Comparison of toxicity of selected mustard agents by percutaneous and subcutaneous routes

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Comparative toxicity of nitrogen mustards (HN-1, HN-2 and HN-3) and sulphur mustard was carried out in mice. Based on LD50, the toxicity pattern was HN-2 < HN-1 < HN-3 < sulphur mustard by percutaneous route whereas, by subcutaneous route the toxicity pattern was sulphur mustard < HN-3 < HN-2 < HN-1. Single dose of 1 LD50 of nitrogen mustards and sulphur mustard was administered percutaneously and various oxidative stress parameters were also evaluated. The weight loss was more in HN-2 on day 3 and in sulphur mustard on day 7. There was a drastic fall of WBC count on day 3 in all groups with a recovery in nitrogen mustard groups on day 7. The RBC count and haemoglobin content showed a significant increase on day 7 in sulphur mustard group. The plasma enzymes (ALT, AST and ALP) showed an increase in all groups on day 3 and day 7. The hepatic GSH and GSSG contents were reduced and MDA content increased in all groups, with a further change in sulphur mustard on 7 day. Extensive DNA fragmentation was observed in all the nitrogen mustard groups compared to sulphur mustard group, on day 3. However, on the day 7 the DNA fragmentation was same in all groups. This study showed that the nitrogen mustards and sulphur mustard were extremely toxic by percutaneous route and caused oxidative stress. Sulphur mustard was more toxic by the percutaneous route and the effects were delayed and progressive.

Keywords: Mechlorethamine, Nitrogen mustard, Oxidative stress, Sulphur mustard, Toxicity

Nitrogen mustards, sulphur mustard, and a number of their analogues are potent alkylating agents\(^1\). Mechloroethamine (HN-2) is the first nitrogen mustard identified as an anticancer agent\(^2\). The derivatives such as phenylalanine mustard (melphalan), chlorambucil and cyclophosphamide are widely used as anticancer drugs despite their non-specific reaction mechanisms\(^3\). The polyfunctional alkylating agents are highly reactive under appropriate conditions. Mechanism of alkylation of nitrogen mustard is mediated through a cyclic immonium ion and sulphur mustard through a cyclic sulphonium ion, and both react at the nucleophilic center. The major nucleophilic centers that might be available for alkylation in biological systems are organic and inorganic anions, amino groups, and sulphide groups\(^4\). The cytotoxic agents probably react indiscriminately with many cellular components, and small concentrations are lethal to all types of cells.

Major site of alkylation of nitrogen mustards and sulphur mustard when incubated with DNA or RNA, was shown to be 7-nitrogen of guanine\(^5\). Alkylation of DNA at other sites has also been demonstrated\(^6,7\). This results in the formation of monoadducts, as well as intrastrand and interstrand cross links\(^8,10\). Although the relationship of these effects to their cytotoxicity is unclear, there is some evidence that inhibition of DNA replication caused by DNA interstrand cross links is of particular importance\(^11\).

Nitrogen mustards and sulphur mustard are qualified as chemical warfare agents. Like any xenobiotic, these mustards can be absorbed through all three major routes viz., oral, dermal and respiratory route. Absorption through dermal route is common in the incidence of mustard agent’s exposure as they are lipophilic compounds\(^12\). Respiratory route or inhalation is one of the major routes of entry for vapours of mustard agents.

It was earlier reported that sulphur mustard toxicity depends upon the route of administration and quite interestingly, the percutaneously administered sulphur mustard was more toxic than subcutaneous route in mice as well as in rats\(^13\). Generally, all chemicals show higher toxicity by subcutaneous route than percutaneous route. An analogue of sulphur mustard chloroethyl ethyl sulphide, though used as a stimulant for sulphur mustard for mechanistic studies and also
for identification of antidotes, does not behave like sulphur mustard\textsuperscript{14-17}.

The objective of this study is to determine the acute toxicity of nitrogen mustards and sulphur mustard by determining LD\textsubscript{50} by percutaneous and subcutaneous routes, supported by biochemical evaluation.

