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Evaluation of mRNA Expression Levels of TNFα, TNFR1 and IL1β in Lung Tissue 20 Years after Sulfur-mustard Exposure

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ABSTRACT

Despite many years having passed since exposure to sulfur mustard (SM) gas, there are many exposed subjects who are still suffering from delayed pulmonary complications. The levels of pro-inflammatory cytokines in the lung of these subjects have not been investigated in delay phase. In this study, we evaluated mRNA expression of pro-inflammatory cytokines (tumor necrosis factor alpha (TNFα), tumor necrosis factor receptor type 1 (TNFR1), and interleukin 1 beta (IL-1β)) in lung biopsy of SM-exposed subjects and compared them with control (non-exposed) subjects. We used formalin-fixed, paraffin-embedded (FFPE) tissue for this purpose.

Lung FFPE blocks of SM-exposed subjects (30 samples) and a control group (30 samples) were collected from archival pathology department. The total mRNA of FFPE tissues were extracted and the mRNA expression of pro-inflammatory cytokines were determined by quantitative Real Time PCR (RT-qPCR). The obtained results from two groups were compared to each other and non-parametric statistical analyses were carried out on them.

Our studies showed that the mRNA expression of TNFα, TNFR1 and IL-1β in lung tissue of SM injured and control people have no significant difference (p-value= 0.159, 0.832 and 0.314 respectivly). TNFR1 showed direct correlation with TNFα (r=0.867, p=0.002) and IL-1β (r= 0.65, p=0.006).

The evaluation of mRNA expression in pro-inflammatory cytokines in lung of SM-exposed subjects after 20 years showed that these mediators are similar to those of non-exposed group and there was no acute inflammation in lung of these patients.

Keywords: Interleukin-1 beta; Lung; mRNA expression; Sulfur mustard; Tumor necrosis factor-alpha; Tumor necrosis factor receptor type 1

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INTRODUCTION

Dysregulated inflammation lead to many disorders and diseases such as autoimmune diseases, metabolic syndromes, neurodegenerative diseases, cancers, and cardio- vascular diseases.[1] In the lung, alveolar epithelial type II cells and circulating and resistant immune cells like lymphocytes, macrophages and plasma cells are able to release inflammatory mediators and cytokines/chemokines such as tumor necrosis factor-alpha (TNFα),interleukin-1 beta(IL-1β) and interleukin-6(IL-6).[2] The role of TNFα and IL-1β in inflammation have been highlighted, but inappropriate or excessive production of them could be harmful. In chronic pulmonary diseases like chronic obstructive pulmonary disease (COPD), pro-inflammatory cytokines, TNFα and IL-6, are increased, particularly during exacerbation phase.[3,4]

The biological activities of TNFα trigger by two functionally distinct receptors named tumor necrosis factor receptor type 1 (TNFR1 (p55)) and tumor necrosis factor receptor type 2 (TNFR2 (p75)). The TNFR1 induced by inflammatory responses and has high level expression on inflammatory cells such as alveolar macrophages and it is a major produced mediator in exposure to toxicants.[5,6] Cho and colleagues showed that knocked out TNFR1 in mice could inhibit lung injuries of pulmonary irritants like ozone, silica, bleomycin and radiations.[7]

Sulfur mustard (bis(2-chloroethyl) sulfide; SM) was used in World War I. It was frequently used in Iraq-Iran War (1980-1988) as a chemical weapon. More than 100,000 Iranians have been exposed to the SM.[8] The exposed people, even once, experienced many acute and delay complications in several organs such as eyes, respiratory tract, dermis, gastrointestinal tract, haematopoietic system, and immune system for many years after exposure.[9] The high-resolution computed tomography (HRCT) and pathological studies have demonstrated the bronchiolitis obliterans (BO) in these patients. The pathogenesis of bronchiolitis obliterans in SM-exposed patients might be due to an inflammatory process, similar to bronchiolitis obliterans after lung transplantation.[10] However, some researchers studied inflammatory markers in serum of SM-exposed subjects and declared that systemic inflammation has the main role in pathogenesis of COPD caused by SM, like COPD by diseases.[11] Ghazanfari and colleagues showed that the serum levels of pro-inflammatory cytokines of SM-exposed people, interleukin-1 alpha (IL-1α), IL-1β, TNFα and interleukin-1 receptor antagonist (IL-1RA) significantly decrease in long term in comparison to the control subjects.[12] Further studies illustrated that these pro-inflammatory cytokines rise in spontaneous sputum of SM-exposed civilians in moderate and severe pulmonary status and in the hospitalized subgroup.[13] Also, Emad and Emad demonstrated that levels of several cytokines, such as IL-1β and TNFα, of SM-exposed subjects with pulmonary fibrosis or bronchiectasis in bronchoalveolar lavage (BAL) fluid are higher than control group.[14]

