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RESEARCH ARTICLE

Conjunctival microbial florae in patients with seriously sulfur mustard induced eye injuries

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Abstract

Introduction: Ocular surface disorders and infections in sulfur mustard (SM) exposed patients are of particular clinical importance. The aim of the present study is to detect the conjunctival bacterial florae in patients with seriously SM induced eye injuries.

Materials and methods: Conjunctival bacterial florae of 143 seriously eye injured subjects as the study group was detected. The results were compared with 26 normal participants. Both groups were matched in age and sex. The samples were taken by sterile swab from interior fornixes of conjunctiva in both groups and were transported to microbiology laboratory by Stuart's Transport Medium. All samples were inoculated onto Blood agar, Mac Conkey agar and Chocolate agar and isolated microorganisms were identified by biochemical tests. The data were analyzed by SPSS and Man Whitney tests.

Results: Nineteen cases (13.39%) and none of the controls (0%) had positive culture results (p = .043). Isolated microorganisms from patients included coagulase-negative staphylococci 10 cases (52.6%), *Staphylococcus aureus* 5 cases (26.3%), non enterobacteriaceae gram negative bacilli 2 cases (10.5%), Penicillium spp. 2 cases (10.5%), Citrobacter sp. 1 case (5.2%), non-spore forming Gram positive bacillus 1 case (5.2%) and α hemolytic streptococcus 1 case (5.2%). Two patients had mixed microorganisms and other patients had just one microorganism. Most of the *S. aureus* isolates were sensitive to usual antibiotics.

Conclusions: The results of this study showed that the prevalence rate of conjunctival bacterial isolates in patients with seriously SM induced ocular injuries are higher and potentially more dangerous than normal controls.

Keywords: Sulfur mustard, eye, ocular surface, conjunctiva, bacterial florae

Introduction

Sulfur mustard (bis-(2-chloroethyl) sulfide, SM) that was widely used during the World War I and in the Iraq–Iran conflict is an alkylating chemical warfare agent. Late complications of SM poisoning in the skin, eyes, and respiratory system are mainly due to its direct toxic effects, however the neuromuscular, hematological and immunological complications are probably the result of systemic toxicity (1). Immune system is compromised to some degrees in exposed patients. The serum IL-1β levels in SM exposed patients with slit lamp findings were significantly decreased (2). Eyes are the most sensitive organ to SM. This marked susceptibility is attributable to several ocular features including the aqueous-mucous surface of the cornea and conjunctiva, as well as the high turnover rate and intense metabolic activity of the corneal epithelial cells (3). Approximately 34,000 Iranians are known to have sustained mustard agent exposure during the Iraq–Iran war since 1983–1988. In order of frequency, these include lesions of the lungs, eyes, and skin (4). Regular

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follow-up of veterans exposed to SM is recommended due to progressive nature of SM complications (5,6). SM is absorbed by inhalation, gastrointestinal tract, skin and anterior surface of the eyes (7). Severity of exposure play important role in cause specific mortality. Milder grades of mustard gas exposures were not associated with any increased risk of cause specific mortality (8). Ocular injuries following SM exposure are characterized by an inflammatory response, observed as eyelid swelling, conjunctivitis, corneal edema and cellular infiltration starting 1–4 h after exposure. Corneal epithelial defects, stromal edema and cellular infiltration appear in 48 h. The clinical signs are significantly dose-dependent and reach at peak within 24–72 h. Epithelial regeneration begins after 72 h. These effects heal partially during the first 1–2 weeks after exposure. The later responses may appear as corneal neovascularization, recurrent erosions and edema (delayed response). The delayed injuries were seen in up to 40% of the eyes with more severe inflammation than the initial ones (9,10). The maximum incidence of delayed keratitis usually occurs 15–20 years after initial exposure, although latency periods as long as 40 years or as short as 6 years have also been reported (11,12). Ocular surface bacterial changes in abnormal conditions may trigger the release of endotoxins, lipopolysaccharides, and/or lipase activation, causing eyelid inflammation, meibomian gland dysfunction, lipidic changes and tear film instability (13).

Conjunctival flora develops soon after the birth and when immune function is compromise, some of the saprophytic conjunctival flora may appear as a pathogen with serious infections (14).Concerning the conjunctival florae, most of the studies represented in the literature have done on normal subjects without control group. Additionally, the studies including the control groups are of the "before and after intervention study" types and are mostly focused on the effects of local antibiotics before intraocular surgeries (15,16).

