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Tear and serum interleukin-8 and serum CX3CL1, CCL2 and CCL5 in sulfur mustard eye-exposed patients

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ABSTRACT

Background: The serum and tear levels of four inflammatory chemokines were evaluated in sulfur mustard (SM)-exposed with serious ocular problems.

Materials and methods: In this study, 128 SM-exposed patients and 31 healthy control participants participated. Tear and serum levels of chemokines were assessed by ELISA method.

Results: There was no significant difference in the serum level of IL-8/CXCL8, CX3CL1/fractalkine, CCL2/MCP-1, and CCL5/RANTES between all SM-exposed subjects and control groups. The tear level of IL-8 in the SM-exposed group was lower than the control group, but the difference was not significant. In the SM-exposed group with the abnormalities in tear breakup time (TBUT) test, fundus and pannus formation were significantly higher than SM-exposed patients without these problems. CX3CL1 levels have significantly increased in SM-exposed group with blepharitis, pterygium, and conjunctival pigmentation as compared with the control group. Besides, significantly higher levels of CX3CL1 were observed in SM-exposed group with or without bulbar conjunctival hyperemia and abnormal vessels a well as with fundus abnormality compared to the control group. Only, SM-exposed group with subconjunctival fibrosis had significantly lower levels of CCL5 than SM-exposed group without this problem.

Conclusion: The higher level of CX3CL1 and consistent levels of IL-8/CXCL8, MCP-1/CCL2, and RANTES/CCL5 in SM-exposed individuals may indicate an anti-inflammatory response against the destructive effects of SM gas. High tear level of IL-8/CXCL8 reflects the severity of ocular surface abnormalities, yet significantly low tear level found in mild SM-exposed subgroup compared with the control group. The lower levels of CX3CL1 and RANTES/CCL5 may represent the different pathophysiology which requires further studies.

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1. Introduction

Sulfur mustard (SM) is a chemical warfare agent used against the Iranian army and civilians by Iraqi military forces from 1980 to 1988 [1]. The main targets of SM are the eyes, skin, and respiratory system [2]. Varying degrees of ocular abnormalities, including chronic blepharitis, meibomian gland dysfunction (MGD), dry eye, limbal ischemia, limbal stem cell deficiency, aberrant conjunctival vessels, corneal neovascularization; and secondary degenerative changes, like lipid and amyloid deposition, corneal irregularity, thinning and scarring has been reported in 75% to 90% of SM-exposed individuals [3]. Photophobia, ocular surface discomfort (burning, itching, and redness), bulbar conjunctival abnormalities, and limbal tissue changes were reported by Ghasemi et al. among SM-exposed victims in Sardasht-Iran cohort study (SICS) [4]. In the previous study, we have shown alterations in the serum levels of chemokines in Sardasht City residents 20 years after SM exposure [5]. In addition, we found a positive association between serum levels of proinflammatory cytokines (interleukin [IL]-1β, IL-8, IL-12 and RANTES [regulated on activation, normal T cell expressed and secreted]) and acne among SM-exposed population in Sardasht City, Iran [6].

Chemokines are a large family of cytokines produced during inflammatory reactions. They participate not only in the regulation of inflammation and various immune responses but also organ development, angiogenesis/angiostasis, leukocyte trafficking and homing, tumorigenesis, and metastasis. Chemokines are classified into four major groups of CXC (α chemokines), CC (β chemokines), C (γ chemokines), and CX3C (δ chemokines) [7]. The CC and CXC chemokines are chemotactic for monocytes and neutrophils, respectively [5]. The expression of CX3CL1 (called also fractalkine) has been reported in a variety of ocular tissues and cells which is thought to be involved in regulating leukocyte migration in inflammatory eye diseases such as anterior uveitis and retinochoroiditis [8]. The contribution of chemokines such as chemokine (C-X-C motif) ligand 8 (CXCL8) or interleukin (IL)-8, CXCL10/IP-10 (interferon g-inducible protein-10), chemokine (C—C motif) ligand 2 (CCL2) or monocyte chemoattractant protein 1 (MCP-1), chemokine (C—C motif) ligand 5 (CCL5) or RANTES (regulated on activation, normal T cell expressed and secreted), and chemokine (C—C motif) ligand 4 (CCL4) or macrophage inflammatory protein-1 beta (MIP-1b) in eye inflammation has been already investigated [9] whereas their involvement in ocular abnormalities in response to SM exposure has not been studied. To evaluate eye injuries of SM exposure, we measured the serum and tear levels of chemokines in the Iranian war veterans exposed to SM with ocular abnormalities. Moreover, assessing the involvement of chemokines in SM-exposed individuals with various ocular abnormalities can help develop new therapies. In this study, the serum levels of four inflammatory chemokines of MCP-1/CCL2, RANTES/CCL5, IL-8/CXCL8, and CX3CL1/fractalkine as well as the tear level of IL-8/CXCL8 were evaluated in SM-exposed individuals with ocular problems.

