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Original Article

Investigating the Association between Angiogenic Cytokines and Corneal Neovascularization in Sulfur Mustard Intoxicated Subjects 26 Years after Exposure

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ABSTRACT

Objectives: This study aimed to evaluate the associations between the concentrations of three major angiogenic cytokines–vascular endothelial growth factor-A165 (VEGF-A165), basic fibroblast growth factor (bFGF), and platelet-derived growth factor-BB (PDGF-BB)–in the tear of sulfur mustard (SM)-exposed subjects and corneal neovascularization (CNV) 26 years after exposure. **Materials and Methods:** The concentrations of VEGF-A, bFGF, and PDGF-BB were measured by enzyme-linked immunosorbent assay (ELISA) in reflex tears of (i) SM-injured patients with CNV (positive case group including 18 individuals) and (ii) SM-injured patients without CNV (negative case group including 22 individuals). Then results were compared to corresponding values obtained from tears of 40 healthy control subjects. **Results:** The mean concentrations of all investigated growth factors, VEGF-A165, bFGF, and PDGF-BB, were significantly higher in positive cases than controls ($P \leq 0.001$, $P = 0.028$, and $P = 0.041$, respectively). Whereas, VEGF-A165 was the only growth factor which displayed significantly elevated concentrations in negative case group compared to the healthy individuals ($P = 0.030$). Additionally, the mean level of VEGF-A165 was also higher in positive patient group than negative patients ($P = 0.022$). Subjects with increased concentrations of tear VEGF-A165 were more than 10 times more likely to suffer from CNV than normal individuals (odds ratio (OR) = 10.43, confidence interval (CI): 2.14–38.46, $P = 0.001$), while elevated levels of bFGF and PDGF-BB increased the risk of CNV by about twofold. **Conclusion:** Although all investigated cytokines had increased in tears of positive patients, VEGF-A was the only one which showed a significant correlation with the severity of CNV, and thus played a crucial role in corneal angiogenesis.

Key words: Basic fibroblast growth factor, corneal neovascularization, platelet-derived growth factor-BB, sulfur mustard, vascular endothelial growth factor

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INTRODUCTION

Sulfur mustard (SM; bis (2-chloroethyl) sulfide) is a highly reactive, alkylating chemical, which has been used as a chemical warfare agent since the early 20th century.[1] The last military use of mustard gas goes back to the 1980s, during the Iran–Iraq conflict in which over 100,000 Iranians were injured.[2] It is estimated that about 45,000 victims are still suffering from its long-term effects.[3] SM

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has destructive effects on skin, bone marrow, mucosal cells of the gastrointestinal tract, respiratory system, and particularly on eyes.[4] The severity of ocular damage is dependent on the dose and the duration of exposure and is classified into three degrees: Mild, moderate, and severe.[5] Clinical complications that are observed in the mild-to-moderate injuries include tearing, photophobia, edema, ocular discomfort, severe pain, blurred vision, dryness, blepharospasm, conjunctival hyperemia, and corneal erosions.[6] Whereas, in the severe degree, other clinical signs such as posterior synechia, full-thickness corneal injury, cataract, and elevated intra-ocular pressure may also emerge.[6] Apart from acute effects, late ocular complications arise in more than 90% of injured cases even 15–20 years after initial exposure. These late ocular complications may appear as blepharoconjunctivitis, increased sensitivity to irritants, abnormal conjunctival vascularization, varices, and delayed keratitis.[7] Delayed keratitisis the most serious sight-threatening disease in the patients suffering from SM. It has been detected in approximately 0.5% of exposed subjects, even up to 40 years after exposure.[8]

Corneal neovascularization (CNV) in humans is developed years after the healing of the acute corneal erosions following chemical burn induced by SM exposure.[9] In general, CNV occurs when the delicate balance between angiogenic and antiangiogenic factors in cornea is tilted towards angiogenic molecules. Vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), and platelet-derived growth factors (PDGFs) play central roles in ocular angiogenesis.[10]