**Materials and Methods**

**Chemicals**—Nitrogen mustards \{HN-1, bis-(2-chloroethyl)ethylamine; HN-2, bis-(2-chloroethyl)methylamine; HN-3, tris-(2-chloroethyl)-amine and sulphur mustard (2,2-dichloroethyl sulphide)\} were synthesised in the Synthetic Chemistry Division, and were found to be above 99\% pure by gas chromatographic analysis. \textit{O}-pthalaldehyde (OPT), glutathione and \textit{4’,6-diamidino-2-phenylindole} (DAPI) were purchased from Sigma Chemical Company (USA). Other chemicals of high purity were from Qualigens (India) or E-Remck (India).

**Animals**—Randomly bred Swiss female mice (25 - 30 g) from the Institute's animal facility were used for the study. The animals were kept in polypropylene cages with sterilised and dry paddy husk as a bedding material. Free access to food (standard pellet diet, Ashirwad Ltd, India) and water were allowed until 2 hr before the experiment. The care and maintenance of the animals were followed as per the approved guidelines of the ‘Committee for the Purpose of Control and Supervision of Experiments on Animals’ (CPCSEA, India). A day before percutaneous exposure, hair on the back of the animals was closely clipped using a pair of scissors. Food and water were allowed 2 hr after the experiment. All animal procedures were approved by the Institutional Animal Ethical Committee.

**Determination of LD\textsubscript{50} dose** —LD\textsubscript{50} of nitrogen mustards and sulphur mustard for two routes viz. percutaneous and subcutaneous was determined by dissolving the nitrogen mustards and sulphur mustard in PEG-300 (polyethylene glycol-300) or DMSO (dimethyl sulphoxide). The animals were exposed to different log doses through the respective routes. For percutaneous route, the diluted solution was uniformly smeared on the back of the animals on a circular area of 1.5 cm diameter, using a gas tight syringe (Hamilton syringe, USA). The animals were held for about a minute for absorption of the chemical, before returning them to cage. Subcutaneous injection was also given using the gas tight syringe on the back of the animals. After percutaneous and subcutaneous administration of nitrogen mustards and sulphur mustard, the animals were kept in a well ventilated room for 24 hr and then shifted to the experimental animal room for further monitoring. The body weight was recorded daily and the animals were observed for mortality for 14 days. LD\textsubscript{50} was determined as per the moving averages method\textsuperscript{18}.

After determination of LD\textsubscript{50} dose of nitrogen mustards and sulphur mustard, group of animals (4 animals per group) were administered single and double dose LD\textsubscript{50} for evaluating the survival time.

**Haematological and biochemical evaluation**—The animals were administered single LD\textsubscript{50} dose of nitrogen mustard or sulphur mustard by percutaneous route. Each group consisted of 6 animals. They were anaesthetised with ether for collection of blood from orbital sinus, and then sacrificed by cervical dislocation for removal of vital organs.

Part of liver tissue was used for biochemical estimations. Hepatic GSH and GSSG contents were estimated fluorimetrically\textsuperscript{19}. For this, 150 mg of liver tissue was homogenized in 4 ml of phosphate EDTA buffer and metaphosphoric acid (25\%), centrifuged and the supernatant was used for the estimation of GSH and GSSG. Hepatic lipid peroxidation was determined by measuring MDA level\textsuperscript{20}. Liver (100 mg) was directly homogenized in 5 ml of thiobarbituric acid reagent and boiled for 30 min, cooled, centrifuged and absorbance of the clear supernatant was measured at 535 nm. Amount of MDA formed was calculated using a molar extinction coefficient of \textit{1.56 \times 10\textsuperscript{5}/M per cm}. For DNA fragmentation assay, the liver from control and treated animals were quickly excised and frozen\textsuperscript{21}. The frozen tissues were homogenized in ice-cold lysis buffer [10 mM tris, 20 mM EDTA; and 0.5\% triton X-100; (pH 8.0)] and then centrifuged at 20000 X g for 30 min. Both pellet (intact chromatin) and supernatant (DNA fragments) were assayed for DNA content fluorimetrically by using fluorescent dye \textit{4’,6-diamidino-2-phenylindol} (DAPI). To 2 ml of reagent, 100 ng/ml of DAPI in 10 mM tris (pH 7.4) containing 100 mM of NaCl and 20 \textmu l of the sample was added, and then fluorescence intensity was measured at 450 nm with excitation at 362 nm. The percentage of fragmented DNA was defined as the ratio of DNA content of the supernatant at 27000 \times g to the total DNA in lysate\textsuperscript{22}. The haematological parameters viz., WBC, RBC and Hb, were analysed by using Beckman Coulter Analyser (USA).
Statistical analysis—The survival data was analysed by Friedman's repeated measures ANOVA on ranks followed by Dunnett’s test. The biochemical variables were analysed by one way analysis of variance with Dunnett’s multiple comparisons 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