2. Materials and methods

2.1. Study design and participants

This study performed on 128 veterans with SM-induced serious eye injuries and 31 healthy control participants after receiving written informed consent. The study design approval received from the Ethics Committee of Janbazan Medical and Engineering Research Center (JRMEC). All controls were male and age-matched. A history of ocular manifestations, including photophobia, ocular surface discomfort (burning, itching, and redness), foreign-body sensation, tearing, pain, blurred vision, and dry eye sensation was obtained. Then, a complete ocular examination was done by the expert ophthalmologist using a slit lamp biomicroscope (NIDEK Co., Japan), including abnormality in lid (meibomian gland dysfunction (MGD), trichiasis, blepharitis and punctal abnormality), tear status (tear break up time [TBUT] test and tear meniscus height), bulbar conjunctiva (limbal ischemia, hyperemia, abnormal vessels, pterygium and subconjunctival fibrosis), limbal tissue (pigmentation, abnormal vessels and pannus), cornea (calcium deposition, melting, verticillata, vascularization, epithelial, stromal, and endothelial abnormalities), lens (nuclear sclerotic [NS], cortical [C] and posterior subcapsular [PS] cataract) and fundus. Evaluation of the posterior segment was carried out using direct and indirect ophthalmoscopy (HEINE, Germany). Final ophthalmologic assessments were categorized into normal, mild, moderate, and severe, based on the chart of the War Veterans Foundation (Iranian Ophthalmic Committee of Chemical Warfare Veterans) [10]. Demographic and clinical data of the studied group were described in our previous study [11].

2.2. Serum preparation

For serum preparation, we have drawn blood samples of all subjects which after clotting, the sera were separated, aliquoted, and kept at −80 °C.

2.3. Tear collection

One drop (200 μL) of sterile 5% sodium chloride instilled in the inferior fornices of both eyes to avoid any ocular surface trauma from capillary or microtubes. The available basal and reflex tears samples collected via the placement of a sterile Weck-Cel sponge in the lacrimal lake for a few minutes from 118 veterans and 20 controls, immediately [12]. Dilution of tear samples conducted in special tubes using 200 μL of RPMI medium was and afterward, centrifuged at 2000 × *g* for 20 min to isolate the tear fluid from the Weck-Cel sponges. Samples stored at −80 °C pending laboratory measurements.

2.4. Chemokine measurement

Four chemokines were selected because they represent markers implicated in infectious disease pathobiology, including inflammation and endothelial activation. The serum concentration of chemokines (IL-8/CXCL8, CX3CL1/fractalkine, CCL2/MCP-1, and CCL5/RANTES) in SM-exposed and control groups were measured using Human DuoSet® ELISA Development kits (R&D Systems, US) according to the manufacturers' instructions as described previously [13]. We validated ELISA kits before use, and we obtained appropriate samples dilutions for each biomarker by testing a dilution curve of serum received from subjects. Chemokine concentrations (pg/mL) were deduced from the equivalent standard curve.

2.5. Statistical analysis

The Mann-Whitney test and Interquartile range compared chemokines levels between the SM-exposed and control groups as data were not normally distributed. A difference of $P \leq 0.05$ was considered to be statistically significant. The data are presented as mean (SD) and median (first and third quartiles). Because of deep departure from a normal distribution, box plots were used to show the data. Statistical analyses were performed in SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Clinical findings

We already described demographic information, ocular history, ophthalmoscopic, and slit-lamp findings of subjects [11]. In brief, all subjects were male with a mean age of 44.86 and 50 years in SM-exposed and controls, respectively. The mean percentage of veterans' disability determined by the chart of the Foundation of Martyrs and Veterans Affairs as 58.85% and the mean duration of their disease was

