PROTECTIVE EFFECT OF CHITOSAN AGAINST SKIN LESIONS INDUCED BY SULPHUR MUSTARD

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Abstract: An experiment was designed to study the sulphur mustard (SM) toxicity and its protection by chitosan. Chitosan was applied dermally once daily in Swiss albino mice after 24 hours of SM exposure. Daily body weight was recorded and animals were sacrificed on day 7 or day 14. Organs and body weights indices were calculated for liver, kidney, spleen and lungs. Hematology assay and histopathological analysis of liver, lung, spleen, kidney and skin were performed. Liver was assayed for oxidized and reduced glutathione and malondialdehyde level. No significant change was observed in absolute body weight, organ body weight indices and hematological parameters in treated and control mice. Liver glutathione (oxidised and reduced) and malondialdehyde levels were not significantly altered compared to control, and this was also supported by normal histology of liver, lung, spleen and kidney after microscopic evaluation. SM exposed and chitosan treated mice skin had shown early formation of eschar, extensive proliferation of collagenous tissue and reepithelialization. The present study suggested protective effect of chitosan against SM induced skin lesions.

Key words: Sulphur mustard, Wound healing, Chitosan

INTRODUCTION

Sulphur mustard (SM), chemically bis (2-chloroethyl) sulphide is a bifunctional alkylating agent that causes serious blisters on contact with human, and in animal causes skin lesions and systemic toxicity [1-2]. The easy availability of precursors, the simple method of synthesis and extremely stable nature makes SM a chemical weapon of choice by the military and terrorist groups. Although decontamination is one of the important aspects of protection against chemical warfare agents but it is effective only when done in first two minutes of the exposure, which is not always possible in field conditions [3]. However, there is always a possibility of wound formation due to absorption of warfare agents during time interval between exposure and decontamination.

Though several antidotes have been reported for the systemic toxicity still there is a possibility that severe skin lesions may develop. There is no specific therapy for SM-induced skin injury, but procedures such as LASER debridement of established burns have shown to increase the rate of burn resolution in animal models and may, therefore, benefit clinically [4]. But, limitation of LASER debriment is the requirement of advanced infrastructure, which is practically not possible in field conditions. To date, no effective and specific antidote for local and systemic toxicity of SM has been recommended, despite scientific research for more than 90 years. Many compounds were tested as antidotes for SM [5-15] and most of them are in the experimental stage. Chitin and its derivatives as wound healing accelerators began with the studies of Prudden et al. [16]. Chitosan is a natural
biopolymer that is derived from chitin polysaccharide made up of β-(1-40-linked Glc Nac and D-glucosamine units, a major component of crustacian outer skeleton. Chitosan had reported to influence all stages of wound repair [17] in mechanical wounds. It possesses biological activities like macrophage activation, stimulate cell proliferation, and induce inflammatory cells migration, increased collagen synthesis with granulation tissue and the formation of the dermo-epidermal junction [18-19]. However, literature for protective role of chitosan and its wound healing activity in SM induced skin lesions remains unexplored. Thus, we focused our experiment for evaluating protective effect of chitosan against SM induced skin injury in a resourceful and consistent mouse model described by us [20].

MATERIALS AND METHODS

Chemicals and reagents: SM was synthesised in the Synthetic Chemistry Division of the Establishment and was found to be above 99% pure by gas chromatographic analysis. Other chemicals were purchased from Sigma (USA) or Merck (India).

Animals: All experiments were performed on randomly bred female Swiss albino mice, weighing 20-25 gm. Animals were obtained from Animal Facility of Defence Research and Development Establishment. The care and maintenance of the animals were as per the approved guidelines of the CPCSEA, and the Institutional animal ethical committee approved the protocol for the experiments. A day before the actual experiment hair from back region of all mice was closely clipped using a pair of stainless steel scissors. Thirty female mice were randomized into five groups.

1. Group I - Acetone (control)
2. Group II - SM application once and sacrificed after 7 days
3. Group III - SM application once and sacrificed after 14 days
4. Group IV - SM application once and chitosan application daily for 7 days
5. Group V - SM application once and chitosan application daily for 14 days

SM was diluted in acetone and applied percutaneously at a dose of 10-mg/kg-body weight. All the safety precautions were taken while handling SM during application. Liberal dermal application of chitosan was done daily after 24 hour of SM exposure.