Nitrogen mustards (HN-1, HN-2 and HN-3) and sulphur mustard caused a decrease in the body weight from 24 hr post exposure onwards and there was a progressive decrease in body weight. Death of mice following subcutaneous route of administration of sulphur mustard occurred between 6 and 8 days, whereas treatment through percutaneous route resulted death between 8 and 12 days which was statistically significant. By percutaneous or subcutaneous routes of administration of nitrogen mustards, the death occurred between 4 and 6 days (Fig. 1).

LD$_{50}$ values of nitrogen mustards and sulphur mustard in different solvents for two routes are given in Table 1. Based on LD$_{50}$, the toxicity pattern was HN-2 < HN-1 < HN-3 < sulphur mustard by percutaneous route, whereas administration through subcutaneous route, the toxicity pattern was sulphur mustard < HN-3 < HN-2 < HN-1. Sulphur mustard was more toxic through percutaneous route than subcutaneous route. HN-3 was equally toxic through both the routes. Toxicity of nitrogen mustards and sulphur mustard was more in DMSO compared to PEG-300.

Body weight of animals in percutaneous route (1.0 LD$_{50}$) treatment varied significantly as compared to controls on 3rd day (Table 2). Percentage weight loss was more in HN-2 compared to HN-1, HN-3 and sulphur mustard on 3rd day, whereas on 7th day, the percentage weight loss was more in sulphur mustard compared to HN-1, HN-2 and HN-3. None of the animals died in nitrogen mustards and sulphur mustard administered groups on 3rd day. Animals treated for 7th day resulted in death of 2 animals each died in HN-1 and HN-2 treated groups, but no death was observed in HN-3 and sulphur mustard treated groups.

The organ to body weight ratio of liver and kidney did not show any significant difference from the controls, but the spleen weight reduced on 3rd day of HN-2 and on 7th day of sulphur mustard administration.

Data on haematological and selected enzyme activities following nitrogen mustards and sulphur mustard administration at a dose of 1 LD$_{50}$ by percutaneous route has been given in Table 3. There was a significant decrease in WBC count on 3rd day after administration in nitrogen mustards and sulphur mustard groups. WBC count remained same in sulphur mustard group and there was a partial recovery in nitrogen mustard administered groups on 7th day. RBC count did not show any significant difference in nitrogen mustards and sulphur mustard administered group except sulphur mustard administered group showed a significant increase on 7th day. The same was reflected in increased level of haemoglobin content on 7th day after sulphur mustard administration. The plasma enzymes showed an increase in nitrogen mustards and sulphur mustard administered groups both on 3rd and 7th day.

Depletion in hepatic GSH content was observed in all nitrogen mustard and sulphur mustard

<table>
<thead>
<tr>
<th>Agent</th>
<th>Percutaneous route</th>
<th>Subcutaneous route</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD$_{50}$ (mg/kg)</td>
<td>Confidence limit (mg/kg)</td>
</tr>
<tr>
<td>In PEG 300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN-1</td>
<td>16.8</td>
<td>11.0 – 25.7</td>
</tr>
<tr>
<td>HN-2</td>
<td>33.6</td>
<td>22.7 – 49.7</td>
</tr>
<tr>
<td>HN-3</td>
<td>23.8</td>
<td>15.6 – 36.3</td>
</tr>
<tr>
<td>SM</td>
<td>8.1</td>
<td>5.3 – 12.3</td>
</tr>
<tr>
<td>In DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN-1</td>
<td>11.9</td>
<td>8.1 – 18.0</td>
</tr>
<tr>
<td>HN-2</td>
<td>20.0</td>
<td>12.7 – 31.5</td>
</tr>
<tr>
<td>HN-3</td>
<td>7.1</td>
<td>4.1 – 12.3</td>
</tr>
<tr>
<td>SM</td>
<td>7.1</td>
<td>3.2 – 15.2</td>
</tr>
</tbody>
</table>