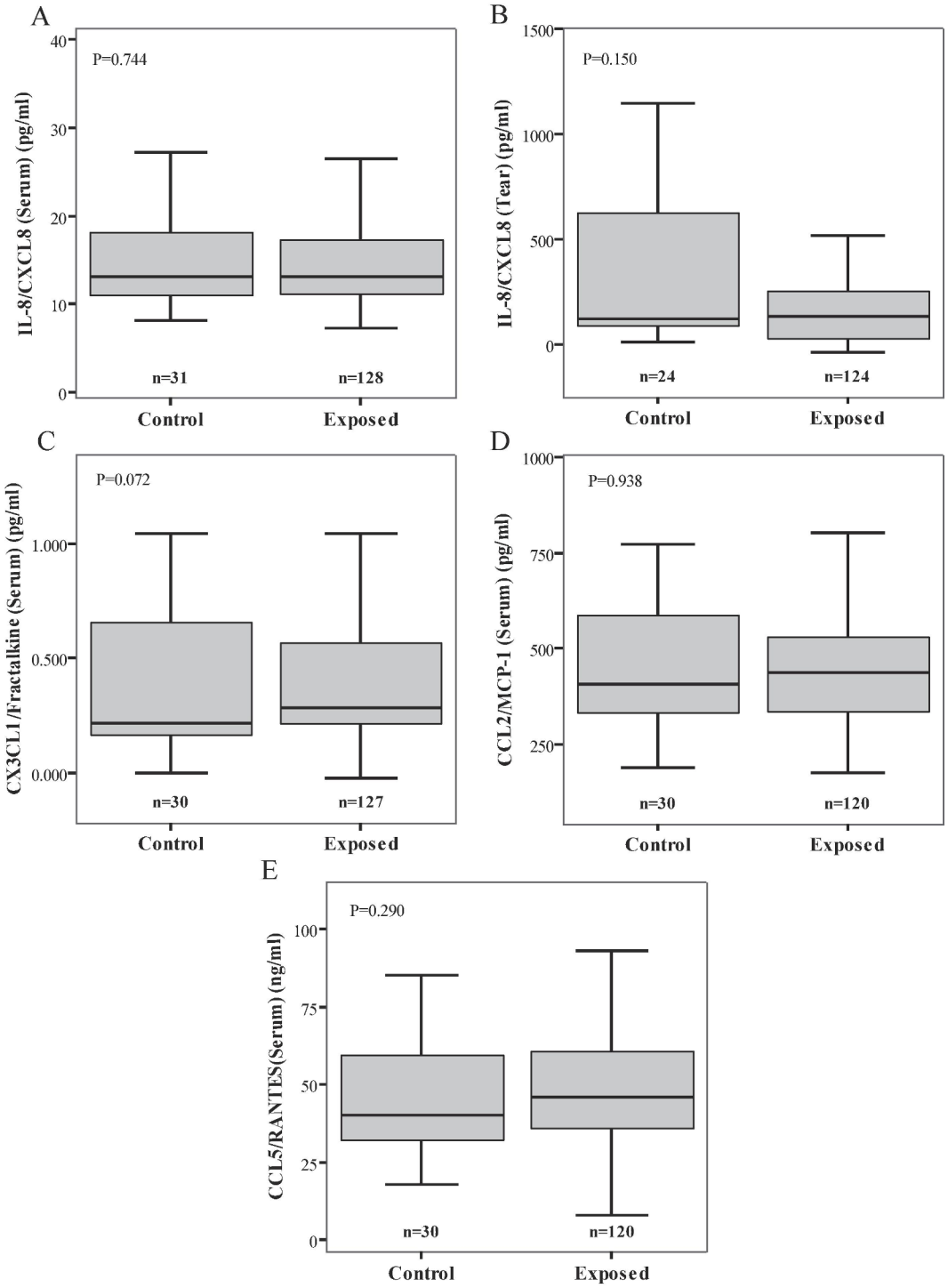

Fig. 1. Comparisons of the levels of chemokines (pg/ml) between SM-exposed and control groups. A: IL-8/CXCL8 in serum, B: IL-8/CXCL8 in tear, C: CX3CL1/Fractalkine in serum, D: CCL2/MCP-1 in serum, and E: CCL5/RANTES in serum. Data represented as Median (first and third quartile).

21.58 years. Frequency of exposure to SM was one, two, and more than two times in 65.2%, 24.3%, and 10.5% of SM-exposed subjects, respectively and the majority of them had at least 3 to 5 symptoms. As shown in the Supplementary Table majority (60.9%) of SM-exposed subjects had severe ocular injuries. Summary of slit lam and ophthalmoscopy examination findings presented in the Supplementary Table. The most common ocular symptoms were photophobia, sense of decreased vision, dry eye sensation, foreign body sensation, tearing, and pain with a frequency of 73.2%, 72.5%, 66.4%, 61.1%, 46.3%, and 43%, respectively.

3.2. Serum and tear levels of chemokines

Fig. 1 shows the comparisons of the tear level IL-8/CXCL8 and serum levels of chemokines (IL-8/CXCL8, CX3CL1/fractalkine, CCL2/MCP-1, and CCL5/RANTES) presented by median (first and third quartile) in SM-exposed and control groups.

There was no significant difference in the serum level of IL-8/CXCL8, CX3CL1/fractalkine, CCL2/MCP-1, and CCL5/RANTES between all SM-exposed subjects and the control group.

Although the tear level of IL-8 in the SM-exposed was lower than

Table 1
Association of chemokines with severity of ocular injury (Mild, Moderate and severe) in Sulfur mustard group and their comparison with control group.

	Ophthalmic complication severity	N	Median	Q_1	Q_3	Mean	SD	P-value[1]	P-value[2]	P-value[3]
IL-8/CXCL8 (Serum) (pg/ml)	Control	31	13.17	10.94	18.36	20.059	22.540			
	Mild	19	12.4	11.68	17.8	17.492	17.840	0.660		
	Moderate	31	13.03	11.45	16.54	16.491	11.030	0.767	0.976	
	Severe	78	13.145	11.18	20.57	20.678	36.789	0.830	0.730	0.898
CX3CL1/fractalkine (Serum) (pg/ml)	Control	30	0.214	0.165	0.656	0.339	0.278			
	Mild	18	0.296	0.205	0.586	0.427	0.370	0.229		
	Moderate	31	0.286	0.219	0.620	0.409	0.311	0.085	0.820	
	Severe	78	0.281	0.214	0.555	0.419	0.343	0.119	0.985	0.848
CCL5/RANTES (Serum) (pg/ml)	Control	30	400.1	321.5	596.3	462.3	194.6			
	Mild	18	473.3	374.7	632.9	474.8	184.9	0.602		
	Moderate	30	513.2	375.6	679.2	548.2	231.8	0.156	0.443	
	Severe	72	453.8	360.1	588.2	489.8	194.3	0.443	0.964	0.290
CCL2/MCP-1 (Serum) (pg/ml)	Control	30	406.7	331.5	587.8	447.9	171.8			
	Mild	18	435.0	338.7	502.1	464.7	192.4	0.749		
	Moderate	30	496.7	294.5	573.9	465.0	176.8	0.668	0.565	
	Severe	72	397.4	348.3	499.4	427.5	139.1	0.840	0.614	0.227
IL-8/CXCL8 (Tear) (pg/ml)	Control	24	119.40	90.34	623.75	419.8	628.9			
	Mild	18	51.98	23.38	172.9	125.4	170.8	**0.010**		
	Moderate	30	142.27	34.78	343.0	1000.1	2999.4	0.520	0.217	
	Severe	76	143.18	31.31	273.2	666.4	2340.6	0.262	0.080	0.966