Six family members or subtypes of the VEGF family are found in human, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF).[11] Of these, VEGF-A (also written simply as VEGF) is the most important member specifically with regard to the pathologic angiogenesis[12] and exists as four isoforms (VEGF-A121, VEGF-A165, VEGF-A189, and VEGF-A206).[13] Among them, VEGF-165 is the most common and the most important isoform for angiogenesis.[14] On the other hand, PDGFs constitute a family with five different isoforms; PDGF-AA, -BB, -AB, -CC, and -DD. Among these isoforms, PDGF-BB shows mitogenic, chemotactic, and migratory effects on corneal keratocytes *in vitro*.[15] This isoform also prevents spontaneous regression of newly formed vessels in multiple models of ocular neovascularization.[16] The best known members of the FGF family are acidic FGF (aFGF) and basic FGF (bFGF) that stimulate the proliferation of endothelial cells and subsequent angiogenesis.[17]

In the present study, the levels of VEGF-A165, bFGF, and PDGF-BB were determined in tears of SM injured patients with or without CNV, and the results were compared with normal controls. These molecules were chosen as three major proangiogenic growth factors that are present in human tear fluid and their levels are critical in maintaining corneal avascularity.

MATERIALS AND METHODS

Study population

This was a case–control, cross-sectional study which was performed between September 2012 and February 2013. Included individuals belonged to either one of the following categories: (i) Positive cases comprised 18 patients who had CNV in at least one of their eyes with documented exposure to SM, (ii) negative cases comprised 22 patients with documented exposure to SM, but no CNV, and (iii) control group included 40 healthy subjects with no history of ocular disease except for refractive errors. Exclusion criteria were: (i) Presence of any acute infectious disease or inflammation at the time of recruitment, (ii) a history of systemic disease, (iii) a history of the following surgeries: Amniotic membrane graft, limbal stem cell graft, corneal graft, punctal plug, punctal occlusion, and lateral tarsorrhaphy, (iv) use of antivascular or immunosuppressive drugs at the time of sampling, (v) contact lens users, (vi) use of artificial tears, lubricants, antibiotics, and steroids at the time of sampling, and (vii) a history of eye trauma. All patients were from Sardasht city located in West Azerbaijan province in northwest Iran, which was attacked with chemical weapons by Iraqi Air Force in 1987. All individuals were informed about the study's objectives and only those who provided a written informed consent were included for further assessments. The Human Ethics Committee in the Medical University of Urmia reviewed the technical and ethical aspects of the investigation and approved the entire protocol. The investigation adhered to the Declaration of Helsinki and subsequent revisions. Demographic and clinical characteristics such as age, smoking habit, blood pressure, medical history, duration of drug exposure, and body mass index (BMI) were all collected from participants' medical files. Smokers were considered as those who smoke at least five cigarettes per day for the past 1 or more years. Hypertension was considered as systolic/diastolic blood pressures higher than 140/90 mmHg. A complete ophthalmologic assessment including ocular history, visual acuity changes, and detailed slit-lamp examinations was achieved on included eyes. Recorded ocular symptoms were as follows: Ocular surface discomfort (including itching, burning, and redness), photophobia, foreign body sensation, dry eye sensation, and blurred vision. The lids, tear meniscus layer, bulbar conjunctiva, limbal tissue, cornea, and anterior segment were all evaluated during slit-lamp examinations. Due to the severity of corneal vascularization, eyes were categorized into two subgroups: Mild vascularized corneas (vascularization affecting <1 mm of cornea) and severe vascularized corneas (vascularization affecting >1 mm of cornea).

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Tear sample collection

Approximately 10 μl of basal tears were collected nontraumatically using glass capillary micropipettes (Drummond Scientific, Belgium) from the external canthus of the inferior fornix. Care was taken to avoid eliciting reflex tearing by coming into contact with the conjunctiva. Tenmicroliters of reflex tear was also collected from the inferior fornix near the inner canthus using a microcapillary tube. Reflex tear was stimulated by shining a bright light into the eyes. No anesthetic drops used during tear collections. All samples were collected by the same experienced investigator at the same time each day to eliminate diurnal effects and were transferred to Eppendorf tubes immediately and stored at −80°C until analysis.

