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13. ABSTRACT *(Maximum 200 Words)*

Studies are in progress to understand the basic mechanism of damage to the eye on exposure to mustard gas and to develop preventive therapies. These studies have been done using rats as experimental animal models and 2-Chloroethyl-ethyl sulfide (half mustard) as the model compound. It is hypothesized that the toxic effects of mustard are caused by reactions at multiple sites, including the sites located out side the cell membrane as well as inside. The manifestations are hence a consequence of derangements starting with the extra-cellular membrane and extending to the various intracellular sites, with adverse effects on the membrane permeability and transport activities, and inhibition of multiple bio-energetic processes, and initiation of oxidative stress. We have hence felt desirable to develop a mixture of compounds capable of simultaneously antagonizing the various adverse biochemical reactions, and to test the efficacy of such a mixture against tissue damage. As described in the report, we have developed a mixture of compounds labeled as VM. It has been found to attenuate corneal damage caused by exposure of the tissue to half mustard. We now plan to extend this study using HD, the real warfare agent.

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PI - Signature Date

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Annual Report USARMY 2001.
Sulfur Mustard Damage to Cornea: Preventive Studies.

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Introduction:

Sulfur mustard (HD) is one of the most common chemical warfare agents that affect the performance of the combat personnel soon after their exposure to this agent, dispersed either as such or in an aerosol mist. It was first used by the German Army in 1917. Subsequently, it has been used in World War II, Iran-Iraq conflict and most recently in the Persian Gulf Operation. The immediate manifestation of exposure to this agent consists of a generalized irritation and intense itching all over the body including the eye. This is soon followed by development of dermal erythema and blisters in various regions of the body and development of corneal haze. The latter leads to the development of impaired vision, which may persist for weeks and months to the permanent lifetime disability. The rapid and more pronounce effects in the eye is related to the availability of the excessive water in the cull de sac, and consequent rapidity with which the incoming mustard can be hydrolyzed to more toxic derivatives.

The mustard (HD) induced ocular symptoms of irritation and itchiness to be followed by tissue necrotic changes in the cornea and conjunctiva can be triggered by exposure to levels as low as 1/10,000. The pathological legions in the cornea as well as in the skin brought about by mustard exposure have been previously well studied in experimental animals as well as humans. General information is also available on the reactivity of mustard at biochemical levels. The toxicity seems to be triggered primarily by its strong alkylating effects, modifying a host of structural and nonstructural but metabolically important cellular constituents. However, development of an effective therapy, prophylactic or post-exposure, against mustard toxicity has been difficult and non is available so far. We propose that such difficulty may be due to use of preparations containing single agents. The failure of treatment by use of such preparations can be linked to the inability of active compounds to exert a more generalized inhibition against alkylation of the diverse of the tissue constituents, at sites extending from the outer limiting cell membranes to the cytosolic, mitochondrial and intra-nuclear regions. Attention has also not been given to toxicity caused by a simultaneously general inhibition of cellular metabolism because of the alkylation of various –SH and –NH- groups of the enzymes and their cofactors, and consequent decreased energy production required to drive transport pumps and sustain biosynthetic and repair activities. The inhibition of metabolism is also known to divert the available oxygen towards generation of free radical derivatives causing oxidative stress to the tissue. The latter leads to further

de-naturation of the enzymatic and non-enzymatic protein constituents, as well as per-oxidation of membrane and cytosolic lipids. We, therefore, hypothesize that the pathological manifestations of mustard on the cornea, conjunctiva and other exposed tissues could be more effectively prevented by topical application of a formulation containing a mixture of compounds selected to inhibit the several toxic biochemical reactions initiated by alkylation with sulfur mustard, as well as capable of promoting tissue regeneration and repair by providing additional metabolic support and preventing oxygen radical induced damage.

It is also clear that the extent of pathophysiological/dysfunctional manifestations in response to mustard exposure should differ widely from tissue to tissue, depending upon their anatomical structure and function, and their underlying biochemical and metabolic make-up and characteristics. The most apparent physiological function of the cornea for example is to restrict (Barrier Function) the movement of charged ions from the tear film to the aqueous humor. It is this involved in the maintenance of corneal detergescence. While all other layers of the corneal structure (epithelium, stroma and endothelium) are also important in maintaining the detergescence of the tissue as a whole, the epithelial layer occupies an important place also in preventing physiological damage caused by external environmental agents. Its damage by such agents leads to hydration and opacity development. The tight packing of the epithelial cells is essential also for physically preventing the internalization of various pathogens. The hydrophobicity linked to the orientation of the membrane lipids also limits the permeability of uncharged molecules such as mannitol, as recently demonstrated in this laboratory. It resistivity to the penetration of charged ions and consequent participation in the maintenance of tissue hydration is related to the anteriorly directed activity of the transport pumps, particularly the Na-K-ATPase.

The early development of edema and haziness in the cornea following the mustard exposure may therefore be triggered by alkylation of various enzymatic and nonenzymatic components of the exposed epithelial cell membrane. This would be followed by alkylation of various cytoplasmic, mitochondrial and intranuclear enzymatic and nonenzymatic constituents inhibiting cellular metabolism involved in energy production necessary to drive the transport pumps and to sustain other biosynthetic and tissue repair processes. As referred above, metabolic inhibition also leads to increased production of toxic oxy-radical derivatives known to inflict tissue damage by oxidative stress. The distribution patterns of various reactive constituents inside and outside the corneal epithelial cells along with the kinetic variations in the reactions involved could be so diverse that a single compound may not be able to prevent mustard damage. We have, therefore, continued to study the biochemical and physiological mechanisms involved in mustard damage to the cornea and to examine if the physiological damage caused by exposure to this gas can be prevented by topical treatment with a preparations consisting of compounds endowed with appropriate biochemical and physiological properties.

(1) Competitively inhibit alkylation at -SH and --NH sites of the cellular constituents,
(2) Cleave –SS-to SH
(3) Scavenge reactive Oxygen species and minimize oxidative stress to the tissue
(4) Provide additional metabolic and regenerative support by supplying additional substrates and maintaining the status of tissue redox

(5) Offer protection against the diverse effects of invading inflammatory cells, including the oxidative stress caused by liberation of oxygen radicals
(6) Decrease prostaglandin synthesis
(7) Help in tissue regeneration.

We have devised such formulation that is effective in inhibiting half mustard-induced damage to the cornea. Preliminary results also demonstrate a potential effectiveness of our formulation against HD induced damage to the eye, conducted in collaboration with USAMRICD.

The proposed mixture consists of the following compounds purporting to perform the functions indicated.

Taurine (Membrane stabilization and inhibition of N alkylation, Anti-oxidant)
N-acetyl cysteine, Penicillamine (Reduce –SS- to –SH, Inhibit S-alkylation).
Alpha-keto-glutarate (Oxyradical scavenger)
Pyruvate (Oxyradical scavenger and metabolic support)
Glucose (Metabolic support)
Insulin (Promote glucose availability to tissue metabolism, mitosis and tissue regeneration)
Patothenate (Provide metabolic support via Coenzyme A)
Salicylate (Inhibit prostaglandin synthesis)
Citrate (Modulate invasion by inflammatory cells)
Indomethacin or Dexamethasone (Inhibit prostaglandin synthesis).
Retinol palmitate or other esters of retinol, (epithelial regeneration)
Free or esterified alpha-tochopherol (Prevent oxidation of ointment base and membrane lipids)

The continued specific aims of the project are hence as follows:

A: Investigations on the physiological and Biochemical mechanism of Mustard toxicity to cornea.

