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Article *in* Journal of Receptor and Signal Transduction Research · October 2011

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Journal of Receptors and Signal Transduction, 2011; 31(5): 367–373

ISSN 1079-9893 print/ISSN 1532-4281 online
DOI: 10.3109/10799893.2011.602415

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RESEARCH ARTICLE

Nuclear factor κB1/RelA mediates the inflammation and/or survival of human airway exposed to sulfur mustard

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Abstract

Context: Sulfur mustard (SM) is known as an effective chemical agent and was used in the 1980s during the Iran–Iraq war against Iranians. At the present time, there are more than 40,000 people suffering from pulmonary lesions due to mustard gas in Iran. Though much is known about the gross pathology of SM damage, the molecular and cellular basis for this pathology is not well understood.

Objective: One of the most important protein groups involved in inflammatory responses is nuclear factor κB protein (NF-κB1) family. They belong to the category of DNA-binding protein factors necessary for transcription of many proinflammatory molecules. In our research, we examined the role of NF-κB1/RelA in the pathophysiology of the lung.

Materials and methods: We investigated 10 normal individuals and 20 SM induced patients. Expression of NF-κB1/RelA in controls and the SM exposed samples was measured by real-time polymerase chain reaction and localization of NF-κB1 protein was detected by immunohistochemistry staining.

Results: Our results revealed that expression levels of NF-κB1 and RelA were upregulated 0.64–6.50 fold and 0.83–8.34 fold, respectively, in the SM exposed patients in comparison with control samples.

Discussion and conclusion: As far as we know, this is the first finding of induction of NF-κB in patients exposed to SM. NF-κB1/RelA may play a major role in inflammation induced by mustard gas or even in cell survival in the bronchial wall of affected patients.

Keywords: NF-κB1, RelA, bronchial wall, sulfur mustard

Introduction

Sulfur mustard (SM) or 2,2-dichlorodiethyl sulfide is avesicant warfare agent with devastating results (1) that was widely used during World War I (1–3) and also in the Iran–Iraq conflict (1980–1988) by Iraqi forces against Iranians (4). This highly reactive toxin binds to the biological compounds and leads to irreversible alkylation of nucleic acids and proteins. Collectively, the alterations of large molecules, especially the structure of DNA, are major cytotoxic and mutagenic effects of sulfur mustard (SM (5)). In addition to the diverse acute casualties, SM-affected individuals suffer from late involvement in various organs, including the skin, eyes and respiratory tract (6,7).

Nowadays, as a consequence of exposure to SM (8), thousands of Iranians are suffering from late complications (9–11), mainly in the respiratory tract. Bronchiolitis obliterans (BO), a type of inflammatory lung disease (12),

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(Received 19 June 2011; revised 00 00 0000; accepted 29 June 2011)

is the most frequent chronic illness among survivors (4,7,13).

Nuclear factor κB (NF-κB) is considered a critical upstream regulator (14,15) for transcription of mediators; it contributes to diverse physiological processes such as differentiation, proliferation (16), cell adhesion (17) and cell survival (18) and plays a prominent role in inflammation (19–21) and immune processes as well (14,22–24). The conserved Rel homology domain, which is responsible for dimerization, nuclear translocation, and DNA binding (14,25,26), consists of NF-κB1 (p50), NF-κB2 (p52 (27)), RelA (p65), RelB and c-Rel (28) which establish different homodimers or heterodimers in different type of cells (25,26). In the basal state, the IκB family of inhibitory proteins bind to NF-κB and keep it in the cytosol as an inactive dimer (19,29–31). Various extracellular stimuli, including proinflammatory cytokines such as tumor necrosis factor-α (TNF-α (32)), oxidative stress, bacterial products (33) and environmental stress (34) proceed through different signaling pathways and cause activation of IκB kinase (IKK (33,35–37)). IκB molecules are rapidly phosphorylated via IKK activation, which leads to ubiquitination and following degradation of IκB (38–40). This alteration allows translocation of NF-κB to the nucleus, where it is permitted to start transcription of target genes (41,42). Promoter regions of some proinflammatory genes such as TNF-α and intercellular adhesion molecule 1 (ICAM-1) contain transactivating binding sites for NF-κB, which enhance transcription of these genes (14,43,44).

