

Comparative Toxicity Studies Of Sulfur Mustard (2,2'-Dichloro Diethyl Sulfide) And Monofunctional Sulfur Mustard (2-Chloroethyl Ethyl Sulfide), Administered Through Various Routes In Mice

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ABSTRACT

Sulfur mustard (SM; 2,2'-dichloro diethyl sulfide) is an alkylating chemical warfare agent and inflicts serious blisters upon contact with human skin. Monofunctional sulfur mustard, 2-chloroethyl ethyl sulfide (CEES) has been used as a simulant for detection, mechanism of action and protection studies of SM. The objective of this study is to compare the toxicity of SM and CEES using oxidative stress markers with reference to the route specific toxicity, through percutaneous (p.c.), subcutaneous (s.c.), oral (p.o.) and intraperitoneal (i.p.) routes in mice.

The LD₅₀ of SM was determined to be 9.7, 13.5, 19.3 and 4.8 mg/kg through p.c., s.c., p.o. and i.p. routes, respectively. Notably, SM through i.p. route was found to be the most toxic, the p.c. route was more toxic than s.c. and p.o. routes. The LD₅₀ of CEES was determined to be 1425, 100, 566 and 17.7 mg/kg through p.c., s.c., p.o. and i.p. routes, respectively. In the case of CEES the s.c. route was more toxic than p.c. route, which is more typical of toxic chemicals. At 1 and 2 LD₅₀, death after CEES administration was within 1 to 2 days, while death following SM administration was much delayed (> 6 days).

Equitoxic doses (0.5 LD₅₀) of SM and CEES were administered through all the routes: and it was observed that the p.c. route showed a significant decrease in body weight in SM. Depletions in reduced and oxidized glutathione, compared to CEES, were also observed 7 days after administration. However, increases in malondialdehyde and reactive oxygen species levels were observed in both SM and CEES through all routes, 7 days after administration. DNA fragmentation was observed in SM through the i.p. route and in CEES through the p.o., i.p. and s.c. routes (also at 0.5 LD₅₀ dose).

This study shows that though SM and CEES induce oxidative stress, their toxicity patterns are different. SM is more toxic through p.c. route compared to s.c and p.o routes, probably due to a different metabolic pathway, and this phenomenon is not observed in the case of CEES. The use of CEES as a simulant of SM for the mechanism of action and antidote evaluation may not be appropriate.

INTRODUCTION

Vesicants, such as mustards and organic arsenicals, are blistering agents and are listed as chemical warfare agent (Marrs, 1996). Vesicant induced injuries are generally characterized by relatively delayed healing. Mustards, such as sulfur mustard (SM, 2, 2'-dichloro diethyl sulfide), are class of vesicants that has gained notoriety during World War I despite the low death rate (Somani, 1992). SM is considered a constant threat to the military owing to its simple method of preparation, low cost, incapacitating potential and ease of concealment. There is also the possibility that the terrorist groups can attempt to use SM. Hence the development of an effective countermeasure against SM and other chemical agents has become a compelling requirement. SM primarily affects the skin, eyes and respiratory tract. SM destroys the epithelial cells, especially the sensitive basal cells and deeper cells by interacting with basement membrane component, initiating an acute inflammatory response, serum leakage, leukocyte infiltration, fibroblast activation, blister formation and desquamation (Dacre, 1996).

It has been established that the key reaction of SM is the intramolecular cyclization to form the electrophilic ethylene episulfonium intermediate and the liberation of the free chloride ion (Wormser, 1991). The conversion to this reaction is temperature dependent and is facilitated in the presence of aqueous solution (Sawyer, 1999). This fact may explain the high vulnerability of warm and wet regions, including the mucosal tissues of eyes and respiratory tract, to SM's effects. The cyclic sulphonium intermediate alkylates the nucleophilic residues of several macromolecules. The major alkylation site of nucleic acid of mammalian origin is the N-7 of the guanine residue. The second chloroethyl moiety of the sulfur mustard can attack an additional N-7 of the guanine to create interstrand and intrastrand cross-linking (Fox, 1980). Additional targets for alkylation are N-3 adenine and O-6 guanine. Thus, chromatid aberrations occur, synthesis of DNA, RNA and proteins are inhibited, and the cells are blocked at the interface of the G₂/M phase of the cell cycle (Fox, 1980).