B. Determine the protective effect of the above mixture against mustard induced damage to the eyes of mice and rats, in vitro.

C. Extend the above studies using the human cornea obtained post-mortem.

D. To determine the protective effect of the formulation against mustard damage to the rat and mice skin in vivo.

E. To implant sub-dermally an osmotic pump containing the formulation near the sites exposed to mustard and follow the process of recovery.

Study During the period under review: Studies are in progress to determine the efficacy of mixtures containing the above ingredients against mustard induced damage to the external eye, particularly the cornea. 2-Chloroethyl-ethyl sulfide (Half Mustard) is used as a model laboratory compound. Its effects are similar to the effects of full mustard (HD). We have discovered that an aqueous preparation (Varma Mixture) containing the above ingredients could be effective against mustard induced damage to the eye as judged by biochemical, morphological and ophthalmologic studies. The biochemical and morphological studies were done using cornea as the representative tissue of the external eye. Emphasis was laid on the cornea also because of its early and direct involvement in the onset of visual disability in the combat personnel exposed to the mustard spray. However, retention of the aqueous preparation in the cul-de-sac is relatively short. Hence a frequent application is required. Therefore, further studies are in progress to improve the rheological properties of the preparation. We are now incorporating the above ingredients into a home made ointment base consisting of liquid paraffin, Tween-80/60, and methylcellulose. Lipid soluble nutritional antioxidants such as retinol and alpha-tochopherol were also added.

The overall composition of the preparation is as follows.

Wash Solution: g/100ml water

Taurine	1.20
Ca-pantothenate	0.35
Na-Pantothenate	0.35
Glucose	0.30
Na-citrate	0.20
EDTA Na (di)	0.025
Na-salicylate	0.25
N-acetyl cysteine	0.25
Na-pyruvate	1.12
Alpha-ketoglutarate	2.40
Choline chloride	0.60
Hydroxymethyl cellulose	0.370
Adjusted pH	7.50
Tonicity	663

Composition of the Ointment: (%)	
Taurine	1.30
Ca-pantothenate	0.36
Na-Pantothenate	0.36
Glucose	0.30
Na-citrate	0.20
EDTA Na (di)	0.02
Na-salicylate	0.25
N-acetyl cysteine	0.25
Na-pyruvate	1.16

Alpha-ketoglutarate	2.45
Hydroxymethyl cellulose	0.370
Adjusted pH	7.50
Tween 60	21
Insulin	.025
Retinol	0.26
Alpha-tochopherol	0.26
Indomethacin	0.13
HPMC	0.16

Mineral Oil	8.4
White Petrolatum	39
Lanolin	4.3
Water	40

Spague-Dawley albino rates weighing about 250 gm were used. They were anesthetized with an intramuscular dose of Ketamine-Xylazine in the dose of 66mg and 6.7 mg /kg body weight. Cornel dryness was prevented by instillation of a few drops of saline or artificial. tear. CEES was dissolved in propylene glycol (20 ul in 200 ul of the latter). 10 ul of this was applied in each eye at 10 AM. After a wait of 10 minutes, eyes were rinsed with about 2 ml. of VM Aqueous. Just before using this solution, 1.2 ml of a 50 % solution of Choline Hydroxide was added to 100 ml and the pH and tonicity adjusted to 7.6 and 600 mOsmoles respectively. The process was repeated again at 20 and 30 minutes. The ointment as described above was applied to eye after another wait of 10 minutes. The application of the ointment was then repeated at intervals of 30 minutes till 6 PM. The total number of application on this day was 12. On subsequent days the ointment was applied 6 times during the daytime only. The treatment was stopped on day 18. The eyes were examined every day for 30 days for corneal and lid edema, corneal ulceration and opacity, neovascularization. and conjunctivitis, starting from the day on which CEES was applied. Final judgment of the efficacy of the treatment however relied on photography, histology and transparencey of the cornea. A parallel group of animals that received CEES were kept as the untreated Controls.

Results and Discussion

Figure 1 describes the results as apparent by visual and pen light inspection of the eye aimed at determining the status of corneal hydration and Opacification The data represent the observations till day 15, the mid point of total number of days for which the animals

were followed. As summarized therein, the cornea of the normal controls animals remained unaffected during the period of observation.
In the CEES group that remained untreated, almost all the corneas lost their transparency by the third day. The loss in transparency was associated with the development of edema and ulceration. There was remarkable swelling of the lids also. It was interesting to note however, that the corneas seem to partially recover their transparency and detergence significantly, starting after about a week, in the animals treated with the ointment, the corneas maintained their transparency almost to normal at least till 10 days. Subsequently, they seemed to develop minor opacity. The extent of opacity however was still very minor. No In another group where the ointment contained a small amount of dexamethasone(D)(0.1%) the scores were similar to that in the animals where the ointment did not contain and dexamethasone.

Development of Corneal opacity and its remission after CEES Exposure

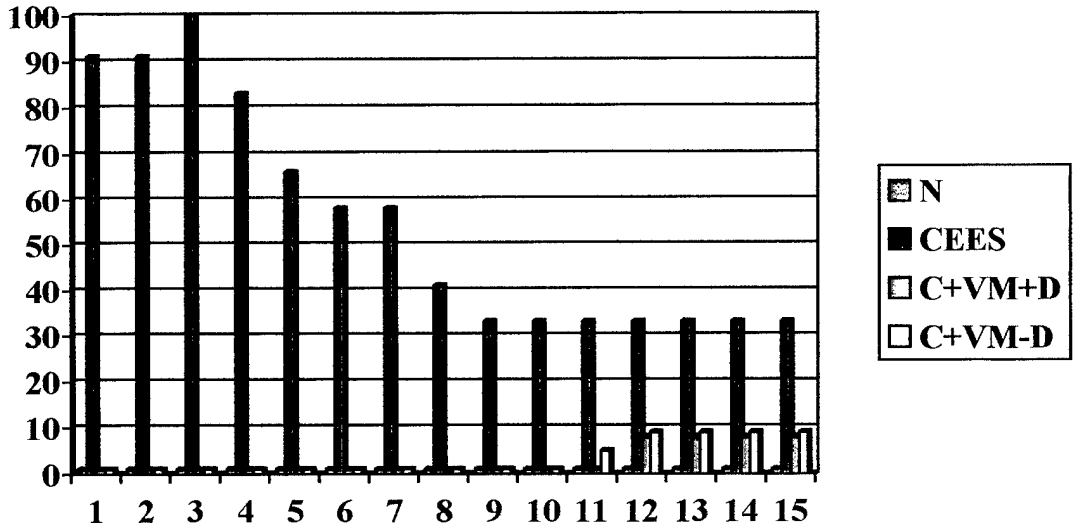


Figure 1. The ordinate represents the percentage of eyes that developed corneal opacity. The abscissa indicates the days following CEES application. Number of animals in each experiment was six, number eyes=12. The experiments were repeated at least three times with similar results.