pg/mL: pico-grams per milliliter; N: number; Q1: First quartile; Q3: Third quartile; SD: standard deviation.
SM-exposed group was categorized in there sub groups (mild/moderate/severe) according to present ocular problems. Cytokine level was compared between all subgroups with control and each other via Mann-Whitney test.
P-value[1]: Comparison of SM-exposed subgroups (mild, moderate, and severe) with control group.
P-value[2]: Comparison of moderate and severe subgroups with mild group.
P-value[3]: Comparison of moderate with sever subgroups.
P-value < 0.05 in bold.

Table 2
Association of serum level of IL-8 (CXCL8) with ocular findings in Sulfur mustard group and their comparison with control group.

Slit Lamp		IL-8/CXCL8 (Serum) (pg/ml)						P-value[1]	P-value[2]
		N	Median	Q_1	Q_3	Mean	SD		
	Control	31	13.17	10.94	18.36	20.059	22.540		
Tear status - tear meniscus height	> 1 mm	13	11.81	10.8	15.43	13.840	5.178	0.354	
	< 1 mm	115	13.13	11.28	19.17	19.796	31.527	0.852	0.388
Tear status – tear break up time	> 10"	20	13.79	10.67	16.07	17.088	11.069	0.736	
	< 10"	108	13.03	11.23	18.48	19.581	32.297	0.771	0.914
Bulbar conjunctiva - subconjunctival fibrosis	No	77	13.54	10.89	20.57	22.177	38.187	0.873	
	Yes	51	13.03	11.49	15.17	14.684	5.475	0.622	0.683
Limbus - abnormal vessels	No	35	13.63	11.49	16.54	14.401	4.742	0.700	
	Yes	93	13.03	11.18	20.19	20.994	34.919	0.795	0.889
Limbus - pannus	No	87	13.54	11.31	19.75	17.551	13.357	0.932	
	Yes	41	12.19	11.18	15.14	22.671	49.491	0.488	0.357
Cornea - verticillata	No	111	13.16	11.18	19.75	19.764	31.924	0.836	
	Yes	17	12.4	11.63	14.58	15.449	10.209	0.477	0.476
Stroma	Normal	17	12.39	11.74	16.53	17.442	13.712	0.889	
	Abnormal	111	13.13	11.18	17.8	19.459	31.763	0.735	0.933
Corneal vascularization	Normal	115	13.13	11.28	17.8	19.656	31.522	0.769	
	Abnormal	13	12.4	10.86	16.96	15.077	6.641	0.728	0.729
Fundus	Normal	119	13.03	11.18	16.96	19.386	31.013	0.702	
	Abnormal	9	13.66	12.26	26.06	16.614	7.794	0.808	0.689

pg/mL: pico-grams per milliliter; N: number; Q1: First quartile; Q3: Third quartile; SD: standard deviation.
Cytokine level was compared between all slit lamp ocular findings (no/yes or normal/abnormal) subgroups with control and each other via Mann-Whitney test.
P-value[1]: Comparison of SM-exposed subgroups with control group.
P-value[2]: In the SM-exposed group comparison between with and without ocular findings.

that in the control group (72.27 vs. 86.04 pg/mL), it was not statically significant (P = 0.15; Fig. 1B).

3.3. Association of chemokines with the severity of the ocular injury

Regarding the serum levels of four studied chemokines, there were no significant differences between SM-exposed subgroups (with mild, moderate, or severe ocular injuries) with the control group (Table 1). However, significantly lower tear levels of IL-8/CXCL8 was observed in the exposed group who had mild ocular involvement compared to the control group (Table 1).

3.4. Association between serum and tear levels of IL-8/CXCL8 and serum levels of other chemokines and slit lamp findings in SM-exposed group

There is no association between serum level of chemokines and slit lamp findings in SM-exposed group (Table 2).

The tear level of IL-8 in the SM-exposed individuals with an abnormality in TBUT, limbal tissue (pannus), and fundus was significantly higher than SM-exposed individuals without these problems (Fig. 2A–C

