

**ORIGINAL ARTICLE**

Iran J Allergy Asthma Immunol  
August 2016; 15(4):283-288.

## **Evaluation of Apoptosis in the Lung Tissue of Sulfur Mustard-exposed Individuals**

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Received: 13 October 2015; Received in revised form: 10 November 2015; Accepted: 23 November 2015

### **ABSTRACT**

Lung exposure to sulfur mustard (SM) results in pulmonary complications, which is the main cause of long-term disability and morbidity. Up to now, the precise mechanisms of SM-induced lung complications has not been identified. The aim of this study was to evaluate apoptosis in the lung tissue of SM-exposed individuals.

The study was performed on archived lung paraffin-embedded tissue specimens of 21 patients suffering from pulmonary complications due to previous SM exposure and 9 unexposed patients who had undergone lung resections for another lung disease. Evaluation of apoptosis in paraffin-embedded lung tissue sections was performed using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and the cleaved caspase-3 immunohistochemistry assays.

TUNEL-positive apoptotic features and caspase-3 expression of specimens were significantly higher in the SM-exposed group compared with the control group. This result demonstrates higher apoptosis rate in the SM-exposed group. Furthermore, the majority of positive cells consisted of alveolar epithelial cells in both methods.

In conclusion, it seems that exposure to SM may result in increased apoptosis in respiratory epithelium. More studies are needed to evaluate the role of apoptosis in SM-induced lung complications in order to design new and effective therapeutic protocols.

**Keywords:** Apoptosis; Lung; Lung disease; Sulfur mustard

### **INTRODUCTION**

Sulfur Mustard (SM) with  $\text{ClCH}_2\text{CH}_2)_2\text{S}$  chemical formula and 159.08 molecular weight is a yellow-

Brown lipophilic agent.<sup>1</sup>It can be rapidly absorbed by target tissues and causes alkylation of proteins, lipids and nucleic acids, resulting in DNA damage and cytotoxicity. SM as a strongly vesicant toxic chemical warfare was for the first time used in the World War I, and during the Iraq-Iran war (1980–1988). SM can affect a number of organs such as skin, eyes, and the respiratory system, among the most important are

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respiratory problems.<sup>3</sup> Many studies have been performed to elucidate the mechanisms of SM-induced lung damages. According to these studies, SM-induced pulmonary complications include laryngitis, tracheobronchitis, bronchiolitis, bronchopneumonia, chronic obstructive pulmonary disease (COPD), bronchiectasis, asthma, large airway narrowing, pulmonary fibrosis, and bronchiolitis obliterans.<sup>4</sup>

Despite many efforts in evaluation of SM-exposure effects, the mechanisms involved in its clinical complications is not clearly known.<sup>5</sup>

Apoptosis is one of the probable mechanisms which may be involved in inflammatory lung diseases and pathogenesis of pulmonary complications induced by SM.<sup>4</sup> Several studies have revealed the occurrence of apoptosis and necrosis after SM exposure in animal models and cultured cells.<sup>6-8</sup> Ray et al. showed that SM induces apoptosis in normal human bronchial epithelial cells (NHBE) and small airway epithelial cells (SAECs) via extrinsic pathway.<sup>9</sup> Also it has been reported that apoptosis is induced in SM-exposed keratinocytes via both mitochondrial and death receptor pathways, and is inhibited by suppressing the extrinsic and intrinsic pathways.<sup>10-12</sup>

Keyser et al. found that apoptosis was inhibited in human airway epithelial cells exposed to SM by Fas receptor (FasR) Small interfering RNA (siRNA), indicating that SM-induced apoptosis in cells happens via the Fas response. Furthermore in an animal model of SM, up-regulation of soluble Fas and active caspase-3 was observed in bronchoalveolar lavage fluid (BALF) cells. Both apoptosis and necrosis were involved in cell death due to SM.<sup>13</sup> In a study on Iranian chemical victims with pulmonary complications; there was substantial increase of Fas and Fas ligand (FasL) level in BALF cells.<sup>14</sup>

Previous researches investigated the role of SM in apoptosis in animal models and human cell culture,<sup>6-8,15-17</sup> the present study was designed to focus on apoptosis in lung tissue from SM-affected injured individuals by caspase-3 immunohistochemistry and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays.

