

Ocular Effects of Sulfur Mustard

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Abstract

Purpose: To review current knowledge about ocular effects of sulfur mustard (SM) and the associated histopathologic findings and clinical manifestations

Methods: Literature review of medical articles (human and animal studies) was accomplished using PubMed, Scopus and ISI databases. A total of 274 relevant articles in English were retrieved and reviewed thoroughly.

Results: Eyes are the most sensitive organs to local toxic effects of mustard gas. Ocular injuries are mediated through different toxic mechanisms including: biochemical damages, biomolecular and gene expression modification, induction of immunologic and inflammatory reactions, disturbing ultrastructural architecture of the cornea, and long-lasting corneal denervation. The resulting ocular injuries can roughly be categorized into acute or chronic complications. Most of the patients recover from acute injuries, but a minority of victims will suffer from chronic ocular complications. Mustard gas keratopathy (MGK) is a devastating late complication of SM intoxication that proceeds from limbal stem cell deficiency (LSCD).

Conclusion: SM induces several different damaging changes in case of ocular exposure; hence leading to a broad spectrum of ocular manifestations in terms of severity, timing and form. Unfortunately, no effective strategy has been introduced yet to inhibit or restore these damaging changes.

Keywords: Sulfur Mustard, Mustard Gas Keratopathy, Corneal Injury, Ocular Complication

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Introduction

Sulfur mustard (SM) is a lipophilic, highly reactive, alkylating chemical, which had been used as a strong vesicant and debilitating chemical warfare agent during the 20th century.¹⁻³ Pure SM was produced by Meyer in 1886,⁴ but it was first effectively applied during World War 1 by German army against English soldiers in a field near Ypres, Belgium.^{5,6} The last military use of mustard gas was in the Iran-Iraq war (1980-1988),⁷ in which over 100,000 Iranians were injured. Unfortunately, SM is still a major potential threat to both civilian population and military staffs^{8,9} as there is not effective antidote nor effective therapeutic agent available.¹⁰⁻¹²

SM has destructive effects on viable tissues only and exposed surfaces of the body including eyes, skin and respiratory system are the major targets for absorption and local toxic effects of mustard gas.¹³⁻¹⁵ Also, it can be absorbed through gastrointestinal system following ingestion of contaminated food. Absorption of large amounts of SM may result in short-term and long-term impairment of immune system due to damaging the rapidly growing cells of bone marrow.^{15,16}

Primary toxic effects of SM generally appear after a variable periods of latency - typically within 2 to 24 hours- depending on the mode of exposure, concentration of SM and exposure duration as well as environmental factors like temperature.^{1,16} However, it was found that a significant but short-term exposure of a subject to mustard gas -like its deployment in battlefields- has massive acute and chronic debilitating effects in humans including ocular and cutaneous damage, respiratory tract injury, reproductive and developmental toxicity, gastrointestinal effects, hematological and immunological impairments, malignancies and psychosocial disorders.^{15,17-19}

The eyes are most sensitive to local damage from SM with a threshold of 12 mg*min/m³, compared to 200 mg*min/m³ for the skin,¹ and even low doses induce incapacitation and visual impairments.²⁰ Acute ocular lesions are characterized by eyelid erythema and edema, photophobia, chemosis, subconjunctival hemorrhage, corneal epithelial erosions and inflammation of the anterior segment.²⁰⁻²³ Presentation of ocular lesions depends on severity of exposure²⁴ but in most

cases, lesions resolve within weeks and a full restoration of visual acuity is observed.^{2,22,25,26}

However, some of the patients develop more severe corneal pathologies, such as chronic keratitis, impaired corneal sensation, recurrent/persistent corneal erosions, limbal vasculature injury and neovascularization, which eventually may lead to significant visual impairments and even blindness.^{2,25} These symptoms comprise the pathologic condition known as mustard gas keratopathy (MGK) that appear immediately after exposure to SM in the form of persistent smoldering inflammation (chronic form) or following a clinically latent period of 0.5 to 40 years (delayed-onset form).^{22,24,25,27-30} Chronic and delayed-onset form are characterized by severe conjunctival, limbal and corneal involvement.^{25,30} Although, different clinical aspects of MGK pathogenesis have been published in Iranian veterans with progressive keratitis following a long asymptomatic period,^{10,25,31,32} the exact etiology and patho-mechanism of MGK is not fully understood yet.

Herein, we aimed to review the literature to provide a comprehensive description of clinical and pathological effects of SM on eye and their associated pathomechanism.

Methods

Literature review of medical articles (human and animal studies) was accomplished using PubMed, Scopus and ISI database. A total of 274 relevant articles in English were retrieved and reviewed thoroughly. The following search terms were used: mustard, mustard gas, sulfur mustard, nitrogen mustard, chemical warfare agent, blister agent, vesicant agent, alkylating agent, corneal burns, corneal transplant, limbal stem cell deficiency, keratitis, mustard gas keratopathy, ocular toxicity, corneal injury, confocal, ocular complication, and delayed keratitis. Quality of the evidence being provided in each article was judged based on the credit of the publishing journal.

