Original Article

Correlation between MMP-9 and MMP-9/ TIMPs Complex with Pulmonary Function in Sulfur Mustard Exposed Civilians: Sardasht-Iran Cohort Study

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Abstract

Background: Matrix metalloproteinases (MMPs) are a family of proteinases and have the vigorous capacity to degrade all parts of the extracellular matrix. MMP enzymes strongly participate in physiological processes such as normal tissue remodeling and wound healing and in pathology of pulmonary diseases. They are released in response to environmental stimuli such as toxins and regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). Sulfur mustard (SM) is a chemical toxic which can cause severe permanent damages to lung tissues. The aim of this study was assessing the possible role of MMP-9 and TIMPs in SM-induced lung symptoms and signs in exposed patients 20 years after exposure.

Methods: Totally, 372 male volunteers with a history of SM exposure and 128 age- and sex-matched unexposed controls participated and were divided into three groups: normal, mild and moderate-severe. All participants underwent clinical evaluation and pulmonary function tests and serum concentrations of MMP-9 and its inhibitors were measured using the ELISA technique.

Results: Serum level of MMP-9 was increased in the SM exposed group who had moderate-severe pulmonary complications compared with the SM exposed with normal lung (2.321 ± 2.836 vs. 1.546 ± 2.176, $P = 0.001$) while only the MMP-9/TIMP-4 complex was elevated in the SM exposed with normal lung individuals compared to its corresponding control group (85 ± 265 vs. 82 ± 222, $P = 0.025$). Although MMP-9 and its inhibitors did not show any correlation with spirometry findings, elevated circulating MMP-9 was detected in SM exposed patients with chronic chough and hemoptysis ($P = 0.013$ and $P = 0.013$ respectively).

Conclusion: High level of tissue disruption and remodeling mediators could influence lung structure in long-term after SM exposure. The correlation of clinical evaluation with these factors efficiently helps us to identify important effectors.

Keywords: Lung, Mustard gas, MMP-9, TMIPs

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Introduction

During the Iraq-Iran war, numerous Iranian were injured due to sulfur mustard (SM) exposure.[1] Currently, after more than two decades, thousands of people suffer from long-term complications, mostly in lung, skin and eyes.[2] Lung involvement is the major cause of morbidity in these casualties. Some pathological pulmonary processes include asthma, emphysema, chronic bronchitis, pulmonary fibrosis, bronchiolitis, chronic obstructive pulmonary disease (COPD) and bronchiolitis obliterans (BO).[2–4] The molecular mechanism of these complications in chemical victims is not clear; hence, the diagnosis and treatment of these diseases is difficult. Sardasht Iran Cohort study (SICS) was launched to clarify the molecular mechanism of SM induced clinical complications. Some reports have been published on inflammatory mediators.[5–8]

Proteases activity is one of significant aspects in acute and long-term sulfur mustard induced complications.[4,9–12] Matrix metalloproteinases (MMPs) are a 23-member family of zinc-dependent endopeptidases that appear in secreted and membrane-bound forms and have the collective ability to degrade all parts of the extracellular matrix.[13] MMP enzymes strongly participate in normal, pathological, physiological, and inflammatory processes such as normal tissue remodeling and wound healing,[13,14] as well as in pulmonary diseases such as COPD and BO.[15–17] MMPs are released in response to environmental stimuli such as toxins, growth factors and cytokines; their activity is firmly regulated by both transcriptional and post-translational mechanisms and endogenous tissue inhibitors of metalloproteinases (TIMPs). Disruption of the balance between MMPs and their endogenous inhibitors can cause dissociation of epithelial cells from basement membranes, destruction of the pulmonary epithelial barrier and pulmonary architecture remodeling.[4,15]

In previous studies, we measured serum level of MMP-1, MMP-2, MMP-8, MMP-9 and TIMPs.[11] Serum levels of these factors were compared between SM exposed groups without

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any symptoms as the control group and those with mild or moderate–severe lung complications based on the Global Initiative for chronic Obstructive Lung Disease (GOLD) classification. Furthermore, MMP-2 and MMP-9 activity was evaluated. Our results showed elevated serum levels of MMP-1 and reduced MMP-2 activity that may have roles in pathogenesis and constancy of lung complications in SM exposed patients. Likewise, serum and sputum level of MMP-9 assessed in SM exposed victims and compared based on hospitalization and GOLD classification.[18] Serum levels of MMP-9 were significantly increased in the more severe (grades 3–4) group, while there was no significant correlation between sputum level of this factor and pulmonary complications in the patients. In these studies, serum level of MMP-9 was evaluated in a small sample size of SICS, and compared based on GOLD classification and hospitalization. Here, we tested this hypothesis in all participants of SICS and serum level of MMP-9 was compared between control and SM exposed group based on a standardized diagnostic protocol approved by Janbazan Organization.