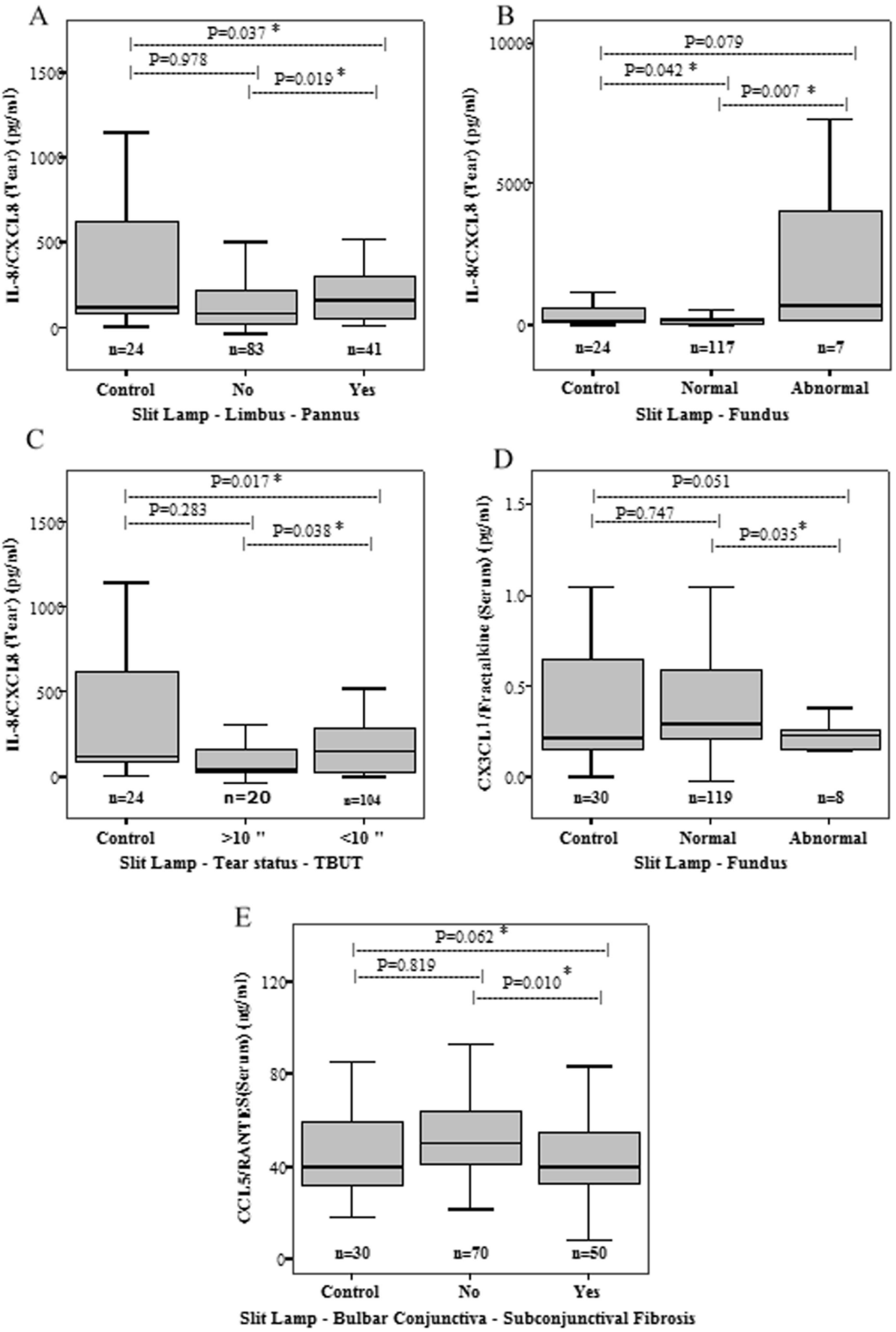

Fig. 2. Comparisons of the levels of chemokines (pg/ml) between SM-exposed with different ocular abnormalities and control groups. *: p-value < 0.05.

and Table 3). The significantly highest tear level of IL-8 belonged to the SM-exposed group with fundus abnormality compared to that in the control group (P = 0.042) and also than SM-exposed individuals without this problem (Fig. 2C, P = 0.007).

However, CX3CL1 levels were significantly higher in SM-exposed individuals with blepharitis (P = 0.042), pterygium (P = 0.019), and conjunctival pigmentation (P = 0.044) as compared with those levels in the control group. In addition, in SM-exposed with or without bulbar conjunctival hyperemia and abnormal vessels, we observed significantly higher levels of CX3CL1 compared to those levels in the control group (P = 0.045, P = 0.049, P = 0.045, and P = 0.044, respectively). As seen in Fig. 2D and Table 4, the development of fundus

Table 3
Association of tear level of IL-8 (CXCL8) with ocular findings in Sulfur mustard group and their comparison with control group.

Slit Lamp		IL-8 (CXCL8) (Tear) (pg/ml)						P-value[1]	P-value[2]
		N	Median	Q_1	Q_3	Mean	SD		
	Control	24	119.40	90.34	623.75	419.8	628.9		
Tear status - tear meniscus height	> 1 mm	13	108.40	25.14	158.20	131.4	141.4	0.124	
	< 1 mm	111	131.78	30.12	284.40	731.5	2479.8	0.189	0.239
Tear status – tear break up time	> 10"	20	41.64	23.30	163.9	213.879	536.340	**0.017**	
	< 10"	104	142.98	33.63	288.2	756.019	2551.370	0.283	**0.038**
Bulbar conjunctiva - subconjunctival fibrosis	No	73	134.78	38.24	292.0	758.2	2722.4	0.204	
	Yes	51	120.50	26.06	233.0	540.3	1707.7	0.156	0.556
Limbus - abnormal vessels	No	33	62.50	23.20	202.8	242.6	477.6	**0.040**	
	Yes	91	137.78	38.24	284.4	823.1	2719.1	0.302	0.111
Limbus - pannus	No	83	79.77	27.46	232.0	482.9	1845.6	**0.037**	
	Yes	41	161.94	62.50	304.2	1044.5	3134.1	0.978	**0.019**
Cornea - verticillata	No	107	120.50	29.18	246.6	641.7	2427.7	0.118	
	Yes	17	149.56	38.24	385.0	837.8	1863.4	0.711	0.311
Stroma	Normal	16	173.65	31.50	313.3	200.7	186.6	0.473	
	Abnormal	108	119.60	29.46	247.6	737.9	2514.1	0.139	0.711
Corneal vascularization	Normal	112	133.28	29.32	288.2	639.3	2304.6	0.154	
	Abnormal	12	118.40	36.52	221.6	941.9	2865.8	0.365	0.823
Fundus	Normal	117	108.40	29.18	233.0	543.1	2170.3	0.079	
	Abnormal	7	686.00	177.70	7308.0	2765.7	4120.5	**0.042**	**0.007**

