleavage, marker of cell apoptosis, compared to TNF-α and NaA-

so₂ alone after being exposed for 6 hr (Fig. 2G). PARP cleavage was increased after 24 hr exposure in NaAsO₂ and TNF-α alone (Fig. 2H) and there were insufficient cells for PARP detection in the combination of NaAsO₂ and TNF-α due to massive cell death. This suggests that NaAsO₂ and TNF-α have an additive effect to induce cell death that clearly involves phosphatidylserine (PS) translocation, rapid depolarization of mitochondrial membrane to initiate apoptosis.

The role of NaAsO₂ and TNF-α on mitogen activated protein kinase (MAPK) pathway

Activation of the MAPK pathway has been shown to be stimulated by several inducers, including chemical carcinogens and inflammatory cytokines such as NaAsO₂. However, the combined effect of these two inducers on MAPK signaling has not been identified. We determined the phosphorylation of p38/ERK/JNK-MAPK after NaAsO₂ and TNF-α treatments, alone and in combination, in transient manner (Fig. 3). We found that exposure of NaAsO₂ stimulated p38 and ERK phosphorylations at 10 min and JNK at 30 min, respectively (Fig. 3A). On the other hand, TNF-α transiently induced phosphorylations of p38/ERK/JNK-MAPK at 10 min followed by dephosphorylation after 20-30 min (Fig. 3B). Interestingly, the combination of NaAsO₂ and TNF-α prolonged the phosphorylation of p38/ERK/JNK-MAPK when compared to TNF-α alone (Fig. 3C). These results suggest that both NaAsO₂ and TNF-α can activate MAPK signaling and

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Fig. 2. The effects of NaAsO₂ and TNF- α on cell apoptosis. Cells were stimulated with 20 ng/mL TNF- α and 50 μ M NaAsO₂, alone and in combination, for 6 hr and 24 hr. Then cells were analyzed for apoptosis by Annexin-V and Dead Cell assay kit using Muse Cell Analyzer. *2A* and *2B*, dot plots showing Annexin-V and 7-AAD positive cells from the representative experiment at 6 hr and 24 hr, respectively. *2C* and *2D*, quantitative analysis of percentage of cell populations, live cell and apoptotic cells, at 6 hr and 24 hr, respectively. *2E* and *2F*, the mitochondrial membrane potential detected by NIR-MTP assay kit in cells treated with 20 ng/mL TNF- α and 50 μ M NaAsO₂ alone and combination, at 6 hr and 24 hr, respectively. Western blot analysis of total and cleaved PARP of TNF- α and NaAsO₂ treated cells were detected after 6 hr (*2G*) and 24 hr (*2H*) exposures. β -actin was used as a loading control. The bars represent the means $\pm$ standard deviation (S.D.) from three independent experiments. * p -value ≤ 0.05 .

Fig. 3. The role of NaAsO₂ and TNF-α on MAPK signaling pathway. Cells were stimulated with *3A*, 50 μM NaAsO₂, *3B*, 20 ng/mL TNF-α and *3C*, the combination (NaAsO₂+TNF-α) at different time points. Whole cell lysates were immunoblotted with specific antibodies indicated on the *left side* of each panel, including anti-specific phospho-p38, JNK, and ERK and their total protein expressions. β-actin was used as a loading control.

NaAsO₂ enhances TNF-α-mediated MAPK phosphorylation resulting in apoptosis induction.

The role of MAPK signaling on NaAsO₂ and TNF-α-induced apoptosis

To examine the role of MAPK signaling on cell apoptosis induced by NaAsO₂ and TNF-α combination, we inhibited MAPK signaling by two methods, pretreating cells with selective inhibitors of MAPK, including p38 inhibitor (SB203580, 20 μM), JNK inhibitor (SP600125, 20 μM) and ERK inhibitor (U0126, 10 μM), for 2 hr (Fig. 4A) and using ten picomole of siRNA against p38, JNK and ERK to pretreat cells for 72 hr (Fig. 4B), before being exposed to NaAsO₂ and TNF-α for 6 hr. We found that p38 inhibitor and siRNA-p38 increased PARP cleavage and this effect was not clearly observed by JNK and ERK inhibitions. In addition, siRNA-p38 but not siRNA-JNK and siRNA-ERK decreased mitochondrial membrane potential (Fig. 4C) and increased superoxide level by dihydroethidium (DHE) staining (Fig. 4D). We obviously showed that inhibition of p38, but not JNK and ERK, increased cell apoptosis induced by NaAsO₂ and TNF-α combination. Thus, it is possible that the activation of p38 by NaAsO₂ and TNF-α combination may

counteract cell death signaling.