Materials and Methods

Study design and participants

Complete information of the study design and methodology of the SICS have been reported previously in the original methodology paper.[19] Briefly, 372 male volunteers from Sardasht with a history of SM exposure in June 1987 and 128 subjects as controls from the town of Rabat were recruited in June 2007. The two groups were matched for sex and age. They were classified into three major subgroups based on pulmonary assessment. There were 85 normal individuals, 30 with mild and 8 with moderate - severe lung complication in the control group, and 204 normal individuals, 100 with mild and 48 with moderate – severe pulmonary problems in the exposed group. Baseline information of participants is summarized in Table 1.

Ethical considerations

The study was approved by the ethical committee of the Board of Research Ethics of Janbazan Medical and Engineering Research Center (JMERC), the Board of Research of Ministry of Health, and Shahed University. We recruited individuals who wished to take part and signed their informed consent.

Clinical evaluation

All study participants were examined; pulmonary symptoms, consisting of chronic cough, sputum, hemoptysis, and dyspnea, and pulmonary findings such as fine crackles, coarse crackles, and wheezing were assessed. Chronic cough was defined as cough continuing for more than 3 weeks. Three successive spirometry measurements (Chest 801 Spirometry) were made for all participants according to the American Thoracic Society Criteria under surveillance of a trained nurse. The appropriate measurement was selected for data analysis. The results of spirometry findings are presented in Table 2.

Categorization by Severity of Pulmonary Complications

Classification of severity of SM lesion is detailed by Khateri *et al.* (2003).[20] Briefly, all participants were categorized by severity of lung into three subgroups: normal, mild-moderate and severe based on a standardized diagnostic protocol approved by Janbazan Organization. Pulmonary complications were determined by spirometry and the presence of abnormal lung sounds on physical examination.

Serum preparation

Blood samples were drawn into Vacutainer tubes (BD Biosciences). The sera were separated by 20 min centrifugation at 2000 ×g at 4°C and kept at -80°C until assessment of factors.

Serum soluble MMP-9 and MMP-9/TIMPs complex assessment

Human MMP-9, MMP-9/TIMP-1, MMP-9/TIMP-2 and MMP-9/TIMP-4 complex DuoSet® ELISA Development Kit (R&D Systems) was employed. Capture antibody was mouse anti-human and biotinyalated goat anti-human was detection antibody. Standards of the kits were prepared by dilution with 1% BSA in PBS. 0.05% Tween 20 in PBS was used as wash buffer, and block buffer was 1% BSA in PBS. This human MMP-9 assay measured the 92 kDa pro-MMP-9 and the 82 kDa active MMP-9. ELISA reader and washer were Stat-Fax 2100 and Stat-Fax 2600, respectively.

Statistical analysis

Statistical comparison between groups was performed using the Kruskal–Wallis test. Correlation between MMP-9 and its inhibitors was computed using Spearman's rank correlation coefficient. Differences were considered statistically significant when $P \leq$

Table 1. Baseline information of study population.

Variables		Control	Exposed	*P*-Value
Sample count		128	372	-
Age (Mean ± SD)		41.7 ± 9.8	43.9 ± 10.7	0.103
Smoking	Yes / Quitted	29 (22.7%)	44 (23.6%)	0.946
	Never	99 (77.3%)	142 (76.4%)	
Pulmonary disease severity	Normal	89(68.8%)	218(58.6%)	0.095
	Mild	31(24.2%)	104(28.0%)	
	Moderate-Severe	8(6.3%)	50(13.5%)	
GOLD classification	Normal	115(89.7%)	313(83.5%)	0.059
	Mild	0(0.0%)	7(1.9%)	
	Moderate-Severe	13(10.3%)	52(13.9%)	

Not exposed and SM exposed participants compared based on age, smoking and two different classification. There was no significant difference between two groups in mention parameters. Pulmonary disease severity categorized based on a standardized diagnostic protocol approved by Janbazan Organization.
GOLD = chronic Obstructive Lung Disease

Table 2. Comparisons of the spirometry findings between study groups.

