Response of Human Alveolar Epithelial (hAELVi) Cells to Heavy Metals in Airborne Particulate

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Research Article

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Abstract

In vitro human alveolar models provide a platform to investigate the cellular behavior and activity of human lung cells for toxicity assays. However, there has been relatively rare research on the new human alveolar epithelial cell line (hAELVi) for the toxicity induced by heavy metal ions. In this study, hAELVi cells were exposed to various concentrations of Cd, As, and Zn for 24h. The morphological changes of hAELVi cells were observed after exposure to heavy metals. A high concentration of Cd, As, and Zn led to cell death. However, the cytotoxicity was not reflected by the transepithelial electrical resistance (TEER) value after exposure to 250 μM of As and Zn. In addition, Cd at a concentration of 200 μM induced the significant production of TNF-α whereas As and Zn did not induce observable secretion of the cytokine. On the contrary, a high concentration of Cd, As, and Zn inhibited the production of IL-6. In addition, there is not a direct relationship between the metal ion cytotoxicity and the ROS production in the hAELVi cells. Accordingly, this study contributes to elucidating the cytotoxicity, TEER variation, ROS production, and cytokine secretion of hAELVi cells to acute exposure to heavy metal *in vitro*. The discrepancy between the cytotoxicity and the TEER result suggests that hAELVi cell cultured on permeable membrane exhibit very different behavior, which is worth pursuing.

Introduction

Ambient particulate matters (PM) have critical impacts on air quality deterioration. [1]. PM is an aggregate of a particle and a large number of chemicals and metal materials, and the compositions of PM can differ by time and place [2]. Several studies have indicated that elevated concentrations of inhalable particles were associated with increased respiratory diseases [3–5]. Epidemiological studies have shown that increases in the ambient nickel (Ni) and vanadium (V) concentrations are significantly associated with an increased probability of wheezing in young children [6]. Increasing concentrations of ambient zinc (Zn) and iron (Fe) have been associated with decrements in lung function indices (FEV1/FVC) in chronic obstructive pulmonary disease (COPD) subjects [7]. Increases in ambient Zn have been associated with increases in asthma emergency department visits [8].

To date, ambient PM pollution is still one of the most concerning environmental issues in Taiwan. In a report by Chen *et al.*, the authors investigated the concentrations and metal compositions of PM2.5 and PM2.5–10 in Yunlin County, Taiwan, during air quality deterioration. The total mean concentrations of metals (such as Cd, Sb, Zn, Pb, Se, As, Mo, Cu, Cr, and Ni) with anthropogenic sources in fine and coarse particles during the episode period were ~1.5 times higher than those during the non-episode period. The mean value of fine-size As (6.67 ng/m^3) obtained from the episode period exceeds the air quality standards in the European Union (6 ng/m^3) [9]. Previous studies have shown that toxic metals (Cd and As) are associated with respiratory diseases. The fumes or gaseous forms of cadmium (Cd) may lead to acute chemical pneumonitis and pulmonary edema or acute tracheobronchitis [10]. Cd inhibits the synthesis and expression of inflammatory cytokines like IL-1β, IL-4, IL-6, TNF-α, IFN-γ, and ICAM-1leading to the prevalence of asthma [11]. Chronic exposure to arsenic (As) has been associated with impaired lung

function, chronic lung disease, respiratory infections, and pulmonary inflammation [12–14]. Lung is the primary target organ of airborne heavy metal-induced toxicity. Alveoli, in which air exchange occurs, are an important part of the respiratory system in lung. Exposure to air pollution can cause respiratory diseases (Fig. 1). Therefore, it is important to investigate the effects of heavy metals on the biological responses of human alveolar cells.

A typical pair of human lungs contain about 480 million alveoli. An adult alveolus has an average diameter of 200 μm [16]. The type I alveolar cell (AT1) cells cover over 95% of the alveolar surface area and form the epithelial component of the thin air-blood barrier [17]. The AT1 cells spread out to be only 0.2 μm thick and provide a large surface area for gas exchange [18]. The alveolar epithelial cells are well known to form intercellular tight junctions [19]. Transepithelial electrical resistance (TEER) is an indicator of the integrity of epithelial tissues and is an effective tool to assess ion transport and permeability of tight junctions [20]. The widely used cell lines for TEER measurement are A549, 16HBE14o- and Calu-3. However, the human alveolar cell line A549 is derived from adenocarcinoma and does not exhibit high TEER values [21]. The 16HBE14o- and Calu-3 monolayers exhibit typical high TEER values, but they are both of bronchial origin [22–24]. Therefore, the above cells may not be suitable as an alveolar epithelium model. Models of the outer epithelia of the human lung have found valid applications in both research and industrial settings as attractive alternatives to animal testing [25]. The human alveolar epithelial cell line (hAELVi – human Alveolar Epithelial Lentivirus immortalized) with AT1 cell characteristics, functional tight junctions, and high TEER (> 1000 $\Omega*cm^2$) [21], may be a good model for an alternative to animal testing to study the toxicity of heavy metals on pulmonary functions.

Pro-inflammatory cytokines, such as TNF-α and IL-6 are elevated in acute respiratory distress syndrome (ARDS) subjects [26]. TNF-α is implicated in various pulmonary diseases, such as asthma, chronic bronchitis (CB), COPD, and acute lung injury (ALI) [27]. IL-6 is induced by environmental insults and play important roles in the respiratory system by stimulating lymphocytes, inducing neutrophils recruitment, and up-regulating mucin secretion [28–30].These studies shows that the cytokines TNF-α and IL-6 play important roles that may be affected by heavy metal exposure. The present study aimed to evaluate the biological responses of heavy metals in hAELVi cells. We examined the effect of heavy metals on the cellular toxicity, TEER variation, ROS production, and cytokine secretion of human alveolar epithelial (hAELVi) cells.

Materials And Methods

Cell culture and maintenance. The immortalized human alveolar epithelial cell line CI-hAELVi (Cat. No. INS-CI-1015) cells were purchased from InSCREENeX (InSCREENeX GmbH, Braunschweig, Germany). hAELVi cells were grown on the huAEC coating solution (Cat. No. INS-SU-1018) coated cell culture flask (Nunc, Roskilde, Denmark) and maintained in hAEC-Medium [Consists of basal medium (Cat. No. INS-ME-1013) and basal supplements (Cat. No. INS-ME 1013BS)]. All media and reagents for cell culture were purchased from InSCREENeX (InSCREENeX GmbH, Braunschweig, Germany).

The cells were placed in an incubator filled with 5% CO_2 atmosphere and maintained at 37°C. The cells were subcultured every 3–4 days and the cells used in the present study were within 10 to 15 passages. The TEER of the monolayer formed by the hAELVi cells was measured and the tight-junction formed among the cells was observed by ZO-1 staining (61-7300, Thermo Fisher Scientific). The cultured cells were routinely tested for mycoplasma using a commercial PCR kit (e-Myco plus, iNtRON Biotech, Korea). All the cells used in the present study were free of mycoplasma contamination.

In vitro **cytotoxicity assay.** The cell cytotoxicity was measured using the Live-Dead cell staining kit (ALX-850-249, Enzo). The kit utilizes Live-dye^{TM}, a cell-permeable green fluorescent dye (Ex (max): 488 nm; Em (max): 518 nm), to stain live cells. Dead cells can be easily stained by propidium iodide (PI), a red fluorescent dye (Ex (max): 488 nm; Em (max): 615 nm).

The hAELVi cells (2×10^4 cells/well) were seeded in 96-well huAEC coating solution-coated black plates (Corning 3603, Costar) and incubated for 24 h in a complete medium at 37°C in a humidified atmosphere of 5% CO_2. After 24 h incubation, the cadmium chloride ($CdCl_2$, 20899, Fluka, referred to as Cd below), sodium (meta) arsenite ($AsNaO_2$, S7400, Sigma-Aldrich, referred to as As below), and zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$, 204986, Sigma-Aldrich, referred to as Zn below) was added to the 96-well black plate at different concentrations (20 μM, 40 μM, 80 μM, 100 μM, 200 μM, and 250 μM) and the cells were further cultured for 24 h at 37°C. The cells in the control group (CTL) were grown in the absence of heavy metals. After exposure to the heavy metals, the medium was harvested and stored at −20°C for cytokines analysis. 100 μL of Live-Dead cell staining reagent was added into each well and incubated for 15 min at 37°C. After the incubation, the fluorescence was measured using a fluorescence microplate reader (Synergy^{TM} 2, BioTek). Results are expressed as the percentage of viable cells compared to untreated cells (0 μM).

Transepithelial electrical resistance (TEER) measurements and crystal violet assay. The TEER values were measured using the ECIS TEER24 (Applied BioPhysics, New York, USA). The hAELVi cells ($1x10^5$ cells/transwell) were seeded on huAEC coating solution-coated transwell inserts with the permeable membrane pore size of 0.4 μm and a growth area of 0.33 cm^2 (Corning 3470). Wells in a TEER24 plate (Applied BioPhysics, T24W) were filled with 1 mL of culture medium. After that, the inserts were put into the wells and incubated at 37°C in a humidified atmosphere of 5% CO_2. After 3 days, the medium in both the upper and lower chamber was replaced with fresh medium.