Body weight and organ weight: Body weights of animals were recorded daily. After 7 days or 14 days the animals were sacrificed and liver, lung, kidney and spleen were dissected out, weighed and preserved in Bouin’s fluid. A portion of skin from SM application site was also taken and preserved in Bouin’s fluid.

Hematology and biochemical analysis: WBC count and RBC count, haemoglobin, haematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were measured on Sysmex Hematology Analyzer (model K4500). Flurometric method of Hisin and Hilf [21] was used for determination of hepatic GSH and GSSG concentration. Lipid peroxidation was determined by measuring the level of malondialdehyde (MDA) according to the modified method of Easterbauer and Cheeseman [22] using molar extinction coefficient of 1.58 ×105 / M per cm.

Histopathology: Tissues collected at the time of sacrifice, freed from adhered tissue, washed with normal saline and fixed in bouins fluid for 12-24 hours. After proper fixation, tissue were washed overnight under running tap water to remove excessive fixative, dehydrated and processed in a graded alcohol and xylene and embedded in paraffin blocks. Multiple tissue sections of 4-5µ thickness were taken with the help of microtome (HM360, Micron), which were further stained with haemotoxylin and eosin (H&E) and analyzed under light microscope (Leica, DMLB).

Statistical analysis: The biochemical variables were analysed using one-way analysis of variance with Dunnett’s test. A probability of 0.05 and less was taken as statistically significant. The analyses were carried out using Sigmastat for Windows version 2.03 (SPSS Inc., USA).

RESULTS

Effects on body weight and organ weight: Figure 1 shows the percent change in body weight of mice in control (group I) and SM exposed animals of group III and V. The animals in SM control group III showed a significant decrease in body weight as compared to the control (group I). However, no control mice
showed the normal renal architecture (Fig. 4). It showed normal glomerulus, bowman’s space and renal parenchyma. Mice with SM (treated with chitosan and without any treatment) exposure showed minimal to mild ballooning of tubular cells and presence of tubular cast in the tubular lumen. These changes were common to all groups, which were considered spontaneous and details are presented in Table 2. Spleen of control (acetone only) mice showed normal splenic histology with germinal center, red pulp, and marginal zone of white pulp. Section of spleen from other groups of SM exposure with and without chitosan treatment showed similarity with the histology of control as shown in figure 4 and Table 2. Histology of lung of control and mice with SM (treated with chitosan and without any treatment) exposure showed normal alveolar pattern with normal alveolar septa, air duct, alveoli and bronchioles with intact epithelium. Microscopic observation of control mice skin (with percutaneous acetone only) showed stratified epithelium that was united from dermis by a thin basal lamina without showing any degenerative changes (Fig. 5A). Following SM exposure the skin showed severe coagulative necrosis of epidermal cells extending to dermis and subsequent loss of epidermis leading to erosions. Transmigration of inflammatory cells with minimal edema was evident on day seven that subsequently got subsided at 14 days. Severity of lesions such as hyperaemia of blood vessels with dermo-epidermal separation and formation of vesicle was reduced from day 7 to day 14. Basal cells showed

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<th>Group</th>
<th>WBC (X10⁹/mm³)</th>
<th>RBC (X10⁹/mm³)</th>
<th>HGB (g%)</th>
<th>HCT (%)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
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<tr>
<td>I (Acetone control)</td>
<td>13.23 ± 3.57</td>
<td>7.64 ± 1.54</td>
<td>11.2 ± 2.17</td>
<td>37.47 ± 7.72</td>
<td>49.00 ± 1.5</td>
<td>14.76 ± 0.63</td>
<td>30.16 ± 0.78</td>
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<td>II (SM + Acetone 7 days)</td>
<td>13.53 ± 2.48</td>
<td>8.13 ± 0.17</td>
<td>12.66 ± 0.24</td>
<td>40.66 ± 0.60</td>
<td>50.13 ± 1.74</td>
<td>15.56 ± 0.60</td>
<td>31.18 ± 0.15</td>
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<td>III (SM + Acetone 14 days)</td>
<td>12.27 ± 1.10</td>
<td>8.31 ± 1.23</td>
<td>12.13 ± 2.25</td>
<td>40.8 ± 7.00</td>
<td>48.9 ± 1.20</td>
<td>14.55 ± 0.55</td>
<td>29.7 ± 0.40</td>
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<td>IV (SM + Acetone + chitosan 7 days)</td>
<td>12.96 ± 2.07</td>
<td>7.62 ± 0.17</td>
<td>11.63 ± 0.56</td>
<td>38.06 ± 1.64</td>
<td>49.86 ± 1.03</td>
<td>15.23 ± 0.44</td>
<td>30.56 ± 0.41</td>
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<td>V (SM + Acetone + chitosan 14 days)</td>
<td>13.36 ± 3017</td>
<td>8.04 ± 0.53</td>
<td>13.36 ± 0.54</td>
<td>45.23 ± 2.16</td>
<td>50.13 ± 0.99</td>
<td>14.86 ± 0.61</td>
<td>29.5 ± 0.65</td>
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Table 1. Effect of SM (10 mg/kg) on hematological variables, 7 and 14 days after administration with and without chitosan treatment. SM was applied once percutaneously and chitosan was applied dermally, daily once. Value= Mean ± SE (n=6) p<.005.