pg/mL: pico-grams per milliliter; N: number; Q1: First quartile; Q3: Third quartile; SD: standard deviation.
Cytokine level was compared between all slit lamp ocular findings (no/yes or normal/abnormal) subgroups with control and each other via Mann-Whitney test.
P-value[1]: Comparison of SM-exposed subgroups with control group.
P-value[2]: In the SM-exposed group comparison between with and without ocular findings.
P-value < 0.05 in bold.

Table 4
Association of serum level of CX3CL1/fractalkine with ocular findings in Sulfur mustard group and their comparison with control group.

Slit Lamp		CX3CL1 (Serum) (pg/ml)						P-value[1]	P-value[2]
		N	Median	Q_1	Q_3	Mean	SD		
	Control	30	0.214	0.165	0.656	0.339	0.278		
Tear status - Tear Meniscus Height	> 1 mm	13	0.284	0.227	0.530	0.413	0.306	0.244	
	< 1 mm	114	0.288	0.214	0.580	0.418	0.341	0.077	0.849
Tear status – Tear Break Up Time	> 10"	20	0.358	0.248	0.616	0.450	0.254	0.080	
	< 10"	107	0.280	0.209	0.506	0.411	0.351	0.101	0.245
Bulbar Conjunctiva - Subconjunctival Fibrosis	No	76	0.277	0.215	0.508	0.393	0.339	0.152	
	Yes	51	0.296	0.206	0.620	0.453	0.333	0.051	0.452
Limbus - Abnormal vessels	No	34	0.296	0.223	0.586	0.395	0.239	0.102	
	Yes	93	0.280	0.209	0.530	0.426	0.367	0.097	0.675
Limbus - Pannus	No	86	0.282	0.226	0.519	0.393	0.272	0.066	
	Yes	41	0.296	0.194	0.600	0.468	0.443	0.194	0.930
Cornea - Verticillata	No	110	0.294	0.211	0.586	0.424	0.344	0.075	
	Yes	17	0.264	0.231	0.334	0.377	0.286	0.236	0.721
Stroma	Normal	17	0.297	0.205	0.637	0.402	0.268	0.215	
	Abnormal	110	0.285	0.215	0.555	0.420	0.347	0.078	0.975
Corneal vascularization	Normal	115	0.284	0.214	0.580	0.416	0.342	0.081	
	Abnormal	12	0.297	0.218	0.575	0.431	0.297	0.200	0.711
Fundus	Normal	119	0.295	0.215	0.600	0.430	0.344	0.051	
	Abnormal	8	0.230	0.159	0.265	0.229	0.080	0.747	**0.035**

pg/mL: pico-grams per milliliter; N: number; Q1: First quartile; Q3: Third quartile; SD: standard deviation.
Cytokine level was compared between all slit lamp ocular findings (no/yes or normal/abnormal) subgroups with control and each other via Mann-Whitney test.
P-value[1]: Comparison of SM-exposed subgroups with control group.
P-value[2]: In the SM-exposed group comparison between with and without ocular findings.
P-value < 0.05 in bold.

abnormality in the SM-exposed group was associated with significantly lower levels of CX3CL1 compared to SM-exposed group without this abnormality (P = 0.035). Only, SM-exposed individuals with an abnormality in bulbar conjunctiva (subconjunctival fibrosis) had significantly lower levels of CCL5 compared to SM-exposed individuals without this problem (Fig. 2E, P = 0.01) (Table 5).

There is no association between serum level of MCP-1/CCL2 and slit lamp findings in SM-exposed group (Table 6).

4. Discussion

Findings of this investigation showed that the serum level of CX3CL1 has increased in SM-exposed individuals. Measurement of the serum level of CX3CL1 in SM-exposed group who had eye abnormalities compared to SM-exposed individuals without these abnormalities and healthy control groups indicate that CX3CL1 level has decreased in the SM-exposed group with fundus abnormality compared with SM-exposed without this abnormality, and significantly increased in SM-exposed with subconjunctival fibrosis as well as in SM-exposed with

Table 5
Association of serum level of CCL5/RANTES with ocular findings in Sulfur mustard group and their comparison with control group.