Growth factor measurement

VEGF-A165, bFGF, and PDGF-BB levels were determined by enzyme-linked immunosorbent assay (ELISA; R and D Systems, Minneapolis, USA) according to the manufacturer's instructions. Before each analysis, the samples were centrifuged at 12,000 rpm for 5 min in order to remove any cellular components or debris from the water phase.

Statistical analysis

Statistical analyses were performed by Statistical Package for Social Sciences (SPSS ver. 17; SPSS Inc, Chicago, USA). A *P* - value less than 0.05 was considered to establish statistical significance. Fisher's exact test was used to compare qualitative parameters; whereas, Mann–Whitney U-test and Kruskal–Wallis one-way analysis of variance were utilized to compare intergroup continuous variables. Univariate analyses were performed to investigate the association of VEGF-A165 concentrations with other variable. For this purpose, Pearson correlation coefficients were calculated to evaluate the relationships between VEGF-A165 concentrations and continuous variables. Meanwhile, Student's *t*-test was used to compare the mean values of VEGF-A165 concentrations in binary variables (such as SM exposure, smoking status, hypertension, sex, photophobia, ocular surface discomfort, foreign-body sensation, etc). Those variables that showed significant association with VEGF levels in univariate analyses were further analyzed by a multiple linear regression analysis to determine independent predictors of tear VEGF-A165 concentrations. Furthermore, a logistic regression model was fitted to examine the independent impact of VEGF on CNV.

RESULTS

There were no significant differences in VEGF concentrations as well as in PDGF-BB levels between basic and reflex tear samples, neither in the total study population nor in the subgroups (data not shown). On the other hand, in none of the basic tear samples could bFGF be detected. Thus, cytokine concentrations presented here are belonged to reflex tear samples.

Baseline characteristics and ocular symptoms of included participants are summarized in Table 1. There was not any significant difference regarding sex, age, smoking status, BMI, and hypertension between exposed patients and normal subjects. Almost all ocular symptoms had higher frequencies in either negative or positive case groups compared to the control group, but these differences were not statistically significant, except for the foreign body sensation and photophobia. These two symptoms had significant higher rate of occurrences in positive case group than control subjects.

Table 1: Comparison of the symptoms, demographic characteristics and clinical parameters between the studied groups

	Controls (*N* = 40)	Negative cases (*N* = 22)	Positive cases (*N* = 18)	*P* value‡	*P* value†
Sex (male/female)	24/16	11/11	12/6	0.593	0.772
Smoker, *N* (%)	8 (20)	2 (9.1)	7 (38.89)	0.311	0.194
Age (years), mean (SD)	58.11 (4.07)	49.11 (5.12)	53.29 (4.78)	0.341	0.511
BMI (kg/m^2), mean (SD)	26.11 (4.24)	24.14 (3.20)	25.31 (3.78)	0.442	0.703
Hypertension, *N* (%)	16 (40)	7 (31.81)	9 (50)	0.591	0.571
Photophobia, *N* (%)	8 (20)	8 (36.36)	9 (50)	0.226	0.030*
Ocular surface discomfort, *N* (%)	6 (15)	6 (27.27)	6 (33.33)	0.317	0.161
Foreign body sensation, *N* (%)	0 (0)	1 (4.5)	3 (16.67)	0.354	0.026*
Tearing, *N* (%)	4 (10)	5 (22.72)	6 (33.33)	0.259	0.055
Dry eye sensation, *N* (%)	2 (5)	1 (4.5)	2 (11.11)	1.000	0.580
Eye pain, *N* (%)	4 (10)	4 (18.18)	3 (16.67)	0.438	0.665
Blurring of vision, *N* (%)	14 (35)	10 (45.45)	9 (50)	0.586	0.385
Blepharitis, *N* (%)	0 (0)	1 (4.5)	1 (5.6)	0.355	0.310
Limbal ischemia, *N* (%)	0 (0)	1 (4.5)	1 (5.6)	0.355	0.310
Vascular abnormalities of conjunctiva, *N* (%)	0 (0)	0 (0)	1 (5.6)	1.000	0.310