NF-κB is often portrayed as a rapid–response gene involved in inflammatory situation, so suppression of NF-κB can be used as a potential pharmacological point for treating human inflammatory diseases (31).

In this study, we sought to address the existence of inflammation and/or cell survival in the airway of SM injured patients at the molecular level, and, for this reason, we chose the NF-κB1/RelA heterodimer, which is the primary mediator of NF-κB (19,45–47), the main transcription factor and coordinating regulator in the expression of inflammatory mediators. This study investigates a new pathway implicated in SM injured patients and a new strategy for treatment of these patients.

Materials and methods

Study design and participants

The present study included 20 male subjects who were exposed to SM 20 years ago during the Iran–Iraq war and 10 unexposed males as control group. All of them signed an informed written consent. Our study was also approved by the Medical Ethics Committee of Baqiyatallah University of Medical Sciences. Participants with the following characteristics were excluded from our survey: History of cigarette smoking, any occupational exposure to pulmonary toxic agents, history of inflammatory lung disease such as asthma or bronchitis before exposure to SM, pre-exposure to tuberculosis, lung cancer or other respiratory infection, and advanced age. Data concerning the gender, age and pulmonary function tests (PFTs) results of the two groups were collected (Table 1).

Bronchoscopy and biopsy

After inhalation of 5-aminolevulinic acid, patients were topically anesthetized. The nasopharynx and larynx were anesthetized by applying 2% lidocaine or aerosolized; after that, patients were lightly sedated with IV midazolam. Supplemental oxygen was provided for each patient, and pulse rates were monitored for oxygen saturation. Patients received supplemental oxygen until they awoke, and alarms started to go off when saturation fell below 90%.

After preparing the patients, flexible fiberoptic bronchoscopies (Olympus BF1T, Tokyo, Japan) were carried out. Using the transnasal route, a flexible fiber passed through the airway to the segmental and subsegmental carinae, and endobronchial biopsies were collected from these areas via bronchoscopic forceps (Olympus, Tokyo, Japan). Obtained biopsies were placed in Tripure Isolation Reagent (Roche Applied Science, Germany) or formalin and were kept in 80 or 4°C, respectively.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of NF-κB1/RelA mRNA expression

Total mRNA of biopsies was harvested with the Absolutely Tripure Reagent (Roche Applied Science, Germany). Biopsy samples were homogenized by a Homogenator (Heidolph, SilentCrusher, Germany), and RNA was purified according to the manufacturer's protocol. After purification, whole-cell RNA was prepared in diluted RNase-free water, and the isolated RNAs were quantified by NanoDrop (ND-1000, Wilmington, DE). Complimentary DNA was reverse transcribed from 300 ng total RNA of each sample using a reverse transcriptase kit, CycleScript RT PreMix (dN6) (Bioneer, Korea) according to the manufacturer's instructions.

Semi-quantitative RT-PCR

Equal amounts of cDNAs served as templates for amplification of each gene with semi-quantitative RT-PCR in a final volume of 25 μL. Reagents and Taq polymerase were purchased from Roche Company (Germany). The

Table 1. Characteristics and pulmonary function test results of SM exposed samples and unexposed controls.

	Control group subjects, $n=15$	SM injured patients, $n=24$	p-value
Age range	22–57	30–58	—
Age (mean ± SD)	43.6 ± 10.92	42.9 ± 5.48	0.83
FVC (mean ± SD)	3.34 ± 0.79	2.87 ± 0.89	0.11
FEV_1 (mean ± SD)	2.71 ± 081	1.92 ± 0.87	0.007*
FEV_1/FVC (mean ± SD)	79.85 ± 6.15	67.88 ± 15.81	0.001*
RV(mean ± SD)	2.38 ± 0.97	3.75 ± 1.75	0.04*

*Statistical significance: $p<0.05$. FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; RV, residual volume.

oligonucleotide primers for PCR were designed as follows: NF-κB1 Forward 5′TGGGAATGGTGAGGTCACTC-3′ and reverse 5′-TCTCATCCTGCACAGCAGTG-3′; RelA Forward 5′-TCAATGGCTACACAGGACCAG-3′ and reverse 5′-TCACACACTGGATTCCCAGGT-3′. The primers for amplifying β actin as an internal control cDNA were: forward 5′-TCATGAAGATCCTCACCGAG-3′ and reverse 5′-TTGCCAATGGTGATGACCTG-3′. The conditions of PCR were as follows: 94°C for 3 min, followed by 30 cycles each consisting of 30 s at 94°C, 30 s at annealing 59°C for NF-κB1 and β actin and 61°C for RelA, 60 s at 72°C, and final extension at 5 min at 72°C. After amplification, PCR products were electrophoresed in a 2% agarose gel (Cinnagene, Tehran, Iran) (7 μL each), and bands were visualized under ultraviolet light in the gel documentation (Biorad, USA) after staining with ethidium bromide.