## MATERIALS AND METHODS

### Study Design and Participant

The study was performed on 30 archived paraffin-

embedded lung blocks. The species were collected from the pathology departments of Tehran hospitals, Iran. The subjects were divided into two groups: (a) 21 subjects exposed to SM in Iraq-Iran war as exposed group, and (b) 9 subjects not exposed to SM as control group; the samples of control group were taken by a pathologist from the normal and near normal areas of their cancerous lung. The Summary of inclusion and exclusion criteria for patient selection is shown in Table 1. The study protocol was approved by the Ethics Board of Shahed University, Tehran, Iran (No.: IR.Shahed.REC.1395.15)

### Immunohistochemistry (IHC) Assay

Immunostaining for identifying caspase-3 was performed on formalin-fixed and paraffin-embedded lung tissues using standard protocols. Briefly, 3µm sequential sections collected on poly-Lysine-treated slides (Sigma-Aldrich) were deparaffinized in xylene, and rehydrated in graded alcohol. Antigen retrieval was performed by boiling the specimens for 35 min in 1mM EDTA buffer (pH 8.0) containing 0.05% Tween-20. Then the slides were rinsed with TBS (Tris buffer solution) and immersed for 10 min in 3% hydrogen peroxide to quench endogenous peroxidase. To block nonspecific binding, the sections were incubated for 20 min at room temperature (RT) in 100-200µl protein block buffer (Biogenex). The sections were then incubated overnight at 4°C in a humidified chamber with primary antibody (Invitrogen) at a concentration of 3µg/ml, and after washing for 35 min in RT with a horseradish peroxidase-conjugated secondary antibody (Biogenex).

**Table 1.** Inclusion and exclusion criteria

Inclusion criteria:
1. SM exposure based on medical records (for exposed group)
2. Age: 30 – 60 years
3. No smoking
4. Removal of malignant samples
Exclusion criteria:
1. Age <30 and >60 years
2. Loss of samples during the experiment

Following a rinse with TBS, the slides were immersed in diaminobenzidine (DAB) solution, counterstained with hematoxyline, and analyzed under a light micro scope (×400).

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### TUNEL Assay

The TUNEL assay was performed by the in situ cell death detection kit (Roche, Germany). The deparaffinized and rehydrated 3 $\mu$ m thick sections of lung tissue were incubated for 10 min in 3% hydrogen peroxide in methanol to suppress endogenous peroxidase activity, followed by proteinase K (20 $\mu$ g/ml in 10mM Tris-HCl, pH 7.4; Roche) for 30 min at 37°C to unmask fragmented DNA 3-OH ends. Following three rinses in TBS adjusted to pH 7.4, the sections were incubated with TUNEL reaction mixture for 60 min at 37°C. Next, they were washed and incubated for 30 min at 37°C in a humidified chamber with converter-POD. After a 6 min exposure to DAB solution as substrate, the slides were analyzed under a light microscope. Negative control was performed by the reaction buffer without TdT enzyme. Positive control was performed by digestion with 500 U/ml DNase (Roche, Germany).

### Statistical Analysis

Data were presented as mean $\pm$ standard deviation (SD). Independent sample t-test was used to compare

the data between the exposed and control groups. Correlation desired data were calculated by Pearson's correlation test, and analyzed using the SPSS software (ver.20.0) (SPSS Inc., Chicago, IL) at a significance threshold of 5% ( $p<0.05$ ).

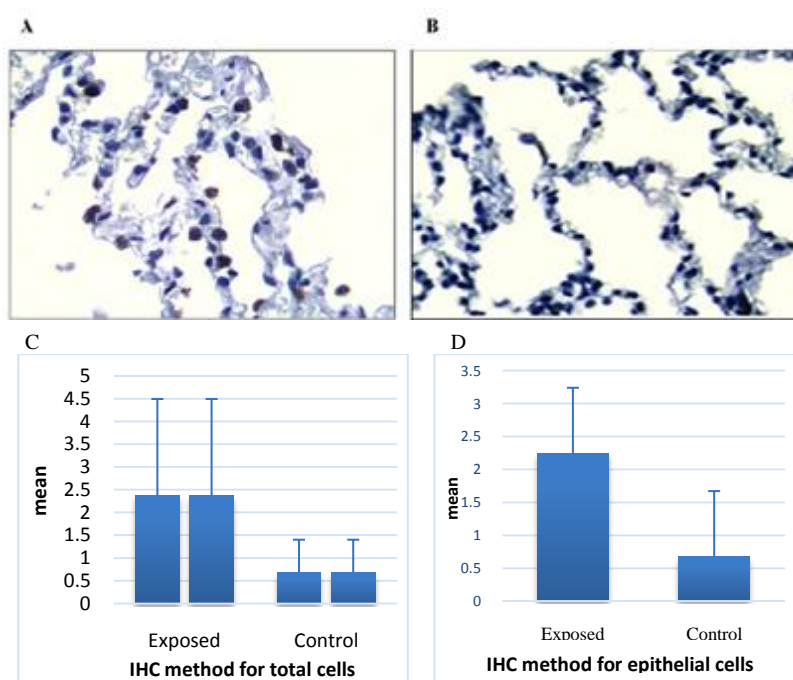
## RESULTS

In this study, apoptosis was evaluated by two methods: TUNEL assay for detecting DNA fragmentation, and immunohistochemistry to recognize activated caspase-3.

