Effects of hexamethylene tetramine on lung tissue macrophages in rats exposed to two different doses of sulfur mustard

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Abstract
Aims: Inhalation of sulfur mustard (HD) gas causes inflammation of airways and bronchioles. Macrophages are phagocytic cells which spread out all over the body tissues. Hexamethylenetetramine [HMT] has been shown to protect human lung cells against HD and phosgene. This study was conducted to investigate the effects of HMT on macrophages of the lung tissue in the rats infected by two different doses of HD.

Methods: 42 male rats with weights of 200±20g were randomly divided into 8 groups of NS, HMT, HD1, HD2, Pre1, Pre2, Post1 and post 2. HD1, Pre1 and Post1 groups received 0.5 %; HD, HD2, Pre2 and Post2 groups received 0.25%; HD and NS groups received Normal Saline (as endotracheal) and HMT group received 7.5mg/kg of this medicine (as intra-peritoneal). 5 groups of HMT, Pre1, Pre2, Post1 and post 2 received the medicine for 14 days.

Results: Results of the counting of macrophages revealed significant increase in the number of macrophages in HD groups comparing with NS group. In addition, macrophages significantly increased in the HD1 comparing with HD2. Macrophages significantly increased in HD1&Pre1 comparing with HMT group. Macrophages significantly decreased in Pre and Post groups comparing with HD groups.

Conclusion: HMT have therapeutic and protective effects on the lung tissue against HD.

Keywords: HMT have therapeutic and protective effects on the lung tissue against HD.

Introduction
Sulfur Mustard (HD) gas is an electrophilic alkylator agent with mutagenic, carcinogenic, cytotoxic and vesicatory properties. The cytotoxic effects of alkylator agents on DNA, RNA and protein can lead to mutagenic injuries and finally cell death [1, 2, 3]. Papirmeister et al. showed that the alkylation dependent cytotoxic effects of sulfur mustard are in target cell and will therefore disrupt the control of normal cell processes [4]. In this process, respiratory tract is influenced more than other parts [5, 6, 7]. Inhalation of sulfur Mustard causes the inflammation of respiratory ways, damage to its epithelial tissue and release of inflammatory mediators in the lung [8]. Infiltration of inflammatory cells and the increased polymorphonucleases that are considered as important mediators in inflammation is among the pulmonary complications due to contamination by this agent [9]. Macrophages are phagocyte cells that are spread out all over the body and form the phagocytic system. Monocytes are mainly the origin of macrophages and circulate in the blood [10].

Research has shown that after exposure of the rat lung to sulfur mustard, the accumulation of inflammatory cells and inflammatory responses start and after 48 hours reach to its maximum level and cell vacuolization and the parenchyma swelling of the lung tissue have been observed after seven days [11]. Few quantitative studies have been conducted on animal models [12, 13] and laboratory models [14, 15] on epithelial respiratory cells as the main target of sulfur mustard. The increased death due to the lung disease and also increased chronic bronchitis has been reported in Japanese workers employed in factories producing chemical gases [16]. Manning et al have shown that pneumonia is the single cause of death in workers in the factories that produce sulfur mustard in Britain [17]. A number of researchers have obtained similar results on the effects of mustard gas on the lung [18, 19].

Hexamethylene Tetramine (HMT) is a drug with anti-inflammatory and antibacterial properties. HMT’s structure consists of four nucleophile nitrogen atoms that appear to have the ability to react with sulfur mustard, and reduce its bad effects on body cells [15, 20, 21]. The protective effects of HMT in the culture setting on nomocyte II cells infected with sulfur mustard has been reported [21]. HMT has also protective effects on body cells against the chemical agent of phosgene [22].

Human studies show that sulfur mustard causes dyspnea, wheezing, irritated larynx, nasal mucosa and respiratory damage and bronchitis accompanied by mucosal necrosis [23]. Inhalation of large amounts of sulfur mustard causes some damages to respiratory
epithelial cells [7, 24]. In this study, HMT was used for the first time to protect and treat the lung tissue in rats exposed to sulfur mustard. The purpose of this study was to investigate the number of macrophages in lung tissue of male rats of different groups as an inflammatory index.