Measurement of NF-κB1/RelACDNA by real-time PCR

Real-time PCR quantification was carried out using a SYBR Green Premix (Takara, Japan) in Rotor-Gene RG 3000 (Corbett Research, Sydney, Australia). Cycling parameters for real-time PCR were started with denaturation at 95°C, 40 cycles of 30 s at 95°C, 30 s at 59°C for NF-κB1 and β actin at 61°C RelA and 30 s at 72°C. As described above, the β actin gene was used to normalize target genes for all PCRs; and a melting curve program was detectable with continuous fluorescence measurement. Each experiment was repeated in triplicate; subsequently, results represent normalized values, and expressions of the target genes were measured via the $2^{-\Delta\Delta CT}$ method.

Immunohistochemical localization of NF-κB

We examined 10 SM injured patients and 10 unexposed controls. In summary, tissues were fixed in 4% buffered paraformaldehyde (Merck, Germany) and, after storage in phosphate buffer saline (PBS) (Takara, Japan) containing 30% sucrose, 15 μm sections were prepared from water embedded tissues via cryostat (Histo line, Italy).

Immunohistochemical analysis for NF-κB1 protein localization was performed as described previously with slight modification (Nourani, 2005). Briefly, sections were incubated overnight with polyclonal rabbit NF-κB1 antibody as the primary antibody at a dilution of 1:200 in PBS (Santa Biotechnology, Inc., USA) at 4°C. For the next step, sections were incubated with secondary antibody. Specific labeling was distinguished with a biotin-conjugated anti-rabbit antibody and avidin-biotin peroxidase complex (MolecularProbe. Eugene, OR). The reaction product was detected using diaminobenzidine (DAB) as a substrate (Vector Laboratory, Burlingame, CA).

Statistical evaluation

To examine the differences between the SM injured group and unexposed group, the two populations were compared with Student's *t* test via SPSS software version 15.0 (SPSS, IL). Data were considered significant at $p < 0.05$, and results were reported as mean ± SD.

Results

This research enrolled 20 SM injured patients and 10 unexposed subjects as controls (Table 1).

The outcome of PFTs is presented in Table 1. Even though forced vital capacity (FVC) in the unexposed group is higher than in the SM exposed group, no significant difference was detected between them ($p = 0.11$). However, forced expiratory volume in 1 s (FEV1) in the chemical exposed group is considerably lower than the unexposed ones ($p = 0.007$). In addition, FEV1/FVC is different in two groups, and it is notably elevated in unexposed volunteers ($p = 0.001$). Among the PFT parameters we evaluated, RV is significantly higher in SM exposed patients in comparison to unexposed cases ($p = 0.43$).

Real-time RT-PCR analysis

To test whether NF-κB1/RelA heterodimer mRNA increases in modulation of the inflammatory process, semi-quantitative RT-PCR was carried out. As Figure 1A and B shows, mRNA expression of NF-κB1 and RelA was upregulated in SM exposed patients compared to the unexposed group.

To analyze the mRNA levels of this inflammatory heterodimer, we employed real-time RT-PCR. Our results obtained from real-time RT-PCR revealed that the mRNA expressions of NF-κB1 and RelA were significantly increased in airway biopsies of SM exposed patients compared with the unexposed group. Figure 2 shows the differences between mRNA levels for NF-κB1 (Figure 2A) and RelA (Figure 2B) of the SM injured population and the unexposed group. Our results also show that the fold changes in mRNA expression of NF-κB1 in SM exposed patients was from 0.64–6.50 fold compared to unexposed participants ($p = 0.049$); for RelA; it was 0.83–8.34 fold in comparison with unexposed subjects ($p = 0.002$).