Each well exhibit TEER values of typically 2000 Ω across the permeable support after 6 days of culture. Then, continuous measurement of TEER value after the exposure with 250 μM of the heavy metals (Cd, As, and Zn) was conducted. To determine the integrity of the cell monolayer, the cellular image was observed after the cells were stained with 1% crystal violet solution (V5265, Sigma-Aldrich). After 24h of the heavy metal exposure, the medium was aspirated and replaced by 50 μL of crystal violet stain and then incubated at room temperature for 20 minutes. Excessive dye was then washed off with

demineralized water and the cells were observed under an inverted phase-contrast microscope (CKX41, Olympus).

Cellular reactive oxygen species (ROS) assay. The intracellular ROS production levels were determined using an oxidation sensitive fluorescent dye, 2´,7´-dichlorofluorescin diacetate (DCFDA, also known as H2DCFDA and as DCFH-DA) (ab113851, Abcam). The fluorogenic dye measures hydroxyl, peroxyl and other ROS activity within the cell [31].

The hAELVi cells (2.5×10^4 cells/well) were seeded in 96-well huAEC coating solution-coated black plates (Corning 3603, Costar) and incubated for 24 h in a complete medium at 37°C in a humidified atmosphere of 5% CO_2. After that, the cells were washed gently with phosphate-buffered saline (PBS) and incubated at 37°C with 25 μM DCFDA for 45 min in the dark. Then, the cells were washed gently with PBS and treated with the heavy metals at different concentrations (20 μM, 40 μM, 80 μM, 100 μM, 200 μM, and 250 μM) and further incubated for 24 h. A common inducer of ROS production tert-butyl hydroperoxide (TBHP), was used as a positive control. The fluorescence of oxidized DCF was measured immediately after the incubation at an excitation/emission wavelength of 488/535 nm using a fluorescence microplate reader (Synergy™ 2, BioTek).

Quantitation of cytokines in the culture supernatants. The levels of TNF-α (ab181421, Abcam) and IL-6 (ab178013, Abcam) in the culture medium were measured by ELISA according to the manufacturer's instructions. After the exposure to the heavy metals, the medium was harvested and centrifuged at 2000 × g for 10 min to remove floating cells. The cell culture supernatants were stored at −20°C until analysis. The absorbance of each well was measured at 450 nm using a microplate reader (SPECTROstar^{Nano}, BMG LABTECH). The data from each exposure condition was from three independent experiments. Each experiment had three technical replicates. The measurement of each independent experiment was repeated at least twice to ensure reproducibility and allow for statistical evaluation.

Statistical analysis

Cell viability parameters were analysed as follows:

(1) $F(518)_{sam}$ is the fluorescence at 518 nm in the experimental group, which is labelled with the green fluorescent dye and PI.
(2) $F(518)_{min}$ is the fluorescence at 518 nm in the control group, where all cells are alive and labelled with PI.
(3) $F(518)_{max}$ is the fluorescence at 518 nm in the control group, where all cells are alive and labelled with the green fluorescent dye.
For cell viability analysis, the percentage of live cells was calculated from the fluorescence readings by the equation:

$$\% \text{ Live cells} = \frac{F(518)_{sam} - F(518)_{min}}{F(518)_{max} - F(518)_{min}} \times 100\ \%$$

For each assay, all conditions were tested in triplicate. All data were expressed as mean ± SEM. Statistical significance was determined using Student's t-test. $p < 0.05$ represents statistical significance. The asterisk (*) denotes $p < 0.05$, double asterisks (**) denote $p < 0.01$ and triple asterisks (***) denote $p < 0.001$.

Results

Morphological changes of hAELVi cells by heavy metals. The morphological change of hAELVi cells exposed to heavy metals was evaluated. The hAELVi cells were seeded on the huAEC solution-coated 96-well plate, and the cell morphology was observed using an inverted phase-contrast microscope. The hAELVi cells were treated with increasing concentrations of Cd, As, and Zn for 24 h. Judging from the cellular images, there is no significant reduction in cell adhesion after exposure to 20 μM of the heavy metals (Fig. 2). The cell morphology remained mostly intact until the concentration exceeded 40 μM (Fig. 2A), 80 μM (Fig. 2B), and 100 μM (Fig. 2C), respectively. From our observation, at a high concentration compared to that of the CTL group (Fig. 2), the hAELVi cells show rounded shape, indicating reduced cell adhesion to the well bottom surface in the 96 well plate.

Cell cytotoxicity of hAELVi after heavy metal exposure. We investigated the effect of heavy metals on the cellular cytotoxicity of the hAELVi cells after exposure to each metal for 24 h. As shown in Fig. 3, compared with the CTL group, Cd, As, and Zn decreased the cell viability in a concentration-dependent manner. At the concentration of 100 μM (Cd) and 250 μM (As), cell viability decreased to about 55%. However, cell viability was reduced to 45% when Zn reached 100 μM. Moreover, Zn showed a more significant reduction on the cell viability than do Cd and As at high concentrations.

Effect of heavy metals on TEER values. We examined whether or not the rapid TEER change is useful for testing the cytotoxicity of heavy metals. The TEER was monitored before, during, and after the exposure with 250 μM of the heavy metals. After 4h of the Cd exposure, the TEER values decreased significantly (Fig. 4) and the adhesions among the cells were broken (Fig. 5) On the contrary, there was no significant TEER difference during, before, and after the As or Zn exposure (Fig. 4) and the integrity of the monolayers were maintained and observed (Fig. 5) until at least 24 h.

ROS production of hAELVi cells after exposure to heavy metals. For further confirming whether or not cytotoxicity is directly or indirectly related to ROS production in hAELVi cells, the ROS levels were detected by a fluorescence microplate reader at different concentrations of the heavy metals. The ROS level induced by the heavy metals were shown in Fig. 6. It can be seen that the ROS generation level was elevated by low concentrations of the heavy metals. DCF fluorescence intensity decreased gradually with

increasing concentrations of the heavy metals; the cell cytotoxicity was negatively correlated with the ROS generation.

Effect of heavy metal exposure on cytokine secretion from hAELVi cells. The expression levels of cytokines in hAELVi cells exposed to heavy metals can provide information on the possible role of heavy metals on respiratory illnesses due to inflammation. TNF-α and IL-6 released or suppressed in hAELVi cells after the heavy metal exposure was further evaluated. The Cd-induced secretion of TNF-α in hAELVi cells were shown in Fig. 7. The absence of basal levels of TNF-α measured in the controls suggests that this pro-inflammatory cytokine is not secreted or present at concentrations lower than 2 pg/ml (the detection limit for the assay employed). Also, the expression levels of TNF-α were lower than the detection limit after the As and Zn exposure, and therefore the data is not shown.

The secretion of IL-6 was reduced in the hAELVi cells after the heavy metal exposure. The most significant effect was observed at the higher concentration of 200 μM (Cd) and 250 μM (As or Zn) (Fig. 8). The levels of IL-6 at the ion concentrations of 200 μM and 250 μM were significantly lower than that of the CTL. Zn decreased the release of IL-6 in a concentration-dependent manner. Cd, As, and Zn suppressed the IL-6 cytokine releases at 200~250 μM, at which concentration these heavy metals also showed inhibition of cell viability in the cell viability assay.

Discussion

In vitro human alveolar models provide a platform to investigate the cellular behavior and activity of human lung cells [32]. The purpose of this study is to investigate the effect of heavy metals on the cellular cytotoxicity, TEER variation, ROS production, and cytokine secretion of human alveolar epithelial (hAELVi) cells *in vitro*. The responses may be correlated to the observed pulmonary physiological responses raised by the heavy metals. The human lung carcinoma cells and human bronchial epithelial cells exposed to heavy metals have been reported [2,33]. In the report by Honda *et al*., the authors have shown that the biological reaction of human bronchial epithelial BEAS-2B cells to metals in the atmosphere can lead to airway damage and the exacerbation of respiratory diseases [2]. In the report by Mu *et al*., the authors determined the biological responses of sodium arsenite on the human alveolar cell line A549 by monitoring the arsenite-induced toxicity [33]. However, the A549 and BEAS-2B are not derived from the normal lung; therefore, these cells may not be suitable as alveolar epithelium models. The hAELVi cells are derived from human alveolar epithelial cells and with functional tight junctions [21]. The hAELVi cells may be suitable for an alternative to animal testing to study the toxicity of heavy metals on pulmonary functions.

