

## ORIGINAL ARTICLE

# Cell Viability and Cytokine Production of Human Alveolar Epithelial Cells Following Exposure to Sulphur Dioxide

SHAHNAZ BAKAND<sup>1,2\*</sup>, CHRIS WINDER<sup>3</sup>, AMANDA HAYES<sup>2</sup>

<sup>1</sup>Department of Occupational Health, Research Center for Occupational Health, Tehran University of Medical Sciences, Tehran, Iran; <sup>2</sup>School of Chemistry, University of New South Wales, UNSW Sydney, 2052; <sup>3</sup>School of Risk and Safety Sciences, University of New South Wales, UNSW Sydney, 2052.

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## ABSTRACT

Exposure to air pollutants is significantly associated with health risks ranging from bronchial reactivity to morbidity and mortality. However, the precise mechanisms are not always fully understood. The aim of this study was to investigate the effects of sulphur dioxide (SO<sub>2</sub>) on cell viability and cytokine production of A549-human pulmonary epithelial cells. Test atmospheres of SO<sub>2</sub> were generated using a direct dilution method and calibrated by ion-chromatography. Test atmospheres were delivered to lung cells cultured on porous membranes (0.4 µm) using Harvard Navicite horizontal diffusion chamber systems. The cytotoxic endpoints were investigated using the MTS (tetrazolium salt; Promega), NRU (neutral red uptake; Sigma) and ATP (adenosine triphosphate; Promega) assays. Expression of inflammatory markers including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were evaluated using double-antibody immunometric assays. Dose-dependent effects of SO<sub>2</sub> were observed in A549 cells using all *in vitro* assays at test concentrations (10-200 ppm). The ATP assay appeared to be the most sensitive test (IC<sub>50</sub> = 48 ± 2.83 ppm) that may related to the impaired metabolic activity of the cells following SO<sub>2</sub> exposure. After analysis of TNF-α, no statistically significant differences were observed between control and exposed cells. However, the IL-6 production in A549 cells was significantly reduced in a dose-dependent manner (*p* < 0.05). These results suggest that SO<sub>2</sub> may induce a functional alteration of cells of the pulmonary epithelial preventing cells to produce adequate amounts of IL-6. IL-6 as a multifunctional proinflammatory cytokine may regulate cellular responses and plays a significant role in inflammation and tissue injury.

**Keywords:** Adenosine triphosphate, Interleukin-6, *In vitro* cytotoxicity, Neutral red uptake, Sulphur dioxide, Tetrazolium salt, Tumor necrosis factor-α

## INTRODUCTION

Occupational and environmental exposure to air pollutants can lead to significant human health risks ranging from bronchial reactivity to morbidity and

mortality due to acute intense or long term low level repeated exposures [1-6]. Sulphur dioxide is one of the major components of urban air pollution and a common industrial air contaminant that can be oxidised to acid aerosols during its atmospheric life. SO<sub>2</sub> is generated as a by-product from diverse industrial processes such as smelting, electrical generation, iron and steel mills, chemical plants, paper manufacturing, and petroleum

\* **Corresponding author:** Shahnaz Bakand, E-mail: sh-bakand@tums.ac.ir

**Table 1.** Calculated concentrations of SO<sub>2</sub> test atmospheres

Calculated Concentration (ppm)	SO <sub>2</sub> flow rate (L/min)	Air flow rate (L/min)	Total flow rate (L/min)
10	0.05	0.95	1
20	0.1	0.9	1
40	0.2	0.8	1
80	0.4	0.6	1
200	1	0	1

refining [7, 8]. The harmful effects of SO<sub>2</sub> and particulates were well documented in several severe air pollution episodes in London during the 1950s [8].

