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Studies are in progress to determine the efficacy and mechanism of a formulation containing anti-alkylating, antioxidants and metabolic accelerators present in VM against mustard induced skin toxicity. A preventive effect has been observed at the level of tissue morphology. Studies are in progress at the level of cellular metabolism. Here, CEES has been used as a representative compound simulating the action of sulfur mustard (HD). It has been demonstrated for the first time that application of this formulation is effective even post exposure to mustard. Prophylaxis is not absolute. VM has been found to be useful even for inducing tissue regeneration adversely affected by the mustard compound

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**Annual Report US ARMY 2004.**
**Sulfur Mustard Damage to Skin. Preventive Studies**

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## Introduction:

Sulfur mustard in one of the well known toxicant used in combat operations. In addition to long term tissue toxicity, its immediate physiological effect is highly disabling because of instantaneous development of a severe generalized body irritation. It is soon followed by appearance of highly painful skin blisters, specially in the moist areas of the surface anatomy. Visual impairment due to corneal hydration is also set in soon. Hence, there is a strong desirability of developing chemical formulation that could inhibit the immediate as well as the long term effects of its exposure. One of the immediate cellular effects of the compound is the alkylation of various ingredients containing free –SH and -$NH_2$ groups. Such compounds present in the cell membranes of the skin epithelium are therefore the first targets of such alkylation. Pathophysiologic evidence of such membrane damage has been previously shown, with respect to cornea as well as skin. Damage to the cell membrane is followed by several well known intracellular metabolic disruptions as evidenced by a decrease in ATP. Such disruptions are induced by oxidative stress caused by the alkylation of several enzymes involved in tissue metabolism and consequent diversion of the excessively available oxygen into the cytochrome independent auto oxidative pathways, generating excessive amounts of reactive oxygen species. Hence the tissue comes into the ambit of a generalized oxidative and metabolic stress. Keeping in view in these biochemical reactions involved in the tissue damaging process initiated by sulfur mustard, studies are in progress to inhibit these biochemical reactions by using a mixture of compounds (Varma Mixture) known to inhibit alkylation reactions as well as oxidative stress caused by oxygen free radicals. The oxygen free radicals are generated not only due to inhibitions in tissue metabolism, but are also derived by the invading neutrophils. In addition, the mixture also contains compounds known to provide metabolic support to the tissue regenerative process. In the earlier studies we have shown that pathological manifestations of topical exposure to CEES (2-Chloroethyl ethyl sulfide, a laboratory model of sulfur mustard) is largely preventable if the said mixture is applied prophylactically. Since a prophylactic use may not be always practical, studies are in progress to determine the efficacy of the mixture after CEES has been applied. We now show that the mixture is effective even when applied after 6 minutes of CEES exposure and without any pre-washing subsequent to CEES application. The efficacy is apparent by the maintenance of normal tissue structure, apparent histologically.

**Methods of Study:**

Studies were conducted using Sprague –Dawley rats weighing about 150 g. They were anesthetized by intra-muscular injection of a mixture of ketamine and xylazine (66mg ketamine and 7 mg of xylazine/kg body weight). Immediately after the onset of anesthesia, a small area was demarcated on the flank and clipped to remove hairs. 10ul of a freshly prepared solution of CEES in propylene glycol (20 microliters CEES per 100 microliters of Propylene Glycol) was painted on the circumscribed area of skin covering 1.5 square centimeters. After 6 minutes of this application, the sites were treated with the VM ointment. The treatment was repeated every hour till 3 hours. They were again treated at 2 hourly intervals till the end of the day. A total six treatments were given on the first day. On subsequent days, four treatments were given at intervals of 1.5 hours. Treatment was continued for six days. After the treatments, the animals were again anesthetized and skin sites cut out and preserved in 10% buffered formalin. They were then processed for histology.

**Results and Discussion:**

The histology of the representative skin samples obtained form normal, CEES treated and CEES plus VM treated groups is described in figures 1, 2, and 3 respectively. Figure 1 shows the histological structure of the normal skin . The structure represents typical thin skin architecture; the epidermis consisting of one or two layers of epithelial cells covered by stratum corneum made up of flattened enucleated keratinocytes. The dermis, as expected, consists of loosely arranged elastin and collagen fibers, fibroblasts, some macrophages, glandular structures and embedded hair follicles (Figures 1,2).