In this study, we examined the effect of Cd, As, and Zn on hAELVi cells. The morphological change of hAELVi cells exposed to Cd, As, and Zn was examined at concentrations of 40 μM, 80 μM, and 100 μM, respectively (Fig. 2). Significant cytotoxicity of these heavy metals on the cells was observed at a concentration exceeding 100 μM (Fig. 3). In a report by Takano *et al*., Cd concentrations causing a 50% loss in cell viability was 220 μM to 250 μM in alveolar type II cells [34]. In our study, we also observed that

250 μM Cd induced a similar level of cell viability reduction in hAELVi cells. In a report by Mu *et al*., the cell viability of A549 cells was decreased to about 70% at the highest concentration of $NaAsO_2$ employed (80 μM) [33]. Our results also indicate that the hAELVi cell viability was similarly reduced to 70% at 80 μM of As.

Several studies have demonstrated that zinc plays a critical role in diverse cellular functions such as growth and development, maintenance and priming of the immune system, and tissue repair [35]. However, zinc is also an important environmental pollutant that becomes dangerous after excessive intake or exposure [36]. In a report by Sharif *et al*., cell growth and viability were decreased by 100 μM of $ZnSO_4$ in the WIL2-NS human lymphoblastoid cell line [37]. In a report by Li *et al*., the authors have found that $ZnSO_4$ (50 to 150 μM) reduced the cell viability of immortalized murine ovarian granular KK-1 cells [38]. As shown in Fig. 3, we observed that the cell viability of hAELVi cells was decreased to about 45% by Zn at a concentration of 100 μM. In our present comparative study of the acute cytotoxicity of heavy metals to hAELVi cells, Zn at concentrations higher than 100 uM was more toxic than the other heavy metals. These results indicated that the cell viability of WIL2-NS, KK-1, and hAELVi cells was all decreased after the treatment with Zn. In summary, all the tested heavy metals show significant toxicity to the hAELVi cells. Zinc ions seemed to be the most toxic metal ions in concentrations higher than 100 μM.

TEER has been widely used to check the level of toxicity of drugs on the epithelial cells [39,40]. However, previous studies have reported that cell viability is a more sensitive indicator of cytotoxicity than TEER [39,41,42]. In a report by Calabro *et al*., the cell viability decreases before the integrity of the monolayer are compromised. In a report by Konsoula *et al*., the authors demonstrated that the MTT assay is a more sensitive indicator of chemical exposure than the TEER measurements. In a report by Mukherjee *et al*., the authors have suggested that TEER mainly represents the tight junctions and does not reflect cell viability. In this study, we demonstrated that the cell viability was not reflected by the TEER value after exposed to 250 μM of As and Zn in hAELVi cells (Fig. 3 and Fig. 4). For the Cd exposure, the TEER value decreased to near zero while the cell viability sustained at 50 %. On the contrary, for the exposure to the other two metal ions, the TEER values remained virtually unaffected while drastic cell viability deterioration down to ~50 % was indeed observed. The electrical resistance of the monolayers was maintained even while the cellular viability had been severely reduced. In summary, the measurement of TEER seems not a reliable method to reflect the toxicity of heavy metals on the hAELVi monolayers. The discrepant result between the two assays may rise from the substrate on which the cells adhere.

The integrity shown in Fig. 5, especially for As and Zn, seems to be in contradiction to the deteriorated cell viability shown in Fig. 2. However, the viability test was done with the cells grown on solid substrate while the cells in the TEER test was grown on porous membrane, which is supposed to be more relevant to the niche for the alveolar cells *in vivo*. The fact that the completeness of the Zn- or As- treated monolayer (Fig. 5) resembles that in the CTL group explains the observed resilient TEER values (Fig. 4). The result suggests that the hAELVi cells cultured on the membrane survived the damages caused by the metals.

The escape from the damage is in consistence with the observation that the level of the cytokine TNF-α was not elevated by As and Zn, as discussed below.

Our results showed that the heavy metals had a significant cytotoxic effect on the hAELVi cells (Fig. 3). However, what mechanisms are involved in the metal-induced cell damage needs to be questioned. In this study, we observed that the ROS level was elevated at low concentrations of 20 μM heavy metals (Fig. 6). However, there is no significant effect on cell viability at this concentration. A significant effect on cell viability was observed at higher concentrations of the heavy metals (100~250 μM) (Fig. 3). Some research efforts suggested that the effect between cytotoxicity and ROS production might not be simple as direct correlation [43]. Previous studies have also reported that the behaviour of some cell lines to ROS-mediated oxidative stress is strongly dependent on the scavenging enzymes, like SOD, Cat, GPx [44–47]. hAELVi cells are alveolar cells, and in principle, they are expected to have a robust antioxidant system since these cells are exposed to gas exchanges *in vivo*. In addition, cell death is one of the many scenarios that could be initiated via activating mitochondrial or alternative pathway apoptosis, or inflammatory phenomenon could exist [48,49]. In a report by Shen *et al.*, the authors suggested that increases in intracellular ROS levels were not the sole cause of cytotoxicity. ROS may be a result of cytotoxicity rather than a causal factor [50]. In summary, the previous reports indicated the biphasic effect of ROS-induced growth, ROS-induced apoptosis, or necrosis. However, our results indicate that the cytotoxicity was negatively correlated with ROS generation in the hAELVi cells. Therefore, we suggest that there is not a direct relationship between cytotoxicity and ROS production on the hAELVi cells, at least by using the analysis method employed in this study.

Tumor necrosis factor (TNF), a vital cytokine in physiology and pathology processes, involves in cell differentiation, apoptotic and necrotic cell death, inflammation, and tumorigenesis [51]. High concentrations of TNF can be deleterious and promotes inflammation and organ injury [52]. Previous report has shown that human peripheral blood mononuclear cells exposed to $CdSO_4$ (1, 5, and 10 μM) or other metal ions produces an increase in the levels of TNF-α [53]. In our study, the TNF-α showed a significant dose-dependent increase in hAELVi cells after exposure to a high concentration of Cd (Fig. 7). In addition, the cell viability decreased after the treatment of the high concentration of Cd (Fig. 3). Previous research has reported that *Taraxacum officinale* induces cytotoxicity through tumor necrosis factor-α (TNF-α) secretion in Hep G2 cells [54]. Our finding also suggested that exposure to Cd induced the secretion of TNF-α by hAELVi cells and the significant increase in cell death and the accompanying drastic TEER decrease. In our study, As did not induce TNF-α production. Similar study on lung cells is rare. As a comparison, a study has shown that 2-8 μM of As increased the expression of TNF-α in human uroepithelial cells, while the expression is decreased under 10 μM As [55]. Apparently, the hAELVi cells respond to As very differently from the uroepithelial cells. Concerning the fact that Zn did not induce TNF-a in the hAELVi cells in this study, there has been relatively rare research on the Zn's effect on the production of TNF-α and therefore is not compared in more depth. Further studies on the toxicity of heavy metal on hAELVi cells are obviously in need.

IL-6 is pleiotropic and acts as both an anti-inflammatory cytokine [56] and a pro-inflammatory cytokine [29], which are mediated by classic signaling and by trans-signaling, respectively [57]. IL-6 has a wide range of biological activities, including immune responses, acute-phase response, and inflammation [58]. In our study, As and Zn at low concentrations (20 μM) increased the levels of IL6. At higher concentrations (250 μM), an opposite effect was seen (Fig. 8), while TNF-α secretion remain un-detectable. A high concentration of Cd significantly increased the secretion of TNF-α but decreased the secretion of IL6 from the hAELVi cells (Fig. 7 and Fig. 8), which is very different from the effect by the other two metal ions.

Previous studies have reported that TNF-α and IL-6 production is mutually counter-regulated [59–62]. In a report by Gonzalez *et al*., the negative relationship between TNF-α and IL-6 in patients with acute stage liver abscess has been observed [59]. In a report by Yimin *et al*., IL-6 has a suppressive effect on TNF-α production and down-regulates granulomatous inflammation reaction [60]. In a report by Diao *et al*., IL-6 plays an important role in the negative feedback of the immune response via suppression of TNF-α production in *Streptococcus pyogenes*-infected mice [61]. In the report by Yimin *et al*., it has been shown that TNF-α and IL-6 production exhibit negatively regulatory effects by each other [62]. These results indicated that the negatively regulated mechanism between IL-6 and TNF-α secretion. In our study, we also observed that the concentration of 250 μM Cd significantly increased the secretion of TNF-α but decreased the secretion of IL6. However, for As and Zn, the decrease of IL-6 was not accompanied by the increase of TNF-α. Previous research efforts suggested that the cytokines assayed were differentially affected by heavy metal exposure [53]. Our results also suggested that the cytokines of hAELVi cells were differentially affected by heavy metal exposure. Therefore, although significant cell death was indeed induced by Zn, there was no corresponding TNF-α secretion. It may be influenced by other cytokines released.