However, anti-inflammatory treatment approaches like inhaled corticosteroids and long-term β2 adrenergic agonists are not helpful and do not adequately improve morbidity and mortality of these patients. For they, a curative treatment is not available up to now.[15,16] In addition, some studies have demonstrated that the levels of local and systemic pro-inflammatory cytokine may be different, which causes different regulation mechanisms in the local and circulating cytokines.[17]

Hence, we evaluated mRNA expression of pro-inflammatory cytokines, *IL-1β*, *TNFα* and *TNFR1*, at the local position in the lung formalin-fixed, paraffin-embedded (FFPE) section of SM-exposed subjects and non-exposed patients. The mRNA expressions were evaluated by quantitative real time PCR (RT-qPCR).

MATERIALS AND METHODS

Study Groups

This study was carried out on paraffin blocks of biopsy lung tissues. These blocks have been collected from archived blocks of pathology department of general hospitals in Tehran, Iran during years 2005 to 2011. According to inclusion and exclusion criteria, the samples were chosen. The exposed samples were selected according to the following criteria: a) having documented exposure to sulfur mustard during the 1980–1988 Iraq-Iran war, b) having chronic respiratory complication (cough, sputum, dyspeniam hemoptosis, pain thorax), c) not having systemic or local diseases which affect a study like acute and chronic infection, autoimmune disease, d) not having history of other toxic gas exposure and occupational pollution. In the control group, the inculsion and exculsion criteria were: a) having lung biopsy with

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normal area, b) not having history of mustard gas exposure or other toxic gas and occupational pollution, c) not having chronic disorders that interfere with our study, such as systemic or pulmonary inflammation diseases, systemic or local acute and chronic infection, autoimmune disease and asthma. Both groups had age between 30-60 years and were not a heavy smoker (>1 packet/day) and were not addicted to opiates or alcohol.

Also some blockes were excluded due to poor quality of the formalin fixation and paraffin embedding process in both groups. More characteristics about samples are shown in Table 1. We selected thirty FFPE lung tissues as the SM-exposed group and thirty FFPE lung tissues as the control group. This study has been approved in the Research Ethics Committee of both Shahed University (41/198215) and Medical Faculty of Tarbiat Modares University (52/1547). For the control group, histopathological near normal adjacent tissue of the pathologic area has been determined by anatomic and clinical pathologist (co-author). The clinical document results of patients have been used for statistical analysis. Severity of pulmonary complications of SM-exposed group has been evaluated by spirometry and by the presence of abnormal lung sounds during physical examination.[18]

RNA Extraction and cDNA Synthesis

RNA extraction from FFPE tissue samples has been described in previous study.[19] Briefly, the total RNA was extracted from the samples by RNeasy FFPE Kit (Qiagene- Germany) with a few modifications in manufacturer's protocol. DNase treatment (Fermentase -USA) was carried out on eluted RNA. After extraction of mRNA from FFPE tissues, their concentration were measured with nanodrop 2000 (Thermo- USA). The samples outed when Optical Density (OD) ratio of 260/280 was less than 1.8.cDNA was synthesized with high capacity cDNA reverse transcription kit (ABI-USA). Finally, cDNA was kept in -20°C for future researches.

Quantitative Real-Time Reverse Transcription-PCR

Our previous study, which its data is not presented here, demonstrated that whenever mRNA is isolated from FFPE tissue, short amplicon length (<120-130 bp) is very important to achieve highest amplification quality at RT-qPCR. Hence, we manually designed primers with amplicon length<125 bp to detect mRNA expression of pro-inflammatory cytokines in this study. Additional information of primers is given in Table 2.