SD = Standard deviation, BMI = Body mass index. *$P \leq 0.05$, ‡*P* value: Comparison between control and negative case groups (Fisher's exact test or Mann-Whitney), †*P* value: Comparison between control and positive case groups (Fisher's exact test and Mann-Whitney)

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As shown in Table 2, slit-lamp findings showed statistically significant differences between positive cases and controls in the bulbar conjunctiva ($P = 0.002$), the limbal tissues ($P = 0.004$), and the cornea ($P = 0.000$). The frequency of the abnormal limbal tissues was also significantly higher in negative cases compared to the control group ($P = 0.041$). All other recorded signs were observed at higher frequencies in both positive and negative patients than controls, but the differences were not significant. Moreover, the tear levels of all investigated growth factors in the exposed groups (positive and negative case groups) with and without slit-lamp findings did not show significant differences (data not shown) except for the cornea. Indeed, abnormal corneas had elevated levels of VEGF-A165 ($P = 0.013$) as well as PDGF-BB ($P = 0.038$) compared to the normal corneas in the positive case group.

Table 3 shows the mean concentrations of the studied growth factors in each group and their pairwise Mann–Whitney *P* - values. The mean concentrations of all investigated growth factors, VEGF-A165, bFGF, and PDGF-BB, were significantly higher in the positive cases than controls ($P \leq 0.001$, $P = 0.028$, and $P = 0.041$, respectively). Whereas, VEGF-A165 was the only growth factor which displayed significantly elevated concentrations in the negative case group compared to the healthy individuals ($P = 0.030$). Additionally, the mean level of VEGF-A165 was also higher in positive case group than negative cases ($P = 0.022$). Meanwhile, among the studied growth factors, only VEGF-A165 showed a significant correlation with the severity of corneal vascularization (Spearman r = 0.322, $P = 0.019$) [Table 4].

A logistic regression analysis with backward selection strategy was conducted to identify effective factors predisposing to CNV [Table 5]. Continuous factors (including VEGF-A165, bFGF, PDGF-BB, current age, and age at exposure) and categorical variables (including smoking, hypertension, and sex) were included in the model as independent variables. Results revealed a strong and independent association between VEGF-A165 and the risk of CNV (odds ratio (OR) = 10.43, 95% confidence interval (CI): 2.14–38.46; $P = 0.001$). bFGF and PDGF-BB were also independently associated with CNV, but with a lower odds ratios (OR = 2.10, 95% CI: 1.13–6.05 and OR = 2.04, 95% CI: 1.25–4.68, respectively).

In order to determine the independent predictors of VEGF-A165 levels in tear fluid, a univariate and subsequently a multivariate regression analyses were conducted. In univariate analysis, tear VEGF-A165 levels were significantly correlated with bFGF levels (r = 0.179, $P = 0.014$). Likewise, the mean VEGF-A165 level was found to be higher in SM-exposed patients and smokers. The results of the stepwise multiple linear regression analysis also admitted these variables as independent predictors of VEGF-A165 in our study [Table 6]. PDGF-BB concentrations did not show correlations either with VEGF-A165 (Spearman r = 0.152, $P = 0.361$) or with bFGF levels (Spearman r = 0.109, $P = 0.289$).

Table 2: Slit-lamp findings in the investigated groups

Slit lamp findings	Controls (*N* = 40)	Negative cases (*N* = 22)	Positive cases (*N* = 18)	*P* value‡	*P* value†
Lids, *N* (%)					
Normal	36 (90)	18 (81.81)	12 (66.67)	0.438	0.055
Abnormal	4 (10)	4 (18.18)	6 (33.33)		
Bulbar conjunctiva, *N* (%)					
Normal	40 (100)	20 (90.9)	13 (72.22)	0.122	0.002**
Abnormal	0 (0)	2 (9.1)	5 (27.78)		
Limbal tissue, *N* (%)					
Normal	40 (100)	19 (86.36)	15 (83.33)	0.041*	0.026*
Abnormal	0 (0)	3 (13.64)	3 (16.66)		
Tear status, *N* (%)					
Normal	36 (90)	19 (86.36)	13 (72.22)	0.690	0.119
Abnormal	4 (10)	3 (13.64)	5 (27.78)		
Cornea, *N* (%)					
Normal	40 (100)	20 (90.9)	10 (55.56)	0.122	<0.001**
Abnormal	0 (0)	2 (9.1)	8 (44.44)		