SO<sub>2</sub> is primarily known as an upper airway irritant. The majority of inhaled SO<sub>2</sub> (>90%) can be scrubbed in the upper respiratory tract due to its high water solubility [7, 9, 10]. Upon inhalation, SO<sub>2</sub> rapidly dissolves into the upper airway lining fluids and dissociates into bisulphite and sulphite ions. Bisulphite is known as a bronchial constrictor particularly in asthmatic individuals [8]. Tissue damage of the pulmonary epithelial surface and airway constriction might be induced by SO<sub>2</sub> in both animals and humans. Human adverse health effects of SO<sub>2</sub> were primarily investigated by pulmonary function tests and mainly focused on bronchial constriction and hyper reactivity [9]. Long-term exposure to SO<sub>2</sub> may impair immune and defence function of respiratory system causing various types of respiratory tract inflammation [11]. However, cellular and molecular mechanisms involved in the harmful effects of SO<sub>2</sub> on the respiratory system needs to be revealed further.

The aim of this study was to investigate further the cytotoxic effects of SO<sub>2</sub> on human alveolar epithelial cells and potential mechanisms responsible for such effects using a range of both *in vitro* cytotoxicity assays and inflammatory cytokines.

**MATERIALS AND METHODS**

All experimental work of this research was conducted at the Chemical Safety and Applied Toxicology (CSAT) Laboratories, The University of New South Wales, during 2006- 2008.

*Chemical compounds*

Sulphur dioxide (SO<sub>2</sub>, CAS No. 7446-09-5, 200 ppm) balanced in synthetic air was purchased from Linde Gas Pty Ltd, Australia. Synthetic air was purchased from Linde Gas Pty Ltd, Australia. *In vitro* assay reagents were purchased from Sigma (USA) and Promega (USA).

*Cell types and culture conditions*

Human pulmonary type II- epithelial cells (A549, ATCC No. CCL-185) were cultured in sterile, vented 75-cm<sup>2</sup> cell culture flasks with Dulbecco’s modified eagle medium: Ham’s F12 nutrient mixture (DMEM/F12; Gibco, USA) supplemented with fetal calf serum (FCS 5% (v/v); JS Bioscience, Australia), and L-glutamine (2 mM), penicillin (100 U/ml),

streptomycin (0.1 mg/ml) solution (Sigma, USA). Cultured cells were kept at 37°C in a humidified 5% CO<sub>2</sub> incubator. These cell lines have been well characterised for their use in respiratory toxicity studies.

For cytotoxicity experiments, human A549 lung cells were grown on porous membranes (0.4 µm) in Snapwell inserts [12-16]. The Snapwell insert is a modified transwell culture insert with a 12 mm diameter providing a growth area of 1.12 cm<sup>2</sup> (clear polyester Snapwell™ insert, 3801, Corning), supported by a detachable ring that was placed in a six well culture plate. Culture media supplemented with HEPES buffer (0.01 M) was added and the Snapwell inserts were incubated at 37°C for 1 hour. Culture media from the top was replaced with fresh media (0.5 ml) containing 25 × 10<sup>4</sup> cells supplemented with HEPES buffer (0.01 M). Cell cultures were incubated at 37°C in a humidified incubator for 24 hours. Before exposure, cell confluence (75-80%) and attachment was observed, the medium was removed, and membranes washed with Hank’s balanced salt solution (HBSS; Gibco, USA). Human alveolar cells grown on porous membranes were exposed to various airborne concentrations of SO<sub>2</sub> directly at the air/liquid interface, using the dynamic direct exposure method [12-14].

*Dynamic exposure protocol*

Different concentrations of SO<sub>2</sub> were generated using a dynamic direct dilution method [12]. The system comprised of a metered test gas source, a metered clean-air source and a mixer to dilute the test gas to the desired concentration. Accurate flow rates were continuously monitored and directed to the dilution chamber in order to produce the final desired concentrations (Table 1).

The Navicyte horizontal diffusion chamber system (Harvard Apparatus, Inc, USA) was adapted for dynamic delivery and exposure of airborne chemicals to human cells [12]. The chamber creates an environment in which the apical surface of the cell monolayer is exposed to airborne contaminants while the basolateral surface is perfused with culture medium. Human cells grown on membranes were exposed to test atmospheres of gaseous contaminant directly at the air/liquid interface [12]. After washing with HBSS, membranes were placed into the horizontal diffusion chambers containing serum free culture media, supplemented with HEPES buffer. The upper compartments were closed and test atmospheres were delivered through the chambers for 1 hour at 37°C. The volume of culture

media in the basolateral compartment was sufficient to keep the cells hydrated. At the same time, humidified atmosphere was supported inside the chambers by keeping exposure chambers at 37°C.