Alkylation of DNA by SM leads to the activation of poly (ADP-ribose) polymerase (PARP) that reduces the availability of oxidized NAD⁺ in cell. This leads to inhibition of glycolysis, activation of hexosemonophosphate shunt, induction of plasminogen activator and ultimately, production of skin lesions (Papirmeister, 1991). This reduction of NAD⁺ accounts for the rapid ATP dependent alterations in the microfilament architecture and function in the keratinocytes (Papirmeister, 1985; Byers, 2000). The energy depletion is responsible for cell death (Papirmeister, 1991).

In the necrotic cells there is a significant depletion in ATP, while in apoptotic cells the ATP remained at the level similar to healthy cells (Dabrowska, 1996).

Preliminary evidence obtained in our laboratory, suggested that SM could induce various biochemical alterations consistent with oxidative stress in several organs of mice and rats (Vijayaraghavan, 1991; Rao, 1999; Kumar, 2001; Gautam, 2005). Butyl 2-chloroethyl sulfide, an analogue of SM, has also been shown to induce oxidative stress (Elsayed, 1989; Omaye, 1991; Elsayed, 1992). Our previous study also suggested DNA damage in various organs distal to the site of exposure of SM (Rao, 1999). Single dermal application of SM resulted in oxidative damage that included lipid peroxidation and glutathione depletion in the liver of mice. We also reported that percutaneously administered SM was more toxic than subcutaneous and oral routes in mice as well as in rats (Vijayaraghavan, 2005). Intratracheal administration of 2-chloroethyl ethyl sulfide (CEES), a monofunctional derivative of SM has also been reported to induce oxidative stress in mice (Das, 2003; Chatterjee, 2004). There are no reports about the percutaneous administration of CEES. Reports are available to explain SM toxicity based on similarities with CEES toxicity (Blaha, 2001). As an alternative to SM, CEES is preferred for various protection studies as it is less toxic, easy to make, stable and is not listed in chemical weapon convention (McClintock, 2002). Though it is used as a simulant of SM for various detection, protection and decontamination approaches (Arroyo, 2000; Panayotov, 2004), we questioned the appropriateness of CEES as a simulant for SM in antidote studies.

The objective of this study is to compare the route specific toxicities of SM and CEES using oxidative stress markers through percutaneous (p.c.), subcutaneous (s.c.), oral (p.o.) and intraperitoneal (i.p.) routes.

MATERIAL AND METHODS

Chemicals:

SM and CEES were synthesized in the chemistry division and was found to be above 99% pure by gas chromatographic analysis. O-thalaldehyde (OPT), reduced and oxidized glutathione, thiobarbituric acid, agarose and ethedum bromide were purchased from Sigma, USA. Other chemicals were purchased from Qualigens or E-Merck (India) with the highest purity available. Utmost care was taken during the synthesis of SM in the declared facility of the Defence Research and Development Establishment (DRDE). The dilution of SM and its administration in animals were carried out in fume hoods.

Animals:

Randomly bred Swiss female mice (25 - 30 g body weight) from DRDE's animal facility were used for the study. The animals were kept in polypropylene cages on sterilized paddy husk as the bedding material. Free access to food (Amrut Ltd, India) and water were allowed until 2 hr before the experiment. After the administration of SM, the animals were kept in well-ventilated area for 24 hr and then kept in the experimental animal room for further monitoring. The care and

maintenance of the animals were as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. A day before p.c. administration of SM and CEES, hair on the back of the animals was closely clipped using a pair of scissors. All animal experiments were carried out with the approval of Institutional Animal Ethical Committee.

LD₅₀ determination of SM and CEES through different routes at different doses:

LD₅₀ of SM and CEES through p.c., s.c., p.o., and i.p. routes were determined in mice by dissolving SM and CEES in PEG-300 (polyethylene glycol 300). The concentrations of SM in PEG-300 were made so that 80 -- 100 µl could be administered per animal. For the p.c. route, the diluted solution was smeared uniformly on the back of the animals on a circular area of 1.5 cm diameter using a gas tight syringe (Harvard Apparatus, USA). The s.c. injections, under the skin on the backs of the animals, were carried out using a gas tight syringe, as were the I.p. injections. A 20 gauge animal oral feeding cannula (Harvard Apparatus, USA) was used for the p.o. route.