*$P \leq 0.05$, **$P \leq 0.01$, ‡*P* value: Comparison between control and negative case groups (Fisher's exact test), †*P* value: Comparison between control and positive case groups (Fisher's exact test)

DISCUSSION

Although several investigations have used tear fluids to investigate the association between ocular diseases and tear protein concentrations;[18] to the best of our knowledge, there are only two studies in the literature which have employed similar approach for SM-exposed patients.[5,6] In one of them, Ghasemi and his colleagues

Table 3: Concentrations of different growth factors in the studied groups

	Controls (*N* = 40)	Negative cases (*N* = 22)	Positive cases (*N* = 18)	*P* value$	*P* value†	*P* value‡
VEGF (pg/mL), mean (SD)	985.23 (213.11)	1139.08 (123.53)	1304.23 (107.42)	0.030*	<0.001**	0.022*
bFGF (pg/mL), mean (SD)	113.53 (24.74)	119.37 (32.36)	143.84 (29.95)	0.168	0.028*	0.436
PDGF-BB (ng/mL), mean (SD)	643.32 (479.45)	615.74 (189.34)	769.88 (175.64)	0.243	0.041*	0.614

VEGF = Vascular endothelial growth factor, bFGF = Basic fibroblast growth factor, PDGF-BB = Platelet-derived growth factor-BB, SD = Standard deviation. *$P \leq 0.05$, **$P \leq 0.01$, $*P* value: Comparison between control and negative case groups (Mann-Whitney), †*P* value: Comparison between control and positive case groups (Mann-Whitney), ‡*P* value: Comparison between positive and negative case groups (Mann-Whitney)

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Table 4: Spearman correlation coefficients between the severity of corneal vascularization and growth factors concentrations

	Corneal vascularization severity (no/mild/severe)	*P* value
VEGF-A165	r=0.322	0.019*
bFGF	r=0.234	0.055
PDGF-BB	r=0.053	0.523

VEGF = Vascular endothelial growth factor, bFGF = Basic fibroblast growth factor, PDGF-BB = Platelet-derived growth factor-BB. *$P \leq 0.05$

Table 5: Risk factors of CNV calculated by logistic regression

Risk factors	β-coefficient	Standard error	Adjusted OR (95% CI)	*P* value
VEGF-A165	3.091	0.732	10.43 (2.14-38.46)	0.001**
bFGF	0.957	0.598	2.10 (1.13-6.05)	0.023*
PDGF-BB	0.741	0.346	2.04 (1.25-4.68)	0.036*

VEGF = Vascular endothelial growth factor, bFGF = Basic fibroblast growth factor, PDGF-BB = Platelet-derived growth factor-BB, CNV = Corneal neovascularization, OR = Odds ratio, CI = Confidence interval. *$P \leq 0.05$, **$P \leq 0.01$

Table 6: Univariate and multivariate regression analysis for VEGF-A165 levels

	Univariate analysis		Multivariate analysis	
	R†	*P* value	β	*P* value
SM exposure	0.287	<0.001**	0.386	<0.001**
bFGF	0.179	0.014*	0.133	0.034*
Smoking	-	0.021*	0.159	0.040*
Sex	-	0.489		
Age	0.042	0.627		
Age at exposure	0.021	0.882		
PDGF-BB	0.069	0.412		
BMI	0.031	0.524		
Hypertension	-	0.389		
Photophobia	-	0.625		
Ocular surface discomfort	-	0.712		
Foreign-body sensation	-	0.269		
Tearing	-	0.743		
Dry eye sensation	-	0.456		
Eye pain	-	0.611		
Blurring of vision	-	0.257		