In terms of ulceration as noted by flourosceine staining, a large number of the animals (80%) developed corneal ulceration within a weak of receiving CEES. However, these ulcers, as expected, started to undergo healing at this time, the process being completed by about the end of the month. But simultaneous with the healing, an aggressive neovascularization of the cornea was set in, the new vessels covering extensive areas in the cornea by the end of the second weak (Figure 2). In the animals treated with VM ointment, 11 out 12 eyes showed no ulceration to start with, and no neovascularization was apparent till at least 13 days. On subsequent days also the extent of vascularization

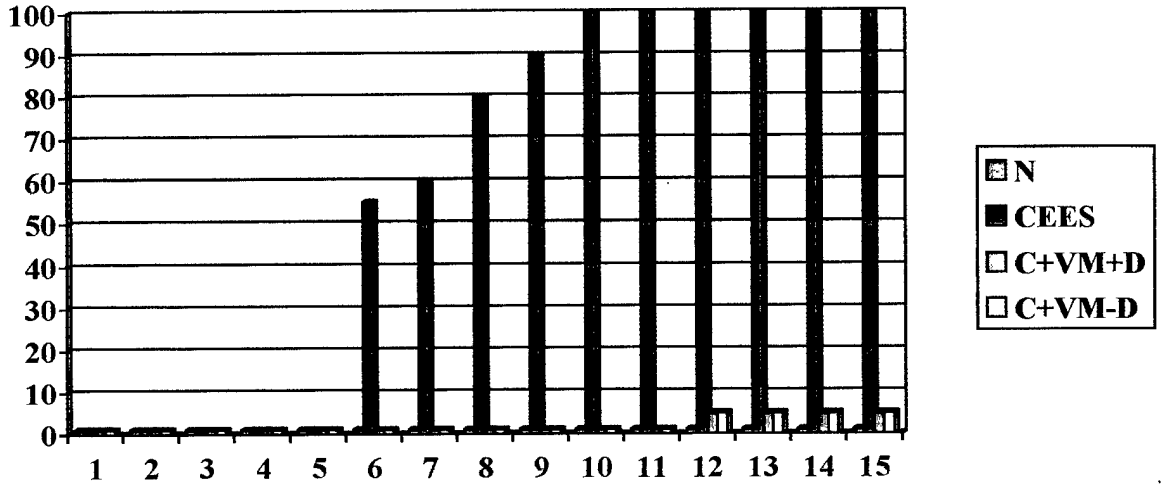
remained minor. Hence VM ointment had a significant beneficial effect against CEES induced neovascularization of the tissue. The results were similar in the group of animals treated with ointment containing dexamethasone .

Photographic data on the status the eye as existed on day 21 are given in Fig 3. Fig 3 A represents the picture of the external eyes in the normal animals. As apparent in Figure 3 B, the formation of abnormal blood vessels in the cornea in the untreated group is quite extensive. The corneas in the two treated groups (Figures 3C and 3D) exhibit cleaner appearance (close to the normal). No neovascularization was apparent at least till two weeks. However, after this time, minor vessels seemed to appear in the group 3 D where the ointment was fortified with 0.1% dexamethasone. The usefulness of this fortification is hence considered un-decisive at present.

More definitive evidence on the preventive effect of VM against corneal damage by CEES was obtained by histological examination of the tissue. Fig 4A represents the histology of the normal cornea, consisting of well organized layers of cells and connective tissue: the layers formed by the epithelium, the Bowman's membrane, stroma, Descemet's membrane and the inner endothelium. As shown in Figure 4B, the epithelium in the CEES group has degenerative changes marked by proliferative cells and the presence of prominent undefined hyaline structures. The layer is also edematous. The stroma is also swollen and has substantial number of inflammatory cells. These changes were prevented from taking place by topical treatment of VM as described above.

In view of these effects we consider that further studies with VM or its modifications may yield preparations suitable for use in preventing ocular damage caused by mustard exposure to the combat personnel. While the present composition of the VM seems adequate to prevent most of the CEES induced changes in the eye, particularly in the cornea and lids, further studies must be continued to increase its effectiveness against neovascularization. Such studies are in progress. Studies with HD are also essential in order to assess the therapeutic potential of VM.

Incidence of Corneal Vascularization during VM treatment after CEES Exposure



Firgure2B: The ordinate represents the percentage of eyes whose corneas showed visible neovascularization. The abscissa represents the days for which observations were made after the application of CEES.

Additional legends:

Figure3. Photographs of the Eyes. A: Normal Eye. B: Eye exposed to CEES and remaining untreated as described in section on methods. C: Eyes treated with VM fortified with dexamethasone after application of CEES. D: Eyes treated with VM alone after application of CEES. All pictures were taken 15 days after the initial application of CEES.

Figure 4: H&E stained sections of the corneas isolated form the above eyes as described in legends to figure 3.

Key Research Accomplishment;

An ointment (VM) has been formulated which inhibits Half-Mustard induced damage to cornea.

Reportable outcome

Morphological Correlates of the Protection Offered by Varma Mixture in Rat Cornea Exposed to Half Mustard (CEES): A proposed new treatment for sulfur mustard toxicity.J. Toxicol. Cutaneous and Ocular Toxicology, 19, 154-163, 2000.
Varma SD, M Henein, Ali H. Ali, PS Devamanoharan, TA Hamilton, JP Petrali.

Conclusion and Summary.

Studies are in progress to understand the basic mechanism of damage to the eye on exposure to mustard gas and to develop preventive therapies. These studies have been done using rats as experimental animal models and 2-Chloroethyl-ethyl sulfide (half mustard) as the model compound. It is hypothesized that the toxic effects of mustard are caused by reactions at multiple sites, including the sites located out side the cell membrane as well as inside. The manifestations are hence a consequence of derangements starting with the extra-cellular membrane and extending to the various intracellular sites, with adverse effects on the membrane permeability and transport activities, and inhibition of multiple bio-energetic processes, and initiation of oxidative stress. We have hence felt desirable to develop a mixture of compounds capable of simultaneously antagonizing the various adverse biochemical reactions, and to test the efficacy of such a mixture against tissue damage. As described in the report, we have developed a mixture of compounds labeled as VM. It has been found to attenuate corneal damage caused by exposure of the tissue to half mustard. We now plan to extend this study using HD the real warfare agent.

References:

1. Sidel, F.R., Smith, W.J., Petrali, J.P., Hurst, (1996). Sulfur Mustard; A chemical Vesicant Model. In " Text Book of Dermato-Toxicology" Edition V. Chapter 9, pages 119- 1299. Ed. F.N. Marzulli & H.I. Maiback. Publ. Taylor and Francis

2. Gilman, A., Philips, F.S. (1946). The biological actions and therapeutic applications of beta-Chloroethyl amines and sulfides. Science 103, 409-415.

3. Lawley, P.D and Brooks, P. (1965). Molecular mechanism of the cytotoxic action of difunctional alkylating agents and of resistance to this action. Nature 206, 480-482.