In conclusion, we confirmed that Cd, As, and Zn induced variable extents of cellular toxicity on hAELVi cells in a dose-dependent manner when the cells were cultured on a solid substrate. However, the cytotoxicity was not reflected by the TEER value after exposure to As and Zn. The discrepancy between the viability assay and the TEER assay may rise from the substrate effect because the TEER was measured with cells cultured on permeable porous membrane, a niche that resembles *in vivo* better than on solid substrate. Further studies observing the cytokine expression from cells cultured on permeable membrane is worth of pursuing. In addition, we found that there is no direct relationship between the cytotoxicity and the ROS production in the hAELVi cells. The exposure to Cd, As and Zn result in differing cytokine secretion of the cells. This study contributes to elucidating the cytotoxicity, TEER values, ROS production, and cytokine secretion of hAELVi cells to acute exposure to heavy metal *in vitro*.

Declarations

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Author contributions statement

HFC designed the study, carried out the experiments, data analysis, and drafted the manuscript. JYC participated in the design of the study, interpreted the data, and helped to draft the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1 Huang, W., Long, E., Wang, J., Huang, R. & Ma, L. Characterizing spatial distribution and temporal variation of PM10 and PM2.5 mass concentrations in an urban area of Southwest China. *Atmospheric Pollution Research***6**, 842-848, doi:10.5094/APR.2015.093 (2015).

2 Honda, A. *et al.* Effects of air pollution-related heavy metals on the viability and inflammatory responses of human airway epithelial cells. *International journal of toxicology***34**, 195-203, doi:10.1177/1091581815575757 (2015).

3 Feng, S., Gao, D., Liao, F., Zhou, F. & Wang, X. The health effects of ambient PM2.5 and potential mechanisms. *Ecotoxicol Environ Saf***128**, 67-74, doi:10.1016/j.ecoenv.2016.01.030 (2016).

4 Costa, A. F., Hoek, G., Brunekreef, B. & Ponce de Leon, A. C. Air Pollution and Deaths among Elderly Residents of Sao Paulo, Brazil: An Analysis of Mortality Displacement. *Environ Health Perspect***125**, 349-354, doi:10.1289/EHP98 (2017).

5 Baxter, L. K., Crooks, J. L. & Sacks, J. D. Influence of exposure differences on city-to-city heterogeneity in PM2.5-mortality associations in US cities. *Environ Health***16**, 1, doi:10.1186/s12940-016-0208-y (2017).

6 Patel, M. M. *et al.* Ambient metals, elemental carbon, and wheeze and cough in New York City children through 24 months of age. *Am J Respir Crit Care Med***180**, 1107-1113, doi:10.1164/rccm.200901-0122OC (2009).

7 Lagorio, S. *et al.* Air pollution and lung function among susceptible adult subjects: a panel study. *Environ Health***5**, 11, doi:10.1186/1476-069X-5-11 (2006).

8 Hirshon, J. M. *et al.* Elevated ambient air zinc increases pediatric asthma morbidity. *Environ Health Perspect***116**, 826-831, doi:10.1289/ehp.10759 (2008).

9 Chen, Y. C. *et al.* Characteristics of Concentrations and Metal Compositions for PM2.5 and PM2.5–10 in Yunlin County, Taiwan during Air Quality Deterioration. *Aerosol and Air Quality Research***15**, 2571-2583,

doi:10.4209/aaqr.2015.04.0261 (2015).

10 Nemery, B. Metal toxicity and the respiratory tract. *Eur Respir J***3**, 202-219 (1990).

11 Chen, Y. *et al.* Summer-winter differences of PM2.5 toxicity to human alveolar epithelial cells (A549) and the roles of transition metals. *Ecotoxicol Environ Saf***165**, 505-509, doi:10.1016/j.ecoenv.2018.09.034 (2018).

12 Ramsey, K. in *Handbook of Arsenic Toxicology* (ed S. J. S. Flora) 335-347 (Academic Press, 2015).

13 Parvez, F. *et al.* Arsenic exposure and impaired lung function. Findings from a large population-based prospective cohort study. *Am J Respir Crit Care Med***188**, 813-819, doi:10.1164/rccm.201212-2282OC (2013).

14 De, B. K., Majumdar, D., Sen, S., Guru, S. & Kundu, S. Pulmonary involvement in chronic arsenic poisoning from drinking contaminated ground-water. *J Assoc Physicians India***52**, 395-400 (2004).

15 Stedman, T. L. *Stedman's medical dictionary*. 27th ed. edn, 1035 (Lippincott Williams & Wilkins, 2000).

16 Ochs, M. *et al.* The number of alveoli in the human lung. *Am J Respir Crit Care Med***169**, 120-124, doi:10.1164/rccm.200308-1107OC (2004).

17 Wang, Y. *et al.* Pulmonary alveolar type I cell population consists of two distinct subtypes that differ in cell fate. *Proc Natl Acad Sci U S A***115**, 2407-2412, doi:10.1073/pnas.1719474115 (2018).

18 Kemp, S. J. *et al.* Immortalization of human alveolar epithelial cells to investigate nanoparticle uptake. *Am J Respir Cell Mol Biol***39**, 591-597, doi:10.1165/rcmb.2007-0334OC (2008).

19 Dekali, S. *et al.* Assessment of an in vitro model of pulmonary barrier to study the translocation of nanoparticles. *Toxicol Rep***1**, 157-171, doi:10.1016/j.toxrep.2014.03.003 (2014).

20 Poenar, D. P., Yang, G., Wan, W. K. & Feng, S. Low-Cost Method and Biochip for Measuring the Trans-Epithelial Electrical Resistance (TEER) of Esophageal Epithelium. *Materials (Basel)***13**, 2354, doi:10.3390/ma13102354 (2020).

21 Kuehn, A. *et al.* Human alveolar epithelial cells expressing tight junctions to model the air-blood barrier. *ALTEX***33**, 251-260, doi:10.14573/altex.1511131 (2016).

22 Ehrhardt, C. *et al.* 16HBE14o- human bronchial epithelial cell layers express P-glycoprotein, lung resistance-related protein, and caveolin-1. *Pharm Res***20**, 545-551, doi:10.1023/a:1023230328687 (2003).

23 Forbes, B., Shah, A., Martin, G. P. & Lansley, A. B. The human bronchial epithelial cell line 16HBE14o- as a model system of the airways for studying drug transport. *Int J Pharm***257**, 161-167, doi:10.1016/s0378-5173(03)00129-7 (2003).

24 Grainger, C. I., Greenwell, L. L., Lockley, D. J., Martin, G. P. & Forbes, B. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm Res***23**, 1482-1490, doi:10.1007/s11095-006-0255-0 (2006).

25 Gordon, S. *et al.* Non-animal models of epithelial barriers (skin, intestine and lung) in research, industrial applications and regulatory toxicology. *ALTEX***32**, 327-378, doi:10.14573/altex.1510051 (2015).

26 Han, S. & Mallampalli, R. K. The acute respiratory distress syndrome: from mechanism to translation. *J Immunol***194**, 855-860, doi:10.4049/jimmunol.1402513 (2015).

27 Mukhopadhyay, S., Hoidal, J. R. & Mukherjee, T. K. Role of TNFalpha in pulmonary pathophysiology. *Respir Res***7**, 125, doi:10.1186/1465-9921-7-125 (2006).

28 Chen, Y. *et al.* Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem***278**, 17036-17043, doi:10.1074/jbc.M210429200 (2003).

29 Thacker, E. L. Lung inflammatory responses. *Veterinary research***37**, 469-486, doi:10.1051/vetres:2006011 (2006).

30 Bautista, M. V. *et al.* IL-8 regulates mucin gene expression at the posttranscriptional level in lung epithelial cells. *J Immunol***183**, 2159-2166, doi:10.4049/jimmunol.0803022 (2009).

31 Figueroa, D., Asaduzzaman, M. & Young, F. Real time monitoring and quantification of reactive oxygen species in breast cancer cell line MCF-7 by 2',7'–dichlorofluorescin diacetate (DCFDA) assay. *Journal of Pharmacological and Toxicological Methods***94**, 26-33, doi:10.1016/j.vascn.2018.03.007 (2018).

32 Evans, K. V. & Lee, J. H. Alveolar wars: The rise of in vitro models to understand human lung alveolar maintenance, regeneration, and disease. *Stem Cells Transl Med***9**, 867-881, doi:10.1002/sctm.19-0433 (2020).

33 Mu, Y., Wang, R. & Wang, H. Programmed Cell Death of Cultured A549 Lung Epithelial Cells Induced by Sodium Arsenite Exposure. *Journal of Medicinal Chemistry & Toxicology***2**, 85-89, doi:10.15436/2575-808X.17.1471 (2017).

34 Takano, Y., Taguchi, T., Suzuki, I., Balis, J. U. & Yuri, K. Cytotoxicity of heavy metals on primary cultured alveolar type II cells. *Environ Res***89**, 138-145, doi:10.1006/enrs.2002.4354 (2002).

35 Truong-Tran, A. Q., Carter, J., Ruffin, R. & Zalewski, P. D. New insights into the role of zinc in the respiratory epithelium. *Immunol Cell Biol***79**, 170-177, doi:10.1046/j.1440-1711.2001.00986.x (2001).