Physico-chemical properties

Mustard agents are characterized by their cytotoxic and alkylating effects¹ and include two principal groups including; sulphur mustard (2,29-dichloroethylsulfide), which is

known for its powerful vesicant effects and used as a chemical warfare agent, and nitrogen mustard that was found ineffective as a chemical agents during World War II.³³ However, nitrogen mustard (Mustargen) and its derivatives (like cyclophosphamide) is currently used as chemotherapeutic agents in the management of malignancies i.e., lymphoma.^{18,34}

SM is not found naturally in the environment. Pure form of SM is colorless, lipophilic and viscous liquid at the room temperature with good solubility in alcohol, fuels and organic solvents.^{1,13,35} Due to impurities of warfare agents they have yellow-brown color and smell like mustard plants, garlic or horseradish, hence the name. The term 'Mustard gas' is a misnomer because in military use SM is delivered in the form of small oily droplets.²⁶ SM droplets will settle in low-lying areas for being heavier than air.

In cold and damp climates, SM can persist for months on the terrain or in porous material. Under average weather, it can last for 24-48 hours in the environment contaminating ground, clothing, foodstuffs, etc.^{8,9,11}

Due to lipophilic nature of SM, its vapor is absorbed through the skin, anterior eye and inhalation. It can also be absorbed from gastrointestinal tract following ingestion of contaminated food. SM vapor is much more toxic to skin with minimum effective dose of 4 $\mu\text{g}/\text{cm}^2$ in comparison to 20 $\mu\text{g}/\text{cm}^2$ of liquid SM for blister formation.³⁶ This significant difference is explained by 80% evaporation of liquid SM prior to penetrating the skin.³⁷ Of the remaining 20% half is absorbed in the skin and the other half into systemic circulation. It should be noted that SM causes systemic effects only at very high doses due to free hydrolyzation.³⁸ Skin penetration and thus toxicity of SM is considerably increased with heat and in occluded conditions.³⁹

Lethal effects of SM is far less than its incapacitating effects as during World War 1 and Iraq-Iran conflict, less than 5% of the injured people were killed.^{19,26,40} In human, the lethal dose 50% (LD50) is about 200 mg if the agent is swallowed, 4-5 g when applied to the bare skin with a long exposure time, and 1500 $\text{mg}/\text{min}/\text{m}^3$ when inhaled.^{2,6,16}

Pathomechanism of sulfur mustard action

Over a century of resilient research and endless dedication, ensued by numerous theories concerning SM's potential threatening impacts on viable tissues, yet the thorough mechanisms of action remains confounded by uncertainty.

Ocular injury, among others, is comprehended to be a major affliction towards patient's overall well-being. Since SM-induced ocular harming effects are mediated through acute as well as chronic phases with discrete mechanisms of action, unraveling SM pathophysiology is arguably coveted by many of the pioneers in the field.^{2,11}

To this date, countless studies have cited potentially destructive mechanisms in the SM pathogenesis. Below, we have an in-depth analysis on this perpetually growing list of mechanisms with specific attention to ocular injury pathogenesis.

1. Biochemical effects

1.1. Acid formation

Advocates of the acid liberation theory, acknowledged the emerging hydrolyzed-SM within the intracellular aqueous environment as a crucial factor in the subsequent formation of hydrochloric acid.⁴¹ However, this view was not shared by majority of the critics due to the fact that vesicant action does not keep up with the pace of acid liberation.⁴²

1.2. Reaction of sulfur mustard with intracellular proteins and enzymes (i.e. hexokinase)

This reaction was assumed to be the most important biochemical damage.⁴³ However, the level of alkylation needed for enzyme inhibition in vitro does not correlate with the vesicant doses in vivo.

Moreover, inhibition of hexokinase due to alkylation should be completed within minutes as would be expected for the resulting cellular damage. In contrast, tissue damage in vitro and in vivo caused by sulphur mustard appears with a substantial delay.⁴⁴

1.3. Intermolecular cyclization of SM

Facilitated conversion of SM to cyclic intermediates in sulfonium and carbenium cations ensured by aqueous solution, an ever-present component of ocular and mucosal tissues alike, is pivotal to the

enhanced vulnerability of these specific areas to intermolecular cyclization.^{45,46} This currently prevails amongst experts as the most consistent theory to successfully explain the root causes of SM-induced toxicity.¹³

The avid reaction of these highly-reactive by-products with cellular particles evidently results in the alkylation of a host of electron-rich bio-molecules, namely sulfhydryl (-SH) and amino (-NH₂) based-groups of proteins, nucleic acids (DNA and RNA), cell membrane and H₂O₂.^{47,48} Under these circumstances one might contemplate that physiologic, metabolic and genetic failures of cellular functioning is already a foregone conclusion.

1.3.1. Alkylation of intracellular proteins and enzymes

Note that the alkylation of enzymes may impair the cellular function from a metabolic point of view, and trigger the process of cell apoptosis.^{13,44}

1.3.2. Alkylation of extracellular proteins (i.e. collagen-mustard compound)

Corneal collagen can be alkylated following exposure to SM in a way that collagenase cannot degrade it. Formation of such collagenase-resistant stromal-SM adducts, results in increasing levels/action of collagenase that may degrade normal stromal collagen, as well as inducing pro-inflammatory responses.⁴⁹