Methods

In this study, 42 three-month male Wistar rats (Pasteur Research Institute; Iran), weighing 200± 20 grams were prepared. Animals were kept in the same conditions in terms of food, light, water and temperature and were randomly divided into 8 groups. Sulfur mustard was prepared as the 0.5% solution (Sulfur mustard by 0.5µl in 100µl of normal saline) and 0.25% solution (Sulfur mustard by 0.25µl in 100µl of normal saline) [25]. For the first group (NS), 100 µl/kg of normal saline was used as endotracheal injection under anesthesia using a catheter with 1.5 mm thickness and 5 cm length. The second group (HMT) received an intra-peritoneal injection of 7.5 mg/kg of HMT (this group included seven rats). The third group (HD1) received an endotracheal injection of sulfur mustard 0.5% with the amount of 100µl/kg once. Group four (Pre1) received an intra-peritoneal injection of HMT an hour before receiving sulfur mustard solution 0.5% in order to protect from lung tissue against Sulfur mustard. The fifth group (Post1), for rapid treatment with HMT, after exposure to Sulfur mustard, HMT drug was injected 10 minutes after receiving sulfur mustard solution 0.5%. Groups 6 (HD2), 7 (Pre2) and 8 (Post2), similar to HD1, Pre1 and Post1 groups received sulfur mustard solution and HMT, but the sulfur mustard solution was 0.25% (the sulfur mustard solution: 100µl/kg and HMT: 7.5mg/kg).

Groups 2, 4, 5, 7 and 8 received an intra-peritoneal dose of HMT each day (Sina drugs; Iran) for 14 days. After 14 days, the animals were killed and samples were taken from the basal part of posterior lobe of the right lung in size of 5 cubic millimeters. Samples were fixed in 10% formalin solution and after tissue processing 5 micron sections were prepared from the samples. For evaluation of lung tissue, Hematoxylin-eosin staining was used. Macrophage count of lung tissue was done using light microscopy (Zenit; Spain) with the zoom of 1000.

Counting was carried out randomly for 20 areas (the surface of each area was equal to 14,100µm²) for each sample. ANOVA and Tukey’s post hoc test were used for the comparison of groups.

Results

The mean number of macrophages in HMT group (76.00±5.47) had a significant increase compared to NS group (50.00 ±4.77) (Table 1).

The mean number of macrophages in HD1 group was significantly higher compared to HD2 group. There was a significant difference between the mean number of macrophages in HD1, Pre1 and Post1 groups. Moreover, the mean difference of the number of macrophages was statistically significant between HD2, Pre2 and Post2 groups. The reduced mean of the number of macrophages in Post1 group compared to Pre1 group was statistically significant but this decrease was not significant in Pre2 group compared to Post2 group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean number of macrophages ± Standard Error</th>
<th>Level of significance compared to NS</th>
<th>Level of significance compared to HMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 NS</td>
<td>50.00±4.77</td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 HMT</td>
<td>76.00±5.47</td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>3 HD1</td>
<td>135.33±6.98</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4 Pre1</td>
<td>103.33±2.72</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5 Post1</td>
<td>70.93±3.73</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>6 HD2</td>
<td>54.53±1.32</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>7 Pre2</td>
<td>26.40±3.49</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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<tr>
<td>8 Post2</td>
<td>28.78±2.36</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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</table>

Discussion

Sulfur mustard leads to alkylation of cell compounds, and production and release of extensive inflammatory mediators. Following these injuries, cell response appears as inflammation and tissue damage [26]. The activated cells in the inflammatory areas cause the release of several toxic agents such as proteases and free radicals that leads to destruction of parenchymal cells. This causes the cell death (apoptosis and necrosis) in pulmonary parenchymal cells and these wounds give rise to an ideal environment for secondary infections in sulfur mustard exposed individuals [11]. In the present study, results showed the increased number of macrophages in sulfur mustard contaminated group compared to normal saline group. This indicates the effect of sulfur mustard on increasing of macrophages in epithelial tissue of lung.