36 Marcinčáková, D. *et al.* Impact of Zinc Sulfate Exposition on Viability, Proliferation and Cell Cycle Distribution of Epithelial Kidney Cells. *Polish Journal of Environmental Studies***28**, 3279-3286, doi:10.15244/pjoes/94045 (2019).

37 Sharif, R., Thomas, P., Zalewski, P., Graham, R. D. & Fenech, M. The effect of zinc sulphate and zinc carnosine on genome stability and cytotoxicity in the WIL2-NS human lymphoblastoid cell line. *Mutat Res***720**, 22-33, doi:10.1016/j.mrgentox.2010.12.004 (2011).

38 Li, Y. *et al.* Zinc inhibits the reproductive toxicity of Zearalenone in immortalized murine ovarian granular KK-1 cells. *Sci Rep***5**, 14277, doi:10.1038/srep14277 (2015).

39 Mukherjee, T., Squillantea, E., Gillespieb, M. & Shao, J. Transepithelial electrical resistance is not a reliable measurement of the Caco-2 monolayer integrity in Transwell. *Drug Deliv***11**, 11-18, doi:10.1080/10717540490280345 (2004).

40 Narai, A., Arai, S. & Shimizu, M. Rapid decrease in transepithelial electrical resistance of human intestinal Caco-2 cell monolayers by cytotoxic membrane perturbents. *Toxicol In Vitro***11**, 347-354, doi:10.1016/s0887-2333(97)00026-x (1997).

41 Calabro, A. R., Konsoula, R. & Barile, F. A. Evaluation of in vitro cytotoxicity and paracellular permeability of intact monolayers with mouse embryonic stem cells. *Toxicol In Vitro***22**, 1273-1284, doi:10.1016/j.tiv.2008.02.023 (2008).

42 Konsoula, R. & Barile, F. A. Correlation of in vitro cytotoxicity with paracellular permeability in Caco-2 cells. *Toxicol In Vitro***19**, 675-684, doi:10.1016/j.tiv.2005.03.006 (2005).

43 Zhu, C., Hu, W., Wu, H. & Hu, X. No evident dose-response relationship between cellular ROS level and its cytotoxicity–a paradoxical issue in ROS-based cancer therapy. *Sci Rep***4**, 5029, doi:10.1038/srep05029 (2014).

44 Ighodaro, O. M. & Akinloye, O. A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine***54**, 287-293, doi:10.1016/j.ajme.2017.09.001 (2019).

45 He, L. *et al.* Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cell Physiol Biochem***44**, 532-553, doi:10.1159/000485089 (2017).

46 Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S. & Kalayci, O. Oxidative stress and antioxidant defense. *World Allergy Organ J***5**, 9-19, doi:10.1097/WOX.0b013e3182439613 (2012).

47 Day, R. M. & Suzuki, Y. J. Cell proliferation, reactive oxygen and cellular glutathione. *Dose Response***3**, 425-442, doi:10.2203/dose-response.003.03.010 (2006).

48 Green, D. R. & Llambi, F. Cell Death Signaling. *Cold Spring Harb Perspect Biol***7**, a006080, doi:10.1101/cshperspect.a006080 (2015).

49 Belizario, J., Vieira-Cordeiro, L. & Enns, S. Necroptotic Cell Death Signaling and Execution Pathway: Lessons from Knockout Mice. *Mediators Inflamm***2015**, 128076, doi:10.1155/2015/128076 (2015).

50 Shen, C. *et al.* Relating cytotoxicity, zinc ions, and reactive oxygen in ZnO nanoparticle-exposed human immune cells. *Toxicol Sci***136**, 120-130, doi:10.1093/toxsci/kft187 (2013).

51 Locksley, R. M., Killeen, N. & Lenardo, M. J. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell***104**, 487-501, doi:10.1016/s0092-8674(01)00237-9 (2001).

52 Steeland, S., Libert, C. & Vandenbroucke, R. E. A New Venue of TNF Targeting. *Int J Mol Sci***19**, 1442, doi:10.3390/ijms19051442 (2018).

53 Maria, B., Koizumi, S. & Jonai, H. Cytokine Production by Human Peripheral Blood Mononuclear cells after Exposure to Heavy Metals. *JOURNAL OF HEALTH SCIENCE***46**, 358-362, doi:10.1248/jhs.46.358 (2000).

54 Koo, H. N. *et al.* Taraxacum officinale induces cytotoxicity through TNF-alpha and IL-1alpha secretion in Hep G2 cells. *Life Sci***74**, 1149-1157, doi:10.1016/j.lfs.2003.07.030 (2004).

55 Liu, S. *et al.* Arsenic induced overexpression of inflammatory cytokines based on the human urothelial cell model in vitro and urinary secretion of individuals chronically exposed to arsenic. *Chem Res Toxicol***27**, 1934-1942, doi:10.1021/tx5002783 (2014).

56 Riemschneider, S., Herzberg, M. & Lehmann, J. Subtoxic Doses of Cadmium Modulate Inflammatory Properties of Murine RAW 264.7 Macrophages. *Biomed Res Int***2015**, 295303, doi:10.1155/2015/295303 (2015).

57 Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta***1813**, 878-888, doi:10.1016/j.bbamcr.2011.01.034 (2011).

58 Kayaalti, Z. *et al.* Effects of the interleukin-6 (IL-6) polymorphism on toxic metal and trace element levels in placental tissues. *Sci Total Environ***409**, 4929-4933, doi:10.1016/j.scitotenv.2011.08.036 (2011).

59 Gonzalez-Amaro, R. *et al.* Plasma levels and in vitro production of tumor necrosis factor-alpha and interleukin-6 in patients with amebic liver abscess. *Rev Invest Clin***46**, 209-213 (1994).

60 Yimin, Kohanawa, M. & Minagawa, T. Up-regulation of granulomatous inflammation in interleukin-6 knockout mice infected with Rhodococcus aurantiacus. *Immunology***110**, 501-506, doi:10.1111/j.1365-2567.2003.01762.x (2003).

61 Diao, H. & Kohanawa, M. Endogenous interleukin-6 plays a crucial protective role in streptococcal toxic shock syndrome via suppression of tumor necrosis factor alpha production. *Infect Immun***73**, 3745-3748, doi:10.1128/IAI.73.6.3745-3748.2005 (2005).

62 Yimin & Kohanawa, M. A regulatory effect of the balance between TNF-alpha and IL-6 in the granulomatous and inflammatory response to Rhodococcus aurantiacus infection in mice. *J*

*Immunol***177**, 642-650, doi:10.4049/jimmunol.177.1.642 (2006).