The LD₅₀ of CEES was also determined in mice by the four routes. For the p.c. route, neat CEES was applied on the back of mice using a gas tight syringe (15 - 45 µl). The p.o. administration of neat CEES was carried out using a 20 gauge animal oral feeding cannula. The s.c. and i.p. injections of diluted CEES in PEG-300 were given using a gas tight syringe. For a comparison, carbon tetrachloride (CCl₄) was administered p.c., s.c., p.o., and i.p. routes as neat liquid. For the determination of LD₅₀, of each chemical and route (p.c., s.c., p.o. and i.p.), 3 to 4 log doses were used, and, for each dose, 4 animals were used. The body weights were recorded daily and the animals were observed for mortality for 14 days. The LD₅₀ was determined by the moving average method of Gad and Weil (1989)

Hematological and Biochemical estimations in mice at 0.5 LD₅₀ of SM and CEES:

Another study was carried out by administering SM and CEES at 0.5, 1 and 2 LD₅₀ dose through p.c., s.c., p.o., and i.p. routes (8 animals per group at 0.5 LD₅₀, and 4 animals per group for 1 and 2 LD₅₀ dose). At 0.5 LD₅₀ dose, 4 animals were sacrificed after one day and the remaining animals were sacrificed 7 days post administration. At 1 and 2 LD₅₀ dose all the animals were observed for mortality pattern. Blood was withdrawn from the orbital plexus under ether anesthesia and the animals were sacrificed by cervical dislocation. The liver was removed, blotted and weighed for biochemical studies. Hepatic GSH (glutathione) and GSSG (the oxidized dimer of GSH) contents were estimated fluorimetrically, as per the method of Hisin and Hilf (Hisin, 1976). For this method 150 mg of liver tissue were homogenized in 4 mL of phosphate EDTA buffer and metaphosphoric acid (25%). The contents of the tube were centrifuged and the supernatant was used for the estimation of GSH and GSSG. Hepatic lipid peroxidation was determined by measuring the level of malondialdehyde (MDA) according to the method of Buege and Aust (Buege, 1978). One hundred mg of liver were directly homogenized in 5 mL of thiobarbituric acid reagent and boiled for 30 min. The contents of the tubes were cooled, centrifuged and

absorbance of the clear supernatant was measured at 535 nm. The amount of MDA formed was calculated using a molar extinction coefficient of 1.58×10^5 /M per cm. Several of the hematological variables known to be affected after SM toxicity, viz.: Hb, RBC and WBC, were analyzed using a Beckman Coulter Analyzer (USA).

A fluorimetric assay was used to determine the relative levels of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radical and hydrogen peroxide. This assay measures the oxidative conversion of stable non fluorescent H₂DCF-DA (2',7'-dichloro dihydro fluorescein diacetate) to the highly fluorescent DCF in the presence of an esterase and ROS, especially hydrogen peroxide (Socci, 1999). Frozen liver samples were weighed (100 mg) and homogenized in 1 mL of ice cold 40 mM tris HCl buffer (pH 7.4). The samples were diluted by 200 times and divided into two equal fractions; one fraction was loaded with 40 μ L of 1.25 mM DCF-DA in methanol. To the remaining fraction was added 40 μ L of methanol, which then served as a blank and the fluorescence was read at 510 nm excitation and 530 nm emission.

Estimation of various oxidative stress marker enzymes after 0.5 LD₅₀ administration of SM and CEES:

Superoxide dismutase (SOD) was measured by the method of Misra and Fridovich (1972). 10 μ L of homogenate (1% in saline) was added in 500 μ L of carbonate buffer (0.1 M carbonate bicarbonate buffer with 0.2 mM EDTA pH 10.2). Epinephrine, (150 μ L of 3 mM epinephrine at pH 2.0) was added to the mixture and the change in OD was measured at 480 nm for 3 minutes. SOD activity is expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50%, each 50% inhibition is equal to one unit. Catalase was determined by the method of Aebi (Aebi 1984) as follows: 50 μ L of tissue homogenate (10% in PBS with Triton-X-100) was taken and 250 μ L of 0.5 M PO₄ buffer was added, and the reaction was started by adding 200 μ L of H₂O₂ (0.066M). The subsequent decrease in absorbance was measured at intervals of 10 sec for 30 sec. An extinction coefficient of 43.6 M cm^{-1} was used to determine the enzyme activity. One unit equals to the number of moles of H₂O₂ degraded /min/mg of protein. Glutathione reductase was assayed by the method of Racker (Racker, 1983) as follows: 50 μ L of tissue homogenate (1% in saline) was mixed with 500 μ L PO₄ buffer (0.1 M pH 7.5), 100 μ L BSA (1% BSA), 100 μ L GSSG (2% GSSG). The reaction was started after adding 50 μ L of 0.001 M NADPH and the decrease in absorbance at 340 nm was monitored for 3 min.

Estimation of DNA damage:

DNA damage was estimated qualitatively by DNA agarose gel electrophoresis. Liver samples of two animals from each group were taken for the electrophoresis. Genomic DNA from the liver of the control and treated animals were extracted (Prigent, 1993) and the damage was assessed qualitatively by agarose gel electrophoresis using 1.0% agarose in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Hyper ladder of DNA from 200-10000 base-pair was used as a DNA marker. The gels were visualized after ethidium bromide staining.

Statistical analysis:

All the variables were analyzed by one-way ANOVA with Student-Newman-Keuls multiple comparison procedure. A probability of < 0.05 is taken as statistically significant. SigmaStat (SPSS Inc., USA) was used for statistical calculations.

RESULTS

Mortality pattern, LD₅₀ and body weight change after SM and CEES administration through different route:

The most toxic route of exposure to SM was the i.p. route, with an LD₅₀ of 4.8 mg/kg. The LD₅₀ of SM by the p.c. route was less than those of the s.c. and p.o. routes, showing that SM through the p.c. route is more toxic [Table 1]. The LD₅₀ of CEES by the i.p. route was the smallest (17.7 mg/kg), less than the p.c., s.c. and by the p.o. routes. In the case of CCl₄ the LD₅₀ by i.p route also was less than p.c., s.c. and p.o. routes.

[Table 2] shows the mortality pattern of mice following SM and CEES administration by different routes. The death after i.p. administration of SM was quicker (2-6 days) than s.c. and p.o. routes (6-8 days). At a dose of 2 LD₅₀ of SM, death occurred only after 8 days in p.c. route, whereas in the case of CEES, death occurred within 1 day, i.e., there was no delayed death. The body weight of mice decreased progressively over time particularly when SM was administered by the p.c. route. At the 0.5 LD₅₀ dose of SM the decrease in body weight was significant in the p.c. route and not in the s.c., p.o. and i.p. routes [Figure 1]. In CEES the p.o. route resulted in a significantly lower body weight compared to the p.c. s.c. and i.p. routes. No significant difference was observed when CEES was administered by the p.c., s.c. and i.p routes compared to the control. Comparing the p.c. routes of administration, SM produced a more severe body weight loss than CEES (<0.05).

Effect of SM and CEES on biochemical variables:

The effects of equitoxic doses (0.5 LD₅₀) of SM and CEES on several oxidative stress markers (GSH, GSSG and MDA) is given in [Table 3]. A substantial decrease in reduced glutathione was observed compared to control in 0.5 LD₅₀ of SM administered through p.c. This depletion, though not significant on 1 day, was significant on 7 days post administration. When SM was administered through s.c., a reduction in GSH (not significant) was observed 1 day after administration and not on 7 days after administration. SM administered through the p.o. and i.p. routes did not produce any significant difference in GSH. CEES administration produced an elevation of GSH levels in the s.c., p.o. and i.p. routes, 1 day after administration, with the p.o. route showing the maximum increase (272 ± 12 %). But there was no significant difference 7 days after CEES administration. The GSSG level increased as a result of all four routes of SM administration on 1 day after, with the p.o. route showing the maximum increase (241 ± 18 %). But the level returned to normal after 7 days. The GSSG level was reduced below the control values in the SM-p.c. group, as did the GSH level, but it was not statistically significant. The GSSG level increased in all the four routes of CEES administration 1 day after, but returned to normal levels, 7 days post administration. The MDA level increased in p.o. route of

administration of SM after 1 day and increased significantly after all four routes after 7 days of administration. The MDA level did not increase after one day of CEES administration, by all the four routes, but was significantly elevated after 7 days. The ROS significantly increased after all the routes of SM and CEES administration, compared to control, 7 days after administration [Figure 2].