Slit Lamp		RANTES/CCL5 (Serum) (pg/ml)						P-value[1]	P-value[2]
		N	Median	Q_1	Q_3	Mean	SD		
	Control	30	400.1	321.5	596.3	462.3	194.6		
Tear status - tear meniscus height	> 1 mm	12	478.6	406.4	588.3	511.0	190.3	0.419	
	< 1 mm	108	459.1	360.1	607.8	501.1	205.3	0.312	0.793
Tear status – tear break up time	> 10"	19	499.8	414.1	679.1	540.0	199.1	0.176	
	< 10"	101	457.6	360.6	604.7	495.0	203.9	0.384	0.316
Bulbar conjunctiva - subconjunctival fibrosis	No	70	501.5	412.2	641.6	538.3	196.6	0.062	
	Yes	50	400.1	328.1	549.4	451.6	203.1	0.819	**0.010**
Limbus - abnormal vessels	No	33	518.8	374.7	663.5	545.3	226.4	0.125	
	Yes	87	457.7	360.6	593.9	485.7	192.4	0.479	0.277
Limbus - pannus	No	80	455.5	376.1	671.4	527.5	219.4	0.136	
	Yes	40	467.0	333.5	553.2	451.3	155.9	0.972	0.142
Cornea - verticillata	No	105	482.3	363.2	632.9	508.8	207.5	0.253	
	Yes	15	428.9	341.0	524.8	455.3	167.7	0.828	0.347
Stroma	Normal	17	518.8	399.1	740.1	548.0	201.5	0.124	
	Abnormal	103	457.7	359.5	599.0	494.5	203.3	0.403	0.332
Corneal vascularization	Normal	109	457.2	359.5	606.8	497.9	207.8	0.377	
	Abnormal	11	545.2	416.4	641.3	544.2	149.6	0.122	0.285
Fundus	Normal	113	457.2	359.5	604.7	497.6	205.5	0.356	
	Abnormal	7	529.6	428.9	740.1	574.6	151.4	0.130	0.208

pg/mL: pico-grams per milliliter; N: number; Q1: First quartile; Q3: Third quartile; SD: standard deviation.
Cytokine level was compared between all slit lamp ocular findings (no/yes or normal/abnormal) subgroups with control and each other via Mann-Whitney test.
P-value[1]: Comparison of SM-exposed subgroups with control group.
P-value[2]: In the SM-exposed group comparison between with and without ocular findings.
P-value < 0.05 in bold.

Table 6
Association of serum level of CCL2/MCP-1 with ocular findings in Sulfur mustard group and their comparison with control group.

Slit Lamp		MCP-1/CCL2 (Serum) (pg/ml)						P-value[1]	P-value[2]
		N	Median	Q_1	Q_3	Mean	SD		
	Control	30	406.7	331.5	587.8	447.9	171.8		
Tear status - tear meniscus height	> 1 mm	12	484.8	386.5	555.8	445.5	131.3	0.717	
	< 1 mm	108	435.0	330.1	522.0	442.1	160.6	0.986	0.460
Tear status – tear break up time	> 10"	19	439.6	370.3	575.1	455.3	144.0	0.652	
	< 10"	101	435.0	331.4	514.8	440.0	160.3	0.976	0.463
Bulbar conjunctiva - subconjunctival fibrosis	No	70	428.4	346.6	514.8	438.3	147.6	0.967	
	Yes	50	436.3	328.8	535.9	448.3	171.6	0.913	0.949
Limbus - abnormal vessels	No	33	436.3	331.5	539.5	461.8	176.0	0.741	
	Yes	87	435.0	338.7	513.0	435.1	150.2	0.963	0.508
Limbus - pannus	No	80	435.7	349.0	537.7	451.7	163.9	0.783	
	Yes	40	436.3	305.7	518.1	424.0	143.9	0.771	0.497
Cornea - verticillata	No	105	435.0	338.7	529.3	444.3	161.9	0.918	
	Yes	15	454.8	299.1	539.5	429.4	125.6	0.942	0.994
Stroma	Normal	17	435.0	370.4	549.8	471.9	170.3	0.565	
	Abnormal	103	436.3	328.8	514.8	437.6	155.6	0.959	0.489
Corneal vascularization	Normal	109	435.0	338.7	532.3	444.2	161.5	0.923	
	Abnormal	11	449.5	296.5	481.2	425.2	114.1	0.941	0.902
Fundus	Normal	113	435.0	328.8	514.8	440.7	160.6	1.000	
	Abnormal	7	476.6	362.5	564.9	470.7	96.1	0.522	0.382

pg/mL: pico-grams per milliliter; N: number; Q1: First quartile; Q3: Third quartile; SD: standard deviation.
Cytokine level was compared between all slit lamp ocular findings (no/yes or normal/abnormal) subgroups with control and each other via Mann-Whitney test.
P-value[1]: Comparison of SM-exposed subgroups with control group.
P-value[2]: In the SM-exposed group comparison between with and without ocular findings.

normal fundus compared with control. CX3CR1 is expressed on all retinal microglial cells [14]. It has been shown that CX3CL1 is involved in severe dry eye disease [15] and binding of CCL2 and CX3CL1 to their receptors, CCR2 and CX3CR1, respectively are involved in the migration of macrophages to tissue lesions or sites of inflammation [16]. It seems that similar to our previous study in Sardasht [13], an elevated level of CX3CL1 in SM-exposed individuals is a defense mechanism against the destructive effects of SM. Also, a reduced immune response (CX3CL1) in SM-exposed with fundus abnormality in comparison SM-exposed without this abnormality may be due to small sample size or a different pathological feature.