<table>
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<tr>
<th>Groups</th>
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<th>Kidney</th>
<th>Spleen</th>
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<tr>
<td></td>
<td>Necrosis/ apoptosis</td>
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<td>Necrosis/ apoptosis</td>
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Table 2. Histopathology of liver, kidney, spleen, lungs and skin in mice (SM dose 10 mg/kg percutaneously) 7 and 14 days after administration with and without chitosan treatment (n=6). SM was applied once and chitosan was applied dermally, daily once. Minimal: less than 22% lesions, ++ Mild: less than 40% lesions, +++ Moderate: less than 60% lesions, ++++ Severe: more than 60% lesions.
Fig. 1: Effect of SM (10 mg/kg percutaneously) on body weight, with and without chitosan treatment. Mean ± SE (n=6). *significant difference from acetone only of the same day. SM was applied once and chitosan was applied dermally, daily once.

Fig. 2: Effect of SM (10 mg/kg percutaneously) on organ weight, 7 and 14 days after administration with and without chitosan treatment. Mean ± SE (n=6). SM was applied once and chitosan was applied dermally, daily once. a. Acetone only, b. Acetone + SM (7 days), c. Acetone + SM (14 days), d. Acetone + SM + chitosan (7 days), e. Acetone + SM + chitosan (14 days).

Fig. 3: Effect of SM (10 mg/kg percutaneously) on GSH, GSSG and MDA, 7 and 14 days after administration with and without chitosan treatment. Mean ± SE (n=6). SM was applied once and chitosan was applied dermally, daily once. a. Acetone only, b. Acetone + SM (7 days), c. Acetone + SM (14 days), d. Acetone + SM + chitosan (7 days), e. Acetone + SM + chitosan (14 days).
Fig. 4: Photomicrographs of SM (10 mg/kg percutaneously) exposed mice liver (A, B, C), kidney (D, E, F), spleen (G, H, I) and lungs (J, K, L) (HandE X40) with and without chitosan treatment. SM was applied once and chitosan was applied dermally, daily once. All the organs showed normal histoarchitecture without showing any pathological changes.
vacuolation and loss of connection from basement membrane (acantholysis) in the area surrounding the wound. These changes were partially recovered with chitosan treatment on day 7. However, reactive growth of epidermocytes was evident on day 14 as hyperplastic basophilic epidermal cells and basal cells of epidermis (acanthosis) was seen. There was severe adenexal atrophy in group II (SM day 7) which got minimized in group III (SM day 14). Minimal lesions were seen on day 14 in group V. Chitosan treated mice skin (group V) showed extensive granulation tissue proliferation, reepithelialisation form the edge of lesion, angiogenesis along with maximum collagen deposition as compared to the other groups on day 7 and 14 both. Details are shown in table 2.

DISCUSSION

SM is a strong alkylating agent, which produces subepidermal blisters, erythema and inflammation after skin contact. Despite the well-described SM-induced gross and histopathological changes [1-2], the exact underlying mechanisms of these events are still a matter of research. Chitosan is a natural biopolymer that is derived from chitin, and reported to show excellent wound healing activity [17]. However, there is no report suggesting protective role of chitosan in SM induced skin lesions till date. Thus, in this study we have focused on the wound healing effect of chitosan in SM-induced skin lesion of Swiss albino mice in a physiologically normal animal model as described earlier [20]. Further, for confirming reliability and reproducibility of the animal model we had considered systemic effects of SM toxicity, if any, and protective effect of chitosan in this study.