4. Wheeler, G.P (1962). Studies related to the mechanism of actions of cytotoxic alkylating agents: a review. Cancer Res. 22, 651-687.

5. Papirmeister, B., Gross, C.L., Meir. H.L., Petrali, J.P and Johnson, J.B. (1985) molecular basis of mustard-induced vesication. Fundamentals and Applied Toxicology 5, S134-S149.

6. Marlow, D.D., Mershon, M.M., Mitcheltree, L.W., Petrali, J.P and Jaax,G.P. (1090)

7. Sulfur mustard induced toxicity in hairless guinea pigs. J. Toxicol & Ocular Toxicol 9, 179-192.

8. Petrali, J.P., Hamilton, T.A., Mills, K.R. and Day, R. (1993). Cell injury and calcium accumulation following sulfur mustard exposure. Proc. 51st Ann. Meeting of Microscopy, Society of America, San Francisco Press, p. 322-323.

9. Varma, S.D., M, Henein., Ali H, Ali., P.S, Devamanoharan., T.A.Hamilton, JP Petrali (2000). Morphological correlates of the protection offered by Varma Mixture in rat cornea exposed to Half Mustard (CEES): A proposed new treatment for sulfur mustard toxicity. J. Toxicol. Cutaneous and Ocular Toxicology, 19, 154-163, 2000.

10. Varma, S.D. P.S, Devamanoharan, Ali, H.Ali. M.Henein, J.Petrali, J.Brozetti, and E, Lenhart. (1998). Corneal damage by half mustard: in vitro Preventive study: Histologic and electron microscopic evaluation. J. Ocular Pharm.Ther. 14, 413-421.

11. Varma, S.D. P.S, Devamanoharan, Ali, H.Ali. M.Henein, J.Petrali, J.Brozetti, E, Lenhart & A.Wier. (1998). Half Mustard induced damage to rabbit cornea: Attenuating effect of taurine, pyruvate. Alpha-ketoglutarate, pantothenate mixture. J. Ocular Pharm.& Ther. 14, 423-427.

Figure 3

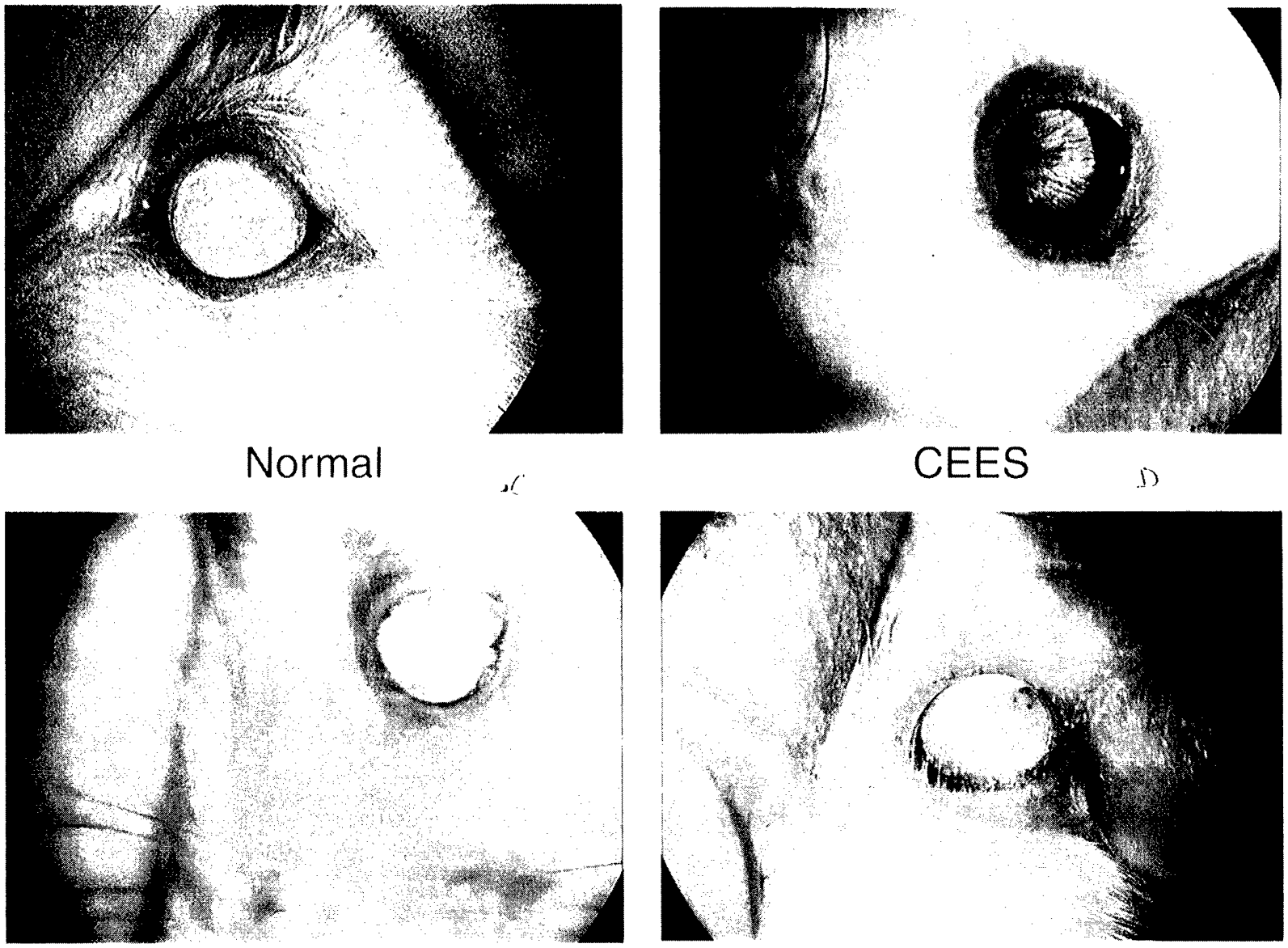
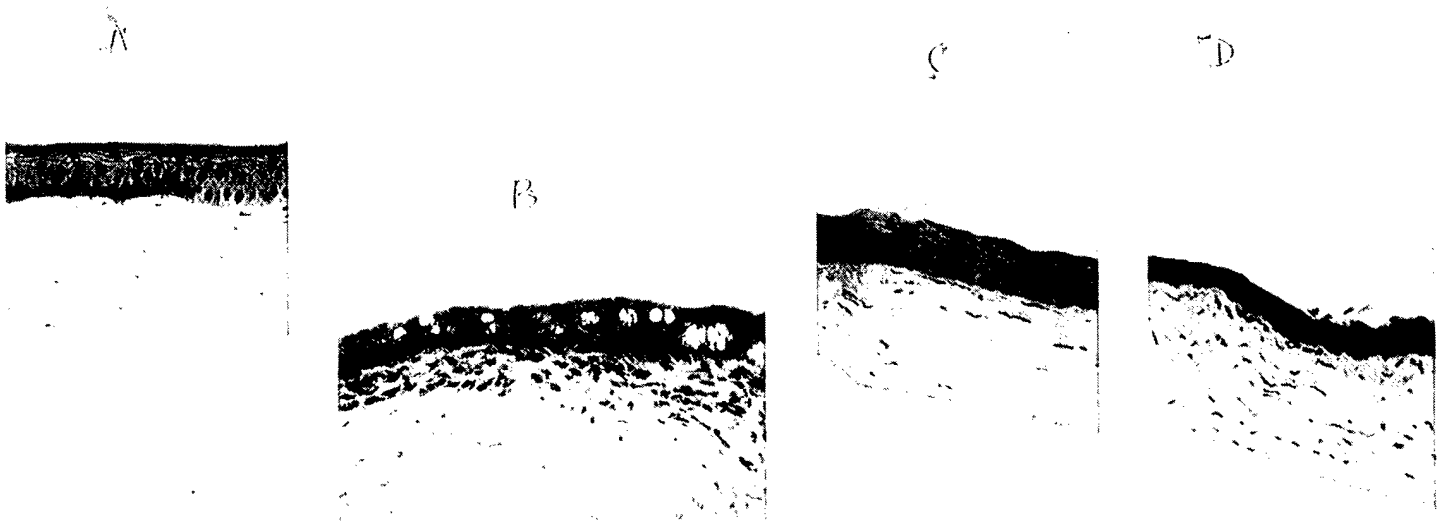