1.3.3. Glutathione (GSH) depletion

GSH as a sulfhydryl-containing protein is virtually second to none in sustaining the appropriate oxidation-reduction state of cellular components. The rapid inactivation of sulfhydryl-containing proteins and peptides would mean a devastating effect on the intracellular reactive oxygen species (ROS) state, hence the inevitable lipid per-oxidation and loss of membrane integrity, which were once denied by a properly functioning GSH.^{50,51} Although an initial up-regulation of antioxidant enzymes (e.g., superoxide dismutase, catalase, and glutathione-S-transferase) suggests the presence of cellular defensive mechanisms, further inhibition of such enzymes, including thioredoxin reductase, by SM aggressively declines the

oxidation-reduction homeostasis.⁵²

However, the big question mark over the verity of this bio-mechanism probably lies in the fact that cell death eludes the instant microscopic detection, a concept that seems rather inconsistent with the rapid effects of SM on GSH depletion.¹³

1.3.4. Formation of reactive oxygen species⁵³

In strong favor of this mechanism, a dramatic increase (30-fold) in copper levels and a decrease in ascorbic acid, with both of these states associated with oxidative stress, are observed within the anterior chamber of the eye following the ocular exposure to mustard compounds.^{54,55}

The responsive corneal inflammations to the exposure of other ocular irritants have never ceased to exist, as in case of alkali substances.^{56,57} The imminent tissue damage after corrosive injury to the cornea is well exacerbated by penetration of ROS to the corneal stroma, leading to the crisis of DNA fragmentation along with polymerization or depolymerization of proteins and hyaluronate as well as destruction of lipid membranes.⁵⁷ ROS reduce ferric iron and copper and produce the highly reactive hydroxyl radicals. These reduced metal ions, in turn, can react with H₂O₂ to create the hydroxyl radical, yielding further damages.⁵⁸

1.3.5. Nicotinamide adenine dinucleotide depletion

Single-stranded breaks within the DNA molecules are conceived initiative to the activation of DNA repair enzymes, notably, poly-adenosine diphosphateribose polymerase (PARP). Excessive PARP activity then may well give rise to the cellular depletion of nicotinamide adenine dinucleotide (NAD⁺), with the simultaneous attenuation of glycolysis and augmentation of hexose monophosphate shunt occurring subsequent to this incident.⁵⁹⁻⁶³

Meanwhile, hexose monophosphate shunt is regarded by many as de facto turning point among the various layers of cell necrosis largely owing to the alleged stimulation of tissue protease activity, followed by disruption of dermal-epidermal attachments and the eventual blister formation.^{64,65}

1.3.6. DNA alkylation and cross-linking

Prima facie evidences have arrived at a consensus to introduce the nitrogen residue of guanine as the principal alkylating site in the nucleic acid of the mammalian origin.⁴⁷

Indeed, the cell nucleus appears as though to be the most susceptible cell area to SM-caused injury.

Additionally, this hypothesis rose to prominence in the case of bifunctional cytotoxic agents (e.g. SM) with two chloroethyl groups,⁶⁶ that directly approach an additional nitrogen residue to secure both inter- and intra-strand DNA cross-linking.^{67,68}

Sixty-one percent of total alkylations is credited to the 7-(2-hydroxyethylthioethyl) guanine (7-HETE-G).⁶⁹ To this effect, one 7-HETE-G per one million nucleotides is produced at a SM concentration of 2.3 M.⁷⁰ Other alkylations incorporate the position 3 of adenine (16%) and 0.1% at the O6 position of guanine.⁷¹

Although SM reacts with RNA, proteins and phospholipids, its DNA alkylating role has gained the highest popularity in delayed healing process.⁷² In all likelihood, the inter-strand DNA cross-linking warranted by bifunctional mustard compounds inflicts a lesion that is more than capable to be lethal at the lowest frequency of occurrence and at the lowest concentration of SM agent.

Ludlum et al believed the formation of O6-(2-ethylthioethyl) guanine is at least, partially, to blame for the mutagenesis of SM.⁷¹ An estimated 17% of total alkylations target two guanines (Galkyl-G) at either the same or the opposite DNA strand.⁷³

Shahin et al identified that 100 μ M SM can result in an approximate, 0.28 crosslinks per 10 kb in the dihydrofolate (DHF) reductase gene of human keratinocytes.⁷⁴ Soon afterwards, DNA damage paves the way for the imminent arrest at certain cell cycle checkpoints. G1 block predominantly occurs at the vesicating doses of >50 μ M whereas concentrations of around 10-fold lower than this, prove sufficient to provoke an instant G2 halt.⁷⁵ Although cellular defensive mechanisms primarily endeavor to remedy the situation, the lethal damages within the DNA molecule may eventually preclude a successful recovery. In other words, DNA alkylation when significant, leads to acute necrosis or chronic cell apoptosis. In this

regard, limbal stem cell deficiency (LSCD) in SM victims with chronic ocular injury could be -partly- attributed to direct alkylating effects of SM on DNA of these cells.⁷⁶

1.4. Nitric oxide toxicity

Perhaps the compelling evidence to prosecute the support of this theory stems back from the well-documented reaction of nitric oxide (NO) with superoxide anion, representing peroxinitrite.

Given the considerable oxidative effects of this nitrating agent, tissues face a formidable challenge ahead to defensively act upon the pursuing oxidative injury.^{59,77}

At this point, it is noteworthy that the acute damage at the corneal surface, mucous membranes and skin after exposure to SM is generated by one or more of these mechanisms. However, at higher dosages of exposure, mechanism other than DNA cross-linking are of paramount importance in that they account for a more rapid cell death.¹³

2. Gene expression and bio-molecular effects

To simply state that experimental studies constitute the cornerstone of our current knowledge about the SM-induced genetic and molecular modifications is massively an understatement.