VEGF = Vascular endothelial growth factor, bFGF = Basic fibroblast growth factor, PDGF-BB = Platelet-derived growth factor-BB, SM = Sulfur mustard, BMI = Body mass index. *$P \leq 0.05$, **$P \leq 0.01$, †calculated for log-transformed data of VEGF-A165

compared tear interleukin-8 (IL-8) concentrations between 37 healthy individuals and 48 SM-intoxicated patients 20 years after exposure.[5] They also assessed serum levels of IL-8 in 370 patients with SM exposure history and 128 unexposed individuals. Their findings showed a significant decrease not only inserum ($P = 0.002$) but also in tear IL-8 ($P = 0.03$) mean levels in the patient group compared to normal subjects. In the other investigation which was conducted by Panahi *et al*.,[6] a total of 50 tear fluids from 18 SM-intoxicated patients and 25 normal individuals were examined for total protein content as well as for the concentrations of VEGF, calcitonin gene-related peptide (CGRP), and substance P. Total protein and CGRP concentrations were found to be lower in tears of the SM-exposed patients vs control group ($P < 0.01$ and $P < 0.05$, respectively); whereas, VEGF showed a statistically significant increase in tears of SM-intoxicated patients compared to control subjects ($P < 0.01$). On the other hand, in an attempt to evaluate the therapeutic effects of an anti-VEGF monoclonal antibody (bevacizumab) on CNV following SM exposure, Kadar and his colleagues applied bevacizumab to rabbit eyes topically or through subconjunctival injection.[18] Based on this study, CNV development was observed in 50–70% of the eyes as early as 2 weeks after exposure and was also strongly associated with increased levels of VEGF. Topical administration and subconjunctival injection of bevacizumab were both beneficial in reducing CNV when given as a symptomatic therapy. Indeed, use of bevacizumab has recently attracted attention as a safe and effective therapeutic option for management of CNV (reviewed in[19]).

It has been reported that the tear VEGF level increases in patients with chronic ocular inflammation such as atopic and vernal kerato conjunctivitis and CNV.[20] Elevated levels of VEGF have also been observedin patients suffering from degenerative diseases (such as pterygium and limbal stem cell deficiency),[10] intraocular vascular diseases (such as macular edema, choroidal neovascularization, diabetic retinopathy, and neovascular glaucoma; reviewed in[21]), and ocular surface diseases (such as dryness).[22] This is in line with the findings of the current study in which the raised concentration of VEGF showed associations with presence as well as with severity of vascularization in tears of SM-intoxicated patients. In fact, our findings showed that subjects with increased concentrations of tear VEGF-A165 were more than 10 times more likely to suffer from CNV than normal individuals (β coefficient = 3.091, standard error (SE) =0.732, OR = 10.43, CI: 2.14–38.46, $P = 0.001$). In this manner, elevated levels of bFGF and PDGF-BB increased CNV risk by about two fold [Table 5]. Tear VEGF-A165 levels in our population were in turn determined by smoking and bFGF concentrations as well as by SM exposure. The interaction of bFGF with VEGF in the production of angiogenesis has been shown by multiple researches both in mouse models and *in vitro* models.[23] There are also evidences that bFGF has regulatory effects on activity or even on expression level of VEGF in smooth muscles[24] as well as in microvascular endothelial cells.[25] Therefore, the possibility of a similar role for bFGF in corneal tissue could not be entirely ruled out. On the other hand, smoking has been shown to cause or worsen several eye disorders from choroidal[26] and retinal neovascularization[27] to age-related macular degeneration (AMD).[28] Some possible mechanisms leading to these conditions are smoking-induced hypoxia,

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ischemia, and microinfarctions.[29] It has also been proposedthat CNV can begin with hypoxia-induced upregulation of proangiogenic cytokines (such as VEGF) and downregulation of antiangiogenic cytokines (such as pigment epithelium derived factor (PEDF)).[30]