SM induced skin lesions damage epidermis and disturbs skin glycolipid barrier leading evaporative fluid loss and decrease in body weight [12]. In this study there was progressive increase in body weight of all the animals from day 0 to 14. However, body weight of SM exposed mice without chitosan treatment showed significant decrease in body weight as compared to the control on day 14. This pattern of body weight change is in agreement with our earlier studies [12]. In contrast to SM control, mice with chitosan treatment showed protection from weight loss that may be due to temporary barrier created by chitosan layer over the skin lesions preventing subsequent water loss.

Animals with percutaneous application of SM showed no significant differences in organ body weight indices and hematological parameters compared to control. In contrast to our findings there was a significant decrease in organ body weight indices of liver, kidney and spleen and subsequent increase in blood hemoglobin concentration, hematocrit, erythrocyte count and reduced number of WBC in topically applied SM diluted in PEG-300 or DMSO [23,24].

Glutathione is an intracellular scavenger of SM. Thus, SM may cause GSH depletion and enhance formation of reactive oxygen species (ROS) [6]. SM in PEG 300 was shown to induce lipid peroxidation and depletion of glutathione [5,25]. In contrast to above finding our results did not reveal any significant change in liver GSH, GSSG levels and in lipid peroxidation at day 7 and day 14 respectively in control and SM exposed both chitosan treated and untreated mice. These findings were consistent with our previous report, and further confirm the use of animal model for studying SM induced skin lesions.

There were no prominent histopathological lesions in the liver, kidney, spleen and lungs of mice after percutaneous administration of SM diluted in acetone as compared to the control animals. In contrast to this higher systemic toxicity of percutaneously administered SM diluted in PEG 300 [24] and by inhalation in lungs [26] was reported earlier. Percutaneous application of SM, diluted in acetone showed a surface spreading and also reduced systemic absorption unlike SM diluted in PEG or DMSO.

Wound healing is a biological process triggered by tissue injury and directed towards the restoration of tissue continuity and its function. The early phase of healing process is inflammation followed by fibroplasia and re-epithelialisation and finally tissue remodeling [27]. These phases overlap and their separation is arbitrary. In this study untreated control mice showed coagulative necrosis of epidermal cells, dermo-epidermal separation followed by sloughing of epidermis leading to ulcer formation that was roofed by an eschar tissue, post SM exposure. Proliferation of immature fibroblasts and collagen synthesis increased from 7 to 14 days after SM exposure. Sprouting of endothelial cells and formation of new blood vessels was also evident from 7 to 14 days post SM exposure. This pattern of SM-induced skin lesions confirm the general pattern of reparative
Fig. 5: Photomicrographs SM (10 mg/kg percutaneously) exposed mice skin (HandE X20). (A) Control mice skin section showing normal arrangement of epidermis, dermis and adenexa (BandC) 7th day post SM exposure showing coagulative necrosis of epidermal cells, edema, infiltration of mononuclear cells and separation of dermo epidermal junction leading to formation of vesicle (D) section of mice skin 14th day post SM exposure showing large vesicle and dead necrotic cells in dermis (E) Section of mice skin 7th day post SM exposure with chitosan treatment showing transmigrated inflammatory cells and granulation tissue formation. (F) 14th day post SM exposure, section of mice skin with chitosan treatment showing excessive fibrous tissue proliferation with hyperplastic epidermis under the eschar.
phase as described by us in the same animal model and in other mammalian species [20].

The Proliferative phase in process of wound healing is characterized by increase in the number of immature fibroblast. Fibroblasts attach to the cables of the provisional fibrin matrix and begin to produce collagen [28]. In the present study amount of collagen was significantly higher in dermis of chitosan treated suggesting its role in healing of SM induced skin injury. Chitosan possesses biological activity of excessive granulation tissue formation and reunion of dermal epidermal junction [18,19], as evident in the present study.

In conclusion, the result of the present study indicates that SM in acetone induces only cutaneous lesion with minimal systemic toxicity in mice. Excessive collagen deposition, reunion of newly formed epidermis (re-epithelialisation) and dermis along with proliferation of basal cells were found to be more prominent feature in chitosan treated mice skin suggesting its wound healing efficacy in SM induced skin lesions. Thus, chitosan can be recommended as a wound healant in SM induced skin injuries.

REFERENCES