Figure 4



Normal | CEES | CEES + VM + DEX | CEES + VM - DEX

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Cutaneous and Ocular Toxicology

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J. Toxicol.—Cut. & Ocular Toxicol., 19(2&3), 153–163 (2000)

MORPHOLOGICAL CORRELATES OF THE PROTECTION AFFORDED BY VARMA MIXTURE IN RAT CORNEA EXPOSED TO HALF MUSTARD (CEES): A PROPOSED NEW TREATMENT FOR SULFUR MUSTARD TOXICITY

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Abstract

Sulfur mustard gas, a bifunctional alkylating synthetic vesicating agent, has been used in warfare since World War I. It continues to be

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The opinions or assertions herein are the private views of the authors and are not to be construed as official or as reflecting views of the Army or the Department of Defense. In conducting this research the investigators adhered to the *Guide for Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources National Research Council.*

a modern chemical warfare threat agent of choice of some factions. Whole-body exposure to sulfur mustard gas, or its laboratory simulant, a monofunctional alkylating sulfur mustard, CEES, induces cutaneous, respiratory, and ocular impairments. Of these, ocular damage resulting from irritation and edema of eyelids, conjunctiva, and especially cornea causes the most immediate incapacitation. This initial ocular pathology may be followed by secondary intracorneal changes leading to severe corneal opacities. Heretofore there has been no specific prophylaxis except avoidance, and no specific therapy except palliative for mustard gas-induced ocular lesions. In the present study, we present morphological correlates of the apparent attenuation of CEES-induced ocular lesions in a rat eye model by a therapeutic mixture compound developed by Varma. Varma mixture consists of compounds known to provide bioenergetic support, reduce oxidative stress, and support tissue metabolism.

Introduction

Whole body exposure to the chemical warfare agent sulfur mustard gas, bis (2-chloroethyl) sulfide (HD), or its laboratory model simulant, half mustard, 2-chloroethyl ethyl sulfide (CEES), induces cutaneous, respiratory, and ocular impairments. Of these, ocular damage causes the most immediate incapacitation with initial symptoms evident within minutes.[1] This incapacitation is the result of irritation and edema of the eyelids, conjunctiva, and especially the cornea. The initial ocular pathology may be followed by a cascade of secondary intracorneal changes to the epithelium, stroma, and endothelium leading to development of corneal edema, opacification, and compromise of corneal physiology.[2] This pervasive corneal pathology subsequently results in a deterioration of corneal transmissive and refractive properties, with predictable untoward effects on visual acuity.

The toxicity of mustard gas is a highly complex and multifaceted biochemical event. The toxicity has been partially attributed to the formation of a transition-state positively charged sulfonium ion. Sulfonium ion is a highly active alkylating agent that can potentially alkylate a wide variety of nucleophilic sites of macromolecular components of tissue, such as peptides, proteins, enzymes, membrane components, and nucleic acids.[3] Although these interactions are the subject of continued investigations, the correlation with tissue injury has not been made nor has the most critical interaction yet been identified. It is conceivable that tissue-injuring effects of sulfur mustard may be the result of a combination or a cascade of biochemical effects.

Heretofore, there has been no specific pretreatment or antidotal therapy for mustard-induced ocular impairment. We describe here for the first time the morpho-

logical correlates of the apparent attenuation of CEES-induced ocular lesions by a topical compound developed by Varma.[4,5] Varma mixture (VM) consists of compounds known to provide bioenergetic support, prevent oxidative stress, modulate membrane permeability, and support tissue metabolism. Compounds used in VM are pyruvate, alpha-ketoglutarate, calcium pantothenate, and taurine.

Materials and Methods

Eyes of anesthetized rats (0.3 ml of a mixture of 8% ketamine and 0.3% xylazine, IM) were exposed in a fume hood to 3μmole CEES (10 μl CEES solution in propylene glycol) applied directly to the corneal surface with a Hamilton microliter pipette. Animals were allowed to recover and returned to housing cages. Animals receiving no exposure, or exposed to propylene glycol alone, served as controls. Following exposure, those animals selected for prevention/treatment studies were administered VM (40 mM sodium pyruvate, 10 mM alpha ketoglutarate, 2.5 mM calcium pantothenate, 75 mM taurine in basal Tyrode's solution) according to the following schedule. Eyes were initially wash-treated with 3 ml VM at 5 min after CEES exposure with subsequent washes continuing every half hour for the first day (8 h) and every hour for the second day (8 h). Some CEES-exposed eyes were washed with basal Tyrode's solution alone to serve as additional positive controls. On the third day, unexposed, propylene glyco-exposed, CEES-untreated and CEES-VM-treated animals were euthanized by CO_2 inhalation in sealed plastic cages followed by incision of the diaphragm muscles. Eyes were then enucleated and corneas removed at the limbus by scalpel. Corneas were processed for routine hematoxylin and eosin light microscopic examination and for macrophotographic examination of transmissive properties. Corneas selected for ultrastructural study were fixed in 50% Karnovsky's fixative (2.5% glutaraldehyde and 1.6% formaldehyde in 0.1M sodium cacodylate buffer), dehydrated in graded ethanol, and embedded in epoxy resin. Thin sections were differentiated with uranyl acetate and lead citrate and analyzed with a JEOL 1200 EX transmission electron microscope.