	Study Groups		*P*-Value	*P*-Value*
	Control N = 128	Exposed N = 372		
FVC%	93.92±17.04	86.68±17.19	**<0.001**	**<0.001**
FEV1%	89.14±19.69	81.08±19.73	**<0.001**	**<0.001**
FEV1/FVC%	98.25±11.03	94.61±13.58	**0.045**	0.061
MMEF%	78.98±34.08	67.12±29.79	**0.008**	**0.030**
PEF%	83.16±20.25	76.91±20.33	**0.004**	**0.012**

Spirometry findings are significantly decreased in SM exposed groups at the time of study (Twenty years after exposure).
FVC = Forced Vital Capacity; FEV1= Forced Expiratory Volume in 1 second.
MMEF = Maximum Mid Expiratory Flow; PEF = Peak Expiratory Flow.
All concentrations are pg/dL.
Bold numbers show significant differences with *P* value ≤ 0.05.
P-Value*: adjusted by age and smoking (ANCOVA: age as covariate, Smoking as fixed effect)

0.05. Data are presented as mean (SD). In order to remove the confounding effect of age and smoking, we used analysis of covariance (ANCOVA) to compare pulmonary function factors between the control and SM exposed groups. Analyses of all the data were performed using SPSS software version 22.0.

Results

Correlation between MMP-9 and MMP-9/TIMPs complexes with spirometry findings

According to Table 3, there was no significant correlation between serum level of MMP-9 and spirometry parameters such as FVC%, FEV1%, FEV1/FVC%, MMEF%, and PEF% in SM exposed group. In addition, we did not find any relation between MMP-9: TIMPs complexes and spirometry parameters.

Comparison of serum levels of MMP-9 and MMP-9/TIMPs complex with pulmonary severity of complications

A significant increase was found in serum level of MMP-9 in exposed group who had moderate-severe lung complications compared to exposed who had normal lung, by removing or without removing smokers; (P = 0.001 and P = 0.003, respectively) (Tables 4 and 5). Also, the data showed a significant elevation in serum levels of MMP-9/TIMP-4 complex in SM exposed individuals (85 ± 265) with normal lung in comparison to the corresponding control group (82 ± 222) regardless of smoking (Table 4); however, the level of this complex declined in SM exposed individuals (89.62 ± 278.16) with normal lung compared to the corresponding control group (90.11 ± 247.12) and SM exposed individuals with moderate-severe lung complication compared to SM exposed with normal lung (P = 0.039)

Association of MMP-9 and TIMPs complexes with pulmonary signs and symptoms.

A significant elevation of serum MMP-9 was detected in SM exposed patient with chronic cough and hemoptysis compared with SM injured people without these symptoms (1.750 ± 2.351 vs. 1.187 ± 2.090 and 1.982 ± 2.482 vs. 1.580 ± 2.255, respectively) regardless of classification based on smoking. However, its concentration did not change significantly in non-

Table 3. Correlation between MMP-9 and MMP-9/TIMPs complexes with Spirometry findings.

		MMP-9		MMP-9/TIMP-1 complex		MMP-9/TIMP-2 complex		MMP-9/TIMP-4 complex	
		Control	Expose	Control	Expose	Control	Expose	Control	Expose
FVC%	**r**	-0.085	-0.098	-0.012	-0.033	-0.059	0.022	-0.078	-0.031
	p	0.350	0.066	0.894	0.536	0.520	0.677	0.393	0.569
FEV1%	**r**	-0.095	-0.090	-0.015	-0.001	0.002	0.046	-0.031	-0.010
	p	0.292	0.091	0.868	0.987	0.984	0.389	0.736	0.851
FEV1/FVC%	**r**	0.118	-0.051	0.178	-0.042	0.136	0.052	-0.008	-0.021
	p	0.340	0.451	0.154	0.537	0.271	0.441	0.947	0.755
MMEF%	**r**	0.015	-0.038	0.212	0.019	0.155	0.075	0.021	-0.103
	p	0.904	0.615	0.088	0.800	0.210	0.325	0.869	0.174
PEF%	r	0.007	-0.021	0.042	-0.004	0.132	-0.040	-0.020	-0.074
	p	0.940	0.710	0.645	0.948	0.148	0.483	0.832	0.201

There is no signification correlation between MMP-9 and its inhibitors with spirometry findings. MMP-9 = matrix metalloproteinase-9, TIMP = tissue inhibitors of metalloproteinase, R = Spearman correlation coefficient , p = *P*-Value.