Effect of SM and CEES on RBC and Hb:

A significant increase in RBC count was observed in SM-p.c., 7 days after administration, but no other significant change was found in other groups. Significant change was found following the p.o administration of CEES, 1 day after administration compared to control. The hemoglobin content increased significantly in all the SM groups, 7 days after administration compared to control. Administration of CEES administration through the p.c., s.c. and p.o. routes also produced an increase in hemoglobin content, 7 days after administration [Table 4]. There was no significant difference in WBC compared to the control (data not given).

Effect of SM and CEES on various oxidative stress enzymes:

The effects of equitoxic doses ($0.5 LD_{50}$) of SM and CEES on several oxidative stress marker enzymes (glutathione reductase, catalase and SOD) are given in [Figure 3]. Significant reductions in glutathione reductase were observed when compared to control by the p.c. route on $0.5 LD_{50}$ of SM, 1 day post administration. This reduction was significant in SM and CEES through all the four routes at 7 days post administration, but the SM-p.o. group showed a significant increase. Catalase activity did not appear to be significantly affected. An increase in catalase activity was seen in the SM-i.p., CEES-p.c. and CEES-s.c. groups, 1 day after administration. In general the catalase activity decreased in SM and CEES through all the routes, 7 days after administration but was statistically significant only in the SM-s.c. and SM-p.o. groups. There was a significant increase in SOD in SM via the p.c. and s.c. routes but no significant change was found in any other group at 1 day post administration. SOD activity returned to normal levels, 7 days after administration.

Effect of SM and CEES on DNA:

Agarose gel electrophoresis of genomic DNA from liver of control mice did not show any damage [Figure 4]. Damage was also not found in the p.c, s.c. and p.o. administration of SM, however, the i.p. administration of SM showed DNA damage. DNA damage was observed after the s.c., p.o. and i.p. administration of CEES, but no DNA damage was observed in the liver after p.c. administration,.

DISCUSSION

Sulfur mustard is a bifunctional alkylating agent. Its vesicant property has been well documented but the mechanism of systemic toxicity has not been clearly delineated. One of the documented mechanisms is alkylation of DNA by SM, subsequently leading to DNA damage and cell death (Papirmeister, 1991). Our earlier study showed that the systemic toxicity of SM is not dependent on its DNA damaging property, and, at lower doses, there was no significant DNA damage (Rao,

1999). Thus DNA alkylation may not play an important role in the cytotoxicity induced by SM. In the present study the dose used was 0.5 LD₅₀ and only the i.p. administration of SM resulted in DNA damage after 7 days. DNA damage was also not observed when CEES was administered through the p.c. route. Though, CEES administration through the s.c., p.o. and i.p. routes showed DNA damage, but the damage did not correlate with the mortality pattern. Also, there are various DNA alkylating agents that do not cause vesication, yet they cause other pathological changes similar to SM. Hence DNA damage is only one of the toxic actions of SM and CEES and may not be the sole cause of the systemic toxicity.

We have already reported that SM is more toxic in mice and rats through the p.c. route compared to the s.c. and p.o. routes (Vijayaraghavan, 2005). The present study shows that the i.p. route is the most toxic route (LD₅₀ =4.8 mg/kg). Following the i.p. route death occurred in a shorter time (<5 days) compared to other routes. The reason may be the direct effect of SM on the mesenteric epithelium. In the case of CEES the most toxic route was also the i.p. route. Unlike SM, the CEES s.c. and p.o. routes were more toxic than p.c. route. CEES is a volatile liquid (b.p. 157°C) compared to SM (b.p. 217°C), and it is expected to get evaporate faster upon topical application when it is applied undiluted. In the present work, lower concentrations (200 mg/kg and less) were also studied diluting CEES in PEG 300. PEG 300 is expected to limit the CEES from vaporization. No apparent effect was observed in those animals. The toxicity of CCl₄ was also greater through the i.p. route, compared to the p.c., s.c. and p.o. routes. Death after CEES administration was within 24 hr, which shows that the mechanism of action of CEES is different from SM (delayed death). The same pattern of toxicity, i.e., the i.p. route is more toxic than the p.c., s.c. and p.o. routes, is also observed in other lipophilic compounds (Lewis, 1996). The mechanism of SM toxicity through the p.c. route is different than that for CEES, in which the death was immediate regardless of the route of administration. A progressive loss in body weight of the animals was a consistent observation after exposure to SM. But no weight loss was observed after CEES administration. Weight loss in the CEES-p.o. group may be due to the local injury of stratified epithelium of esophagus and inner mucosal layers of stomach.