Figures

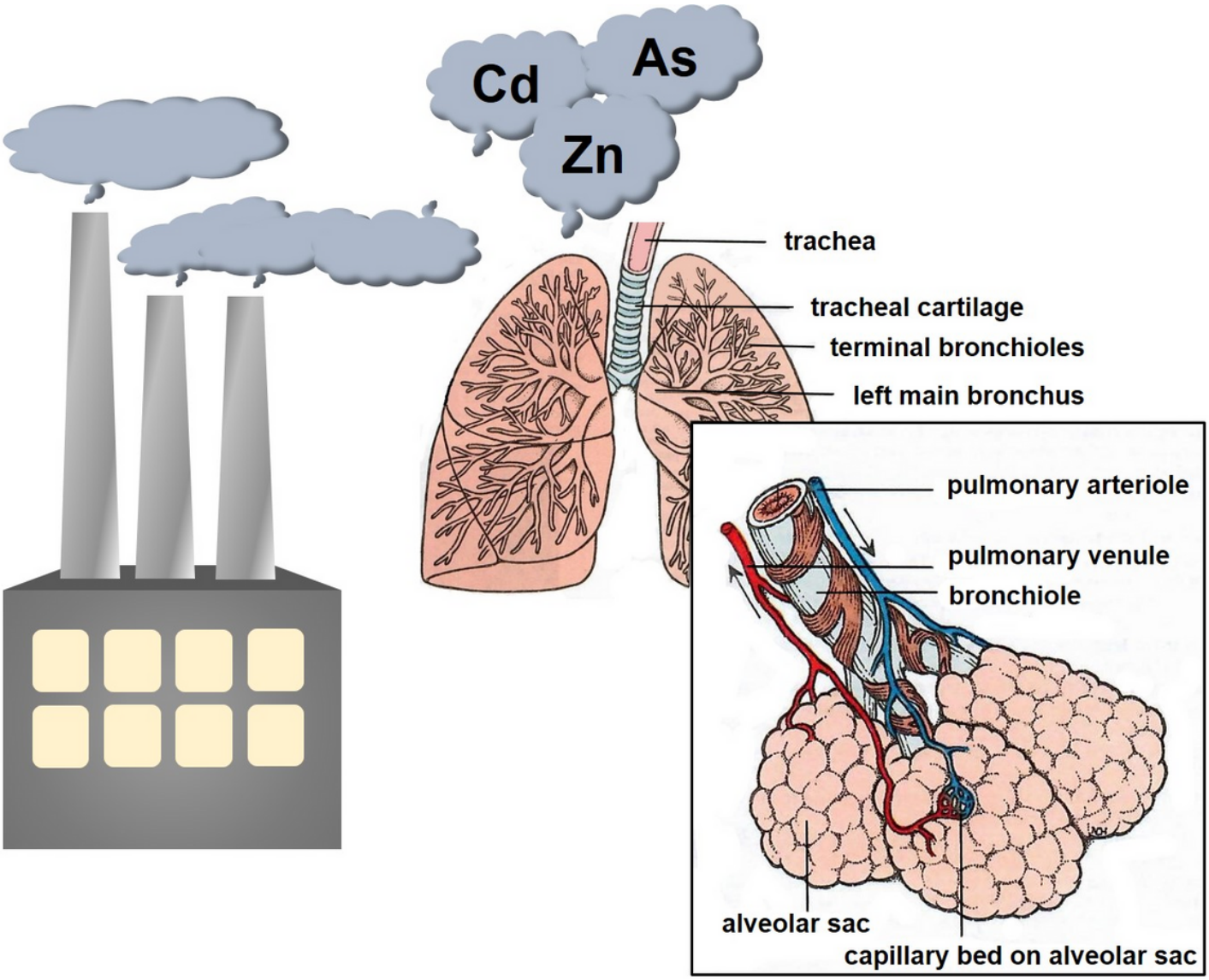

Figure 1

The respiratory system can suffer effects after exposure to air pollution from factories (modified from Stedman's Medical Dictionary 15).

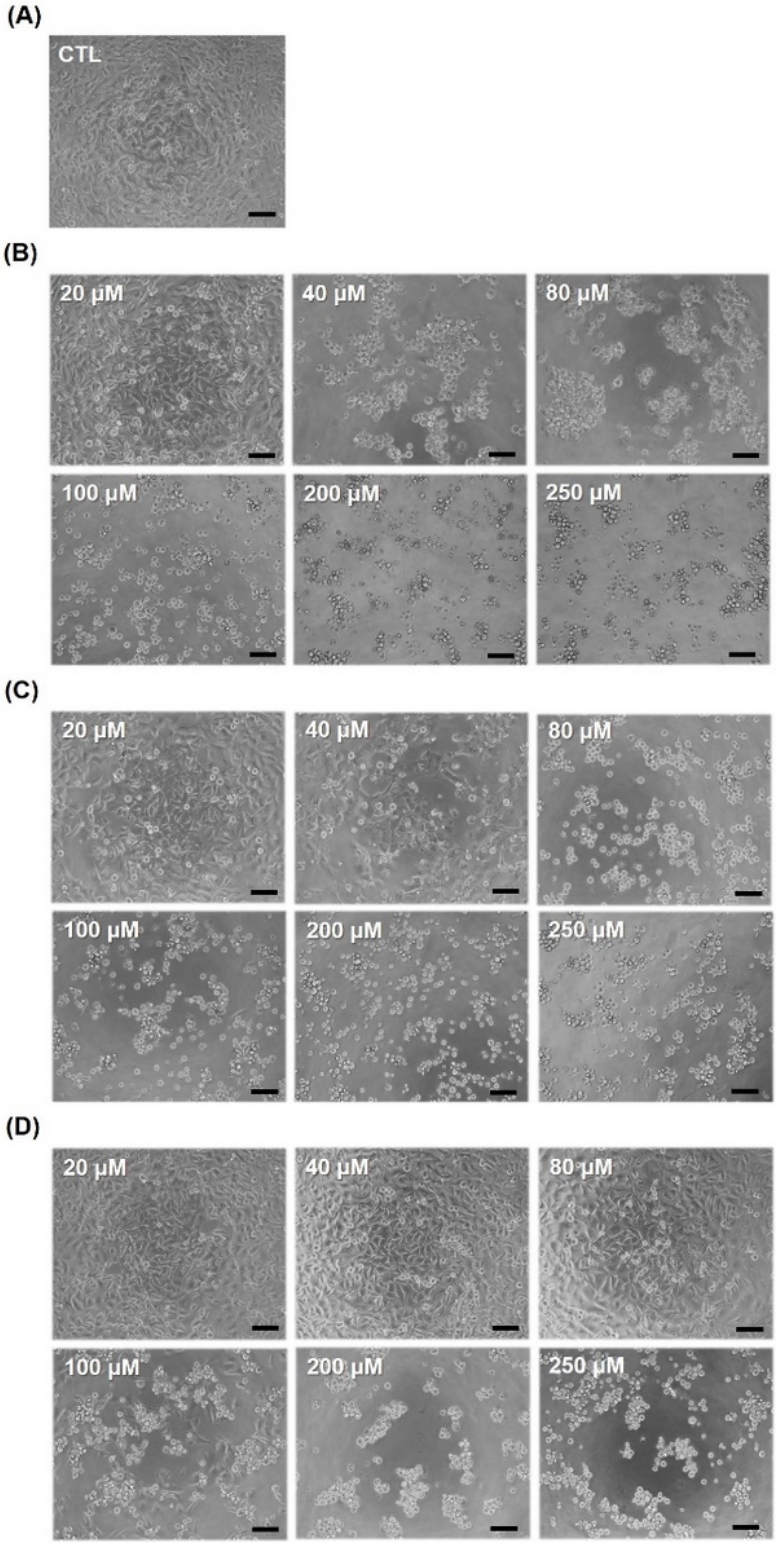

Figure 2

Micrographs of hAELVi cells exposed to heavy metals for 24 h. Cell morphology changes relative to concentrations (20, 40, 80, 100, 200 and 250 µM) of (A) absence of heavy metals (CTL) (B) Cd, (C) As, and (D) Zn respectively. All micrographs are at the same magnification; Scale bars, 50 µm.

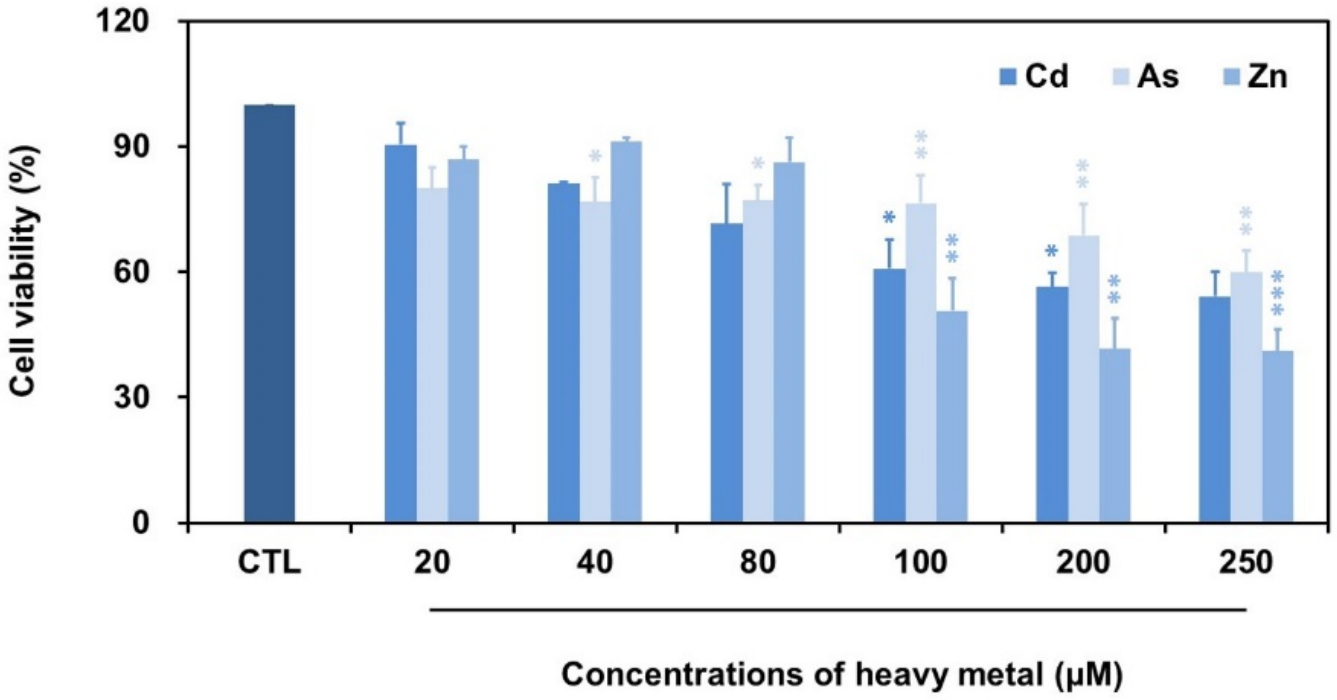

Figure 3

The cell viability of hAELVi cells after exposure to various doses of air pollution-related heavy metals. The hAELVi cells were incubated in the presence of Cd, As, and Zn for 24 h.