After exposure, membranes were placed in six well plates. Fresh culture media was added to the bottom part of the membranes and the MTS, NRU and ATP cytotoxicity assays were performed. For cytokine analysis, after exposure of human A549 cells to SO<sub>2</sub>, samples of supernatant were collected from the top part of the membrane following 1 and 24 hours incubation, and kept at -20 °C until required for cytokine measurement.

### Cytokine measurement

#### Tumor necrosis factor- $\alpha$ (TNF- $\alpha$ )

The production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by A549 cells was evaluated using a TNF- $\alpha$  (human) EIA Kit (Cat No. 589201, Cayman Chemical Company, USA) which measures the TNF- $\alpha$  within the range of 0-250 pg/ml with a detection limit of 1 pg/ml. This immunometric assay was based on a double-antibody sandwich technique in which a monoclonal antibody specific for TNF- $\alpha$  was coated on each well contained in a 96 well microtitre plate and was available to bind any TNF- $\alpha$  introduced into the well. Aliquots of 100  $\mu$ l of each sample were introduced per well and an acetylcholinesterase: Fab' Conjugate (ACHE: Fab', 100  $\mu$ l) which binds selectively to a different epitope on the TNF- $\alpha$  molecule were also added to each well. The plate was incubated overnight at 4°C to allow a sandwich formation of two antibodies binding on opposite sides of the TNF- $\alpha$  molecule. The sandwich was immobilised and the excess reagents were washed away. The concentration of the analyte was then determined by measuring the enzymatic activity of the ACHE. The Ellman's reagent (200  $\mu$ l) was added and after 3 hours incubation in a dark orbital mixer the absorbance level was recorded at 405 nm (Multiskan Ascent, Thermo Labsystems, Finland).

#### Interleukin-6 (IL-6)

The production of interleukin-6 (IL-6) by A549 cells was evaluated using an Interleukin (human) EIA Kit (Cat No. 583361, Cayman Chemical Company, USA) according to the manufacturer's protocol and similar to the procedure explained for TNF- $\alpha$ . However, in order to accommodate the samples with higher level of Interleukin-6 the calibration curve of IL-6 standards was extended over the linear range up to 1.25 ng/ml.

### Cytotoxicity assessment

#### Tetrazolium salt assay (MTS)

The Promega CellTiter 96<sup>®</sup> AQueous Non-Radioactive Cell Proliferation Assay was used to measure the cytotoxicity of test gas by determining the number of viable cells in culture [17]. The MTS (3-(4,

5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product. After exposure, the MTS/PMS reagent was added and cell cultures were incubated at 37°C for 1 hour [12, 16]. After the incubation period, aliquots of 40  $\mu$ l were transferred to the 384 microwell plates and absorbance was recorded at 492 nm (Multiskan Ascent, Thermo Labsystems, Finland).

#### Neutral red uptake (NRU)

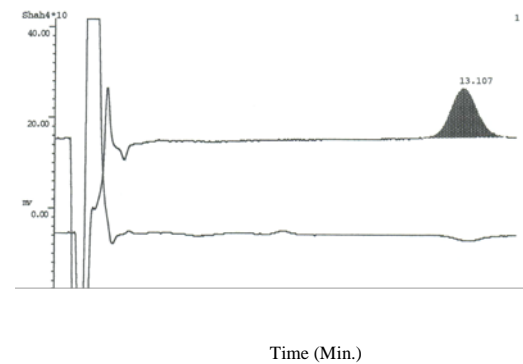
The Neutral red (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride) uptake (NRU; Sigma) assay is a cell survival/viability technique based on the ability of viable cells to incorporate and bind supravital neutral red dye [18, 19]. After exposure, fresh media was added to the bottom and the NRU solution was added to the top part of the membranes in six well plates and cells were incubated for 3 hours at 37°C [12, 16]. The medium was then removed and cells fixed with fixative solution for no longer than 30 sec. Membranes were rinsed with HBSS and assay solubilisation solution was added. The plate was shaken for 10 minutes, aliquots of 100  $\mu$ l were transferred into a 96 microwell plate and absorbance was recorded at 540 nm (Multiskan MS, Labsystems, Finland).