Table 4. Comparison of the serum levels of MMP-9 and MMP-9/TIMPs complex with pulmonary complications severity based on pulmonary assessment.

		Control				Exposed			
		N	Mean ± SD	Median	*P*-Value[1]	N	Mean ± SD	Median	*P*-Value [1]	*P*-Value [2]
MMP-9(µg/mL)	**Normal**	85	1.058±0.661	0.841		207	1.546±2.176	0.819		0.968
	Mild	30	1.156±0.853	0.889	0.705	100	1.779±2.376	0.851	0.296	0.832
	Moderate – Severe	8	1.304±1.074	0.932	0.593	48	2.321±2.836	1.164	**0.001**	0.337
MMP-9/TIMP-1 complex(µg/mL)	**Normal**	85	31.677±36.139	18.553		207	29.220±28.559	20.288		0.874
	Mild	30	28.852±34.826	14.197	0.64	100	34.680±40.880	19.058	0.898	0.436
	Moderate – Severe	8	20.098±15.648	19.432	0.479	48	42.311±73.308	26.197	0.207	0.219
MMP-9/TIMP-2 complex(µg/mL)	**Normal**	85	2.938±16.437	0.113		207	1.911±12.846	0.271		0.075
	Mild	30	2.533±9.519	0.247	0.08	100	1.400±3.848	0.352	0.516	0.842
	Moderate – Severe	8	0.654±0.838	0.295	0.385	48	0.835±2.078	0.209	0.566	0.618
MMP-9/TIMP-4 complex(ng/mL)	**Normal**	85	82.91±222.11	18.518		207	85.54±265.15	24.502		**0.025**
	Mild	30	43.73±53.63	23.874	0.221	100	102.42±287.64	24.816	0.916	0.865
	Moderate – Severe	8	149.68±260.67	25.750	0.197	48	52.28±87.63	20.727	0.211	0.293

There is significant rise in serum level of MMP-9 in SM exposed patients with moderate to severe pulmonary complications in comparison with SM exposed group with normal lung. Also, elevated level of MMP-9/TIMP-4 exhibit in SM exposed individuals with normal lung related to its correspond in control group. Bold numbers show significant differences with *P*-Value ≤ 0.05.
MMP-9 = matrix metalloproteinase-9, TIMP: tissue inhibitors of metalloproteinase
P-Value [1] = Comparison with normal group (Mann-Whitney)
P-Value[2] = Comparison of case with its correspond control (Mann-Whitney)

Table 5. Comparison of the serum levels of MMP-9 and MMP-9/TIMPs complex with pulmonary complications severity based on pulmonary assessment in non-smoker participants.

		Control				Exposed				
		N	Mean±SD	Median	*P*-Value[1]	N	Mean±SD	Median	*P*-Value[1]	*P*-Value[2]
MMP-9(µg/mL)	**Normal**	66	1.093±0.677	0.877		168	1.536±2.162	0.828		0.813
	Mild	25	1.153±0.834	0.999	0.807	75	1.676±2.269	0.838	0.586	0.83
	Moderate – Severe	3	2.009±1.469	1.354	0.112	28	2.580±3.140	1.266	**0.003**	0.688
MMP-9/TIMP-1 complex(µg/mL)	**Normal**	66	31.923±38.332	18.337		168	27.106±25.934	19.563		0.949
	Mild	25	28.143±34.355	14.977	0.788	75	33.653±42.959	18.409	0.906	0.744
	Moderate – Severe	3	22.142±5.300	21.890	0.856	28	28.351±24.251	26.692	0.544	0.789
MMP-9/TIMP-2 complex(µg/mL)	**Normal**	66	3.179±18.325	0.142		168	2.218±14.275	0.271		0.135
	Mild	25	2.883±10.236	0.318	0.122	75	1.492±4.250	0.363	0.68	0.688
	Moderate – Severe	3	0.264±0.203	0.295	0.601	28	0.756±2.467	0.170	0.236	0.734
MMP-9/TIMP-4 complex(ng/mL)	**Normal**	66	90.11±247.12	17.57		168	89.62±278.16	24.82		**0.007**
	Mild	25	46.03±58.29	23.87	0.182	75	109.84±309.81	27.01	0.998	0.74
	Moderate – Severe	3	19.60±11.77	20.73	0.858	28	36.77±70.91	18.83	**0.039**	0.89