Regarding normal conjunctival florae, very few case control studies are presented in the literature on patients with especial underlying disorders and even fewer in the case of SM exposed patients. The aim of this study was to evaluate the usual conjunctival florae and their antibiotics sensitivity in patients with seriously SM induced ocular injuries comparing to healthy controls.

Material and method

Study design and participants

In this case control study, the conjunctival florae of 143 patients with serious SM induced ocular injuries were compared with 26 healthy individuals. The needed numbers for the controls were determined by the statistical methods. The two groups were matched by age and all of them were male. None of the cases had tuberculosis, diabetes, hypertension and asthma. Those patients who were under treatment with local or systemic antibiotics during the recent 4 weeks were excluded from the study. All participants signed a written informed consent before the study. The study protocol was approved by the Iranian Ministry of Health and the Ethics Board of Janbazan Medical and Engineering Research Center.

Clinical evaluation

A case history was obtained about the ocular manifestations such as pain, tearing, foreign body sensation, dry eye sensation, sense of decreased vision and photophobia. Ophthalmologic assessment included complete exam of the lids, tear status, bulbar conjunctiva, limbal tissue, cornea, and anterior segment, using a slit lamp biomicroscope (Nidek model, Gamagori, Japan). Evaluation of the posterior segment was made using direct and indirect ophthalmoscope. Histories of all ocular surgeries were recorded. Finally, the severity of involvement was grouped based on the chart of the Foundation of Martyrs and Veterans Affairs (Iranian Ophthalmic Committee of Chemical Warfare Veterans) as follows: Symptomatic (no sign): Photophobia, foreign body sensation, burning, itching, lacrimation, redness, and dryness; Mild: Symptoms in addition to conjunctival signs of microaneurysm, telangiectasia, tortuosity, segmentation (venous beading), vascular dilatation, ischemia, and dry eye; Moderate: Mild form in addition to limbal ischemia and corneal involvement (opacity, and neovascularization); and Severe: Moderate form in addition to severe corneal involvement (melting, thinning, hyaline deposition, or diffuse opacity) and any related corneal operations (17,18). Based on this classification 82 cases were severe (57.3%), 36 cases were moderate (25.2%), 24 cases were mild (16.8%), and only 1 case was normal (0.7%).

Sample collection

Conjunctival samples were taken by sterile swab from inferior fornixces of the eyes and immediately were transported to microbiology laboratory by Stuart's Transport Medium.

Microbiological study

All samples were inoculated onto Blood agar, MacConkey agar, and Chocolate agar. Inoculated Blood agar and MacConkey agar were incubated in aerobic condition for 48 h at 37°C and Chocolate agar were incubated in microaerophilic condition for 48 h at 37°C. Microaerophilic condition was created by gas pack (Anaerocult C, Merck Co., Darmstadt, Germany) and jar. Isolated microorganisms were identified by biochemical methods as possible. Bacterial susceptibility to routine antibiotics was tested by the Kirby-Bauer disk diffusion method. In brief, to calculate the colony forming units per mL (CFU/mL), bacterial suspensions with 10^6 CFU/mL were spread on the surface of Mueller-Hinton agar plates. Within 15 min after the surface of the agar has been inoculated, antimicrobial disks were applied with sterile forceps. The plates were inverted and incubated at 35°C for 18 h and then diameter of each zone of inhibition was measured. All discs were purchased from Mast Group Ltd. (UK).

Statistical analysis

Data was presented as frequency (percentage). Positive cojunctival florae were compared between control and exposure group with χ^2 test. Statistical analysis test were done in SPSS 13 (SPSS Inc, Chicago, IL, USA) software.