#### Adenosine triphosphate (ATP)

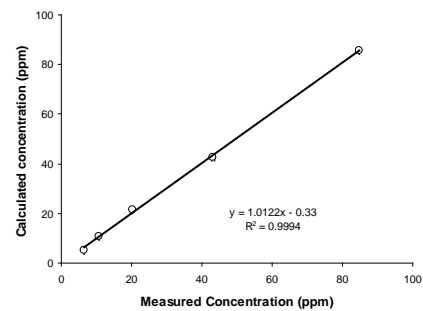
ATP content was measured using the CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay [20]. In this assay, the addition of CellTiter-Glo<sup>®</sup> Reagent to cells in culture induces cell lysis and generation of a luminescent signal proportional to the amount of cellular ATP content. After exposure, fresh media was added to the bottom (2 ml) and top (0.25 ml) of the membranes in six well plates. The CellTiter-Glo<sup>®</sup> reagent was prepared [12], and an equal volume of CellTiter-Glo<sup>®</sup> reagent (0.25 ml) was added to the membranes. The plate was shaken for 2 minutes and kept at room temperature for 10 minutes to stabilise the luminescence signal. Aliquots of 100  $\mu$ l were transferred into 96-well opaque-walled microtitre plates and the luminescence level was recorded (Berthold Detection Systems, Germany).

### Controls

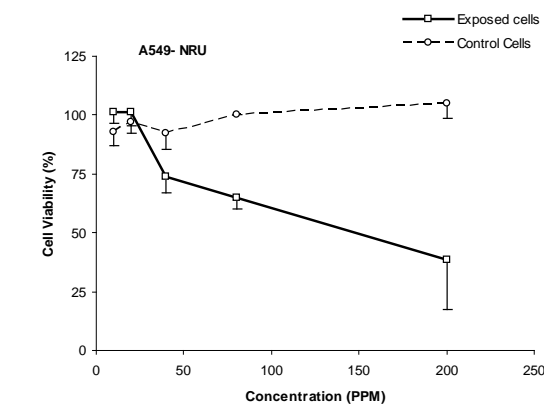
For all *in vitro* experiments, three controls were set up including two incubator controls (media only, IC<sub>100</sub>, 100% inhibitory concentration and cell only, IC<sub>0</sub>, 0% inhibitory concentration), and a synthetic air control which was exposed to a dynamic flow of air during the exposure time to consider any cell viability reduction induced by the dynamic air flow. For all *in vitro* experiments, a flow rate of 25 ml/min was used in which cell viability of A549 cells did not reduce significantly during the exposure time [12].



**Fig. 1.** An example of an SO<sub>2</sub> chromatogram plotted as total sulphate (SO<sub>4</sub>)



**Fig. 2.** Calibration curve of SO<sub>2</sub> test atmospheres



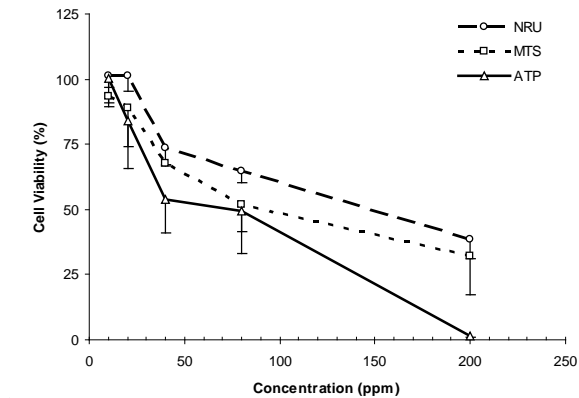
**Fig. 3.** Cell viability of control and exposed lung cells using the NRU assay