Resembling classification regardless of smoking, in non-smoker participants, there is significant increased level of MMP-9 in serum of SM exposed patients with moderate to severe pulmonary complications in comparison with SM exposed group with normal lung. Unlike previous classification, reduced level of MMP-9/TIMP-4 exhibit in SM exposed individuals with normal lung related to its correspond in control group. Also, sera level of MMP-9/TIMP-4 complex decreased in SM exposed victims with moderate to severe pulmonary complications in comparison with SM exposed group with normal lung. Bold numbers show significant differences with *P* value ≤ 0.05.

MMP-9 = matrix metalloproteinase-9, TIMP = tissue inhibitors of metalloproteinase

P-Value[1] = Comparison with normal group (Mann-Whitney)

P-Value[2] = Comparison of case with its correspond control (Mann-Whitney)

Table 6. Association of the serum levels of MMP-9 and MMP-9/TIMP complexes with pulmonary signs and symptoms in the SM exposed group.

	Chronic cough				
	Yes (N = 333)		No (N = 23)		
	Mean±SD	Median	Mean±SD	Median	*P*-Value
MMP-9	1.750±2.351	0.894	1.187±2.090	0.620	**0.013**
MMP-9/TIMP-1 complex	33.521±42.013	21.068	19.738±17.375	13.842	0.065
MMP-9/TIMP-2 complex	1.591±10.183	0.295	1.945±6.867	0.247	0.555
MMP-9/TIMP-4 complex	83.71±240.77	24.82	115.62±418.30	24.50	0.368
	Sputum				
	Yes (N = 320)		No (N = 36)		*P*-Value
MMP-9	1.681±2.220	0.889	2.004±3.218	0.760	0.194
MMP-9/TIMP-1 complex	33.522±42.659	20.646	24.708±19.905	18.268	0.411
MMP-9/TIMP-2 complex	1.546±10.301	0.271	2.206±6.796	0.341	0.868
MMP-9/TIMP-4 complex	84.56±244.00	24.50	96.67±343.41	24.50	0.720
	Hemoptesi				
	Yes (N = 118)		No (N = 238)		*P*-Value
MMP-9	1.982±2.482	0.935	1.580±2.255	0.822	**0.013**
MMP-9/TIMP-1 complex	37.343±55.147	22.918	30.283±31.607	19.941	0.770
MMP-9/TIMP-2 complex	1.548±4.495	0.247	1.646±11.823	0.295	0.781
MMP-9/TIMP-4 complex	90.01±260.89	24.82	83.73±253.16	24.50	0.764
	Dyspnea				
	Yes (N = 338)		No (N = 18)		*P*-Value
MMP-9	1.724±2.337	0.881	1.519±2.391	0.759	0.297
MMP-9/TIMP-1 complex	32.868±41.789	20.585	27.784±19.361	20.288	0.750
MMP-9/TIMP-2 complex	1.654±10.235	0.271	0.828±1.886	0.247	0.499
MMP-9/TIMP-4 complex	81.23±237.34	24.50	175.69±495.25	24.50	0.932
	Pulmonary Auscultation				
	Normal (N = 274)		Abnormal (N = 82)		*P*-Value
MMP-9	1.632±2.253	0.823	1.985±2.592	0.964	0.090
MMP-9/TIMP-1 complex	28.669±28.753	19.971	45.691±65.733	26.213	0.092
MMP-9/TIMP-2 complex	1.671±11.218	0.259	1.426±3.852	0.330	0.529
MMP-9/TIMP-4 complex	83.10±266.52	24.50	94.83±215.36	25.76	0.295

Elevated of the serum levels of MMP-9 exhibit in SM exposed participants who have (Yes) chronic cough and hemoptysis in comparison who does not have. There is not significant change in MMP-9/TIMPs complex in SM exposed victims with pulmonary sign and symptoms. MMP-9 = matrix metalloproteinase-9, TIMP = tissue inhibitors of metalloproteinase.
MMP-9 = matrix metalloproteinase. Bold data shows significant p value.

smoker SM exposed with the mentioned symptoms. The results presented in Table 6 show no statistically significant association between the serum levels of MMP-9 and TIMPs complex and other respiratory symptoms, dyspnea or pulmonary auscultation in the SM exposed group.