In a well-known hypothesis of SM toxicity, GSH may act as an alternative intracellular site or scavenger for SM (Papirmeister, 1991). We earlier reported that significant depletion of GSH was found when SM was administered via the p.c. route. In the present study GSH depletion in SM also was observed 1 day, as well as 7 days, post administration by the p.c. route. CEES administration by various routes showed an elevation of GSH, 1 day after, but the level was normal 7 days after, showing that unlike SM the toxicity of CEES is not due to the effect on GSH. The elevation of GSH after 1 day may be due to the body defense mechanism. Unlike GSH, the oxidized glutathione (GSSG) increased in all the groups on 1 day after both SM and CEES administration and returned to normal levels, 7 days post administration, except in the SM-p.c. group, where a decrease was observed. Malondialdehyde (MDA) is the byproduct of lipid peroxidation of mainly phospholipids of cell membrane; the level of MDA is expected to increase in oxidative stress. Since the

MDA level is not significantly affected 1 day after administration, but is significantly elevated 7 days after SM or CEES administration, the oxidative stress appears to be induced at a later period. The same trend is reflected by the increase in ROS, 7 days after both SM and CEES administration by all routes.

Oxidized glutathione is reduced by a multi-step reaction in which the first step is the reduction of glutathione reductase (GR) by NADPH. The reduced GR reacts with GSSG resulting in a disulfide interchange and produces a molecule of GSH and GR-SG complex. Finally, GR is restored and another GSH molecule is released (Janes, 1990; Wang, 1998). The possibilities of decreased activity of GR may be due to the direct interaction of the toxic metabolite of SM with the GR active site containing thiol moieties, or by any mutagenic changes in GR genes which affect the expression of active GR enzyme. We have also estimated the activities of various cytosolic antioxidant enzymes including SOD and catalase. These enzymes are generally known to be involved in oxidative stress. Increased SOD and catalase activity could reflect a cellular adaptive response against the formation of reactive oxygen species induced by SM or CEES administration, or could be associated with inflammation and influx of inflammatory cells. Alternatively, increased antioxidant enzyme activities can up-regulate the enzyme expression, as a result of a signal transduction pathway, activated by the changes in the cellular redox state. Increases in RBC count and Hb concentration are common observations in SM toxicity. This is due to the increase in permeability of endothelial cells that in turn reduce the plasma volume, so the blood becomes viscous and RBC count and Hb concentration increases. This change is observed 7 days after both SM and CEES administration through all routes, corresponding with the increased level of MDA and ROS. The increase in RBC count and the Hb content was higher in the SM-p.c. group, also showing that the toxic effect is severe by p.c. route.

For any topically applied compound to gain entry into the systemic circulation, it is necessary to penetrate three different layers of the skin, the dermis, epidermis and subcutaneous fats. It was suggested by Hambrook *et al.* (Hambrook, 1993) that, after SM application, the skin contains a reservoir or depot of unchanged SM from which there is continual uptake of SM in the blood during the first few days after the application. But this theory is difficult to explain as we were not able to protect the animal by the amino thiol antidotes such as, DRDE-07 developed by us. The animals are protected when the antidote was administered prophylactically but not therapeutically, even 30 min after SM administration (Vijayaraghavan, 2001). DRDE-07 failed to protect the animals when SM was administered through the s.c., p.o. and i.p. routes. DRDE-07 also failed to protect from CEES toxicity by all routes (unpublished observation). This provides additional evidence that on the whole the mechanism of toxicity of SM through p.c. route is different from any other routes. Also the explanation of SM toxicity on the basis of CEES toxicity is not appropriate (Blaha, 2000).