Results

Mean age of the patients was 45.47 years. The mean duration of disease was 21.58 years. Clinical examination revealed higher prevalence rate of lid and tear abnormalities in the cases including meibomian gland dysfunction (96%), blepharitis (67.1%), punctal abnormality (59.1%), trichiasis (8.1%), tear break up time test less than 10 s (83.8%), and tear meniscus layer less than 1 mm (90.6%). Cojunctival isolates in nineteen (13.39%) out of 143 cases and in 0 (0%) out of 26 controls showed positive results in cultures. This difference was statistically significant ($p = .043$). In cases with positive culture, six patients were exposed two times and two patients were exposed five times to SM. The severities of the organs involvement, ocular symptoms and the ocular signs of this group of patients have showed in Tables 1–3. Isolated microorganisms from patients included coagulase-negative staphylococci in 10 cases (52.6%), *Staphylococcus aureus* in 5 cases (26.3%), none enterobacteriaceae gram negative bacilli in 2 cases (10.5%), *Penicillum* spp. 2 cases (10.5%), *Citrobacter* sp. 1 case (5.2%), non-spore forming gram-positive bacillus 1 case (5.2%), and α hemolytic streptococcus 1 case (5.2%). Two patients had mixed microorganisms and other patients had just one microorganism. Amongst the isolated microorganisms in exposed, antibiogram was done for *S. aureus* because of the potentially dangerous nature of this isolate (Table 4). One isolate of *S. aureus* was missed.

Table 1. Severity of organs involvement in SM exposed patients with positive conjunctival cultures.

Organs	Normal (*N*)	Mild (*N*)	Moderate (*N*)	Severe (*N*)
Eye (right)	0	4	5	10
Eye (left)	0	2	6	11
Skin	8	6	3	0
Lung	4	6	2	4

N, Number of patients.

Discussion

This study showed that a high percent of war veterans with eye injuries caused by SM suffered from photophobia, foreign body sensation, decreased visual acuity, dry eye sensation, tearing, blepharitis, conjunctival hyperemia, or abnormal vasculatures and corneal abnormalities. We

Table 2. Ocular symptoms in SM eye injured patients with positive conjunctival cultures.

Ocular complaints	No (*N*)	Yes (*N*)
Pain	12	7
Tearing	10	9
Foreign body sensation	7	12
Dry eye sensation	4	15
Sense of decreased vision	6	13
Photophobia	5	14

N, Number of patients.

Table 3. Ocular signs in SM eye injured patients with positive conjunctival cultures.

Ocular findings	Eye	Yes (*N*)	No (*N*)
Trichiasis	R	2	17
	L	3	16
Blepharitis	R	10	9
	L	12	6
Punctal occlusion	R	10	8
	L	9	9
Peterigium	R	3	16
	L	2	17
Conjunctival hyperemia	R	16	2
	L	16	2
Vascular abnormalities	R	16	3
	L	11	5
Epithelial abnormalities	R	17	1
	L	15	2

L, Left; *N*, Number of patients; R, Right.

Table 4. Results of antibiogram for isolated strains of S. aureus.

ID numbers (strains)*	62	96	138	139
Antibiotics	Sensitivity			
Gentamycin	S	S	R	S
Oxacillin	S	S	R	S
Tetracyclin	S	R	R	S
Quinupristin/ Dalfopristine	S	S	S	S
Amoxicillin/ Sulbactam	S	S	S	S
Erythromycin	I	I	S	I
Rifampin	S	S	S	I
Vancomycin	S	I	S	S
Linezolid	S	I	S	S
Co-Trimoxazol	R	S	I	R
Clindamycin	I	I	S	I
Ciprofloxacin	I	I	I	S

I, Intermediate; ID, Identification; R, Resistant; S, Sensitive.
*One isolate of *S. aureus* was missed.

found that the numbers of positive conjunctival culture in 143 cases with serious eye injuries induced by SM were significantly higher than the healthy controls (13.39% vs. 0%).

Exposure to mustard gas may affect microbiological flora of the eyelid margin. In the study of Karimian et al. amongst 289 SM exposed patients, 150 (52.0%) cases had chronic blepharitis (19). They also reported significantly higher rates of isolated *Staphylococcus epidermidis* (78%) and *S. aureus* (57%) on eyelid margins in their cases. Meanwhile, isolated fungi were more frequent in the cases compared with controls (30% vs. 4%), with predominance of Cladosporium and Candida sp. Also, isolated *S. aureus* were more resistant to common antibiotics compared with control group (19). While in present study, coagulase-negative staphylococci (52.6%), *S. aureus* (26.3%), none enterobacteriaceae gram-negative bacilli (10.5%), *Penicillum* spp. (10.5%), *Citrobacter* sp. (5.2%), non-spore forming gram-positive bacillus (5.2%) and α hemolytic streptococcus (5.2%) were in order of predominance, but none of the controls showed positive culture (0%). In parallel with the study of Karimian et al., blepharitis was frequent in our cases as well (67.1%). However, higher rates of isolated microorganisms from the lid margins in the study of Karimian et al most probably refers to more contaminated lid margins in contrast to conjunctival surface (which was used for sampling in our study instead of lid margins) and/or other causes such as culture techniques. Furthermore, in normal conditions, tear fluid constituents such as mucins, lysozyme, lactoferrin in addition to corneal and conjunctival epithelial products including antimicrobial peptides, constantly neutralize and wash out the microbial colonization in contrast to the lid margins which lack such complete defensive mechanisms (20).