We found no difference in serum levels of all four measured chemokines (IL-8/CXCL8, CX3CL1, MCP-1/CCL2, and RANTES/CCL5) between the SM-exposed and unexposed groups of study participants. But serum RANTES/CCL5 level in SM-exposed group with an abnormality in bulbar conjunctiva (subconjunctival fibrosis) was lower in comparison with SM-exposed group without this abnormality. In contrast, the serum level of RANTES/CCL5 in SM-exposed group without defect in bulbar conjunctiva (subconjunctival fibrosis) was higher than healthy individuals (control group). RANTES/CCL5 recruits monocytes, lymphocytes, basophils, mast cells, and eosinophils in inflammatory disorders [17]. High serum level of RANTES/CCL5 in SM-exposed group

without abnormality in bulbar conjunctiva (subconjunctival fibrosis) indicates an immune response against exposure to SM. However, this response seems to be suppressed in SM-exposed group with an abnormality in bulbar conjunctiva (subconjunctival fibrosis). The results of chemokine evaluations on Sardasht population 20 years after SM exposure showed elevated levels of MCP-1/CCL2 and reduced levels of IL-8/CXCL8 and RANTES/CCL5 in the SM-exposed group compared to the unexposed control group [5]. Another explanation for this inconsistency is the difference in the geographical environment and the number of people exposed to SM gas. Unlike Sardasht study, the participants of this investigation were not limited to a specific part of our country and one time exposure to SM gas. Beside, SM-exposed individuals in Sardasht had mild ocular complications, while at the present study, SM-exposed individuals with ocular complications were intentionally invited, and most of them had severe ocular involvement.

Tear level of IL-8/CXCL8 in SM-exposed groups with an abnormality in TBUT, limbal tissue (pannus), or fundus were higher than SM-exposed group without these abnormalities. Lam et al. also showed that individuals with dysfunctional tear syndrome (DTS) had higher IL-8/CXCL8 tear level [18]. IL-8 has been detected in the tear of patients with inflammatory ocular surface diseases, such as seasonal allergic conjunctivitis and vernal and atopic keratoconjunctivitis [19]. It shows a correlation between the severity of ocular surface epithelial disease with various chemokines such as IL-8 [18] and ocular pain levels with tear IL-8 [20].

Comparison of tear level of IL-8/CXCL8 between the control group and SM-exposed group with mild, moderate, or severe ocular injuries reveals that SM-exposed group with mild ocular injuries has a significantly lower level of IL-8/CXCL8 than the control group ($P = 0.010$, Table 1). This finding, which is consistent with obtained results of our previous study in Sardasht demonstrates the inhibition of this inflammatory cytokine (IL-8/CXCL8) in patients with mild ocular injuries while SM-exposed group with moderate or severe ocular injuries have a higher level of IL-8/CXCL8 than the control group (Table 1), although these higher levels are statistically insignificant.

IL-8 can be produced by inflamed epithelial cells and might indicate ocular surface stress [18]. One hypothesis to explain the increased tear level of IL-8 is upregulation of IL-8 gene in the conjunctival epithelium, which causes increased production of IL-8 in tear. It should be noted that the above hypothesis has been proposed for the increased concentration of cytokines in tears of individuals with dry eye [21]. Increased tear level of IL-8/CXCL8 in SM-exposed groups with an abnormality in TBUT, limbal tissue (pannus) or fundus might provide a target to develop specific chemokine antagonists for the treatment of individuals with these ocular abnormalities. In our previous study tear IL-8 level in SM-exposed cases without any ocular surface pathology in the eyelids, bulbar conjunctiva, cornea, tear status, limbus and slit lamp examination was significantly lower than that in the normal controls [22]. An explanation for this observation might be the local immunosuppressive effect of SM in exposed subjects without any ocular abnormalities and with mild ocular involvement.

One limitation of this study is the type of study which is the case-control. Since longitudinal studies provide more reliable results over time, comparisons of findings over multiple time intervals can lead to safer conclusions and subsequently more effective decision making in this chronic complication. Another limitation of this study is the lack of assay of tear level of cytokines other than IL-8. Because the serum level of a cytokine does not necessarily reflect its level in tears. Especially in SM-exposed to subjects in this study who had a wide range of chronic ocular manifestations caused by mustard gas exposure. Therefore, longitudinal studies, as well as future tear level measurements of these cytokines, could provide researchers with a clearer picture of the pathogenesis of these chronic ophthalmic complications to develop more effective therapeutic strategies. Since chemical victims often suffer from multiple complications in the eyes, skin and lungs, and because veterans with severe eye complications were considered in this study, one limitation of this study was the absence of people with only eye problems and most people were with lower levels of other problems, and so serum levels of cytokines could be affected to some extent of mentioned problems.

5. Conclusion

The serum level of CX3CL1 has increased in SM-exposed individuals, while other chemokines, including IL-8/CXCL8, MCP-1/CCL2, and RANTES/CCL5 remained unchanged. The elevated levels of CX3CL1 may indicate an anti-inflammatory response against the destructive effects of SM gas. Further analysis of above chemokines in the two SM-exposed groups (with/without ocular abnormalities) showed an increase in tear level of IL-8/CXCL8 and decrease serum level of CXCL1 and RANTES/CCL5 in subjects with ocular abnormalities. High tear level of IL-8/CXCL8 is consistent with other studies that suggest high tear level of IL-8/CXCL8 reflects the severity of ocular surface abnormalities [18]. However, the decreased levels of CXCL1 and RANTES/CCL5 may represent the different pathophysiology which requires further studies such as molecular mechanisms involved in long-term clinical manifestations of SM exposure.

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Declaration of competing interest

The authors report no conflict of interest in this study.

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