**Calibration of SO<sub>2</sub> test atmospheres**

The measurement of SO<sub>2</sub> concentration was carried out by the ID-104 OSHA analytical method with a detection limit of 0.01 ppm in 60-L air [21]. A calibrated personal sampling pump was used to transfer a known volume of air through a midjet impinger containing 10 ml of hydrogen peroxide (0.3 N). Collected air samples were filtered (0.45 μm; Millipore) and analysed as total sulphate (SO<sub>4</sub>; Fig. 1) by an ion chromatograph equipped with a conductivity detector (Waters IC PAK-A Column; model 430 Conductivity & 484 Absorbance Detectors). Calibration of SO<sub>2</sub> test atmospheres indicated a high correlation between calculated and measured SO<sub>2</sub> test concentrations ( $R^2 = 0.99$ ; Fig. 2).

**Statistical analysis**

Statistical analyses were performed using Microsoft Excel 2002 and SPSS (version 12.0) Software. Experimental results were expressed as mean ± standard deviation ( $m \pm SD$ ) for three different replicates at each test concentration. For all *in vitro* assays, percentage of cell viability at each test concentration was calculated [16]. After testing the homogeneity of variances using the F test, the Student t-test was used to compare the averages of cell viability or cytokine production in two



**Fig. 4.** Cytotoxic effects of SO<sub>2</sub> on A549 cells measuring different biological endpoints

groups. Differences were considered as statistically significant at  $P < 0.05$ .

**RESULTS**

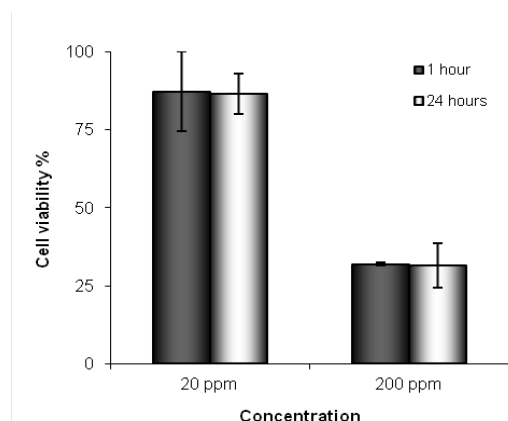
**Cytotoxicity assessment**

**Cell viability of control cells exposed to dynamic air flow**

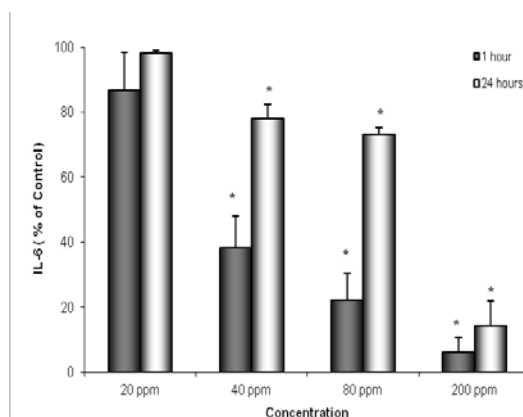
The effects of dynamic airflow rates in cell viability of human cells were studied previously [12]. For all *in vitro* experiments a flow rate of 25 ml/min was used in which cell viability of human A549 cells was not significantly affected during the exposure time. Cell viability of A549 control and exposed cells was compared using the NRU assay (Fig. 3).

**Concentration-dependent effects of SO<sub>2</sub> on human A549 cells**

Cytotoxic effects of SO<sub>2</sub> in human A549-lung derived cell lines were studied using the MTS, NRU and ATP assays (Fig. 4). Cell viability was reduced in a dose-dependent manner immediately after 1 hour exposure of human A549 cells to test concentrations of SO<sub>2</sub> (10-200 ppm). Cellular ATP content appeared to be the most sensitive test for cytotoxicity of SO<sub>2</sub> to human A549 cells among selected *in vitro* assays.