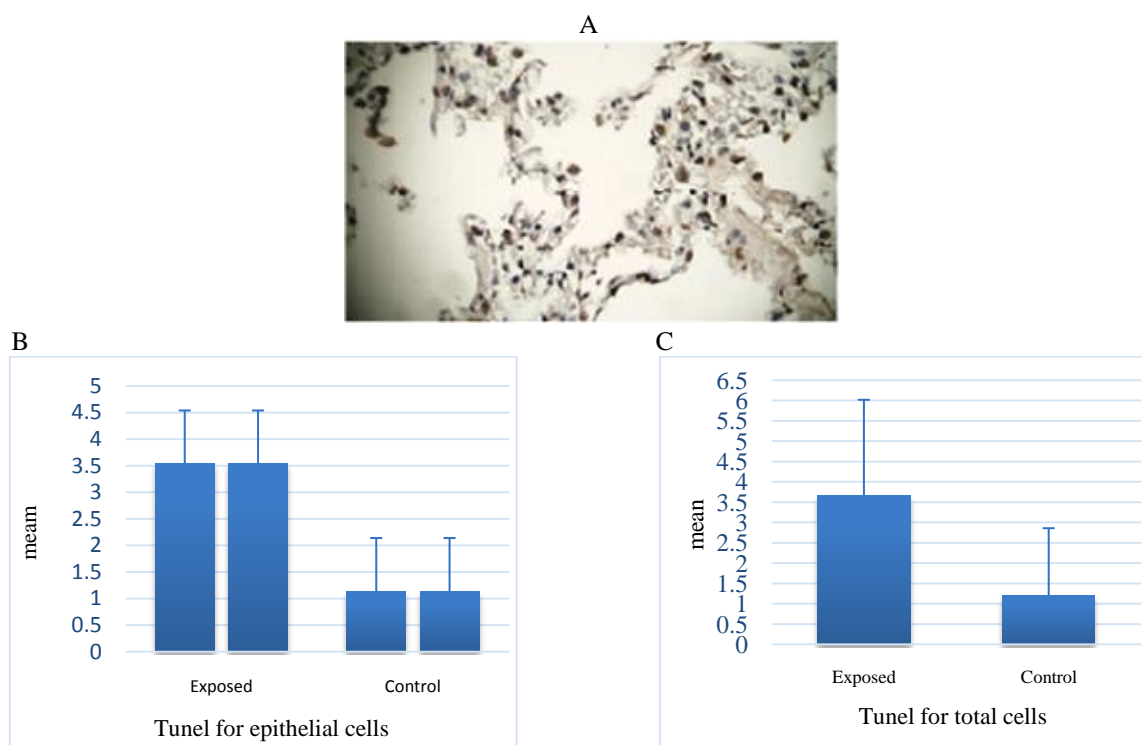
### Caspase-3 Immunohistochemistry

Figure 1A shows caspase immunostaining of apoptotic pneumocytes in a lung tissue section from a SM-exposed specimen, and Figure 1B shows the negative control. As illustrated in Figure 1C, caspase-3 expression in the SM-exposed group is significantly higher than the control group ( $p=0.02$ ), and also the majority of positive cells are alveolar epithelial cells (Figure 1D).

Caspase expression is shown as brown staining in the cells.



**Figure 1. Immunohistochemical assay. Immunohistochemical staining of cleaved caspase-3 in human lung tissue in the exposed (A) and control (B) groups. Detection of positively stained cells per high-power field (original magnification $\times$ 400; n=20 high-power fields per section). (C) Quantitative analysis (mean $\pm$ SD) of cleaved caspase-3 expression ratio (positive cells/total cells). (D) The majority of positive cells are alveolar epithelial cells.**



**Figure 2. Evaluation of Apoptosis by TUNEL assay. (A) Apoptosis in-situ was assessed using TUNEL labeling on paraffin-embedded lung sections in the exposed and control groups ( $\times 400$ ). Negative nuclei appear in blue (hematoxylin counterstaining), whereas TUNEL-positive nuclei are brown. (B) TUNEL data are represented as mean $\pm$ SD of the exposed and control groups. (C) The majority of positive cells are alveolar epithelial cells.**

### TUNEL Assay

The positive stained nuclei for the TUNEL assay are shown in Figure 2A. As shown in Figure 2B and 2C, the TUNEL-positive cells in the SM-exposed group are significantly higher than those in the control group ( $p=0.02$ ). The majority of TUNEL positive cells are alveolar epithelial cells.