One of the most significant and striking effects of CEES application, as reported previously, is the separation of the entire epidermis from the underlying dermis. The space in between is filled with fluid and some cellular debris, characteristic of blister formation. (Figures 3,4). As might be notable, in some places, the entire epidermis becomes exfoliated, leaving the dermis bare. Also, the dermis becomes enriched with additional amounts of inflammatory cells and debris. It is striking however to note that application of VM, as described above, prevented most of the CEES induced structural alterations. The epithelium remained intact, remaining fully adherent to the dermis along the basement membrane. Blister formation was completely prevented. The glandular structure in the dermis also appeared healthy.
It is however interesting to note that the epithelial layer, specially in the glandular areas, appeared hyperplastic and maturing faster. This may represent an injury response.

It was previously thought that the protective effect of VM was supplementary to the effect of washing the site of CEES application. The VM was applied after washing the CEES applied site. This would be a drawback in its practical use. In the current studies, however, the CEES applied areas were not washed and VM was applied directly. Additionally, the time of application was also delayed till 5 to 6 minutes demonstrating the usefulness of the preparation after significant lapse of time following exposure to the

agent. The findings therefore reinforce the hypothesis that the protective effect of VM against damage is due to the interference it offers against the initiating reactions. In addition, the findings provide evidence to its effectiveness in promoting the regenerative and healing mechanisms. A study of the mechanism of VM action is under progress. While studies with CEES appear promising for its use against damage by the real mustard compound (HD), it is considered necessary to study the effectiveness of the preparation or its derivatives against direct and actual HD damage as well.

**Key Research Accomplishment:**

One of the major lacunae of the earlier studies was with respect to the post exposure wash of the site after CEES application, before applying VM. Hence there could be limitation on its use in actual field conditions. In addition, we had used prophylactic application of VM before CEES application. In the present studies we have demonstrated that treatment post CEES application is also effective, indicating clearly that the effect of VM is not supplementary to washing. It can be used even directly without a wash. Additionally, treatment can be commenced even after a significant lapse of time after the exposure. Furthermore, the agent is considered useful in stimulating the healing process subsequent to initiation of blister formation.

**Reportable Outcome:**

The study will become reportable after showing the efficacy of the preparation against a direct HD damage and further verification of the mechanism of its action.

**Conclusion and Summary:**

Studies are in progress to determine the efficacy and mechanism of a formulation (VM) containing inhibitors of alkylation, antioxidants and metabolic accelerators against mustard induced skin toxicity. A preventive effect has been observed at the level of tissue morphology. Studies are in progress at the level of cellular metabolism. Here, CEES has been used as a representative compound simulating the action of sulfur mustard (HD). It has been demonstrated for the first time that application of this formulation is effective even post exposure to mustard. Prophylaxis is not absolute. VM has been found to be useful even for inducing tissue regeneration adversely affected by the mustard and other toxic compounds.

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**Legends to Figures:**

Figures 1 and 2: Histology of normal flank skin: Skin was isolated after anesthetizing the rats and clipping the hairs. The isolated sample was fixed in 10% buffered formalin and paraffin embedded for sectioning in dorso-ventral direction and subjected to hematoxylin-eosin staining following standard procedure. Notable is a single layer of nucleated epithelial cells covered by keratinized stratum. The dermis consists of fibrous material containing fibroblasts. Hair follicles can be seen in certain regions.

Figures 3 and 4: The skin samples were isolated, sectioned and stained as described above. Notable features are (fig. 3): Separation of the epidermis from the dermis and creation of a large fluid filled zone resembling a blister. Fig. 4 shows dermal infiltration with inflammatory cells.

Figures 5 and 6: Effect of VM treatment. The histologic structure in this case appears normal consisting of a compact epidermis-dermis combination, without any separation similar to that in normals. The glandular structures are also normal. Notable however is the presence of double layer of nucleated epithelium in the epidermis. This may be related to the direct favorable effect of VM ingredients on cellular differentiation, in addition to its effect against the reactions initiated by CEES.