**Fig. 5.** Time course effects of SO<sub>2</sub> on A549 cells using the MTS assay



**Fig. 6:** IL-6 production of A549 cells exposed to SO<sub>2</sub> (Significant level; \*  $p < 0.05$ ; \*\*  $p < 0.01$ )

### Time-dependent effects of SO<sub>2</sub> on human A549 cells

The cytotoxic effects of SO<sub>2</sub> in human A549 cells were compared after 1 and 24 hours post incubation using the MTS assay (Fig. 5). Cell viability was reduced in a dose-dependent manner after either 1 or 24 hours exposure of human A549 cells to test concentrations of SO<sub>2</sub>. However, no further significant reduction in cell viability of human A549-lung derived cells was observed after 24 hours post incubation.

### Modulation of cytokine production Tumor necrosis factor- $\alpha$ (TNF- $\alpha$ )

Modulation of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was studied in human A549 cells exposed to SO<sub>2</sub> after 1 and 24 hours incubation. After analysis, a similar range of TNF- $\alpha$  release was measured in both exposed cells (17.5 - 63.5 pg/ml) and control cells (16 - 64.5 pg/ml). No statistically significant differences were observed between the TNF- $\alpha$  production of exposed cells and control cells at test concentrations. Increasing the incubation time did not influence the TNF- $\alpha$  release of human A549 cells.

### Interleukin-6 (IL-6)

Variation of interleukin-6 (IL-6) levels was studied in human A549 cells exposed to SO<sub>2</sub> after 1 and 24 hours incubation. The IL-6 production of human A549 cells was significantly impaired in a dose dependent manner after exposure to SO<sub>2</sub> (Fig. 6). After 1 hour exposure to SO<sub>2</sub> at 40 ppm a significant decrease in IL-6 release of human A549 cells was measured ( $P < 0.05$ ). Following the 24 hours incubation, the IL-6 production of A549 cells appeared to recover considerably, but not to the control levels.

## DISCUSSION

While the harmful effects of SO<sub>2</sub> have been documented in severe air pollution episodes [8], the

precise mechanisms involved in such effects of this major indoor and outdoor air pollutant need to be elucidated further. The toxic effects of SO<sub>2</sub> were studied in human A549 alveolar epithelial cells using multiple *in vitro* cell viability assays. To gain more understanding, the potential effects of SO<sub>2</sub> on cytokine levels of lung epithelial cells were also investigated. Test atmospheres of the selected gaseous contaminant were produced using a dynamic direct dilution method and calibrated via ion chromatography method. Generated test atmospheres were delivered to human cells using the dynamic direct exposure model [12]. In this exposure system, human lung cells were grown on porous membranes and exposed to test concentrations of SO<sub>2</sub> directly at the air/liquid interface using a horizontal diffusion chamber system. The toxic effects of SO<sub>2</sub> was studied in human A549 alveolar epithelial cells using the MTS, NRU and ATP cell viability assays and evaluation of TNF- $\alpha$  and IL-6 cytokines.

Cytotoxic effects of SO<sub>2</sub> were studied in human A549 lung derived cells immediately after 1-hour exposure to 10-200 ppm (Figures 3 and 4). Cell viability of human A549 cells reduced in a dose-dependent manner with the MTS, NRU and ATP assays. The concentrations of SO<sub>2</sub> used were so low that the observed effects were not related to the direct effects of SO<sub>2</sub> dissolving in biological fluids to produce an acid environment. Among selected *in vitro* cytotoxicity assays, cellular ATP content appeared to be the most sensitive biological endpoint influenced by SO<sub>2</sub> exposure. Cell viability of human A549 cells reduced to approximately 50% ( $53.82 \pm 12.57$  %) immediately following 1 hour exposure to 40 ppm SO<sub>2</sub> using the ATP assay. However, different results of biological endpoints may provide an indication of the possible mechanisms responsible for the cytotoxic effects leading to a better understanding of mechanisms involved in the toxicity of the test chemical [5, 22].