Details of the genetic transcription with respect to SM skin injury in mice have revealed that these modifications are both time and dose dependent. Pilot studies have determined the 0.04 mg dosage of SM exposure preliminary to elicit a response by alteration of the inflammation, apoptosis (e.g., calpain 6, caspase-8 associated protein 2, and programmed cell death 10), and cell cycle regulation genes.⁷⁸

At 0.08 mg dosage, an "increase in the expression" of these genes is quite readily observed. Additionally a total number of 6 genes are modified at the level of 0.16 mg SM exposure. However, this exposure does not seem to affect the aforementioned genes as opposed to the earlier findings at the lower doses of 0.04 and 0.08 mg. This manifestation provides the prime example of what is defined as "dose dependent" effect.⁷⁸

Besides, certain studies have closely monitored the SM associated pathological situation either in vitro or in vivo, with an eye towards understanding whether these

changes are "time dependent". Although some of the previously mentioned surveys hardly concealed their interest away from addressing the issue in the under-24 hours interval post-SM exposure, there remain a sheer determination amid the larger portion of authors to assess the condition in their corresponding papers within the time periods of either on a par with or greater than 24 hours following SM injury.⁷⁸

Since the introduction, this view has met generally positive reviews. As reflected in experimental studies comprising of rabbits with SM-induced corneal impairment, a significant shift towards the positive values in interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF), calcitonin gene-related peptide (CGRP), prostaglandin E, matrix metalloproteinases (MMP-2, and MMP-9) levels/activities in the aqueous humor were observed.⁷⁹⁻⁸²

To realize the enormous extent of SM damage all the way down to the posterior part of cornea, coupled with a substantial increase in the secretion of cytokines/chemokine, and the resultant activation of stromal degrading collagenases (i.e., MMP-2 and MMP-9), provides a rational approach to implicitly approve this theory.^{80,81}

More recently, pro-inflammatory cytokines have been the primary focal point of SM dermal injury. Nevertheless, other biological responses encompassing apoptosis and cell cycle regulation have been noted in skin and keratinocyte cultures exposed to SM.⁸³⁻⁸⁵

These biological responses engage in offering the decisive steps to develop a genomic profile for SM induced ocular injury. This genetic fingerprint helps attain new biomarkers to recognize the potential medical countermeasures against SM-induced skin injury.

3. Immunologic effects

SM has been demonstrated to be highly influential on humeral and cellular immune system alike.⁸⁶⁻⁸⁹ The perilimbal site of the lesions, their similarity to Mooren's ulcer, and mixed inflammatory infiltrate within substantia propria immediately implies the immunological basis of SM-induced delayed keratitis.^{25,76,90} Depression of cell-mediated immunity was also reported. Total white blood cell count along with monocyte percentage and CD3+

lymphocytes are strongly associated with SM-exposed patients.⁸⁷ In addition, the percentage of NK cells (CD16+) is remarkably lower in patients with severe respiratory complications up to 20 years post-SM exposure.^{88,91} A meaningful correlation onto the positive direction has been found between hemoglobin levels and the intensity of ocular complications.⁹² Increased levels of IgG, IgM, and C3 have been detected in the majority of SM-exposed patients during the first weeks stretching up to the sixth month after exposure.^{91,92}

Another fascinating observation is the alleged significantly superior levels of IgG, IgM and IgE in the exposed patients vis-a-vis the control group following a 20 years interval.^{26,87,88,91}

However, it remains to be seen whether similar to Mooren's ulcers, SM-damaged corneal stroma elude alloreactive responses. One dispute is already resolved though; abnormalities in the immune system are expected to contribute to the recurrent infections, septicemia, and a higher incidence of malignancies in these patients.^{26,88,93}

4. Ultrastructural effects

Recent experimental studies have demonstrated specific patterns of ultrastructural changes in SM-induced corneal lesions. Early after exposure, the corneal epithelium and stroma are separated within lamina lucida. On day 2, intercellular desmosomal attachments and the highly convoluted basal membrane of epithelial cells show migration of an epithelial sheet. Nascent hemidesmosomal densities in the basal membranes of corneal epithelial cells and the associated anchoring plaques in the Bowman-like layer will become completely apparent by the end of day 4. However, it takes nearly seven days for the stratification of epithelial layer with maturing zone architecture. On the contrary to epithelial healing, stroma constantly depicts signs of edema and the exudates of immune cell infiltration, accompanied by necrotic fibrocytes distributed through the all parts of stromal volume. By fairly reliable accounts, this incident underlies the exposure site to appear as soon as 1 day after SM exposure.^{80,94}

But, MGK corneas exhibit signs of severe ultrastructural degeneration including extensive disruption of the basement membrane zone, necrotic and apoptotic corneal epithelial cell particles, incomplete and disorganized stratification of the corneal epithelium, and a prodigious stromal edema. Likewise, the stroma is marked by full-thickness edema, disorganized collagen fibrils, distorted lamella, heterophil infiltrates, and necrotic fibrocytes. Persistent edema was seen in animals with unresolved lesions, similar to observations in patients with delayed MGK.^{80,94}

Based on ultrastructural findings, this is hypothesized that persistent edema of Bowman-like layer interferes with proper remodeling of the basement membrane, which results in chronic cellular necrosis, inflammation, impaired corneal epithelial adhesion and degeneration of cell anterior cornea. The whole process manifests as cyclic sloughing, edema and failure of reepithelialization, which further aggravates by progressive stromal edema due to delayed SM endothelial toxicity and penetration of protein-rich fluid into stroma.^{80,94}