Table 1. Histopathological characteristics of the sulfur mustard-exposed and control groups

		SM-exposed (n=30)	Control (n=30)
Sex	Male	30	21
	Female	0	9
Age		43.33±8.55	46.40±14.9
Smoking		4 (15.4%)	5 (19.2%)
Pathology Diagnosis			
Respiratory-Bronchiolitis		1	1
Constrictive-Bronchiolitis		21	1
Chronic Bronchiolitis		5	0
Bronchiectasis		1	0
Anthracosis		0	2
BenignTumor		0	1
Malignant-tumor		0	12
Bulla		0	2
Interstitial lung disease (ILD)		0	4
Idiopathic pulmonary fibrosis (IPF)		0	2
Pulmonary congestion		1	0
Sever acute inflammation with abscess formation		1	0
Trauma		0	2
Spontaneous pneumothorax		0	3

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Table 2. The sequences of designed primers for housekeeping and pro inflammatory genes to evaluate mRNA experssion of pro inflammatory cytokines in sulfur mustard-exposed lung

Gene	Forward (3>5)	Reverse (3>5)	Amplicon length (bops)	Efficiency (%)	GenBank, accession number
Actin	CGTCTTCCCCTCCATCGTG	GGTGAGGATGCCTCTCTTGCTC	111	95.29	NM_001101.3
Phosphoglycerate kinase 1 (PGK1)	GGCATACCTGCTGGCTGGATG	ACAGGACCATTCCACACAATCTGC	104	96.06	NM_000291.3
Tumor necrosis factorα (TNFα)	ACCCCGAGTGACAAGCCTGTAG	ATCTCTCAGCTCCACGCCATTG	117	90.59	NM_000594.3
Tumor necrosis factorα receptor 1 (TNFR1)	CAAGCCACAGAGCCTAGACACTG	TCGATCTCGTGGTCGCTCAGC	120	89.99	NM_001065.3
Interleukin 1 β (IL-1β)	TACAGTGGCAATGAGGATGACTTG	TGGTCGGAGATTCGTAGCTGG	122	96.46	NM_000576.2

All primers were designed based on different exon of mRNA sequence. Thermodynamic state and secondary structure of primers were determined by Gene Runner 5.0.1 software and online oligo calculator software.[20] The primers were synthesized commercially (TAG Copenhagen- Denmark). The RT-qPCR was then performed with SYBR Green I dye on the Applied Biosystems StepOnePlus™ Real-Time PCR System-USA. Our previous study showed that *ACTIN* and *PGK1* are suitable as housekeeping genes for Real Time PCR test.[19] So cytokines expression were normalized to mRNA expression mean of *ACTIN* and *PGK1* houskeeping genes.

To analyze the relative quantification of RT-qPCR, Pfaffl equation was used.[21] The real time PCR efficiency of the primers was evaluated using 1:10 dilution of PCR products of each gene.

Statistical Analysis

The threshold cycle (CT) value of each sample was determined by ABI Step One 2.3 software (ABI-USA). To estimate the expression change between the control and SM-exposed groups, Pfaffl method was used.[21] Statistical analysis was performed using the SPSS 22.0 software (SPSS Inc., USA). The Kolmogorov-Smirnov test showed a non-normal distribution of sample data for all cytokines in both of the groups. The median of each group was calculated and evaluated by non-parametric tests. Spearman correlation and Mann-Whitney test were used to evaluate the correlation between cytokine expression and clinicopathologic situation. The relationship between nominal variables were analyzed by Chi-square test. Multivariate analyses were performed to eliminate the confounding effects of sex, smoking, and drugs. In this analysis, the rank of CT value was considered as a dependent variable. For all tests, p-value<0.05 was considered statistically significant**.**

RESULTS

Clinical Information about Samples

Severity of pulmonary complications of SM-exposed group was determined. 40% of subjects in SM-exposed group had normal, 44% had mild, and 16% had moderate-severe pulmonary complications. The pulmonary complication symptomes of the samples are shown inTable .

mRNA Expression of TNFα, TNFR1, IL-1β

The concentration of extracted mRNA was measured. The mean±SD of mRNA concentration in SM-exposed and control groups was 279.04±140.24 ng/µL and 346.34±298.8 ng/µL, respectively. There was no significant difference in any of markers between two studied groups (*p values*=0.57).