The time dependent effects of SO<sub>2</sub> exposure in human alveolar epithelial cells were investigated after 1

and 24 hours post incubation using the MTS assay (Fig. 5). No further significant reduction in cell viability was observed after 24 hours incubation which compared favourably to Knorst *et al.*, who studied the toxic effects of SO<sub>2</sub> in human alveolar macrophages. Up to 9.5% reduction in cell viability was measured immediately after 30-minutes exposure of human alveolar macrophages to SO<sub>2</sub> concentrations up to 5 ppm but no further reduction was reported after 24 hours incubation [9].

Moreover, the effects of SO<sub>2</sub> on alteration of inflammatory cytokine release were investigated. Cytokines are small soluble proteins secreted by many cells in addition to those of immune system serving as communication signals in both physiological and pathophysiological pathways [23]. The role of cytokines in tissue injury, inflammatory conditions and immune response is multifaceted. Therefore, understanding the complex role of this diverse class of proteins in the respiratory tract and its response to inhaled pneumotoxic agents needs further investigations. In this research, alteration of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) was studied in human alveolar epithelial cells following exposure to SO<sub>2</sub>.

After analysis of TNF- $\alpha$ , no statistically significant differences were observed between the TNF- $\alpha$  production of SO<sub>2</sub> exposed cells (17.5 - 63.5 pg/ml) and control cells (16 - 64.5 pg/ml) at test concentrations. Further increasing the incubation time to 24 hours did not influence the TNF- $\alpha$  release of human A549 cells. However, the IL-6 production of human A549 cells was significantly impaired in a dose dependent manner after exposure to SO<sub>2</sub> (Fig. 6). After 1 hour exposure to SO<sub>2</sub> at 40 ppm a significant decrease in IL-6 release of human A549 cells was measured ( $P < 0.05$ ). By increasing the concentration IL-6 production was also impaired at the higher significant level ( $p < 0.01$ ). Although the IL-6 production of A549 cells appeared to be recover considerably following the 24 hours incubation, the measured cytokine concentration was still significantly different from the control cytokine levels ( $p < 0.05$ ).

Reduction of cytokine expression has also been reported in human alveolar macrophages following SO<sub>2</sub> exposure [9]. The ability of alveolar macrophages to release tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) was significantly reduced following a 30-minutes exposure to SO<sub>2</sub>, whereas their capacity to produce IL-6 and transforming growth factor- $\beta$  (TGF- $\beta$ ) was not affected significantly. However, the effects of SO<sub>2</sub> exposure in human alveolar macrophages appeared to be different from those of the alveolar epithelial cells potentially due to the difference of the features between the two immune and nonimmune cell types. Although, exposure of human lung epithelial cells to SO<sub>2</sub> resulted in a dose-dependent decrease in IL-6, the underlying signaling pathways need to be investigated further.

In this study, the cytotoxic effects of SO<sub>2</sub> exposure in human alveolar epithelial cells were determined using multiple *in vitro* assay systems. Cell viability of human A549 cells reduced in a dose-dependent manner with the MTS, NRU and ATP assays. Among selected *in vitro* cytotoxicity endpoint assays the cellular ATP content appeared to be the most sensitive test for cytotoxicity testing of SO<sub>2</sub> in human A549 cells, IC<sub>50</sub>;  $48 \pm 2.83$  ppm, probably due to impaired metabolic activity of the cells following exposure to SO<sub>2</sub>.

The results of the present study suggest that significant damage to pulmonary epithelial cells can occur after short term exposure to SO<sub>2</sub> low level concentrations and cellular injury induced by SO<sub>2</sub> appeared to be in a dose dependent manner. While exposure of human epithelial cells to different concentrations of SO<sub>2</sub> did not appear to alter the TNF- $\alpha$  release, the ability of epithelial cells to release IL-6 was impaired significantly in a dose dependent manner. These results suggest that SO<sub>2</sub> may induce a functional alteration of cells of the pulmonary epithelial preventing cells to produce adequate amounts of IL-6. IL-6 as a multifunctional proinflammatory cytokine may regulate cellular responses and plays a significant role in inflammation and tissue injury.

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