Correlation between MMP-9 and TIMPs complexes.

There was a significant positive correlation between the serum level of MMP-9 with MMP-9/TIMP-1 and MMP-9/TIMP-2 complexes in the exposed group. Also, significant correlations were observed between serum levels of MMP-9/TIMP-4 complex with MMP-9/TIMP-1 complex and MMP-9/TIMP-2 complex in the SM exposed subjects (Table 7). The results presented in Table 7 show that the same was observed in the unexposed control group.

Discussion

The molecular mechanism of long-term complications of SM injured victims has not been clarified. MMP enzymes strongly contribute to tissue remodeling.[13,14] The extracellular matrix (ECM) remodeling is vital for adapting the morphogenesis of lungs. MMPs are released in response to environmental stimuli such as toxins, growth factors and cytokines and their activity is firmly

Table 7. Correlation between MMP-9 and TIMPs complexes levels in serum.

Study Groups			TIMP-1	TIMP-2	TIMP-4
Exposed	MMP-9	r	0.509**	0.127*	0.050
		p	<0.001	0.014	0.341
	TIMP-1	r		0.079	0.214**
		p		0.127	<0.001
	TIMP-2	r			0.452**
		p			<0.001
Control	MMP-9	r	0.442**	0.165	0.051
		p	<0.001	0.064	0.576
	TIMP-1	r		0.008	0.236**
		p		0.925	0.008
	TIMP-2	r			0.590**
		p			<0.001

There is significant positive correlation between MMP-9 and MMP-9/TIMP1 and TIMP2 complexes in SM exposed victims. Also, a significant positive correlation between MMP-9/ TIMP-4 and MMP-9/TIMP1 and TIMP2 complexes detects. MMP-9 = matrix metalloproteinase-9, TIMP = tissue inhibitors of metalloproteinase.
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs).[14] Dysregulation of ECM compound, structure, firmness and affluence by disruption of the balance between MMPs and their endogenous inhibitors contributes to several pathological conditions, such as fibrosis, asthma and COPD.[15,21] The aim of this study was to investigate the role of MMP-9 in long-term pulmonary complications in SM injured victims and association of regulatory factors in modulation of its responses.

The results showed that MMP-9 increased in SM victims with moderate-severe pulmonary complications independent of smoking (Tables 4 and 5). Also, the serum level of MMP-9/TIMP-4 complex was decreased in SM exposed group with normal lung and those with moderate-severe pulmonary complications. So, it seems that SM could affect the production of activated form of TIMP-4 even in SM-exposed peoples with normal lung and probably suffering from other complications and its impact can be more intensive in patients with moderate-severe pulmonary problems. Elevation of MMP-9/TIMP-4 complex in SM exposed group with normal lung, including smokers, is probably caused by smoking. Increased serum level of TIMP- 4 has been reported in COPD patients who have lung problems, often due to smoking.[22] Also, no significant associations were found between MMP-9 and its inhibitors and spirometry parameters.

Similar increases in expression of MMP-9 have been described in bronchoalveolar lavage fluid (BAL) and lung tissue derived from SM treated rats and guinea pigs.[12,23] Study on intratracheally SM exposed rats exhibited a dose- and time-dependent rise in MMP-9 expression in the lung which mostly pertained to the bronchiolar epithelium and alveolar macrophages. In addition, MMP-9 protein and gelatinase activity were increased in BAL.[12] Furthermore, similar evidence on unchanged level of TIMP-1 and 2, indicates an imbalance between MMP-9 and its inhibitors causing tissue proteolytic disruption.[24] Animal model studies have investigated MMP-9 in acute phase of exposure but this work reported its role in long-term phase; however, these data are consistent. In the present study, the severity of pulmonary complication was classified by spirometry and clinical findings. Hence, association between these injuries and remodeling factors with spirometry parameters and clinical findings such as chronic chough, hemoptysis, dyspnea and pulmonary auscultation were evaluated. Recently, we showed the long-term pulmonary problems in sulfur mustard injured group in SICS.[25] In that group, which is the same as the group in the present study, chronic cough, cough severity, sputum, hemoptysis, dyspnea, pattern of dyspnea, severity of dyspnea, and chest pain were statistically different between the SM exposed and control groups. Wheezing was the most common respiratory finding. Although MMP-9 and MMP-9/TIMP complexes did not have any correlation with spirometry findings, a significant elevation was shown in MMP-9 in the exposed group with chronic chough and hemoptysis in comparison to the exposed participants without these symptoms. Thus, considering spirometry findings alone is not enough and pulmonary signs and symptoms must be taken into account in investigation of SM injuries.