In a large sample of patients, candidate for cataract surgery, conjunctival florae in diabetics showed a significantly higher prevalence of *S. aureus*, *Enterococci*, certain *Streptococci*, and *Klebsiella* sp. than nondiabetics. Also those with higher blood creatinine levels had an increased conjunctival bacterial prevalence (21). In parallel, SM exposed cases had significantly higher rates of conjunctival isolates than healthy controls. While *S. aureus* and gram-negative bacteria were significantly more common in patients with chronic conjunctivitis, coagulase-negative species of the Micrococcaceae family were significantly more prevalent in conjunctival florae of healthy controls. In patients with chronic conjunctivitis the risk of multidrug resistant coagulase-negative staphylococci increased (22). In contrast, present study showed that in exposed patients, coagulase-negative staphylococci was the most prevalent isolate followed by *S. aureus*, none enterobacteriaceae gram-negative bacilli and *Penicillum* spp. These differences may refer to the nature of conjunctival inflammation between the two studies. Systemic autoimmune disorder such as Behcet syndrome may have different conjunctival flora compare to normal controls. *S. aureus* (24%), *Moraxella* spp. (16%), and *Streptococcus* spp. (16%) colonization was significantly higher in the conjunctival flora of Behcet patients (23). In 30 patients with vernal conjunctivitis, although the prevalence of coagulase-negative staphylococci was high in the controls, the prevalence of Staphylococcus aurous was significantly higher (24). These studies showed that in especial immune conditions, conjunctival florae differ from normal conditions. In parallel, the present study showed that the conjunctival florae in the cases were significantly different from the controls. On the other hand, among the positive cultures (13.39%), frequency of coagulase-negative staphylococci (52.6%) was as twice as *S. aureus* (26.3%). Given the conjunctival florae, many studies presented in the literature showed predominance of coagulase-negative staphylococcus or other staphylococcus species (25–32). In contrast to the present study, most of them lack a control group and have a higher rate of positive results. These differences may be caused by different cultivation techniques such as using transport media, time intervals between the sampling and culturing in each study, the types of studied samples, or other possible factors. Fahmy et al. believed that the conjunctival florae were correlated with patients' gender and age, environmental conditions such as season, temperature and humidity, as well as number of polymorphonuclear neutrophils of the conjunctival fluid (33). Moeller et al. showed that using both liquid and solid media can increase the chances of isolate recovery in conjunctival flora. Gram-positive cocci show significantly higher growth in brain heart infusion broth medium, while gram-positive bacilli show a significantly higher bacterial growth in solid Blood agar medium (34). When the immune system is compromised; some saprophytic conjunctival flora may appear as a pathogen with serious infections. In diabetics, conjunctival florae differ from that of nondiabetic subjects and *S. epidermidis* and *S. aureus* are the two most frequent conjunctival florae isolates. The numbers of eyes with growth of S aureus are significantly higher in the type 2 DM patients (14). In this study, *S. aureus* as a potentially dangerous saprophyte, was isolated from conjunctival florae in 26.3% of the cases. This finding in addition to higher rate of ocular surface abnormalities and suppression of immune system in SM exposed patients (2,35), may make them more susceptible to serious ocular surface infections. Fortunately, the isolated *S. aureus* strains were sensitive to most of the antibiotics (Table 4).

Conclusion

The results of this study showed that the possibility of ocular infection in SM exposed war veterans are higher than normal controls. Regarding the importance of the visual system, prophylactic hygienic modalities such as daily washing and scrubbing of the lid and regular observation of these groups of veterans are highly recommended.

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Declaration of interest

The authors declare no conflicts of interest.

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