Results

Light Microscopic Examination

Transmissive studies of exposed, unexposed, untreated and treated corneas demonstrated the protective effect of VM against the almost complete opacification induced by CEES (Fig. 1). On histopathological examination, corneas exposed

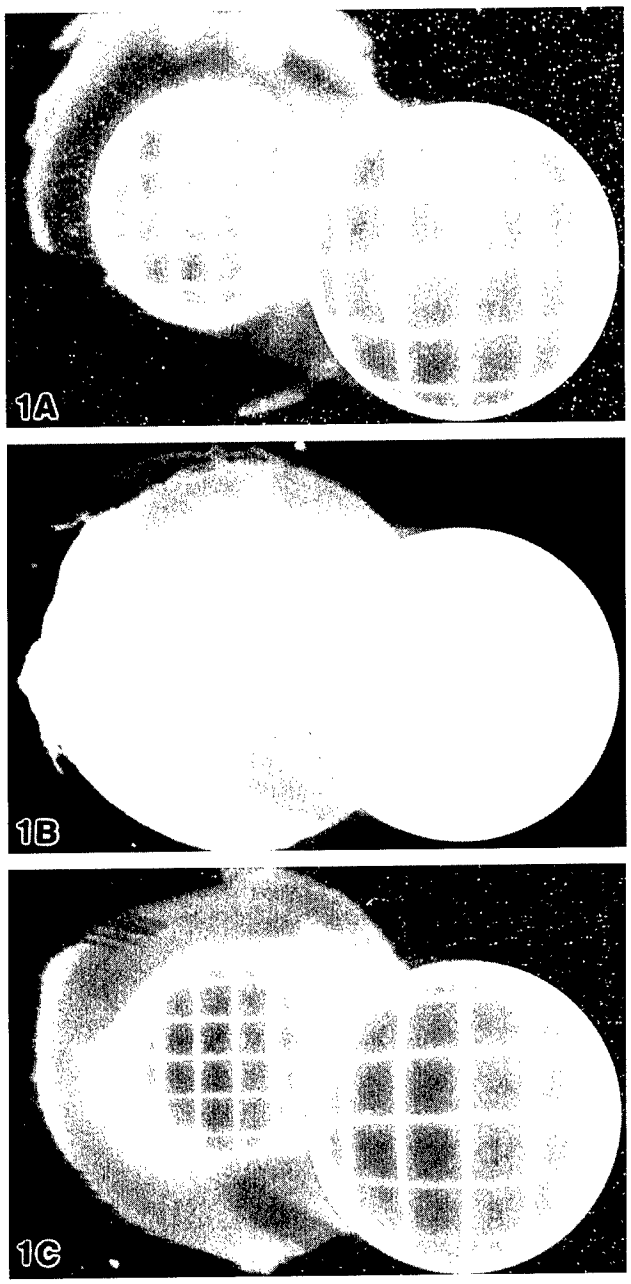

Figure 1. Macrophotography of transmissive properties of rat eye corneas. (A) Geometric pattern (copper specimen grid) photographed through control cornea. (B) Geometric pattern photographed through CEES-exposed cornea. (C) Geometric pattern photographed through CEES-exposed cornea treated with VM.

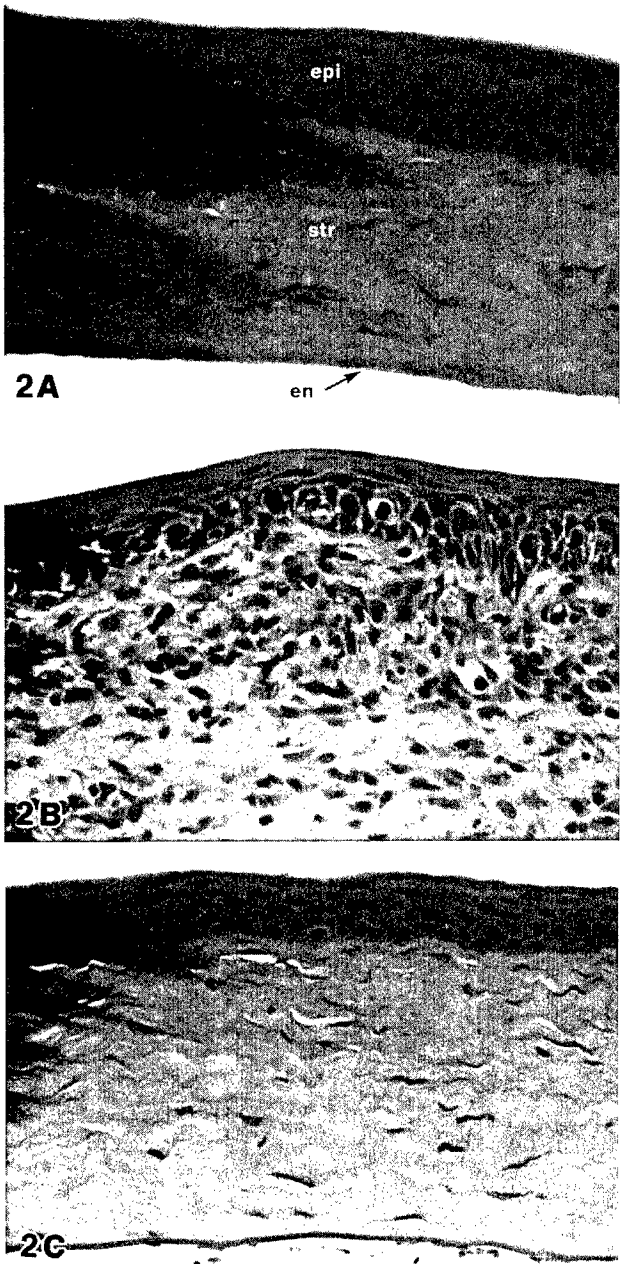

Figure 2. Histopathological appearance of rat corneas. (A) Control cornea with intact epithelium (epi), stroma (str) and endothelium (en), (B) Cornea exposed to CEES. Epithelium undergoing degenerative changes. Stroma edematous with many infiltrating neutrophils. (C) CEES-exposed cornea treated with VM. Epithelium is protected and well-organized while stroma remains slightly edematous. Endothelium is focally detached.

to CEES alone presented pathological changes to epithelium, stroma, and endothelium (Fig. 2). Degenerative epithelial changes included desquamation of surface epithelial cells, basal cell injury of the stratum basalis, and focal cleaving of the epithelium from the basement membrane (Bowman's membrane). Edema and inflammatory cellular infiltrates were persistent stromal changes at sites of exposure. The endothelium was focally detached from Descemet's membrane, with most of the remaining endothelial cells presenting evidence of nuclear pyknosis and cytoplasmic vacuolation. After treatment with VM, corneal epithelium, stroma, and endothelium appeared protected from the effects of CEES and were similar to unexposed cornea. Unexposed and propylene glycol-exposed corneas were unremarkable. Corneas washed in basal Tyrode's alone were not protected against the effects of CEES.

Transmission Electron Microscopic Examination

CEES-exposed corneas presented a progressive degenerative cytopathology of superficial and basal cells of the epithelium leading to epithelial sloughing and a pervasive cleaving of the epithelium from Bowman's membrane at the level of the lamina lucida (Figs. 3–5). Basal cell cytopathological findings included condensation and margination of nuclear chromatin, mitochondrial swelling and densities, rarefaction of cytoplasm, necrosis, and intercellular edema. Large expanses of denuded stroma were present as a result of a degenerative acantholysis of basal cells and a disabling of hemidesmosomes. Cellular fragments, remnants of hemidesmosomes, anchoring filaments, and other cellular debris remained attached to the lamina densa. Stromal edema was evidenced by exaggerated spacing between collagen lamellae. The leading inflammatory cellular infiltrates at sites of stromal edema were identified as neutrophils. Lymphocytes and neutrophils were especially concentrated along the stromal side of Bowman's membrane and along the endothelial side of Descemet's membrane in association with degenerating endothelial cells. Fibroblasts presenting evidence of degeneration were usually found in close association with inflammatory cells. VM-treated corneas appeared protected from the effects of CEES and presented ultrastructural features typical of unexposed corneas.