The mRNA expression of pro-inflammatory cytokines, *TNFα, TNFR1, and IL-1β*, was evaluated by RT-qPCR in all samples of SM-exposed and control groups. It was observed that there is no significant statistical diference in studied cytokines between SM-exposed and control subjects. We found that the

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expression of *TNFα* and *IL-1β* in lung tissue of SM-exposed subjects were 1.82 and 0.73 times lower than that of control people. Moreover, the expression of *TNFR* in SM-exposed subjects was 0.29 times higher than that of control group (according to median of data). However, these differences were not significant (*p* values=0.159, 0.832, 0.314 for *TNFα, TNFR1*, and *IL-1β*, respectively). The boxplot curves of mRNA expression of the pro-inflammatory cytokines have been shown in Figure 1.

Table 3. Clinical Status and pulmonary symptoms of control and sulfur mustard-exposed subjects

Topices	Exposed Num (%)	Control Num (%)
Cough	23 (76.7%)	15(76.7%)
Sputum	24(76.7%)	6(76.7%)
Hemoptesis	19(76.7%)	4(76.7%)
Dyspenia	19(76.7%)	18(76.7%)
Pain Thorax	5(76.7%)	6(76.7%)
Air Trapping (HRCT Test)	10(76.7%)	*

*The HRCT tect was done only for exposed subject

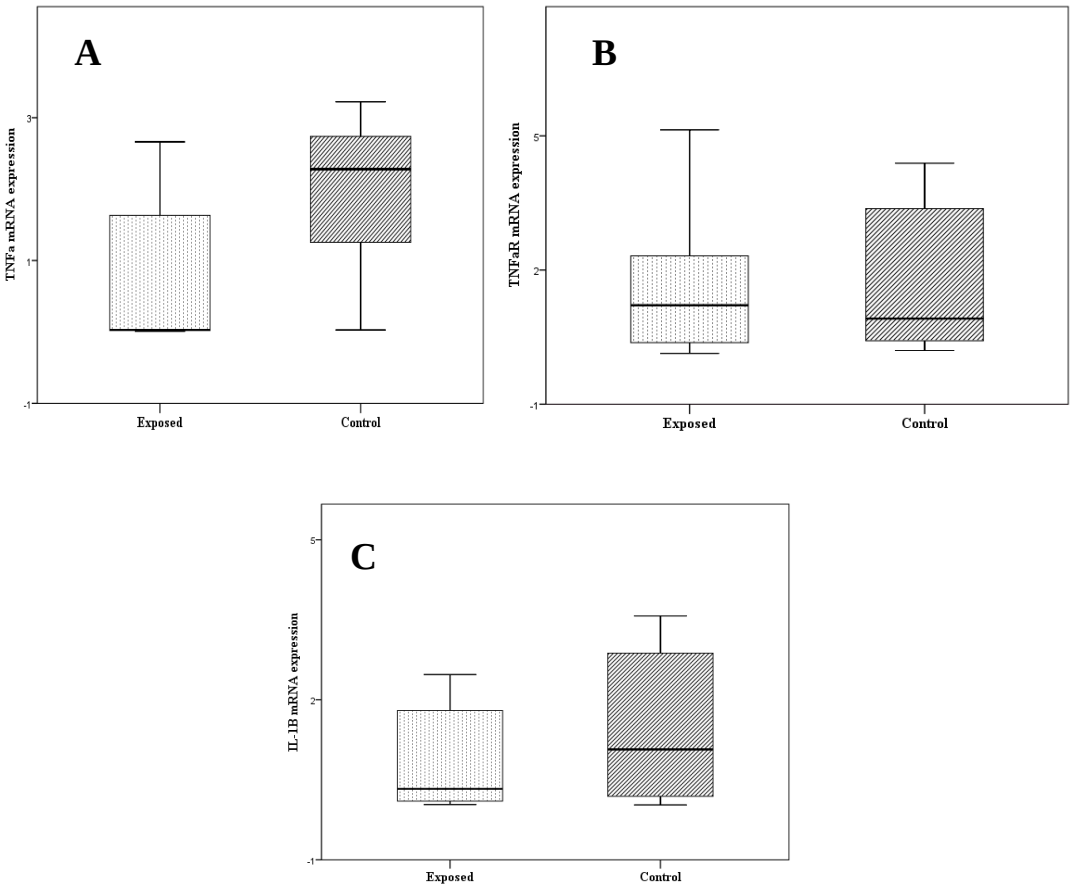

Figure 1. The mRNA expression of tumor necrosis factor alpha (TNFα) (A), tumor necrosis factor receptor type 1 (TNFR1) (B), and interleukin 1 beta (IL-1β) (C) in lung tissue of Sulfur-mustard-exposed subjects and non-exposed control group. mRNA expression of 60 samples (30 SM-exposed and 30 control) was evaluated by RT-qPCR. Expression of these pro-inflammatory cytokines had no significant different between SM-exposed and control groups.