Cell apoptosis induced by the combination of NaAsO₂ and TNF-α depends on ROS reaction

It has been known that ROS production induces oxidative stress and promotes cell apoptosis by inducing mitochondrial membrane permeabilization. We investigated the role of ROS in NaAsO₂ and TNF-α combination-induced apoptosis by measuring the level of superoxide products. Dihydroethidium (DHE) is a cell-permeable fluorescent dye that reacts with superoxide anion to form fluorescent products. The percentage of superoxide products in response to NaAsO₂ and TNF-α exposure was significantly increased compared to NaAsO₂ and TNF-α alone (Fig. 5A) and an increase of superoxide level was sustained until 6 hr (Fig. 5B). We also monitored the levels of superoxide products in TNF-α and NaAsO₂ alone and found that they started to rise after 1 hr (Fig. 5C and D).

To confirm that NaAsO₂ and TNF-α-induced apoptosis involves ROS production, we used *N*-acetyl-L-cysteine (NAC) as an antioxidant to suppress oxidative stress. Cells were pretreated with NAC for 1 hr before being exposed to NaAsO₂ and TNF-α combination and then ROS products, cell viability and PARP cleavage were

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Fig. 4. The role of MAPK signaling on NaAsO₂ and TNF- α combination-induced apoptosis. To inhibit MAPK pathway, cells were pretreated cell with 20 μ M SB203580, 20 μ M SP600125 and 10 μ M U0126 for 1 hr (4A), transfected with 100 pmole siRNA-p38, siRNA-JNK, siRNA-ERK, and scramble control for 72 hr (4B), then cells were exposed to combination of NaAsO₂+TNF- α for 6 hr. Total and cleavage PARP, and β -actin were detected by western blot analysis. The mitochondrial membrane potentials were measured by NIR-MTP assay kit (4C) and superoxide levels were detected by DHE staining assay (4D) of cells as described above. The bars represent the mean $\pm$ S.D. of three independent experiments. * p -value $\leq$ 0.05.

Fig. 5. Cell apoptosis induced by the combination of NaAsO₂ and TNF- α depends on ROS reaction. Cells were treated with NaAsO₂ and TNF- α , alone and in combination. 5A, the levels of superoxide were measured by DHE dye staining after 1 hr of treatment. Hydrogen peroxide (H₂O₂) was used as a positive control. Superoxide levels at different time points from 1 hr to 6 hr were detected after treatment with NaAsO₂+TNF- α (5B), TNF (5C), and NaAsO₂ (5D) alone. The bars represent the mean $\pm$ S.D. of three independent experiments. * p -value $\leq$ 0.05.

monitored. NAC reduced the percentage of superoxide (Fig. 6A), and inhibited cell death (Fig. 6B). In addition, NAC clearly decreased cleavage of PARP and phosphorylation of p38, as demonstrated in Fig. 6C. This suggests that NaAsO₂ and TNF- α combination-induced apoptosis mediated by ROS reaction.

DISCUSSION

It has been shown that arsenite is partially responsible for the initiation of vascular inflammation and vascular disease through proinflammatory cytokines (Tsou *et al.*, 2005). Arsenic stimulates TNF- α release from mononuclear cells, causing cytotoxic effect in T-helper cell apoptosis (Yu *et al.*, 2002). However, the effect of excess

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Fig. 6. *N*-acetyl-L-cysteine (NAC) prevents NaAsO₂ and TNF- α combination-induced apoptosis. Cells were pretreated with 10 mM NAC for 1 hr before being exposed to the combination of NaAsO₂ and TNF- α . Superoxide levels were detected by DHE dye staining after 1 hr exposure (6A). Cell viability was examined by MTT assay after 24 hr exposure (6B). After 6 hr of treatment, anti-specific phospho-p38, total and cleavage PARP and β -actin were detected by immunoblotting (6C). The bars represent the mean $\pm$ S.D. of three independent experiments. **p*-value $\leq$ 0.05.

NaAsO₂ on TNF- α overproduction resembling tissue injury on the cell death process and its intracellular mechanism is not well understood. In this study, we demonstrated the intracellular signaling and cell death process

in response to NaAsO₂ and TNF- α combination in HeLa cells. Our preliminary results discovered that the cytotoxicity of NaAsO₂ was observed at 10 μ M at 24 hr of treatment. However, we are interested in examining the modification of TNF- α in the presence of NaAsO₂ at toxic levels and exploring the involved signaling pathway. A study by Nuntharatanapong *et al.* (2005) also reported the effect of high concentration of NaAsO₂ (50 μ M) on p21^{Cip/Waf1} upregulation and endothelial cell apoptosis. It is important to note that the antiproliferative effect of NaAsO₂ at lower concentration (10 μ M) may involve distinct mechanism that remains elusive.