Discussion

Sulfur mustard gas is a synthetic vesicating agent that gained notoriety[8] as the major chemical warfare agent during World War I, killing approximately 600 US soldiers and injuring over 27,000. It has been used since as a weaponized chemical against military and civilian personnel and continues to be a chemical warfare threat agent of choice of some factions. In spite of its long history of use and many decades of investigative efforts to develop effective countermeasures there remains no specific prophylaxis, except avoidance, and no therapy except palliative for those who

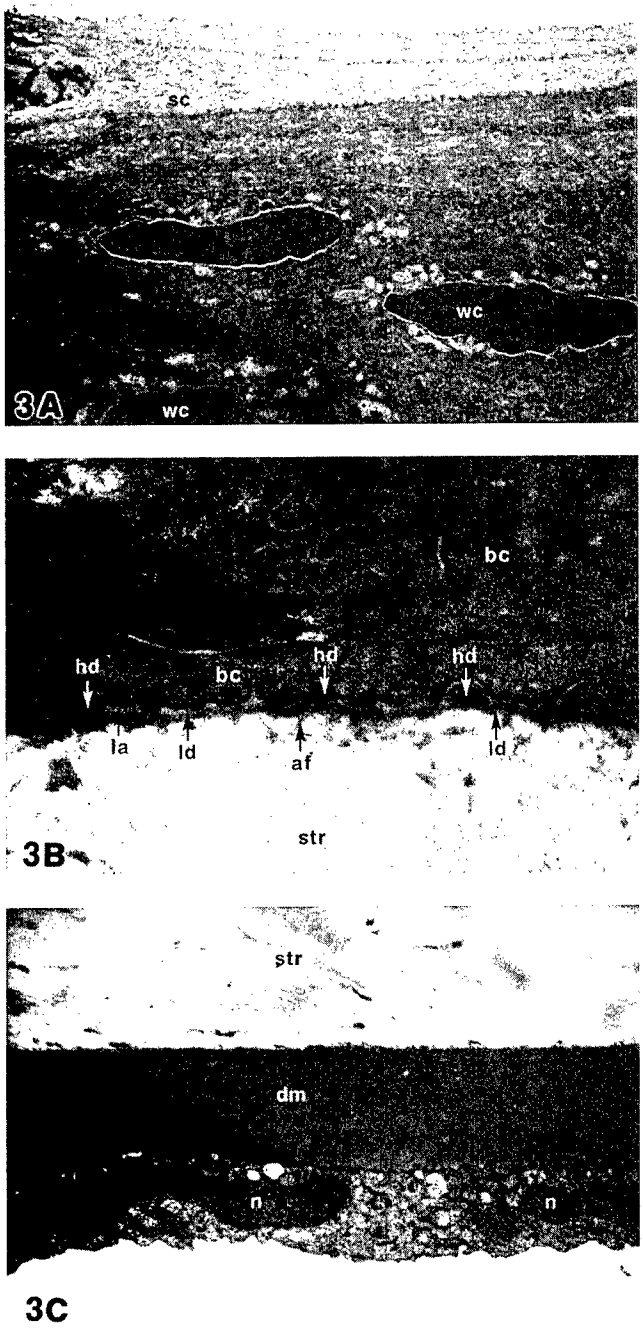

Figure 3. TEM of unexposed control rat cornea. (A) Typical organization of corneal epithelium: superficial cells (sc), wing cells (wc). (B) Epithelial basal cells at the region of Bowman's membrane: basal cell (bc), hemidesmosomes (hd), anchoring filaments (af), lamina lucida (la), lamina densa (ld), stroma (str). (C) At the level of endothelium: Descemet's membrane (dm), stroma (str), nuclei of endothelial cells (n). (Original magnifications ×9,000, ×9,000, and ×60,000 in A, B, and C, respectively.)

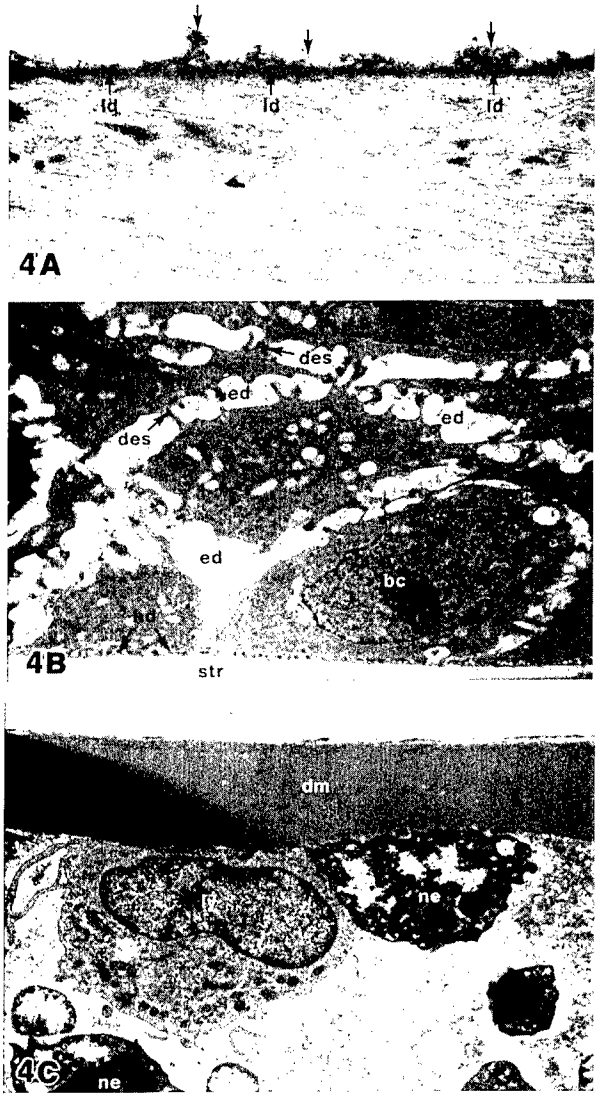

Figure 4. TEM of CEES-exposed cornea. (A) Epithelium completely detached from Bowman's membrane at level of lamina lucida: cellular debris and anchoring filaments (arrowheads) remain attached to denuded lamina densa (ld). (B) Islands of remaining epithelial cells present widened extracellular edematous spaces (ed) in association with strained desmosomal connections (des): basal cell nucleus (bc), stroma (str), hemidesmosomes (hd). (C) Degenerating endothelium replaced by infiltrating inflammatory cells; Descemet's membrane (dm), neutrophil (ne), lymphocyte (ly). (Original magnifications, ×30,000, ×12,000, and ×12,000 in A, B, and C, respectively.)