5. Neurotrophic effects

The other mechanism that seems to play significant role in the pathogenesis of ocular lesions by SM (specially chronic and delayed keratitis) involves significant and long-lasting denervation of cornea. Intact innervation is crucial for keeping corneal clarity, wound healing and providing suitable niche for limbal stem cells.^{79,95}

Degeneration of nerve fibers is available to see within a few hours after exposure to SM, beginning at the nerve endings of the epithelium/sub-epithelium plexus and then progressing retrogradely, in a typical Wallerian degeneration manner.⁷⁹ The damage is initially done at the erosion area, however, in a total contrast with the fact that corneal erosions heal by 96 hours post-SM exposure, corneal nerves persist in degeneration for weeks after the primary exposure.^{79,80,94}

Histo-pathologic findings

Like clinical presentation, timing and severity of histopathologic changes are dose dependent.⁹⁴ Generally, early microscopic

findings of SM toxicity occur within the first 48 hours with epithelial destruction, stromal edema and superficial infiltration of inflammatory cells (esp. eosinophil). Often, endothelial cells are spared the effects of SM. Spontaneous healing of epithelium starts at 72 hours after exposure. Intact and normal corneal epithelial cells have the capability to regenerate normal epithelium. However, scattered presence of goblet cells within newly developed epithelium in case of diffuse corneal epithelium injury, reveals the role of conjunctival cells and their migration to restore corneal epithelial layer.⁹⁶

Typical vesication of cornea manifests with separation of corneal epithelium and stroma, forming small to large vesicles with corneal nerve free endings exposed beneath. Pathologies are more pronounced at palpebral fissure due to higher exposure. In more severe exposures, vasculitis and the subsequent ischemia of conjunctiva with loss of goblet cells and mucous is observed.⁹⁰

Examination of the cornea of patients with delayed and chronic form of SM keratitis, revealed the progressive and distinctive pathology of MGK. Corneal pathologies include demolition of the epithelial and Bowman's layer, loss of keratocytes, conjunctivalization, superficial and stromal vascularization, squamous metaplasia, focal corneal thinning and ulceration, acute and chronic infiltration of inflammatory cells, lipid/amyloid deposition, endothelial cell loss, calcific band keratopathy, and scarring in the stroma. Corneal squamous metaplasia might be seen in cases with LSCD.^{8,9,25,58,90}

Conjunctival findings in patients with MGK include loss of mucous and goblet cells, chronic inflammation, vasculitis and perilimbal ischemia, telangiectasis, tortuous hemorrhagic vessels, dilated lymphatic vessels, thinning and thickening of epithelium and scarring of lamina propria. Conjunctival dysplasia has not been reported.^{8,9,25,58,90}

Clarification of ocular histopathologic responses following SM exposure has also been done through experimental studies.^{54,79-81,94} In rabbit model, the SM-induced ocular injuries are portrayed by a biphasic inflammatory response, embarking upon a few hours after exposure trailed by an ongoing chronic inflammation which last for weeks after the initial exposure.^{79,94} Injury

progression may follow either resolution within 1-2 weeks, persistent keratitis or a delayed keratopathy 3-5 weeks after exposure.

Acute toxicity results in sloughing of corneal epithelium through lamina lucida of the basement membrane, corneal edema and inflammation due to necrosing corneal tissues. Eyes with chronic and delayed injury reveal recurrent corneal epithelial erosions, inflammatory cell infiltrates, bullae, pronounced corneal edema, disorganized architecture of stroma and degradation of basement membrane area.^{54,79}

Confocal microscopy

SM is a lipophilic substance, its deep corneal penetration at the time of exposure is difficult.^{13,79} Nevertheless, confocal microscopy of subjects with MGK revealed pathologies of all the corneal layers with predominant involvement of anterior and middle layers.⁹⁷⁻⁹⁹ Findings in confocal microscopy are compatible with clinical manifestations and histopathologic findings.

In confocal microscopy, the mosaic pattern of basal epithelial cells is lost and pleomorphic cells with ill-defined borders or high reflective boundaries are present instead. Observation of spindle-like keratocytic nuclei within the anterior corneal stroma probably represents keratocyte necrosis. The gradient of keratocyte density is reversed in patients with MGK compared to normal anterior-posterior decreasing pattern.^{98,99} In these patients keratocytes density is least in the most anterior layers of the corneal stroma. Additionally, bizarre and abnormally enlarged pleomorphic keratocytes are observed in all cases with MGK, which could be defended by teratogenic and mutagenic effects of SM.⁹⁸

Foci of amyloid degradation and lipid deposition, as well as stromal neovascularization are observed. On the other hand, intrastromal necrotic tissue represents as microdots within the stroma. These pathologies can impair repopulation of stromal keratocytes.⁹⁸