The animals were observed for mortality for 14 days and the LD$_{50}$ was estimated by Gad and Weil method (1989).
Fig. 1—Survival plot of mice administered with LD₅₀ (1 or 2 doses) of nitrogen mustards (HN-1, HN-2 and HN-3) and sulphur mustard (SM). [The data was analysed by Friedman’s repeated measures ANOVA on ranks followed by Dunnett’s test (n = 4). *Significant from 1 LD₅₀ to 2 LD₅₀. **Significant from per cutaneous route for the same dose, p.c.- percutaneous; and s.c.-subcutaneous]
administered groups 3 days after one LD<sub>50</sub> dose. Nitrogen mustard showed a partial recovery in GSH content after 7 days, but sulphur mustard administered group showed a further decline in GSH content (Table 4). GSSG content also showed a similar pattern to GSH content. HN-1, HN-2 and HN-3 showed a recovery of the GSSG content during 3 to 7 days after administration, while sulphur mustard showed a further decline in the content. There was an increase in MDA level in all nitrogen mustards and sulphur mustard groups after 3 days of treatment. MDA level of nitrogen mustards administered groups remained same on 7<sup>th</sup> day, but sulphur mustard groups showed a further increase in MDA level.

Table 2—Percent body weight and organ to body weight ratio of female mice administered with 1 LD<sub>50</sub> of nitrogen mustards (HN) or sulphur mustard (SM) by percutaneous route. The animals were sacrificed 3 and 7 days after administration.

<table>
<thead>
<tr>
<th>Agent</th>
<th>mortality (%)</th>
<th>BW change (%)</th>
<th>Liver Wt (%)</th>
<th>Spleen Wt (%)</th>
<th>Kidney Wt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100.3 ± 1.5</td>
<td>5.48 ± 0.34</td>
<td>0.49 ± 0.06</td>
<td>1.19 ± 0.06</td>
</tr>
<tr>
<td>3 days after administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN-1</td>
<td>0</td>
<td>85.2 ± 1.9*</td>
<td>4.74 ± 0.07</td>
<td>0.31 ± 0.04</td>
<td>1.36 ± 0.06</td>
</tr>
<tr>
<td>HN-2</td>
<td>0</td>
<td>73.9 ± 3.2*</td>
<td>4.67 ± 0.19</td>
<td>0.17 ± 0.02*</td>
<td>1.59 ± 0.09*</td>
</tr>
<tr>
<td>HN-3</td>
<td>0</td>
<td>88.8 ± 1.2*</td>
<td>5.12 ± 0.26</td>
<td>0.34 ± 0.06</td>
<td>1.18 ± 0.06</td>
</tr>
<tr>
<td>SM</td>
<td>0</td>
<td>82.8 ± 0.7*</td>
<td>4.49 ± 0.11*</td>
<td>0.60 ± 0.08</td>
<td>1.35 ± 0.04</td>
</tr>
<tr>
<td>7 days after administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>33</td>
<td>76.6 ± 7.9*</td>
<td>4.93 ± 0.44</td>
<td>0.44 ± 0.14</td>
<td>1.30 ± 0.06</td>
</tr>
<tr>
<td>HN-2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>33</td>
<td>77.8 ± 5.8*</td>
<td>5.10 ± 0.20</td>
<td>0.66 ± 0.08</td>
<td>1.19 ± 0.12</td>
</tr>
<tr>
<td>HN-3</td>
<td>0</td>
<td>88.2 ± 3.2*</td>
<td>5.65 ± 0.25</td>
<td>0.79 ± 0.11*</td>
<td>1.22 ± 0.04</td>
</tr>
<tr>
<td>SM</td>
<td>0</td>
<td>57.9 ± 3.2*</td>
<td>4.42 ± 0.37*</td>
<td>0.26 ± 0.03</td>
<td>1.51 ± 0.09*</td>
</tr>
</tbody>
</table>

LD<sub>50</sub> : HN-1 = 11.9; HN-2 = 20.0; HN-3 = 7.1; SM = 8.1 mg/kg.
HN-1, HN-2 and HN-3 were diluted in DMSO, and SM was diluted in PEG 300.
*Statistically significant from control.
Control weight of mice = 26.1 ± 0.9 g

Table 3—Haematological variables and few enzymes of mice administered with LD<sub>50</sub> (1 dose) of nitrogen mustards (HN) or sulphur mustard (SM) by percutaneous route. The animals were sacrificed 3 and 7 days after administration.