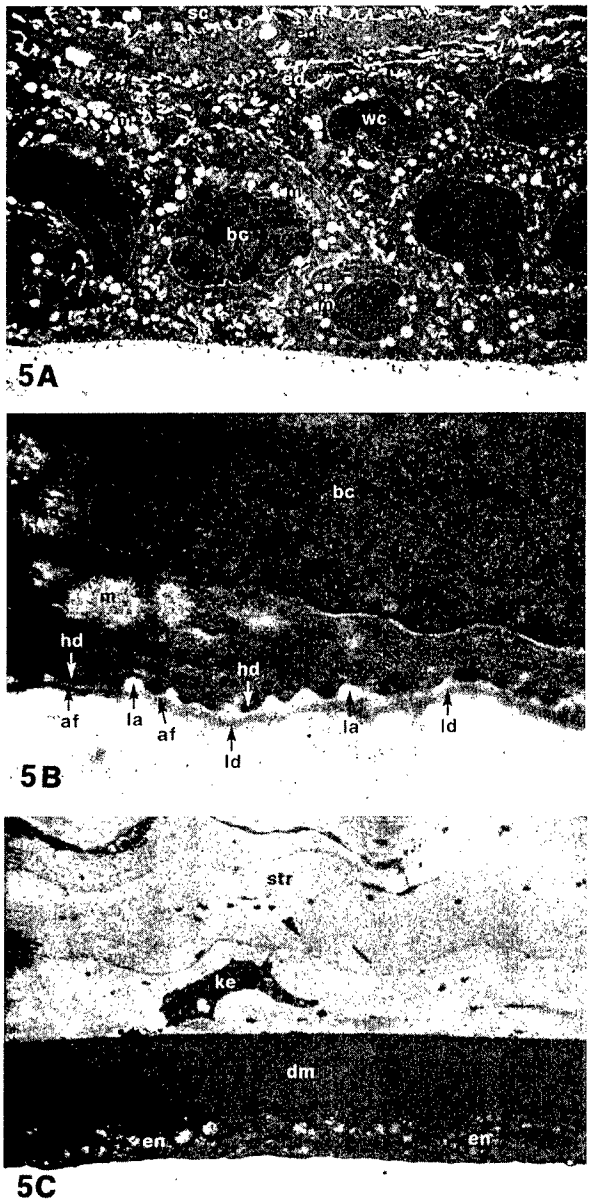

Figure 5. TEM of CEES-exposed cornea treated with VM. (A) Epithelium is intact with mild extracellular edema (ed) and dilated mitochondria (m): basal cell (bc), wing cell (wc), superficial cell (sc). (B) At Bowman's membrane, basal cell with loosened but intact hemidesmosomal connections to basement membrane: basal cell nucleus (bc), dilated mitochondria (m), lamina lucida (la), lamina densa (ld), hemidesmosome (hd), anchoring filaments (af). (C) Endothelium (en) appears protected with strong fidelity to Descemet's membrane (dm). Stroma (str) unaltered and free of edema: keratocyte (ke). (Original magnifications, ×7,500, ×60,000, and ×9,000 in A, B, and C, respectively.)

may become exposed.[9] Vision impairment is one of the most immediate dysfunctional consequences of battlefield and civilian sector casualties of sulfur mustard exposure. Ocular impairment is due to the potent alkylating effect of sulfur mustard on exposed ocular tissues to include the eyelid, conjunctiva, and cornea. Of these, corneal lesions cause the most extended visual incapacitations. In the present study, the use of the laboratory counterpart of sulfur mustard, CEES, resulted in rat corneal pathological changes that simulated the effects of sulfur mustard exposure. A consequence of the exposure was the severe progressive opacification of the cornea with epithelial detachment, stromal edema, inflammation, and loss of integrity of endothelial cells. These pathological findings are consistent with a previous study in which rabbit eyes were subjected to sulfur mustard exposure.[2]

The complexity and multifaceted nature of the mechanisms of action of sulfur mustard alkylations encouraged the development of a new candidate therapeutic compound composed of several pharmacological agents working in concert to attenuate the various induced pathological changes. Such a compound, VM, was tested in this present study as a therapeutic agent against the ocular effects of CEES. The components of VM are salts of pyruvic and alpha-ketoglutaric acids, which promote glycolysis and scavenge various oxy-radical species; taurine, which modulates membrane permeability and competitively inhibits alkylations; and calcium pantothenate, which supports overall tissue metabolism by being a moiety of coenzyme A.[4–7] In the present study, the use of VM as a postexposure topical eye wash attenuated the corneal damage induced by CEES in rat eyes. As such, the mixture was effective in maintaining corneal transparency as well as maintaining the now-protected structural integrity of corneal epithelium, stroma, and endothelium to near-normal appearance, as apparent by light microscopic and ultrastructural study.

Conclusion

This morphopathological study of the ocular effects of the mustard gas simulant, CEES, supports the very real possibility that a specific prophylactic or therapeutic strategy now in development will prevent or attenuate the ravaging effects of ocular exposure to sulfur mustard gas.

Acknowledgment

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References

1. B. Papermeister, A.J. Feister, S.I. Robinson, and R.D. Ford, *Medical Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological Implications*, CRC Press, Boca Raton, FL, 1991, pp. 1–7.
2. J.P. Petrali, F.J. Miskena, T.A. Hamilton, A.V. Finger, and S.J. Janny, Sulfur mustard toxicity of the rabbit eye: An ultrastructural study, *J. Toxicol. Cut. Ocular Toxicol. 16*(4) 227–237, 1997.
3. F.N. Marzulli and H.I. Maibach, *Dermatoxicology*, 5th ed., Taylor and Francis Publishers, Washington, D.C., 1996, pp. 119–130.
4. S.D. Varma, P.S. Devamanorahan, A.H. Ali, M. Hencin, J. Petrali, J. Brozetti, and E. Lehnert, Corneal damage by half mustard (2-chloroethyl ethyl sulfide, CEES) *in vitro* preventative studies: A histologic and electron microscopic evaluation, *J. Ocul. Pharm. Ther. 14*:413–421, 1998.
5. S.D. Varma, P.S. Devamanoharan, A.H. Ali, J. Brozetti, J. Petrali, E. Lehnert, and A. Weir, Half mustard (CEES)-induced damage to rabbit cornea: Attenuating effect of taurine-pyruvate-alpha ketoglutarate-pantothenate mixture, *J. Ocul. Pharm. Ther. 14*:423–428, 1998.
6. S.D. Varma and S.M. Morris, Peroxide damage to eye lens in vitro. Prevention by pyruvate, *Free Radical Res. Commun. 4*:283–290, 1988.
7. S.D. Varma, S. Ramachandran, P.S. Devamanoharan, S.M. Morris, and A.H. Ali, Prevention of oxidative damage to rat lens in vitro by pyruvate: Possible attenuation *in vivo, Curr. Eye Res. 14*: 643–649, 1995.
8. R. Zajtchuk, ed., *Medical Aspects of Chemical and Biological Warfare*, Office of the Surgeon General Publishers, 1997, pp. 9–86.
9. J.P. Petrali and S. Oglesby-Megee, Toxicity of mustard gas skin lesions, *Microsc. Res. Techn. 221–228*, 1997.