Other findings include lack of subbasal nerve plexus, which could be due to destruction of Bowman layer and subepithelial fibrosis. But, confocal microscopy in patients with MGK showed increased thickness of midstromal irregular circular node-like nerve

structures.⁹⁷⁻⁹⁹

Clinical presentation

As it was mentioned above, if SM is released in the air like that in battlefield deployment, eyes, skin and respiratory system are subjected to local toxic effects of the agent.^{2,13} Thus, victims may present with multiple sites of injury, including ocular, cutaneous and lung injury.^{19,92} However, the severity of injury depends on the dose, mode of exposure, method of protection, environmental factors (like temperature and humidity) and vulnerability of victims in terms of age, gender, height, etc.^{13,19,26} Typically, symptoms and signs of acute injury appear following a variable period of latency.^{2,19,26} However, some individuals are more sensitive to effects of mustard gas and may develop symptoms sooner. Mustard gas has cumulative toxic effects and given its unspecific smell, subjects may not be aware of being exposed.¹³ Absorption of high doses may lead to occurrence of systemic toxic effect including the development of neuropsychiatric disorders, bone marrow suppression and immune system defects.^{11,13,19,26}

The eyes are the most sensitive organs to local toxic effects of mustard gas that can be attributed to the aqueous-mucous surface of the cornea and conjunctiva, high turnover rate and intense metabolic activity of corneal epithelial cells.^{21,100}

The reported fatality rates are less than 2% of the exposed soldiers during World War I and 3% to 4% in the Iraq-Iran conflict.^{1,18,40} Death usually results from either respiratory failure due to chemical bronchitis/pneumonia or bone marrow suppression.^{2,15}

Most of the victims will survive acute toxic effects of mustard gas, however a subject may suffer from chronic debilitating effects such as ocular^{3,96,101,102} and cutaneous damage,^{13,19,32} respiratory tract injury,^{14,15,103-105} reproductive and developmental toxicity, gastrointestinal effects, hematological and immunological impairments,^{88,91,106} and malignancies.¹⁰⁷⁻¹¹¹ Studying about 34,000 Iranians who survived exposure to mustard gas, revealed presence of symptoms in lungs among 42.5% of subjects, eye injury in 39.3% and cutaneous involvement in 24.5% of the studied population a decade after exposure.¹⁰⁵

Ocular manifestations

Eyes are most susceptible to local toxic effects of mustard gas. This is attributed to the moistness of the ocular surface, which facilitates intracyclization of SM and its conversion to reactive intermediates. Moreover, extreme lipophilic nature of the gas, the high turnover of corneal epithelial cells as well as their intense metabolism are associated with sensitivity of eye to toxic effects of MG.^{31,112}

Following deployment of mustard gas by Iraqi troops, about 90% of Iranian casualties presented acute ocular manifestations of mustard gas toxicity.^{26,96,113} The acute injuries usually recover completely without further lesions within 2-6 weeks. Sometimes, a corneal opacity may persist that often does not interfere with subjects' visual acuity. Also, photophobia may persist for a longer period after clinical resolution of acute lesions.^{2,11,96,101}

About 16% of patients with moderate or severe exposure (exceeding 100 mg min/m³) to mustard gas show more significant involvement of the eye, which is known as MGK.² Patients with MGK usually are characterized by chronic keratitis, impaired corneal sensation, recurrent/persistent corneal erosions, limbal vasculature injury and neovascularization, which eventually may lead to significant visual impairments and even blindness.^{3,25,27,90,114} MGK comprises two trajectories of presentation; either with a persistent keratitis that eventually leads in corneal degeneration (chronic form) or with a clinically silent period followed by reemergence of severe and progressive ocular lesions (delayed form). Unfortunately, there is no effective therapeutic management for MGK as the exact etiology and pathomechanism of this pathology is not fully understood yet.^{26,96}

Khateri and his colleagues evaluated about 34,000 Iranians who had exposure to mustard gas and survived over a decade afterwards.¹⁰⁵ They found that 61% of them were symptom free, 35% had mild findings (i.e. dry eye, diminished visual acuity and conjunctival scarring), 3.6% had moderate ocular involvement and severe symptoms were observed in less than 1% of the subjects.¹⁰⁵

Acute phase

The latent period before presence of ocular symptoms is shorter than skin injury, that can be attributed to higher vulnerability of eyes to effects of SM.²⁶ Iranian casualties reported burning and eye pain within minutes after the exposure, however it may be due to impurities of Iraqi mustard gas or caused by dust in the battlefield after explosions.² Ocular symptoms and timing are variable depending on the severity (dose and duration) of exposure to the agent.^{11,38}

Generally, within the first hour after exposure, the patient presents with the complaint of foreign body sensation and progressive ocular pain and discomfort. Local hyperemia gives the appearance of a bloodshot, which afterwards proceeds to edema and complete presentation of acute conjunctivitis.^{2,11,96,101} At 2-6 hours after exposure effects of intoxication will progress and encompass a group of debilitating symptoms including severe ocular pain, lacrimation, photophobia, blepharospasm, inflammation of anterior chamber, decreased vision and sometimes temporary blindness.^{2,101} Ocular toxic effects are maximal at 24-48 hours after exposure. The gradual spontaneous regression of symptoms usually occurs after 48 hours. Complete restoration of vision and corneal epithelial healing fulfills within 4-5 days, but patients may not be symptom free until 6 weeks or later.^{2,11,19} Regarding the severity of exposure, immediate lesions can be classified as mild, moderate and severe.^{2,26}

Exposure to 12-70 mg/min/m³ of mustard gas has mild ocular toxicity, in which usually cornea is spared and manifestations are mainly limited to as a slight eyelid erythema/swelling and conjunctival engorgement without chemosis.² Within a few days, this mild conjunctivitis will recover completely.^{2,115}

Subjects who are exposed to 100-200 mg/min/m³ of mustard gas, develop moderated ocular lesions that are characterized by involvement of eyelid, conjunctiva, and cornea.^{2,26,115} Within several hours after exposure, the corneal epithelium starts to vesicate and slough that is more prominent in the palpebral fissure. Symptoms include dry sensation, severe ocular pain, photophobia, and severe blepharospasm.