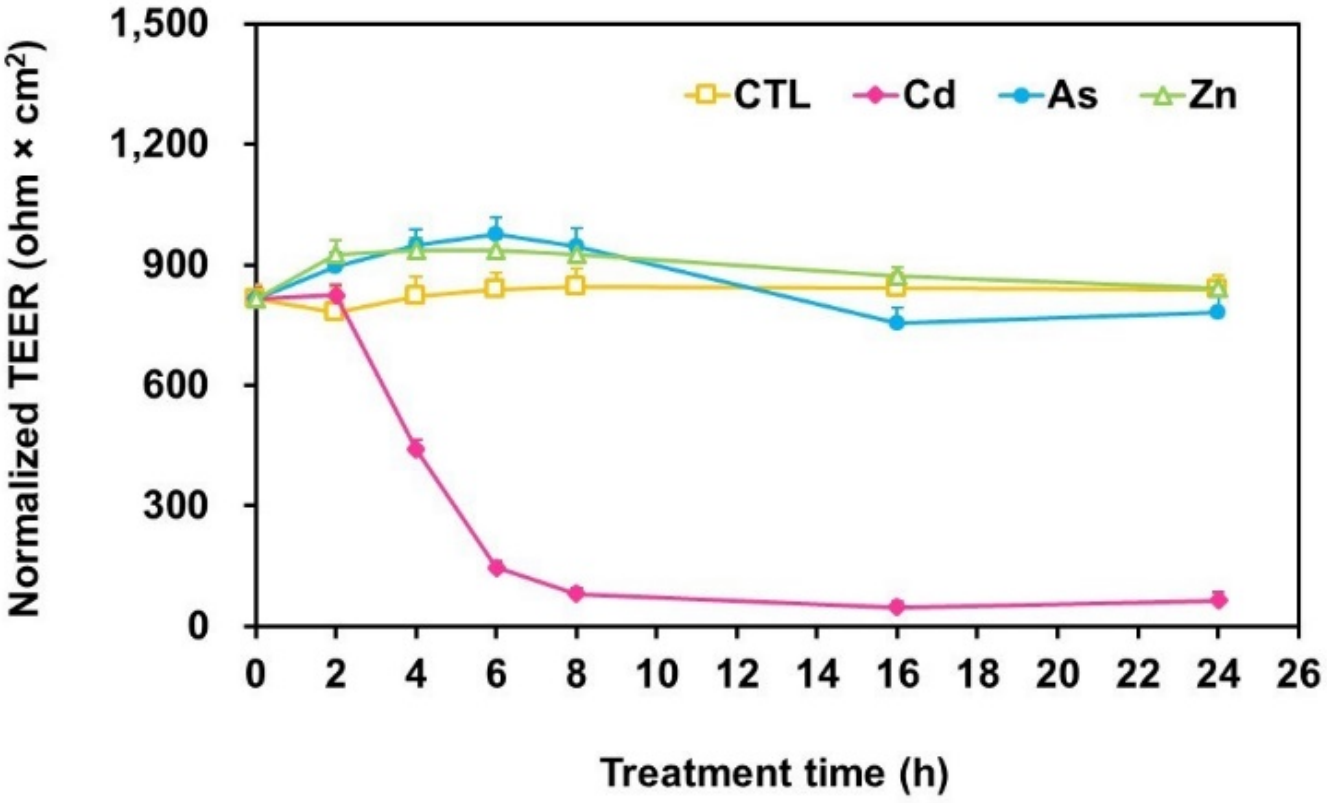

Figure 4

TEER values change of hAELVi cells after exposure to 250 μM of heavy metals. The hAELVi cells were treatment without (CTL) and with Cd, As, and Zn for 24 h.

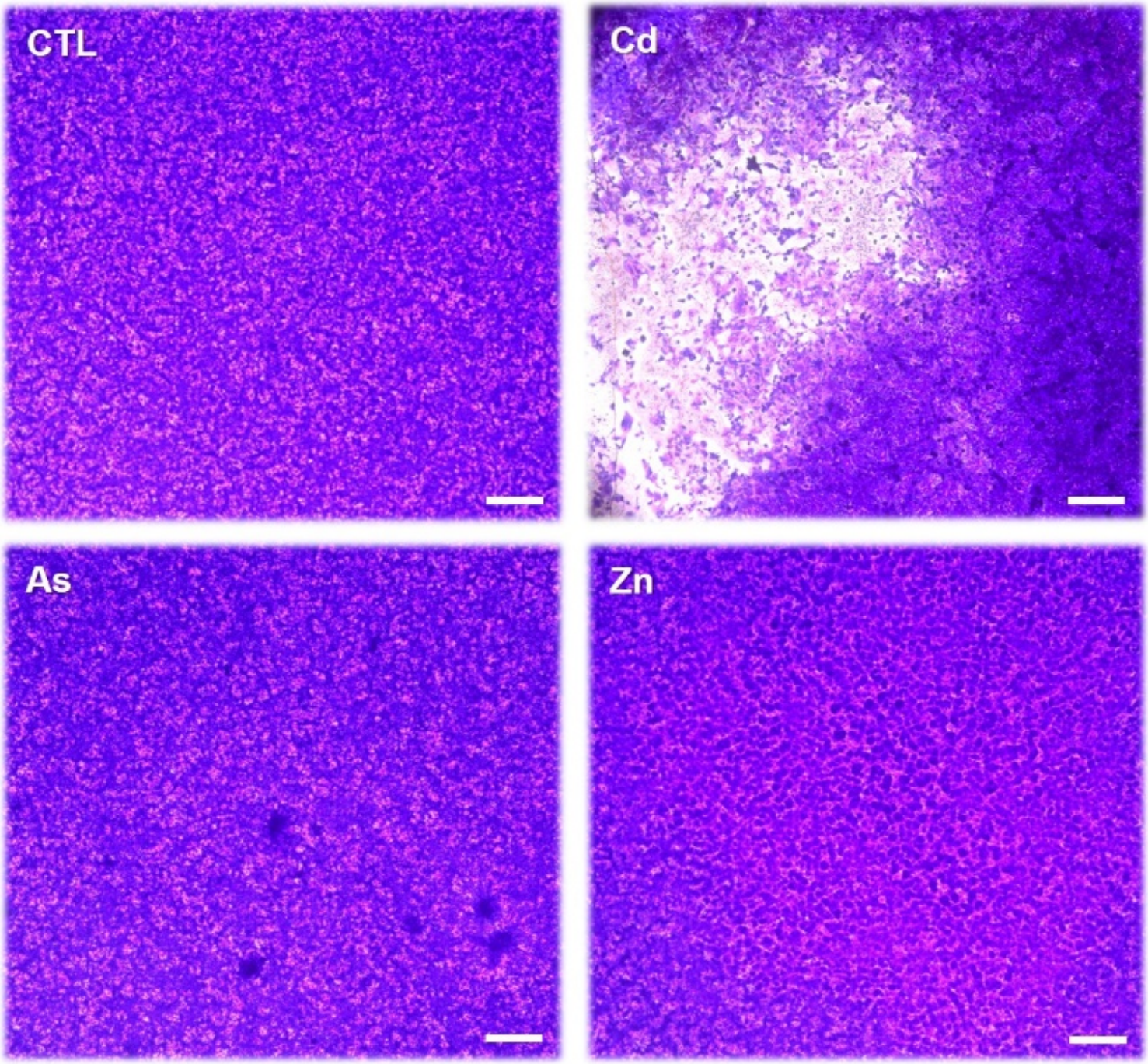

Figure 5

The integrity of hAELVi cells monolayers after exposure to 250 μM of heavy metals. The hAELVi cells were treatment without (CTL) and with Cd, As, and Zn for 24 h. The cells were stained with crystal violet. All micrographs are at the same magnification; Scale bars, 200 μm.