### DISCUSSION

It has been reported that apoptosis plays a significant role in tissue damage during lung injury.<sup>18</sup> The present study aimed to evaluate the impact of apoptosis as one of the possible mechanisms involved in SM-induced lung injuries.

In this study, TUNEL assay and caspase-3 immunohistochemistry were used for detecting the apoptotic cells. The results of TUNEL staining demonstrated that in the SM-exposed injuries, apoptosis was significantly higher than in the control

group. These results are consistent with the previous studies. According to Rosenthal et al. as a result of exposure to SM, apoptosis was induced via both mitochondrial and death receptor (DR) pathways in keratinocytes.<sup>11,12,15</sup> Steinritz, et al. investigated apoptotic cell death in the pulmonary A549 cells exposed to SM, and observed an increase of TUNEL-positive cells, and also PARP cleavage, indicating the activation of the effector caspase-3/7.<sup>19</sup> Furthermore, in our previous study, serum soluble FasL in the SM-exposed group with pulmonary problems was significantly higher than their corresponding counterparts in the control group.<sup>20</sup>

In the current study, positive cells for TUNEL assay were predominantly detected in the lung epithelial cells, and significantly increased in the SM-exposed group compared with control group. Herein to confirm the TUNEL results, the cleaved caspase-3 was detected as a downstream target of the initiator caspase. The results of activated caspase-3 also confirmed verified

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the TUNEL results, and reveal that probably caspase-dependent apoptosis occurs in lung tissue. Thus it can be confirmed that the brown nuclei in the TUNEL assay are apoptotic features, and not necrotic cells. Investigations of apoptosis in cell culture and animal model by several researchers have demonstrated caspase-3 activation, which is consistent with our results regarding caspase-3 activation after SM-exposure.<sup>6,9,13,21,22</sup>

Some other researchers evaluated the effects of SM on apoptosis with other methods and concluded that SM is an inducer of apoptosis.<sup>7,23-25</sup> Pirzad et al. explored Fas and FasL levels and caspase-3 activity in the bronchoalveolar lavage (BAL) fluid, and showed significantly higher levels of Fas and FasL, but caspase-3 activity was not different between the groups.<sup>14</sup> The results of FasL in Pirzad et al.'s study are consistent with our previous Sardasht Iran cohort study, which indicated significantly higher serum levels of sFasL in the SM-exposed group with pulmonary problems compared to their counter parts in the control group.<sup>14,21</sup> The different results of caspase-3 immunohistochemistry of the present study and the work of Pirzad et al. could be due to the different samples used in the two studies, namely lung tissue versus BAL.

In the present study, the correlation between TUNEL assay and caspase-3 activation in the control and SM-exposed groups was positive and negative, respectively, but not statistically significant in both instances. In the case of epithelial cell apoptosis in the SM-exposed group, the correlation between the two methods was negative and statistically significant ( $p=0.05$ ).

The fact that in the SM-exposed specimens, the results of the TUNEL assay were more prominent than in the caspase-3 evaluation, may be related to caspase-independent pathways of apoptosis such as granzyme or endonuclease G and AIF pathway.<sup>26</sup> In some cases when the TUNEL result is lower than that of caspase-3, it can be referred to the regulation of caspase activity, including posttranslational modifications and protein/protein interactions, which have been previously hypothesized.<sup>27</sup> On the other hand, detecting the apoptotic features may depend to the stage of apoptosis process, which may take only a few hours from the initial trigger to the cell destruction.<sup>28</sup>

One of the limitations of this study was the number of samples; with more samples, better results could

have been obtained. Another restriction was utilization of 10-year-old paraffin-embedded lung tissue samples available in the archive of hospitals. Due to ethical limitations, we were not able to use fresh specimens, which could have resulted in more reliable findings.

Taken together, this study revealed that SM-exposure may result in apoptosis induction in the respiratory epithelium even after a long time since exposure. More studies are needed to evaluate the role of apoptosis in SM-induced lung complications in order to use the obtained results in designing new and effective therapeutic protocols. Also other apoptosis mechanisms such as Fas/FasL, initiator caspases, and cytochrome-c should be considered.

### ACKNOWLEDGEMENTS

This study was carried out by the Immunoregulation Research Center, Shahed University.

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