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Table 4. Differential mRNA expression of pro inflammatory cytokines genes in lung tissues of sulfur mustard-exposed and control groups

	Exposed				Control					
	Median	Q1	Q3	Mean±SD	Median	Q1	Q3	Mean±SD	*p*-value[1]	*p*-value[2]
TNF	0.028	0.015	2.663	1.325±2.223	1.860	1.255	2.738	1.827±1.158	0.159	0.477
TNFR	1.212	0.254	2.346	1.713±1.83	0.914	0.419	3.374	2.469±3.459	0.832	0.989
IL-1	0.331	0.100	1.798	2.641±6.551	1.070	0.190	2.873	5.497±13.852	0.314	0.303

p-value[1]: compering between control and exposed(Mann-whitney)

p-value[2]: Univariate

Table 5. Correlation between pro inflammatory cytokines genes and severity of pulmonary involvement in sulfur mustard-exposed subject

Severity	Normal	Mild	Moderate-severe	*p*-value
	Mean±SD	Mean±SD	Mean±SD	
TNF	2.244±3.005	0.024±0.006	1.633±1.457	0.373
TNFR	2.155±2.205	0.748±0.528	2.125±2.014	0.513
IL-1	4.289±10.254	1.274±1.778	0.473±0.469	0.915

p-value: compering between 3 groups (Kruskal Wallis test)

More statistical analysis showed that age, gender, common drugs for chronic respiratory diseases such as Fluticasone/Salmeterol, Salbutamol, N-Acetyl cysteine, Atrovent, Prednisolone and smoking condition have no influence on the obtained results (*p* values=0.477, 0.989, 0.303 for *TNFα, TNFR1*, and *IL-1β*, respectively).

Relationship between Expression of Pro-inflammatory Cytokines and Patient's Clinicopathologic Situation

Inter-Correlation between the Expression of Cytokines

The cytokines correlation was obtained by Spearman correlation. The results showed that there is a significant direct correlation between *TNFα* and *TNFR1* (r= 0.867, *p*=0.002) in the SM-exposed group. In addition, *TNFR1* had direct correlation with *IL-1β* (r=0.65, *p*=0.006). The correlation among other cytokines were not siginificant. Details have been shown in Table 4.

Relationship between Expression of the Cytokines and Clinical Conditions

Surprisingly, *TNFR1* had inverse correlation with Forced Expiratory Volume in 1 second (FEV1) spirometry index in the SM-exposed group (r=-0.604, *p*=0.017). Moreover, subjects with sputum had less *IL-1β* amount in the control group (*p*=0.053). But, there was not any relation between pro-inflammatory cytokines and air trapping, cough, hemoptysis, dyspnea, and thoracic pain in both groups. These cytokines did not have correlation with the severity of pulmonary involvements in SM-exposed subjects. Details have been shown in Table 5

DISCUSSION

The purpose of this study was to evaluate the mRNA expression of pro-inflammatory cytokines in lung tissue of subjects with a sulfur mustard (SM) exposed with a history about 20 years ago. Our results have shown that there is no significant difference between pro-inflammatory expression of cytokines in lung tissue of SM-exposed subjects after many years and non- exposed subjects.

In a study on bronchoalveolar lavage fluid (BAL), Emad and colleagues showed that SM-exposed patients have an ongoing local inflammatory process of the lower respiratory tract, which results in the development of pulmonary fibrosis years after initial exposure. Levels of pro-inflammatory cytokines in these patients were higher than the control group.[22,23] But, present results demonstrated that level of pro-inflammatory cytokines in lung tissue of chemically injured people are similar to the control group. The

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type of the studied samples may justify this difference. Emad and colleagues had only selected SM-exposed subjects with clinical diagnosis of pulmonary fibrosis or bronchiectasis in thier studies. But, the present study have examined SM exposed subjects with a wider range of pathological lesions. Also, they had a healthy control group and the cytokine level of SM- exposed BAL had compared to normal BAL. Due to ethical considirartion, we were not able to have normal lung tissue samples for control group. The best alternative was the use of a near normal area of the lung of pulmonary patients. This area was far from pathologic lesion and its molecular pattern may be similar to healthy or pathology pattern. There was no other way and this is a limitation of the biopsy tissue study.