Skin contains several enzymes similar to those in liver tissue and can metabolize certain inactive molecule such as polycyclic hydrocarbons to active

carcinogens. It is possible that since SM is a bifunctional compound, it could undergo activation in skin and since CEES is a monofunctional compound it may not be similarly activated. The CEES toxicity pattern is also different from SM and is similar to other chlorine containing compounds, e.g., CCl₄. Another possible reason for SM toxicity is the production of inflammatory mediators such as COX-2 and other prostaglandins (Wormser, 2004). Substance P released from sensory nerve endings in skin leads to inflammatory response resulting in edema (Casbohm, 2004). The release of inflammatory mediators and cytokines in later days may be responsible for the toxicity and delayed death observed when SM is administered through the p.c. route.

A number of analogues of SM, such as CEES, BCS (butyl 2-chloroethyl sulfide) and CECBS (2-chloroethyl 4-chlorobutyl sulfide), are used for the evaluation of antidotes against SM. Mostly these agents are used either *in vitro* or *in vivo* and given by parenteral routes and at very high doses. Intratracheal infusion of CEES resulted in the accumulation of TNF- α and it was prevented by pretreatment with N-acetylcysteine (Das, 2003; Chatterjee, 2003). A monofunctional sulfur mustard analogue, BCS when given by the s.c. route has been reported to induce oxidative stress distal to the site of exposure (Elsayed, 1989; Elsayed, 1992). Another analogue, 2-chloroethyl 4-chlorobutyl sulfide (CECBS), given at a dose of about 200 mg/kg (5 μ L per mouse) subcutaneously, induced oxidative stress (Elsayed, 2004). The present work shows that the SM administered by p.c. route is more toxic than the s.c. or p.o. routes and this pattern is not the same as that observed with CEES. The nature of the toxicity of BCS, CECBS and also nitrogen mustards needs to be evaluated by various routes to get more information for the development of antidotes for SM, as well as nitrogen mustards.

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Table 1: Toxicity of SM, CEES and CCl₄ by various routes in mice.

Groups	SM (LD ₅₀ mg/kg)	CEES (LD ₅₀ mg/kg)	CCl ₄ (LD ₅₀ mg/kg)
pc	9.7 (6.1 - 15.2)	1425 (671 - 3029)	> 5000
sc	13.5 (7.2 - 26.0)	100 (47.7 - 209)	> 5000
po	19.3 (11.8-31.5)	566 (400-800)	> 5000
ip	4.8 (2.9-7.8)	17.7 (12.5 - 25.0)	566 (400 - 800)

3 to 4 log doses were used and for each dose 4 animals were used.

Animals were observed for mortality for 14 days.

Figures in parenthesis are confidence limits.

Table 2: Time course of mortality of mice following p.c., s.c., p.o. and i.p. administration of SM, CEES and CCl₄.

Group/ Route	Dose (LD ₅₀)	Number of mice died (cumulative) out of four after SM, CEES or CCl ₄ administration (days)						
		2	4	6	8	10	12	14
SM-pc	0.5	0	0	0	-	-	-	-
	1	0	0	0	0	2	3	3
	2	0	0	0	1	2	4	4
SM-sc	0.5	0	0	0	-	-	-	-
	1	0	0	2	2	2	2	2
	2	0	0	2	4	4	4	4
SM-po	0.5	0	0	0	-	-	-	-
	1	0	0	0	0	2	3	3
	2	0	0	2	4	4	4	4
SM-ip	0.5	0	0	0	-	-	-	-
	1	0	0	2	2	2	2	2
	2	2	3	4	4	4	4	4
CEES-pc	0.5	0	0	0	-	-	-	-
	1	2	3	3	3	3	3	3
	2	4	4	4	4	4	4	4
CEES-sc	0.5	0	0	0	-	-	-	-
	1	2	2	2	2	2	2	2
	2	4	4	4	4	4	4	4
CEES-po	0.5	0	0	0	-	-	-	-
	1	2	2	2	2	2	2	2
	2	4	4	4	4	4	4	4
CEES-ip	0.5	0	0	0	-	-	-	-
	1	2	2	2	2	2	2	2
	2	4	4	4	4	4	4	4
CCl ₄ -ip	400 mg/kg	0	0	0	0	0	0	0
	800 mg/kg	4	4	4	4	4	4	4

0.5 LD₅₀ animals were used for biochemical estimations. 8 animals per group were used. 4 animals were sacrificed, 1 day after administration and the remaining 4 animals were sacrificed on 7 days after administration.