Studies have shown that in response to lung injury induced by sulfur mustard, inflammatory cells migrate to the injured area and release inflammatory mediators and will ultimately lead to return of the extracellular

Table 1- The mean number of macrophages in each group
mat. The inflammatory phase begins mediated by endothelial or epithelial injury followed by the invasion of inflammatory cells to the alveolar interstitial space. Mediators’ release facilitate the polymorphonucleases and blood monocytes’ call and ultimately macrophages and lymphocytes will be present at the site and inflammatory mediators will be released [26, 27, 28, 29].

Levitt observed that sulfur mustard in the doses of 25 and 50 μmoles causes the primary stimulation of phagocytic cells in production and secretion of chemical oxygen-dependent mediators, but at doses of 50 and 100 μmoles will cause the apoptosis of these cells [30]. Other studies have also proved that the damage caused by endotracheal injection of 0.5 mg/kg of CEE (sulfur mustard analog) depends on the dose and duration of infection [31, 32].

Lardot showed that one Mole of sulfur mustard increases the secretion of IL-8 by humanized keratinocytes, while the concentrations of 1 and 10 μmoles did not induce a significant change compared to the control group [33]. In the present study, the increase in the number of macrophages in the HD1 group was observed compared to HD2 group that this is due to the increased dose of sulfur mustard.

Animal model study of both skin and lung exposure to sulfur mustard showed that it causes the leukocyte infiltration, which starts in a short time after exposure and steadily continues. Sulfur mustard causes the release of inflammatory cytokinases and NF-κB activity in keratinocytes and macrophages [32, 34, 35, 36, 37, 38]. Sulfur mustard also causes the stimulation of Phagocytosis [9] and in a long run will lead to increase of CD8+ T-cells and monocytes and decreased percentage of CD4+15 T-cells in patients [39]. On the other hand, Cowan showed that sulfur mustard causes the increased secretion of interleukin-8 by human epithelial keratinocyte cells in the culture environment that this increase is considered as a marker for the pre-inflammatory effect of sulfur mustard [40]. In another study, it was observed that the progressive lung injury and pulmonary fibrosis caused by sulfur mustard are the result of the disorder in pulmonary macrophage-keratinocyte system control [6]. Ahmadi et al. observed that the monocyte-macrophages system has an important role in the initiation of inflammatory responses and tissue damages by secretion of more than 150 secretory materials [41].

It was seen in this study that the results of macrophages counting in pre and post groups show reduction in these groups compared to HD groups, which shows the HMT positive performance in reducing the number of macrophages in these groups. Since the HMT molecule contains four nucleophile nitrogen atoms, it seems that these atoms react with episulfonium ion induced by sulfur mustard outside the cells and prevent sulfur mustard from entering the cells [21]. On the other hand, HMT prevents cellular infiltration due to its disinfecting property and thus prevents the inflammation in lung epithelium, causing lung protection against the toxic effects of sulfur mustard. On the other hand, HMT plays more protective role in glass and zooid [15, 21, 22], that is consistent with the results of the reducing of the number of macrophages in pre groups and shows the protective role of HMT against sulfur mustard.

Some of the studies conducted on the other different drugs against sulfur mustard, have shown that NAC reduces the secretion of many inflammatory mediators and several studies have reported the protective effect of NAC; but no therapeutic effect has been reported of NAC [42, 43]. Moreover, other studies indicate that macrolides and antioxidants may also improve the respiratory symptoms and pulmonary function in patients exposed to sulfur mustard and this is due to the anti-inflammatory effects of it [44]. A number of researchers, in a study that conducted on chemical veterans reported that the administration of Gamma-interferon can be helpful in reducing of tissue inflammation in this group of veterans [45]. Other studies indicate the protective role of NAC, Doxycycline and other drugs [43, 46, 47, 48, 49]. These studies support the results of this study that represent the protective effect of HMT against the toxicity of sulfur mustard. The research conducted in culture medium on lung epithelial cells showed that addition of HMT after sulfur mustard exposure has no therapeutic effect on these cells [15]. These findings are inconsistent with the results of present study, because the results indicate the therapeutic role of HMT.

Conclusion

Hexamethylene Tetramine has protective and therapeutic effects on the lung tissue.

References

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