<table>
<thead>
<tr>
<th>Agent</th>
<th>WBC</th>
<th>RBC</th>
<th>Hb</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.1 ± 0.8</td>
<td>8.9 ± 0.5</td>
<td>12.4 ± 1.1</td>
<td>89.2 ± 6.5</td>
<td>35.7 ± 2.9</td>
<td>248.6 ± 18.3</td>
</tr>
<tr>
<td>3 days after administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN-1</td>
<td>5.7 ± 0.3*</td>
<td>9.8 ± 0.3</td>
<td>13.8 ± 0.6</td>
<td>113.2 ± 14.8</td>
<td>44.3 ± 5.1</td>
<td>315.8 ± 30.5</td>
</tr>
<tr>
<td>HN-2</td>
<td>2.4 ± 0.9*</td>
<td>10.7 ± 0.2*</td>
<td>14.0 ± 0.6</td>
<td>178.6 ± 34.6</td>
<td>87.2 ± 12.6*</td>
<td>320.4 ± 47.7</td>
</tr>
<tr>
<td>HN-3</td>
<td>3.8 ± 0.5*</td>
<td>10.0 ± 0.5</td>
<td>13.9 ± 0.5</td>
<td>128.9 ± 19.0</td>
<td>44.2 ± 6.1</td>
<td>275.8 ± 26.1</td>
</tr>
<tr>
<td>SM</td>
<td>4.3 ± 0.4*</td>
<td>10.1 ± 0.4</td>
<td>14.0 ± 0.5</td>
<td>171.7 ± 11.7</td>
<td>99.8 ± 5.2*</td>
<td>293.4 ± 34.3</td>
</tr>
<tr>
<td>7 days after administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6.7 ± 1.3*</td>
<td>8.5 ± 0.3</td>
<td>12.8 ± 0.6</td>
<td>152.2 ± 29.0</td>
<td>113.6 ± 38.4*</td>
<td>344.7 ± 47.8</td>
</tr>
<tr>
<td>HN-2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>11.6 ± 1.9</td>
<td>8.3 ± 0.3</td>
<td>11.6 ± 2.0</td>
<td>252.1 ± 29.8*</td>
<td>78.9 ± 5.1</td>
<td>285.6 ± 34.5</td>
</tr>
<tr>
<td>HN-3</td>
<td>12.4 ± 2.2</td>
<td>8.4 ± 0.5</td>
<td>10.8 ± 0.3</td>
<td>106.1 ± 10.5</td>
<td>41.0 ± 6.9</td>
<td>225.6 ± 31.2</td>
</tr>
<tr>
<td>SM</td>
<td>4.5 ± 0.9*</td>
<td>12.7 ± 0.8*</td>
<td>16.2 ± 0.9*</td>
<td>279.4 ± 58.6*</td>
<td>164.8 ± 8.6*</td>
<td>293.4 ± 34.3</td>
</tr>
<tr>
<td>F</td>
<td>14.79</td>
<td>8.63</td>
<td>3.86</td>
<td>5.22</td>
<td>15.05</td>
<td>1.10</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

LD<sub>50</sub> : HN-1 = 11.9; HN-2 = 20.0; HN-3 = 7.1; SM = 8.1 mg/kg.
HN-1, HN-2 and HN-3 were diluted in DMSO, and SM was diluted in PEG 300.
*Statistically significant from control.
[WBC = ×10<sup>3</sup> cells/µl; RBC = ×10<sup>6</sup> cells/µl; Hb = gm/dL; AST = U/L; ALT = U/L and ALP = U/L]
DNA fragmentation up to 79.4, 76.4, and 78.1% was observed for the treatment of HN-1, HN-2, and HN-3, respectively, as compared to sulphur mustard administered group (57.1%) after 3 days of treatment with one LD₅₀ dose. However, on the 7th day after administration, the DNA fragmentation was more or less same in all nitrogen mustards and sulphur mustard groups (Table 4).