Results of immunohistochemistry

For localization of NF-κB1 protein expression in airway biopsies, immunohistochemistry analysis was carried out. Only low NF-κB1 immunoreactivity was detectable in unexposed airway epithelial cells, especially in the basal (germinal) layer of epithelium (Figure 3A), whereas, in SM exposed sections, more expression of NF-κB1 protein was recognized in the bronchial epithelial cells (Figure 3B).

Discussion

Nearly 40,000 Iranians are suffering from chronic and disabling respiratory complications more than 20 years after exposure to SM (9). Most of this population exhibit clinical symptoms of inflammatory lung disease, mainly of BO (7). Because little is known about the nature and molecular mechanism of pathophysiology of SM, NF-κB1/RelA was examined as a benefit candidate of this study (45) and a common nuclear factor for many proinflammatory molecules that fortify the existence of inflammatory mechanisms in SM exposed patients.

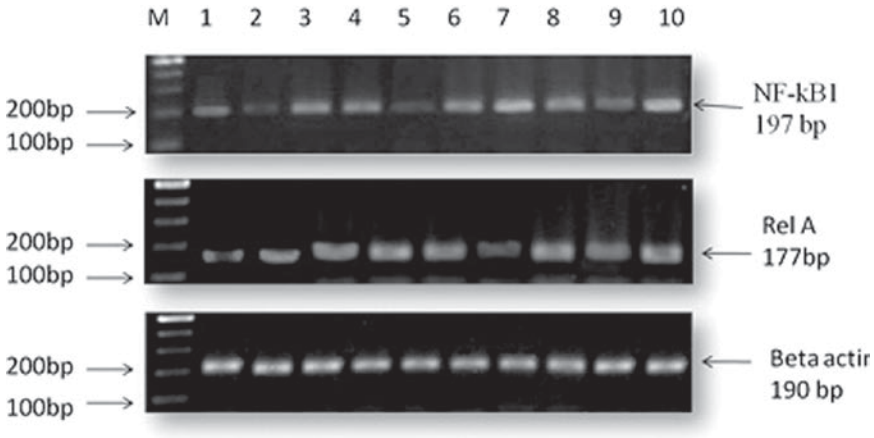

Figure 1. Expression levels of NF-κB1 and RelA in bronchial wall of patients with long-term exposure to sulfur mustard. Total RNAs were purified and then analyzed by semi-quantitative RT-PCR. This panel shows gel bands in order to PCR amplification products of NF-κB1 (197 bp), RelA (177 bp) and β-actin (190 bp) transcripts. (A) A marked increase in NF-κB1 expression levels of SM exposed patients (Lanes 3–10) was recognized in comparison to the expression level of unexposed ones (Lanes 1&2). (B) The density band of RelA in SM exposed patients also shows a clear difference from the mRNA of the unexposed group. (C) β-actin was used as an internal control. Lane M shows DNA ladder (100 bp).