Some evidence suggests a possible role for MMP-9 in pathogenesis of other respiratory problems such as COPD and BO which have similar pathologic features with SM induced pulmonary complications.[10,26] Navrativola and colleagues[22] described a statistically significant elevation of serum level of MMP-9 and TIMP-1 and 4 in 74 COPD patients compared with 20 control subjects. The increased concentration of MMP-9 paralleled GOLD stage. In this study, measurement was performed using the multiple microsphere technology. However, contradicting results were reported by Pinto-Plata[27] and D'Armiento[28] w2ho had greater sample sizes and used the ELISA technique. Higashimoto and colleagues[29] and Olafsdottir *et al.*[30] demonstrated an indirect correlation between the serum level of MMP-9 and FEV1 ($r = -0.28$ and $r = -0.11$ $P < 0.01$). However, Bolton and colleagues,[31] described no correlation between the level of MMP-9 with FEV1 in 70 patients with COPD. Contradictory results obtained from COPD patients may be due to the difference in study population and techniques; our methodology is more similar to that used in the studies by Pinto-Plata and D'Armiento.

The disparity between our data and recent reports could have two reasons. First, according to our previous reports,[32] the systemic conditions of SM injured patients are different from individuals suffering from COPD. Furthermore, the severity of pulmonary complications was classified based on spirometry and

physical exam findings in this study, while COPD patient are categorized based on Global initiative for chronic Obstructive Lung Disease (GOLD) classification and spirometry data alone. For this reason, our group also reported no correlation between MMP-9 and its inhibitors with pulmonary complication severity in SM exposed patients categorized by the GOLD classification.[33] Likewise, serum levels of MMP-9 and TIMP-2 were not different in BO syndrome (BOS) patients compared to the control group. In contrast, the BAL MMP-9 and TIMP-1 levels were significantly elevated in BOS subjects compared to the control population.[34] Our previous study showed no correlation between sputum level of MMP-9 and spirometry findings. It could be suggested that evaluation of MMP-9 and its inhibitors in BAL and locally tissue samples may provide a better perspective on their probable role in pathogenesis of mustard lung.

Owing to MMPs potential to have tremendous effect on structural and biochemical units to abrupt microenvironment, precise regulation of metalloproteinase activity is vital for tissue homeostasis.[13] Tissue inhibitors of matrix metalloproteinases (TIMP) are chief endogenous inhibitors of MMP in tissue. Four similar proteins known as TIMP-1, TIMP-2, TIMP3 and TIMP-4 have been recognized. MMPs form a noncovalent complex with TIMPs in a 1:1 ratio with high dissociation constant Kd (10-9–10-10).[35,36] TIMP-1 favorably constitutes a complex with MMP-9.[36] This is supported by our findings that MMP-9 has a significant correlation with MMP-9/TIMP-1 complex in SM injured patient. Likewise, TIMP-2 forms complexes with all MMPs but it preferentially binds with MMP-2. Although TIMP-2 did not have a powerful relationship with MMP-9 like TIMP-1, its significant correlation with MMP-9 indicates its remarkable role in inhibition of this enzyme activity in SM exposed patients.

In conclusion, elevation of tissue disruption and remodeling mediators could affect lung structure in long-term stage of chemical victims like the acute phase. Furthermore, molecular features of circulating biomarkers of SM injured patients may differ from similar pathologic diseases such as COPD and BO and further investigation is needed to identify effective factors on mustard gas exposed tissues in order to offer proper treatment.

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Declaration of interest

The authors report no conflict of interest in this study

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