### Discussion

It has been reported earlier that sulphur mustard is more toxic in mice and rats through percutaneous route compared to subcutaneous and oral routes. A monofunctional sulphur mustard (chloroethyl ethyl sulphide) do not show this pattern and an analogue of amifostine, DRDE-07, is able to protect percutaneously administered sulphur mustard and not monofunctional sulphur mustard toxicity. In this study, similar results were observed that sulphur mustard was more toxic by percutaneous route than subcutaneous route in both PEG-300 and DMSO as solvents. In the case of nitrogen mustards, subcutaneous route was more toxic as compared to percutaneous route. The days of death following subcutaneous route was shorter (<6 days) compared to percutaneous routes in all nitrogen mustards and sulphur mustard. Death following sulphur mustard by subcutaneous route occurred before 8 days, but in percutaneous route the death occurred only after 8 days. Significant cytotoxic effect was observed in nitrogen mustard and sulphur mustards through percutaneous route. Cytotoxic nature of sulphur mustard was high in percutaneous route and it is progressive. Mechanism by which this pattern occurs is not clearly understood.

Mustards treatment through percutaneous and subcutaneous route caused a progressive decrease in body weight of the animals. Decrease in body weight was slow and progressive following percutaneous administration particularly in sulphur mustard. This pattern has been reported in earlier studies. One of the hypotheses for sulphur mustard toxicity is related to glutathione. GSH may act as an alternative intracellular site or 'scavenger' for sulphur mustard and inhibition of GSH by mustards leads to a variety of complications. A significant depletion of GSH in all nitrogen mustards as well as sulphur mustard is observed in this study. GSH has important function in protecting the cell from oxyradicals and reactive chemicals. Its depletion is related to cytotoxicity. Depletion of GSH is expected to expose protein sulphydrils that may get damaged via reaction with toxicant or with endogenously produced oxygen species. It is also possible that in the absence of GSH, the partially reduced oxygen species will be transformed into highly toxic oxidants, which may further react with membrane phospholipids initiating

<table>
<thead>
<tr>
<th>Agent</th>
<th>GSH GSSG MDA DNA damage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.29 ± 0.28 2.36 ± 0.17 3.21 ± 0.27 24.5 ± 3.46</td>
</tr>
<tr>
<td>After 3 days</td>
<td></td>
</tr>
<tr>
<td>HN-1</td>
<td>1.73 ± 0.12* 1.43 ± 0.11* 4.41 ± 0.42* 79.4 ± 3.3*</td>
</tr>
<tr>
<td>HN-2</td>
<td>1.86 ± 0.38* 1.10 ± 0.14* 5.86 ± 0.22* 76.4 ± 3.5*</td>
</tr>
<tr>
<td>HN-3</td>
<td>2.24 ± 0.44* 3.02 ± 0.44 5.03 ± 0.60* 78.1 ± 3.1*</td>
</tr>
<tr>
<td>SM</td>
<td>2.58 ± 0.21* 1.90 ± 0.06 5.05 ± 0.59* 57.1 ± 2.1*</td>
</tr>
<tr>
<td>After 7 days</td>
<td></td>
</tr>
<tr>
<td>HN-1#</td>
<td>2.86 ± 0.53* 2.33 ± 0.42 5.26 ± 0.43* 65.6 ± 8.7*</td>
</tr>
<tr>
<td>HN-2#</td>
<td>1.20 ± 0.11* 2.50 ± 0.24 5.22 ± 0.11* 79.6 ± 3.8*</td>
</tr>
<tr>
<td>HN-3</td>
<td>3.02 ± 0.30* 2.93 ± 0.17 5.26 ± 0.31* 79.3 ± 3.3*</td>
</tr>
<tr>
<td>SM</td>
<td>1.65 ± 0.17* 1.16 ± 0.12* 7.06 ± 0.52* 74.8 ± 2.9*</td>
</tr>
<tr>
<td>F</td>
<td>9.25 10.65 6.01 25.25</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001 &lt;0.001 &lt;0.001 &lt;0.001</td>
</tr>
</tbody>
</table>

| Values are mean ± SE (n = 6; #n = 4) |

LD₅₀: HN-1 = 11.9; HN-2 = 20.0; HN-3 = 7.1; SM = 8.1 mg/kg.
HN-1, HN-2 and HN-3 were diluted in DMSO, and SM was diluted in PEG 300.
*Statistically significant from control.
GSH = ×10⁻³ μmoles/g of tissue; GSSG =×10⁻³ μmoles/g of tissue; and MDA = ×10⁻⁴ nanomoles/g of tissue.
lipid peroxidation. Significant decrease in hepatic GSH content in the present study was indicative of high affinity of all nitrogen mustards towards GSH. Nitrogen mustards showed GSH depletion in 3 days with a partial recovery in 7 days, whereas sulphur mustard showed a progressive GSH depletion that was maximum on 7 day.