Our results clearly showed the additive effect of NaAsO₂ and TNF on cell death, including decrease of cell viability, phosphatidylserine (PS) translocation and the permeability of mitochondrial membrane, as well as elevation of cleaved PARP. For its intracellular signaling, we discovered prolonged phosphorylation of the MAPK signaling family, including JNK, p38 and ERK. We used pharmaceutical inhibitors and small interfering RNA against MAPK members to investigate the involvement of the three MAPK members, JNK, ERK, p38, in NaAsO₂ and TNF- α combination-induced apoptosis. Surprisingly, only p38 inhibitions (SB203580 and siRNA-p38) increased cell apoptosis indicated by the enhancements of PARP cleavage and superoxide productions in comparison to the controls (without inhibitor). There are studies demonstrating that p38 is involved in cell apoptosis, for example, SB203580 blocked caspase activity in TRAIL/Apo2L exposure (Lee *et al.*, 2002), the inhibition of p38 prevented matrine-induced caspase-dependent cell apoptosis in NSCLC (Tan *et al.*, 2013) and endothelial cells (Grethe *et al.*, 2004). However, there is evidence indicating the antiapoptotic role of p38, depending on the cell type and stimuli (Lenassi and Plemenitas, 2006; Ricote *et al.*, 2006). Our findings are in agreement with the study showing the activation of p38 after the exposure of 8-methoxypsoralen and ultraviolet-A radiation (PUVA) combination in Jurkat T cells and T-lymphocytes and that treatment of SB203580 also induced apoptosis in PUVA treated cells (Cappellini *et al.*, 2005). The association of p38 and myocyte enhancer factor 2 (MEF2) prevented apoptosis during neuronal differentiation of P19 cells (Okamoto *et al.*, 2000). Therefore, our study suggests that p38 may act as an antagonist to cell death in response to NaAsO₂ and TNF- α co-exposure.

Reactive oxygen species (ROS), reactive chemical species containing oxygen, are a key factor in cell proliferation, gene expression, and cell death. It can induce oxidative stress leading to apoptosis and necrosis in cell biological systems (Hancock *et al.*, 2001). NaAsO₂ and

TNF- α were shown to induce ROS generation (Blaser *et al.*, 2016; Shi *et al.*, 2004). TNF- α stimulation induces the formation of TNFR1-TRADD-TNF receptor-associated factor 2 (TRAF2), and then activates Fas-associated death domain protein (FADD) and pro-caspase-8 which subsequently triggers ROS modulators-1 located in mitochondria to reduce mitochondrial membrane potential, leading to apoptotic cell death (Kim *et al.*, 2010). Arsenic was also found to stimulate the generation of ROS and RNS (reactive nitrogen species) to induce DNA damage and changes of gene expression (Shi *et al.*, 2004). Here, we showed that high levels of superoxide were detected in NaAsO₂ and TNF- α combination, compared to single exposure, in time-dependent manners. This remarkable increase was reduced by NAC, an antioxidant. Moreover, NAC prevented cell death, PARP cleavage and p38 activation, suggesting that ROS also play a key role in cell apoptosis induced by NaAsO₂ and TNF- α combination.

In addition, we demonstrated that the inhibition of p38 increases NaAsO₂ and TNF- α combination-induced apoptosis and ROS is essential for this event due to its adverse effect by NAC. Since we observed an increase of apoptosis after p38 inhibition in NaAsO₂ and TNF- α co-exposure, it is likely that the induction of ROS resulting from the treatment may activate other signaling pathways such as JNK, because the phosphorylation of JNK was sustained after p38 and/or ROS inhibition (data not shown). It has been shown that JNK promotes cell apoptosis via ROS generation (Nakano *et al.*, 2006; Santabábara-Ruiz *et al.*, 2015). Although, p38 activation has been shown to be a necessary regulator of cell death and cell survival, this depends on cell type and stimuli. Therefore, the activation of p38 may mediate cell survival during the combination of NaAsO₂ and TNF- α -induced apoptosis

In conclusion, the excess of NaAsO₂ in TNF- α over-expression can cause cell death, which implies the rapid tissue injury after NaAsO₂ exposure. This effect involves ROS production and MAPK signaling pathway, especially p38. Hence, the therapeutic effect of NaAsO₂ and TNF- α exposure should be considered regarding the apoptosis induction and the role of ROS reaction.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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