Figure 1

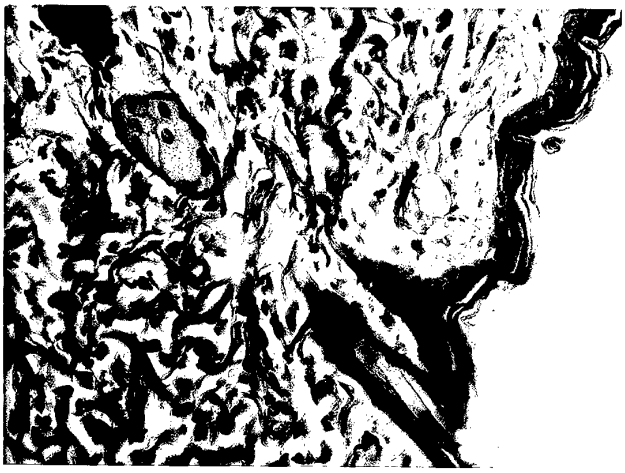


Figure 2

**Normal**

Figure 3

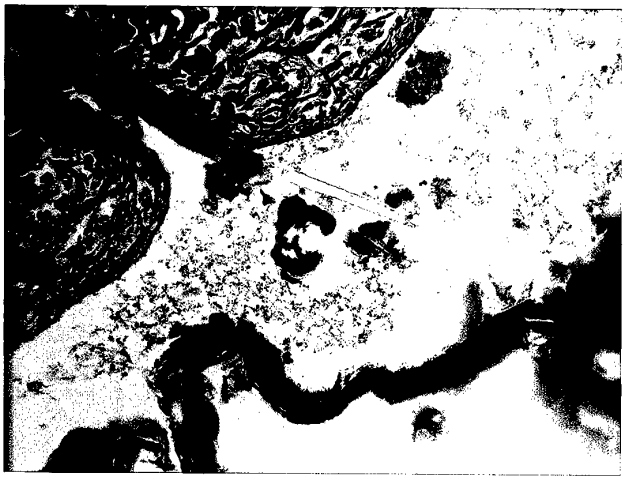


Figure 4

**CEES**

Figure 5

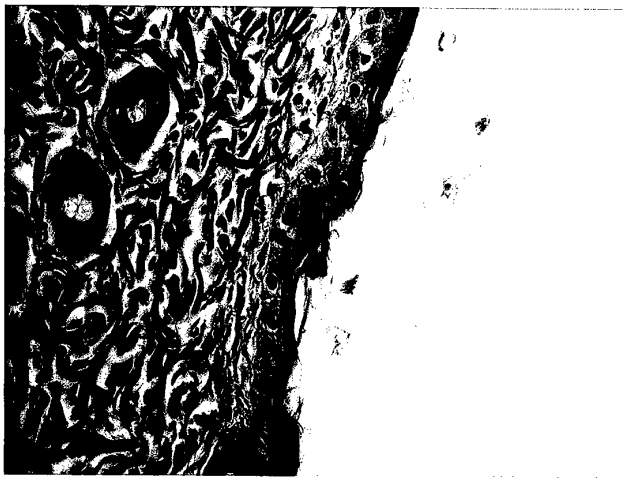
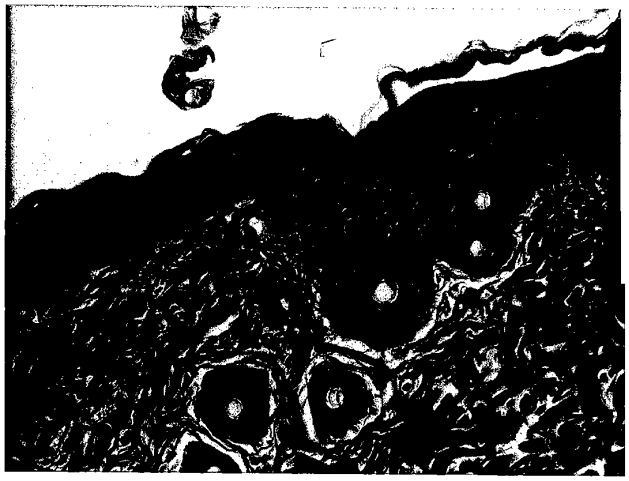


Figure 6



**CEES + VM**