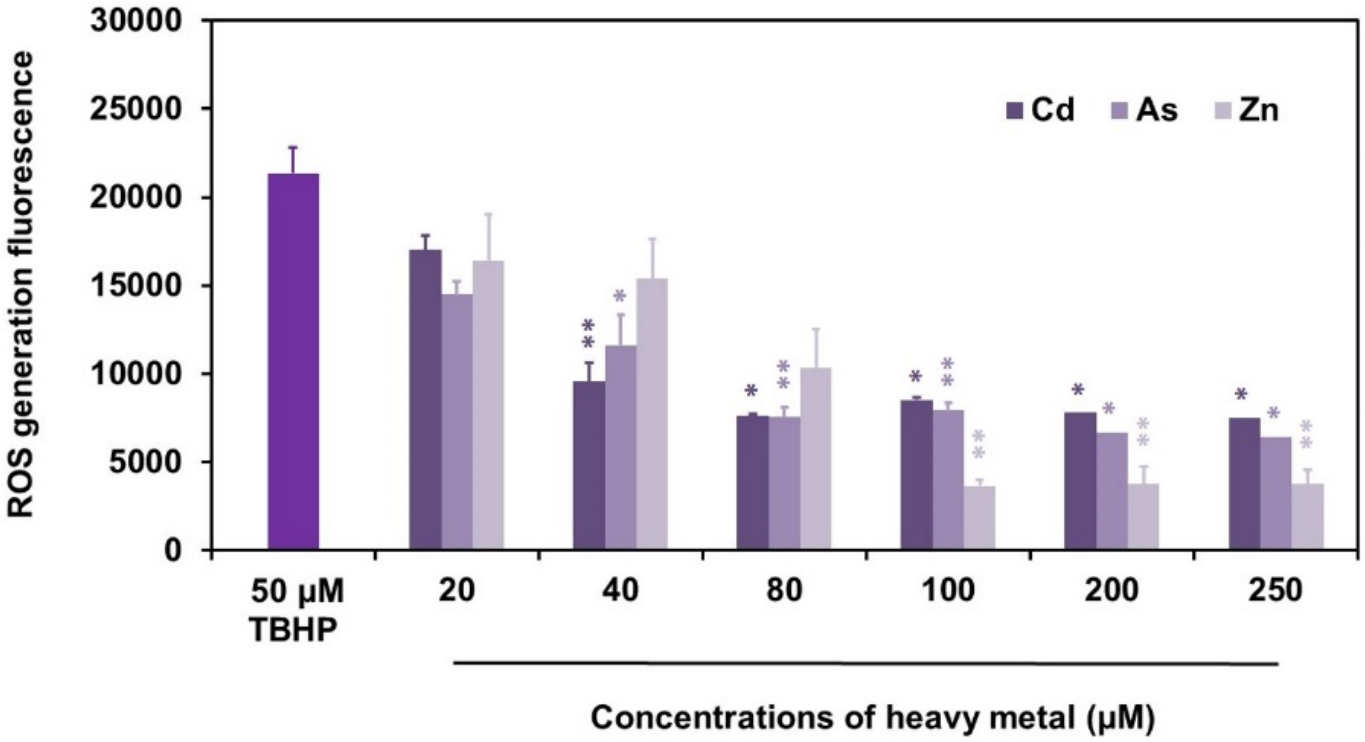

Figure 6

Production of ROS in hAELVi cells. The hAELVi cells were incubated in the presence of Cd, As, and Zn for 24 h. 50 µM Tert-Butyl Hydrogen Peroxide (TBHP) was used as a positive control.

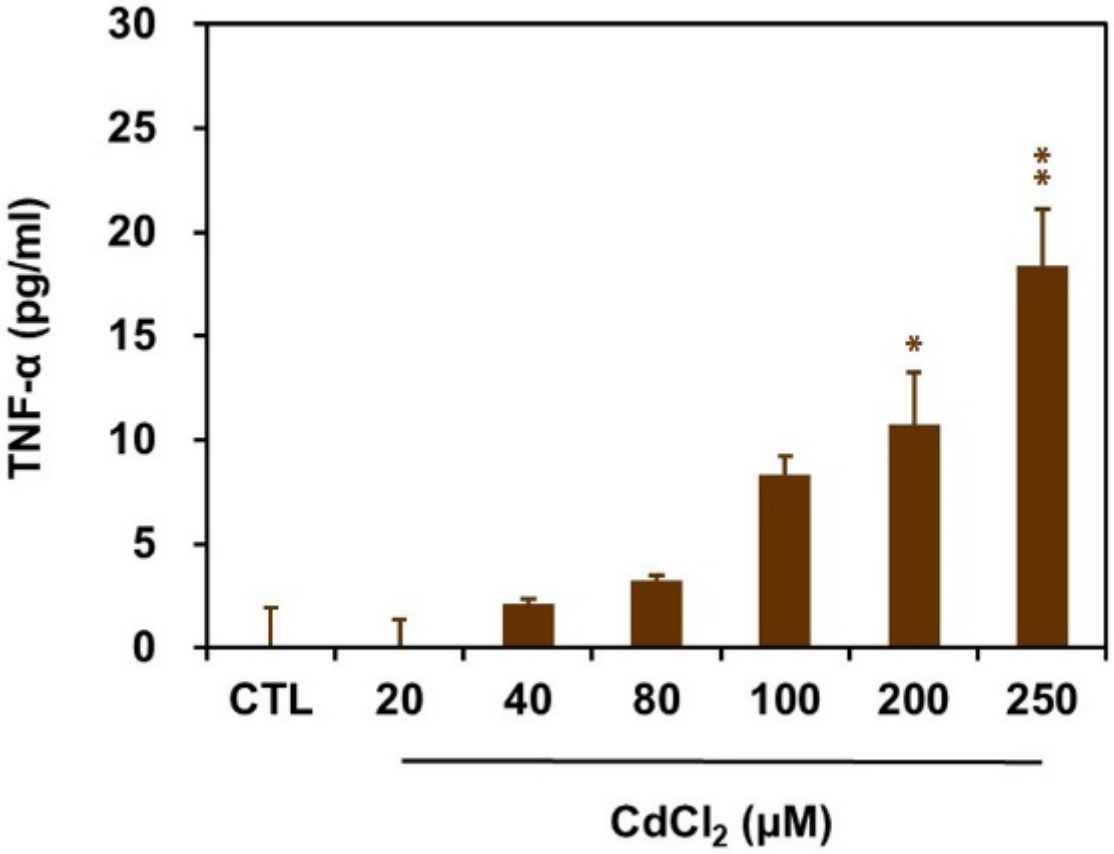

Figure 7

Secretion of TNF-α by hAELVi cells treated with CdCl2. The hAELVi cells were incubated in the presence of Cd for 24 h and TNF-α concentrations measured in the supernatants.

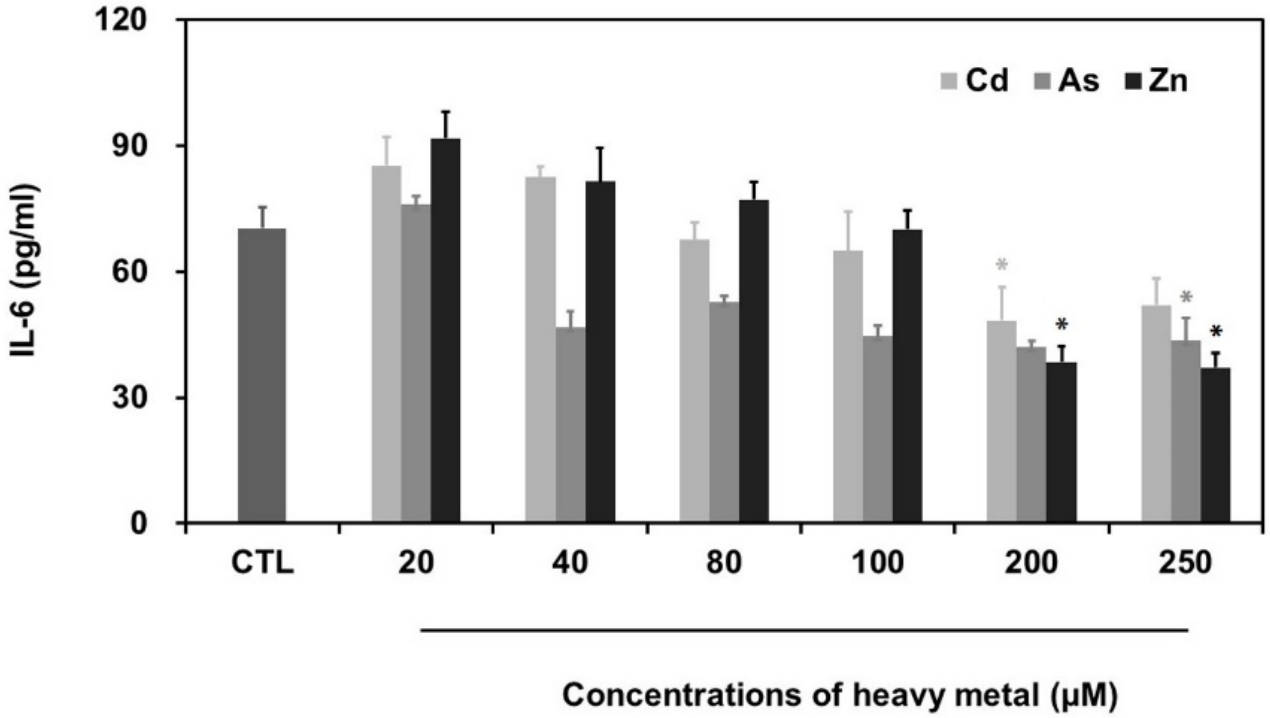

Figure 8

Secretion of IL-6 by hAELVi cells treated with heavy metals after 24h. The hAELVi cells were incubated in the presence of Cd, As, and Zn for 24 h and IL-6 concentrations measured in the supernatants.