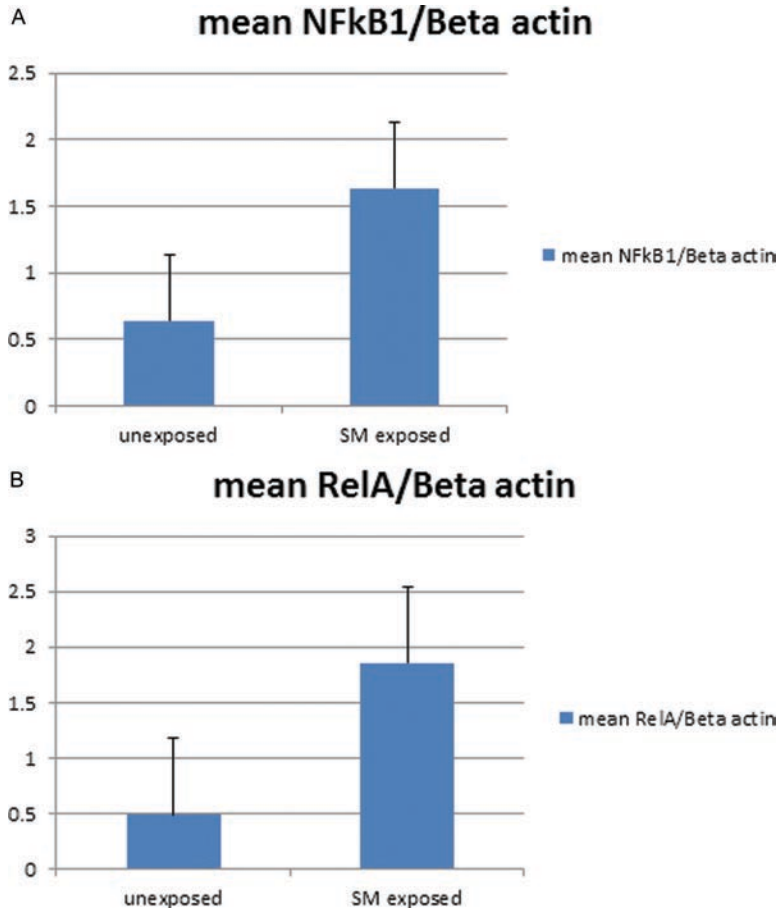

Figure 2. The mean expression of target genes in SM exposed patients and unexposed ones. (A) The mean expression of NF-κB1 in SM injured patients is increased significantly compared to unexposed samples. (B) The mean expression of RelA in SM injured samples is also increased significantly in comparison with unexposed group.

Our data indicate that the expression of NF-κB1 and RelA at mRNA level in bronchial biopsies is significantly elevated in the SM exposed group in comparison to the unexposed group. In addition, our results obtained from immunohistochemical analysis of NF-κB1 indicate the positive immunoreactivity of this protein in the airway walls of SM exposed patients. As far as we are aware, there is very little published data about the molecular mechanisms of the delayed complications of SM, and no data have been reported about the presence of transcription

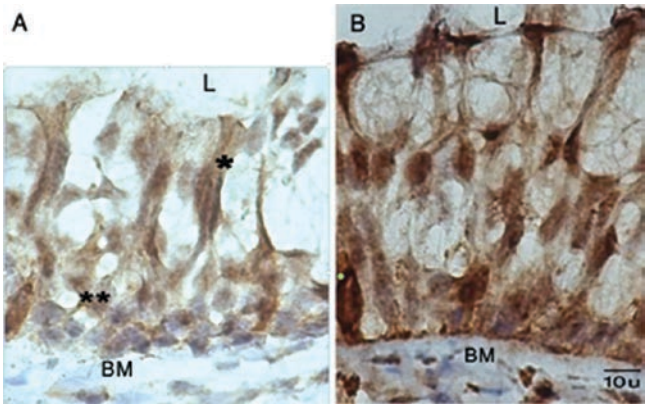

Figure 3. Light micrograph of NF-κB1-immunopositive cells in the bronchial epithelium. (A) NF-κB1-immunoreactivity in bronchial epithelial cells of the control group. NF-κB1-immunopositivity is weakly demonstrated in a substantial number of epithelial cells (single asterisk) and only rarely in the basal border of epithelial cells (double asterisk). (B) immunoreactivity intensity is increased throughout the section in the bronchial epithelial cells of chemical injured patients. Note that the thickness of epithelium due to chemical injury in the experimental group is higher than that in the control group. BM, basement membrane; L, luminal border.

factor of inflammatory molecules. Furthermore, previous studies support our finding in the upregulating expression of NF-κB1 and RelA is implicated in lung disease. For instance, translocation of NF-κB and its binding to an enhancer of target genes elevates in airway epithelial cells isolated from bronchial biopsies and in alveolar macrophages collected from sputum of asthmatics (48). In addition, airway irritants are able to exacerbate asthma symptoms and initiate inflammation. Of these irritants, ozone has been reported to activate NF-κB. Rats exposed to ozone exhibit activation of NF-κB and reveal penetration of neutrophils and monocytes into the lavageable airspace in order to activate chemokines (49). Allergens, other airway irritants, activate NF-κB in the airway epithelium via recruitment of inflammatory cells. Moreover, activation of NF-κB has been reported in the airway epithelium of animal models of allergic airway inflammation (49). NF-κB1 (p50) or c-Rel knockout mice demonstrate airway inflammation less than other mice when exposed to an antigen challenge, highlighting the critical role of NF-κB in inflammation 50. Inhalation of diesel exhaust as a pollutant particle induces expression of cytokines such as IL-8, activates NF-κB in the bronchial epithelium, and increases transcription of ICAM-1, leading to a strong inflammatory response in the airway (51). Other airway irritants, asbestos fiber and iron, which is the main component of asbestos fiber, lead to cellular redox changes by production of intracellular reactive oxygen species which in turn cause activation of NF-κB. Moreover, it has been demonstrated that the activation of RelA increases in airway epithelial cells of rats that have inhaled asbestos (52–55).