Nitrogen mustards are extremely toxic bifunctional alkylating agents. Though, the vesicant ion property of the nitrogen mustard have been documented, but the mechanism of systemic toxicity is not clearly understood. One of the well documented mechanism of mustards is alkylation of DNA, subsequently leading to DNA damage and cell death\(^\text{27}\). Though, the nitrogen mustards are well known DNA alkylating agents, the systemic toxicity is not only dependent on DNA damaging property but also on other factors. DNA alkylation may play an important role in cytotoxicity induced by these agents. There are DNA alkylating agents that do not cause vesication, but induce other pathological changes similar to that of nitrogen mustards\(^\text{28,29}\). This shows that DNA damage is one of the toxic manifestations of all nitrogen mustards and sulphur mustard, but may not be the principle cause of systemic toxicity. The present study showed that DNA damaging property of nitrogen mustards was more than sulphur mustard. Significant DNA damage was observed on 3\(^\text{rd}\) day in nitrogen mustard administered groups compared to sulphur mustard group and the latter showed damage after 7\(^\text{th}\) day of treatment.

Certain compounds after percutaneous absorption may bind in part to serum albumin at the inflamed site. Bound compounds are presumably inactive, protected from biotransformation and able to diffuse out little of the skin into the blood vessel lumina. Since the binding is reversible, the protein - compound complex serves as a local tissue reservoir, which slowly releases the chemical into the circulation\(^\text{30}\). It was also proposed that basement membrane components of the skin such as fibronectine, heparan sulphate proteoglycan and, lamulin, a thiol rich adhesive protein are molecular targets for modification by mustard agents\(^\text{31,32}\). It was suggested that skin contained a reservoir or depot of unchanged mustard agents from which there is continued uptake of these agents in the blood during few days of application\(^\text{33}\). Recently, it was proposed that sulphur mustard may form reservoir within stratum corneum and upper epidermis before a toxic dose has been absorbed by viable epidermis\(^\text{34}\). But, it is difficult to understand that several cytoprotective agents are able to protect only when they are administered prophylactically against percutaneously administered sulphur mustard, and these antidotes are ineffective when they are administered after the administration of sulphur mustard\(^\text{12,35,36}\). These agents are also ineffective when sulphur mustard is administered subcutaneously.

Skin plays important role in transforming topically applied xenobiotics by direct biochemical alteration. It is also observed that skin contains many of the enzymes as in liver tissue and capable of oxidation, reduction, hydrolysis and possible conjugation reactions. Within the skin the enzyme activities are higher in epidermis than in dermis and can convert certain inactive molecules such as polycyclic hydrocarbons into active carcinogen after percutaneous application\(^\text{37}\).

Different metabolites of nitrogen mustards and sulphur mustard through different routes are possible. It has been reported that exposure of rats to sulphur mustard through different routes, (intravenous and intraperitoneal) leads to formation of different metabolites\(^\text{38}\). Glutathione - bis - chloroethyl sulphide is the main metabolite after intravenous administration and sulphone is the major metabolite after intra dermal administration of sulphur mustard. It is possible that after percutaneous route of administration, the mustards are metabolised to another toxic metabolite or intermediate. Nitrogen mustard sulphone have same vesicant properties as neat nitrogen mustard. One hypothesis which requires further confirmation is that the formation of highly electrophilic carbonium ion through percutaneous route of application may be more than other route of exposure. Since the maximum cell division take place in skin, and it contains maximum number of metabolically active and rapidly dividing cells, this may be a possible mechanism of action. In the present study only sulphur mustard showed higher toxicity by percutaneous route than subcutaneous route and not nitrogen mustards. HN-3 showed almost equitoxicity by percutaneous and subcutaneous routes. The present study showed that nitrogen mustards and sulphur mustard were significantly toxic by percutaneous route and cause oxidative stress. Sulphur mustard was more toxic through percutaneous route and the effect was delayed and progressive.
References

33. Hambrock J L, Howells D J & Schock C, Biological fate of sulphur mustard (1,1-V-thiobis(2-chloroethane)):