In contrast to bFGF and PDGF-BB, a statistically significant elevation of VEGF-A165 was observed in tears of negative patient group compared to normal subjects; however, this increase was not followed by CNV formation in the negative patients. Conversely, positive patients had raised concentrations of not only VEGF-A165 but also bFGF and PDGF-BB in their reflex tears compared to the normal group. Therefore, it seems that SM-induced CNV requires synergistic effects of these growth factors and VEGF-A165 elevation do not result in vascularization *per se*. Indeed, numerous investigations reported that the efficacy of anti-VEGF therapy is enhanced by inhibiting PDGF-BB signaling pathway in choroidal and CNV models, showing a synergistic relationship between these two cytokines.[31] In fact, PDGF plays an important role in vessel maturation by pericyte recruitment to the angiogenic vessels. Pericytes, in turn, stabilize vessels; and VEGF acts more effectively on vessels stabilized by pericytes.[30] There are also evidences of synergistic effects of bFGF and PDGF-BB on angiogenesis in mouse cornea[32] as well as on arteriogenesis and muscle regeneration in animal models of hindlimb ischemia.[33]

In summary, the results of the present investigation indicated that mean concentrations of bFGF, PDGF-BB, and particularly VEGF-A165 were higher in tears of SM-intoxicated patients who had CNV in at least one of their eyes compared to healthy individuals. Elevated levels of VEGF-A165 were also found in tears of SM-intoxicated patients without CNV (negative cases) in comparison to the normal subjects. In addition, VEGF-A165 was the only investigated cytokine that exhibited a significant correlation with the severity of corneal vascularization. On the other hand, neither bFGF nor PDGF-BB showed associations with vascularization severity in our study, but these two cytokines, along with VEGF-A165, were regarded as risk factors of CNV development. Thus it seems that, combinatorial antiangiogenic therapeutic strategies with emphasis on anti-VEGF therapies may open an investigative field in suppressing the angiogenic effects of SM gas on intoxicated eyes. In this line, further preclinical studies and prospective investigations are needed to evaluate more accurately the roles and relations of additional pro- and antiangiogenic factors in the pathogenesis of SM-induced CNV.

REFERENCES

1. Graham JS, Schoneboom BA. Historical perspective on effects and treatment of sulfur mustard injuries. Chem Biol Interact 2013;206:512-22.
2. 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;45:1136-43.
3. Wattana M, Bey T. Mustard gas or sulfur mustard: An old chemical agent as a new terrorist threat. Prehosp Disaster Med 2009;24:19-29.
4. Javadi MA, Yazdani S, Sajjadi H, Jadidi K, Karimian F, Einollahi B, *et al*. Chronic and delayed-onset mustard gas keratitis: Report of 48 patients and review of literature. Ophthalmology 2005;112:617-25.
5. Ghasemi H, Ghazanfari T, Babaei M, Soroush MR, Yaraee R, Ghassemi-Broumand M, *et al*. Long-term ocular complications of sulfur mustard in the civilian victims of Sardasht, Iran. Cutan Ocul Toxicol 2008;27:317-26.
6. Panahi Y, Eftekhari Milani A, Sahebkar A, Naderi M, Babaei M, Beiraghdar F, *et al*. Tear total protein analysis in patients with late sulfur mustard-induced ocular complications: A cross-sectional study. Cutan Ocul Toxicol 2012;31:104-10.
7. Pickard H. Ocular action of dichlorethylsulphid (mustard gas). Am J Ophthalmol 1919;2:136-7.
8. Solberg Y, Alcalay M, Belkin M. Ocular injury by mustard gas. Surv Ophthalmol 1997;41:461-6.
9. McNutt P, Hamilton T, Nelson M, Adkins A, Swartz A, Lawrence R, *et al*. Pathogenesis of acute and delayed corneal lesions after ocular exposure to sulfur mustard vapor. Cornea 2012;31:280-90.
10. Lim P, Fuchsluger TA, Jurkunas UV. Limbal stem cell deficiency and corneal neovascularization. Semin Ophthalmol 2009;24:139-48.
11. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999;13:9-22.
12. Avery RL, Pearlman J, Pieramici DJ, Rabena MD, Castellarin AA, Nasir MA, *et al*. Intravitreal bevacizumab (Avastin) in the treatment of proliferative diabetic retinopathy. Ophthalmology 2006;113:1695.e1-15.
13. Shinkaruk S, Bayle M, Lain G, Deleris G. Vascular endothelial cell growth factor (VEGF), an emerging target for cancer chemotherapy. Curr Med Chem Anticancer Agents 2003;3:95-117.
14. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 1992;267:26031-7.
15. Kim WJ, Mohan RR, Mohan RR, Wilson SE. Effect of PDGF, IL-1alpha, and BMP2/4 on corneal fibroblast chemotaxis: Expression of the platelet-derived growth factor system in the cornea. Invest Ophthalmol Vis Sci 1999;40:1364-72.
16. Jo N, Mailhos C, Ju M, Cheung E, Bradley J, Nishijima K, *et al*. Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization. Am J Pathol 2006;168:2036-53.
17. Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L. Basic fibroblast growth factor induces angiogenesis *in vitro*. Proc Natl Acad Sci U S A 1986;83:7297-301.
18. Kadar T, Amir A, Cohen L, Cohen M, Sahar R, Gutman H, *et al*. Anti-VEGF therapy (bevacizumab) for sulfur mustard-induced corneal neovascularization associated with delayed limbal stem cell deficiency in rabbits. Curr Eye Res 2014;39:439-50.
19. Chang JH, Garg NK, Lunde E, Han KY, Jain S, Azar DT. Corneal neovascularization: An anti-VEGF therapy review. Surv Ophthalmol 2012;57:415-29.