Nevertheless, the obtained results are valuable. It shows that there is no remarkable difference between pro-inflammatory cytokines of SM lung injured and other pulmonary diseases in control group. Therefore, these cytokines could not lead to the unique pathology which is observed in the SM-exposed lung. Hence, findings of this study may elucidate ineffectiveness of the current practice in management of the COPD and IPF which did not take in to account this undeylying immunopathological mechanism. Currently, there is no the Food and Drug Administration (FDA) approved protocol for treatment of the SM induced pulomonary complications[16] and this novel finding may help to establish a desired treatment protocol.

Yaraee and colleagues showed that pro-inflammatory cytokines in the spontaneous sputum of SM-exposed individuals were higher at hospitalized group with moderate and severe pulmonary status compared to mild disorders.[24] This difference could be due to lack of control group in their study. Also, this difference could be justified by origin of the samples. The source of spontaneous sputum is upper respiratosy tract and biopsy of parenchymal lung tissue is from lower respiratosy tract. In another study, Yaraee and colleagues reported that these cytokines have significantly decreased in serum of SM-exposed subjects.[25] This result is identical to our finding.

Some studies consider that pathology of SM is similar to COPD and Asthma.[26] Accurate study on pathology of these pulmonary diseases show the pattern of the inflammatory process and the therapeutic response of they are markedly different from SM delay mechanism.[27] The studies often evaluate inflammatory cytokines in BAL, sputum and serum samples, and present study is the first local study on lung tissue of chemical injured people for determination molecular mechanism of SM in delayed phase. Our results are closer to the fact.

On the other hand, Aghanouri and colleagues showed that transforming growth factor beta (TGF-β) in lung tissue of SM-exposed patients at delay phase is higher than that of control people.[28] The high level of TGF-β , as an anti-inflammatory cytokine, could be a reason for low pro-inflammatory cytokine in lung of SM-exposed subjects.

The evaluation of pro-inflammatory cytokines mRNA in lung of the SM-exposed patients revealed that the expression of *TNFα*, *TNFR1*, and *IL-1β* has no difference with those of control subjects. It seems that the inflammatory cytokines are not affected in the delayed pathogenesis of SM-exposed pulmonary tissue. Hence, it is recommended that effectiveness of pro-inflammatory blockers such as corticosteroids or anti TNFα in treatment of pulmonary complications for SM injured people should be further investigated before using them as a certain cure.

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REFERENCES

1. Murakami M, Hirano T. The molecular mechanisms of chronic inflammation development. Frontiers in Immunology. 2012;3(323).
2. Moldoveanu B, Otmishi P, Jani P, Walker J, Sarmiento X, Guardiola J, et al. Inflammatory mechanisms in the lung. Journal of inflammation research. 2009;2:1-11.
3. Aaron SD, Angel JB, Lunau M, Wright K, Fex C, Le Saux N, et al. Granulocyte inflammatory markers and airway infection during acute exacerbation of

chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2001;163(2):349-55.
4. Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. Thorax. 2000;55(2):114-20.
5. Bradley JR. TNF-mediated inflammatory disease. J Pathol. 2008;214(2):149-60.
6. Sunil VR, Patel-Vayas K, Shen J, Gow AJ, Laskin JD, Laskin DL. Role of TNFR1 in lung injury and altered lung function induced by the model sulfur mustard vesicant, 2-chloroethyl ethyl sulfide. Toxicol Appl Pharmacol. 2011;250(3):245-55.
7. Cho HY, Zhang LY, Kleeberger SR. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-alpha receptors. Am J Physiol Lung Cell Mol Physiol. 2001;280(3):L537-46.
8. Kehe K, Szinicz L. Medical aspects of sulphur mustard poisoning. Toxicology. 2005;214(3):198-209.
9. Balali-Mood M, Mousavi S, Balali-Mood B. Chronic health effects of sulphur mustard exposure with special reference to Iranian veterans. Emerging health threats journal. 2008;1:e7.
10. Ghanei M, Adibi I. Clinical review of mustard lung. Iranian Journal of Medical Sciences. 2007;32(2):58-65.
11. Lari SM, Attaran D, Towhidi M. COPD Due to Sulfur Mustard (Mustard Lung). Chronic Obstructive Pulmonary Disease - Current Concepts and Practice. 2012:231-9.
12. Ghazanfari T, Faghihzadeh S, Aragizadeh H, Soroush MR, Yaraee R, Mohammad Hassan Z, et al. Sardasht-Iran cohort study of chemical warfare victims: design and methods. Archives of Iranian medicine. 2009;12(1):5-14.
13. Pourfarzam S, Yaraee R, Hassan ZM, Yarmohammadi ME, Faghihzadeh S, Soroush MR, et al. Chemokines, MMP-9 and PMN elastase in spontaneous sputum of sulfur mustard exposed civilians: Sardasht-Iran Cohort Study. International immunopharmacology. 2013:968–73.
14. Emad A, Emad V. Elevated levels of MCP-1, MIP-alpha and MIP-1 beta in the bronchoalveolar lavage (BAL) fluid of patients with mustard gas-induced pulmonary fibrosis. Toxicology. 2007;240(1-2):60-9.
15. Poursaleh Z, Harandi AA, Vahedi E, Ghanei M. Treatment for sulfur mustard lung injuries; new therapeutic approaches from acute to chronic phase. Daru. 2012;20(1):27.
16. Weinberger B, Malaviya R, Sunil VR, Venosa A, Heck DE, Laskin JD, et al. Mustard vesicant-induced lung injury: Advances in therapy. Toxicol Appl Pharmacol. 2016;305:1-11.
17. Vernooy JH, Küçükaycan M, Jacobs JA, Chavannes NH, Buurman WA, Dentener MA, et al. Local and Systemic Inflammation in Patients with Chronic Obstructive Pulmonary Disease. American Journal of Respiratory and Critical Care Medicine. 2002;166(9):1218-24.
18. Khateri S, Ghanei M, Keshavarz S, Soroush M, Haines D. Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard agent. J Occup Environ Med. 2003;45(11):1136-43.
19. Eghtedardoost M, Hassan ZM, Askari N, Sadeghipour A, Naghizadeh MM, Ghafarpour S, et al. The delayed effect of mustard gas on housekeeping gene expression in lung biopsy of chemical injuries Biophysic and Biochemistry Reports. 2017;11: 27-32.
20. www.biotools.nubic.northwestern.edu/OligoCalc.
21. Pfaffi MW. A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Research 2001;29(9):1-6.
22. Emad A, Emad Y. CD4/CD8 ratio and cytokine levels of the BAL fluid in patients with bronchiectasis caused by sulfur mustard gas inhalation. J Inflamm (Lond). 2007;4:2.
23. Emad A, Emad Y. Levels of cytokine in bronchoalveolar lavage (BAL) fluid in patients with pulmonary fibrosis due to sulfur mustard gas inhalation. Journal of Interferon & Cytokine Research. 2007;27(1):38-43.
24. Yaraee R, Hassan ZM, Pourfarzam S, Rezaei A, Faghihzadeh S, Ebtekar M, et al. Fibrinogen and inflammatory cytokines in spontaneous sputum of sulfur-mustard-exposed civilians--Sardasht-Iran Cohort Study. Int Immunopharmacol. 2013;17(3):968-73.
25. Yaraee R, Ghazanfari T, Ebtekar M, Ardestani SK, Rezaei A, Kariminia A, et al. Alterations in serum levels of inflammatory cytokines (TNF, IL-1alpha, IL-1beta and IL-1Ra) 20years after sulfur mustard

exposure: Sardasht-Iran cohort study. International immunopharmacology. 2009;9(13):1466-70.
26. Khazdair MR, Boskabady MH, Ghorani V. Respiratory effects of sulfur mustard exposure, similarities and differences with asthma and COPD. Inhalation toxicology. 2015;27(14):731-44.
27. Ghanei M, Harandi AA. Molecular and cellular mechanism of lung injuries due to exposure to sulfur mustard: a review. Inhalation toxicology. 2011;23(7):363-71.
28. Aghanouri R, Ghanei M, Aslani J, Keivani-Amine H, Rastegar F, Karkhane A. Fibrogenic cytokine levels in bronchoalveolar lavage aspirates 15 years after exposure to sulfur mustard. Am J Physiol Lung Cell Mol Physiol. 2004;287(6):L1160-4.