Corneal injury may further progress into superficial punctate keratitis, corneal abrasions, superficial infiltrations, corneal ulcers, and even perforation.⁹⁶ These findings are more common in the middle and inferior cornea, whereas superior cornea is protected by the upper eyelid. Symptoms usually show gradual regress after 48 hours, followed by the epithelial regeneration in 4-5 days. Symptoms may last up to six weeks or longer until they completely resolve.^{2,101}

Exposure to doses exceeding 200 mg/min/m³ bring about with severe ocular lesions as well as systemic toxicity in the skin, respiratory system, gastrointestinal tract, etc.^{2,26} Generally, lesions are more severe in the palpebral fissure and tempo-nasal zones due to higher exposure. Limbal vascular injury usually presents with whitening and necrosis of nasal and temporal limbi. Severe intoxication affects internal layers of cornea and may ulcerate the eyelids. Apart from marked congestion and chemosis, severe ischemic/necrotic conjunctival lesions are found.^{2,11,26} Adhesions between globe and the eyelids are unlikely because conjunctival lesions are mainly limited to palpebral fissure. In doses greater than 400 mg/min/m³ presentation also include low-grade iridocyclitis (usually without synechia), cataract and full-thickness corneal injury and sometimes a temporary increase in intraocular pressure. Major corneal epithelial defects as well as stromal edema resemble an orange peel appearance that usually does not stain with fluorescein. Also, nerve endings undergo degeneration, which further impairs corneal privilege and ocular protection.^{2,25}

Severely damaged eyes are susceptible to super-infection with bacterial agents such as *Pseudomonas aeruginosa*.²⁵ Other findings include miosis, chemical anterior uveitis and iris vasodilatation, hemorrhage, and necrosis. Corneal neovascularization may even progress during recovery phase causing intracorneal bleedings that leave white opacities after vessels degeneration. Within 1-2 weeks, resolution of corneal edema and uveitis indicate beginning of recovery.^{2,11,96} Otherwise, long-lasting corneal inflammation or delayed-onset corneal injury may ensue in severe cases.

Chronic phase

Less than 1% of victims of ocular SM exposure develop an irreversible, idiopathic corneal inflammation presenting after acute intoxication or following a clinically asymptomatic period of years.^{2,3,11,23,25,26,102,115} Collectively, these chronic forms of ocular injury are known as MGK. Although MGK has been primarily introduced in WW1 veterans, still, the pathways of this ocular pathology are not fully understood.^{79,94,96,102} Since then, chronic ocular injury following mustard gas exposure has been studied in human victims as well as in experimental animals.^{22,25,79,90,94} Studying Iranian casualties who presented with delayed keratitis after a long asymptomatic period provides most of our current clinical knowledge.⁹⁶ Unlike acute effects, MGK usually leads to permanent vision impairment and even blindness.^{3,25,114} Findings of recent experimental studies suggest that chronic effects of SM are fundamentally different from its acute injuries.^{26,79,94}

Generally, MGK is characterized by chronic blepharitis, xerophthalmia, redness, meibomian gland dysfunction, limbal ischemic injury, LSCD, corneal neovascularization, corneal amyloidosis, lipid deposition and corneal thinning and scarring.²⁵

In human, the chronic form of MGK manifests as prolongation of acute inflammatory signs and symptoms including photophobia, injection, lacrimation and a sensation of grittiness.^{2,25,96} Imperfect resolution of acute lesions results in chronic melting inflammation of the cornea. The significant corneal epithelial lesions during the acute phase, accompanied by compromised corneal sensation due to degeneration of nerve endings and limbal stem cell irritation because of limbal vascular injury and ischemia, further interfere with the process of re-epithelialization. Additionally, edema and infiltration in the corneal stroma plus to the abovementioned epithelial defects result in corneal irregularity, thinning, and neovascularization, which makes the eyes prone to descemetocele formation and a subsequent corneal perforation.^{25,96,97}

Intrastromal deposition of amyloid and plasma lipids may also be found as a result of new vessel formation.¹¹⁶

The delayed-onset form of MGK, appears with progressive ocular discomfort following a clinically asymptomatic period of 0.5 to 40 years. Patients usually complain of foreign body sensation, tearing, injection and photophobia.^{2,25}

Corneal lesions are more prominent in the nasal and temporal quadrants that can be attributed to higher exposure of interpalpebral area to mustard gas. Accordingly, limbal vascular injury and the subsequent limbal ischemia are more severe in these zones. However, it is suggested that susceptibility of temporal and nasal limbi to detrimental effects of mustard gas is due to existence of fewer number of stem cells rather than higher exposure.^{76,79,96}

In initial stages, surrounding of porcelain-like limbal ischemic areas by blood vessels is frequently observed.^{2,11,25} Mustard gas induced chronic vasculitis leads to ischemia within limbal areas surrounded by varicose leaking fragile vessels that form blood islands and cause subconjunctival hemorrhages. This ischemic-hemorrhagic process results in amyloid and lipid deposition in the adjacent cornea and occurrence of a subsequent stromal inflammation and thinning.¹¹⁶⁻¹¹⁸