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20. Zakaria N, Van Grasdorff S, Wouters K, Rozema J, Koppen C, Lion E, *et al*. Human tears reveal insights into corneal neovascularization. PLoS One 2012;7:e36451.
21. Miller JW, Le Couter J, Strauss EC, Ferrara N. Vascular endothelial growth factor a in intraocular vascular disease. Ophthalmology 2013;120:106-14.
22. Enriquez-de-Salamanca A, Castellanos E, Stern ME, Fernandez I, Carreno E, Garcia-Vazquez C, *et al*. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. Mol Vis 2010;16:862-73.
23. Onguchi T, Han KY, Chang JH, Azar DT. Membrane type-1 matrix metalloproteinase potentiates basic fibroblast growth factor-induced corneal neovascularization. Am J Pathol 2009;174:1564-71.
24. Stavri GT, Zachary IC, Baskerville PA, Martin JF, Erusalimsky JD. Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. Circulation 1995;92:11-4.
25. Mandriota SJ, Pepper MS. Vascular endothelial growth factor-induced *in vitro* angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. J Cell Sci 1997;110:2293-302.
26. Chheda LV, Ferketich AK, Carroll CP, Moyer PD, Kurz DE, Kurz PA. Smoking as a risk factor for choroidal neovascularization secondary to presumed ocular histoplasmosis syndrome. Ophthalmology 2012;119:333-8.
27. Zhong ZL, Han M, Chen S. Risk factors associated with retinal neovascularization of diabetic retinopathy in type 2 diabetes mellitus. Int J Ophthalmol 2011;4:182-5.
28. Thornton J, Edwards R, Mitchell P, Harrison RA, Buchan I, Kelly SP. Smoking and age-related macular degeneration: A review of association. Eye (Lond) 2005;19:935-44.
29. Klein R, Lee KE, Gangnon RE, Klein BE. The 25-year incidence of visual impairment in type 1 diabetes mellitus the wisconsin epidemiologic study of diabetic retinopathy. Ophthalmology 2010;117:63-70.
30. Rolfsen ML, Frisard NE, Stern EM, Foster TP, Bhattacharjee PS, McFerrin HE Jr, *et al*. Corneal neovascularization: A review of the molecular biology and current therapies; 2013.
31. Chaoran Z, Zhirong L, Gezhi X. Combination of vascular endothelial growth factor receptor/platelet-derived growth factor receptor inhibition markedly improves the antiangiogenic efficacy for advanced stage mouse corneal neovascularization. Graefes Arch Clin Exp Ophthalmol 2011;249:1493-501.
32. Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, *et al*. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med 2003;9:604-13.
33. Li J, Wei Y, Liu K, Yuan C, Tang Y, Quan Q, *et al*. Synergistic effects of FGF-2 and PDGF-BB on angiogenesis and muscle regeneration in rabbit hindlimb ischemia model. Microvasc Res 2010;80:10-7.

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