However, other studies demonstrate NF-κB as a mediator in the exacerbation of chronic obstructive pulmonary disease (COPD) in cigarette smokers. For example, NF-κB dimer activation has been detected in bronchial biopsies of smokers (56).

Di Stefano et al. (57) confirmed that NF-κB is activated in segmental and subsegmental bronchial biopsies in COPD participants and healthy smokers in association with elevation of lipid peroxidation products. They also demonstrated increased expression of RelA (p65) protein and its positive reaction with immunostaining in the bronchial epithelium (57). Other research using sputum immunocytochemistry technique revealed activation of p65 in alveolar macrophages extracted from sputum through COPD exacerbations (58). In addition, Caramori and colleagues illustrated p65 expression in leucocytes collected from the sputum of exacerbated COPD subjects and exhibited transcription of p65 in macrophages (58). Furthermore, studies using Guinea pigs showed that exposure to cigarette smoke enhances the expression of IL-8 due to NF-κB activation (59).

Joseph et al. illustrated activation of NF-κB in patients who suffer from cystic fibrosis (CF). An *in vitro* study after infection of primary CF epithelial cell cultures by *Pseudomonas aeruginosa* showed enhancement of the baseline translocation of NF-κB to the nuclei (60). In CF subjects, suppression of NF-κB activation could be beneficial to decrease airway inflammation and sputum viscosity and to recover lung function (59).

NF-κB is also implicated in acute respiratory distress syndrome (ARDS), another inflammatory lung disease. Studies have detected higher activation of NF-κB in alveolar macrophages in patients who suffer from ARDS than in participants without acute lung injury (50). Furthermore, the level of subunits p50, p65, and c-Rel increases in alveolar macrophages in nuclei in ARDS; this finding indicates that the association of NF-κB with ARDS may cause deterioration in this illness (61).

As cited above, NF-κB is one of the transcription factors, which has a specific site on the enhancer of inflammatory molecules such as ICAM-1. Upregulation of ICAM-1 has been reported in epithelial cells and the serum of asthmatics, which shows that ICAM-1 is associated in pathogenesis of asthma (50).

Based on the pivotal role of NF-κB in inflammatory situations, the current study focused on expression of the primary heterodimer, NF-κB1/RelA at the mRNA level and

NF-κB1 at the protein level. Our results show that, in comparison to unexposed subjects, expression of NF-κB1/RelA at the mRNA and protein levels is statistically significantly upregulated in patients after long-term SM exposure who have delayed complications in their airways.

The results of our study support the hypothesis that the inflammatory pathway might be activated in bronchial biopsies of long-term SM exposed patients due to activation of NF-κB1/RelA. Evaluation of this molecule can be a better marker for the existence of inflammation.

However, because the mortality of SM exposed patients is less than that with other inflammatory lung diseases, we must consider that activation of NF-κB1/RelA in airway wall of SM exposed patients might cause cell survival. Liu X and colleagues have demonstrated that cigarette smoke extract activates NF-κB and inhibits cell death following DNA damage after cigarette smoke exposure in human bronchial epithelial cells. It has also been reported that the inhibition of NF-κB activity by a pharmacologic inhibitor (curcumin) or suppression of p65 by siRNA causes a noteworthy enhancement in apoptotic cell death in response to cigarette smoke exposure, demonstrating that NF-κB regulates cell survival in human bronchial epithelial cells after cigarette smoke-induced DNA damage (60).

Conclusion

From a practical point of view, this study can open a new area of treatment for the patients who are suffering from SM lesions, especially BO. Nevertheless, it is required to detect the activation of other inflammatory mediators and cell survival molecules in downstream of NF-κB such as ICAM-1, which is a proinflammatory molecule, and Bcl2 which negatively regulates apoptosis, in order to determine the exact mechanisms in the pathway activated in late SM toxicity.

Acknowledgements

We thank members of our laboratory in Chemical Injury Research Center (CIRC) Baqiyatallah University of Medical Sciences, Dr. Barbara Lee Smith Pierce (University of Maryland University College Scientific and Medical Editing Baltimore, USA) for editorial work in the preparation of this manuscript.

Declaration of interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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