In advanced stages, amyloid and lipid deposits cover vascularized scars of the cornea that further worsens corneal opacities. This is followed by secondary degenerative changes that may lead to recurrent ulcerations, thinning and sometimes perforation and phthisis bulbi. Typically, superior segments of cornea are less involved due to protective effects of upper eyelid during primary toxicity of mustard gas. Unfortunately some of these lesions may recur even after penetrating keratoplasty.¹¹⁷

SM-delayed keratitis can be graded as:^{25,76}

- Mild: conjunctival vessel deformation including tortuosity, segmentation and telangiectasia without involvement of adjacent cornea
- Moderate: conjunctivalization, limbal ischemia and peripheral vascular invasion with/without corneal opacity
- Severe: conjunctival ischemia, corneal neovascularization, corneal thinning and melting, and secondary degenerative changes.

Although the severity of deficiency of limbal stem cell is associated with the grade of keratitis, it seems that other underlying mechanisms also play a role in the pathogenesis of delayed MGK.^{76,79} This assumption is supported by clinical and histo-pathological findings of several human and experimental animals. Demonstration of goblet cells on the cornea corresponds to LSCD in patients with delayed MGK.^{25,76} but, other conjunctival presentations are not typical in these cases, i.e. instead of a total vascularized pannus formation, peripheral corneal invasion with telangiectatic, leaking, torturous aberrant vessels is frequently seen. Likewise, corneal manifestations are not compatible with findings in LSCD. Distinctive features include epithelial irregularity, recurrent or persistent epithelial erosions, neovascularization, scarring, thinning, and lipid and amyloid deposition.^{25,76}

It is suggested that ocular presentations in delayed-keratitis are the consequence of three underlying mechanisms: (1) progressive deficiency of limbal stem cell that initially is incomplete and irregular and finally cause total depletion of stem cells. Ongoing loss of stem cells triggers a second cascade of pathologies manifested by conjunctivalization, neovascularization and sustained epithelial erosions. Also, it is suggested that chronic limbal ischemia can lead to neurotrophic and trophic changes including corneal thinning, descemetocoele formation and perforation.^{2,76,96} (2) Persistent, progressive and severe limbal and perilimbal ischemia that is predominantly seen in the palpebral fissure. This represents with stromal thinning and epithelial degeneration of the adjacent cornea. This seems to be secondary to SM-induced vasculitis of limbal vessels.^{25,76} (3) Secondary degenerative changes in the cornea due to lipid and amyloid deposition. This mechanism is related to chronic melting stromal inflammation and thinning.¹¹⁶

The exact effect of SM on limbal stem cells is still unknown. Generally, stem cells have low mitotic activity and they should be quite resistant to direct effects of mustard gas.^{79,119} However, it is possible that alkylation of DNA of limbal stem cell impairs sufficient production of epithelial cells, contributing to persistent epithelial defects.⁷⁹ Experimental studies also proposed presence of a

secondary detrimental process in the vicinity of limbal stem cells that causes LSCD, rather than direct effects of SM.⁷⁹ This secondary process seems to be the result of prolonged impaired innervation as well as ischemia of limbus, chronic inflammation, decreased growth factors, and activation of matrix metalloproteinase that produce a pathologic niche for limbal stem cells.^{79,96}

Intact corneal innervation is crucial for maintaining corneal health through its trophic effects in protecting normal epithelial integrity and its role in the process of wound healing.⁹⁵

Studying rabbit model of MGK revealed severe retrograde degeneration of corneal nerves with a typical Wallerian degeneration, which occurred within hours after exposure and did not heal even after months.⁷⁹ Consistent to these findings, corneal anesthesia/hypoesthesia was observed in victims of ocular SM that contributed to neurotrophic keratitis in some of the casualties.^{25,95,97,120,121}

Additionally, a marked inflammatory response develops in eye and skin of animals with exposure to SM. Nevertheless, treatment with anti-inflammatory agents is partially effective in ameliorating acute and delayed corneal injuries as well as inhibiting neovascularization.^{1,80,94,122-124}

Future studies are necessary to assess the possible role of angiogenic factors in the pathogenesis of SM-induced keratitis especially regarding its neovascularization feature.

It has also been postulated that autoimmune reactions to the altered corneal proteins (collagen-mustard compound) can lead to delayed keratitis. In a recent study by Naderi et al,⁴⁹ they observed inefficacy of collagenase -a critical enzyme for maintaining corneal stromal health- to degrade altered stromal collagen due to SM exposure. A respective increased in expression of collagenase may lead to degradation of normal stromal collagen and increase production of pro-inflammatory cytokines.⁹⁴

Conclusion

In summary, SM when delivered in the form of vapor can induce debilitating ocular injuries both acutely and chronically. Ocular injuries are mediated through different toxic mechanism including: biochemical damages, biomolecular and gene expression modification, induction of immunologic and inflammatory reactions, damaging ultrastructural architecture of cornea, and long-lasting corneal denervation. Most of the patients recover from acute intoxication with SM, but a minority of victims will suffer chronic ocular injury (i.e. MGK). The exact pathogenesis of MGK is not well-known, but it seems that chronic loss of limbal stem cells is the chief cause of signs and symptoms. LSCD may occur due to direct DNA alkylating effect of SM, and indirectly through inducing chronic corneal denervation and inflammation. Consequent presentation of severe progressive ocular complications may threaten victims' vision.

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