1 and 2 LD₅₀ – 4 animals per group.

Table 3: Effect of SM and CEES on GSH, GSSG and MDA at 0.5 LD₅₀ of dose.

Groups	GSH (%)		GSSG (%)		MDA (%)	
	1 day	7 day	1 day	7 day	1 day	7 day
Control	100.0 ± 8.6	100.1 ± 3.5	99.7 ± 0.75	99.7 ± 5.7	99.8 ± 1.2	99.8 ± 6.7
SM – pc	64.9 ± 6.7	55.3 ± 6.4*	178.9 ± 22.7*	65.2 ± 7.0	101.8 ± 8.8	162.2 ± 5.6*
SM – sc	69.2 ± 11.6	99.2 ± 2.5	225.0 ± 7.8*	95.1 ± 8.9	120.3 ± 8.7	147.7 ± 3.9*
SM – po	112.5 ± 3.6	77.2 ± 13.2	241.4 ± 17.9*	83.9 ± 7.7	135.2 ± 0.95*	166.6 ± 10.4*
SM – ip	93.5 ± 7.4	114.2 ± 7.3	223.4 ± 9.9*	109.9 ± 8.1	116.6 ± 0.32	160.3 ± 6.6*
CEES – pc	146.7 ± 25.8	86.3 ± 4.3	213.3 ± 1.0*	123.8 ± 18.6	102.9 ± 8.2	182.8 ± 9.6*
CEES – sc	210.4 ± 50.4*	104.0 ± 4.9	212.0 ± 8.4*	110.5 ± 6.7	81.0 ± 8.0	193.5 ± 5.3*
CEES – po	272.0 ± 12.3*	96.3 ± 10.6	188.5 ± 6.9*	130.7 ± 21.7	91.8 ± 1.8	209.5 ± 10.6*
CEES - ip	216.7 ± 22.0*	109.9 ± 10.2	180.5 ± 0.45*	98.8 ± 4.4	88.7 ± 1.9	177.4 ± 33.3*

* Significantly different from control (P< 0.05).

mean ± SE (n=4)

Control values : GSH = 4.61 ± 0.09 μmoles/gm of tissue
 GSSG = 1.95 ± 0.30 μmoles/gm of tissue
 MDA = 2.57 ± 0.17 nanomoles/gm of tissue.

Table 4: Effect of SM and CEES on RBC and Hb at 0.5 LD₅₀ of dose.

Groups	RBC (%)		Hb (%)	
	1 day	7 day	1 day	7 day
Control	100.0 ± 2.9	118.0 ± 15.6	100.0 ± 1.8	100.6 ± 2.4
SM – pc	81.9 ± 5.0	168.1 ± 6.2*	89.3 ± 0.9	156.7 ± 5.8*
SM – sc	92.3 ± 0.9	141.9 ± 2.6	96.2 ± 0.4	132.8 ± 3.0*
SM – po	92.3 ± 4.5	141.4 ± 4.9	96.2 ± 3.9	139.0 ± 4.4*
SM – ip	93.8 ± 3.6	140.0 ± 7.5	95.0 ± 3.3	130.6 ± 6.5*
CEES – pc	87.6 ± 6.5	144.9 ± 6.8	87.8 ± 7.1	134.1 ± 6.4*
CEES – sc	91.8 ± 2.9	137.0 ± 3.0	96.2 ± 1.3	126.6 ± 3.7*
CEES – po	126.8 ± 14.3*	133.1 ± 9.4	104.6 ± 2.6	123.3 ± 6.9*
CEES - ip	99.5 ± 2.0	120.1 ± 8.6	100.4 ± 1.9	99.3 ± 5.3

* Significantly different from control (P< 0.05).

mean ± SE (n=4)

Control values: RBC = 9.5 ± 0.4 x10⁶ cells/mL
Hb = 12.9 ± 0.3 g/dL

Figures.

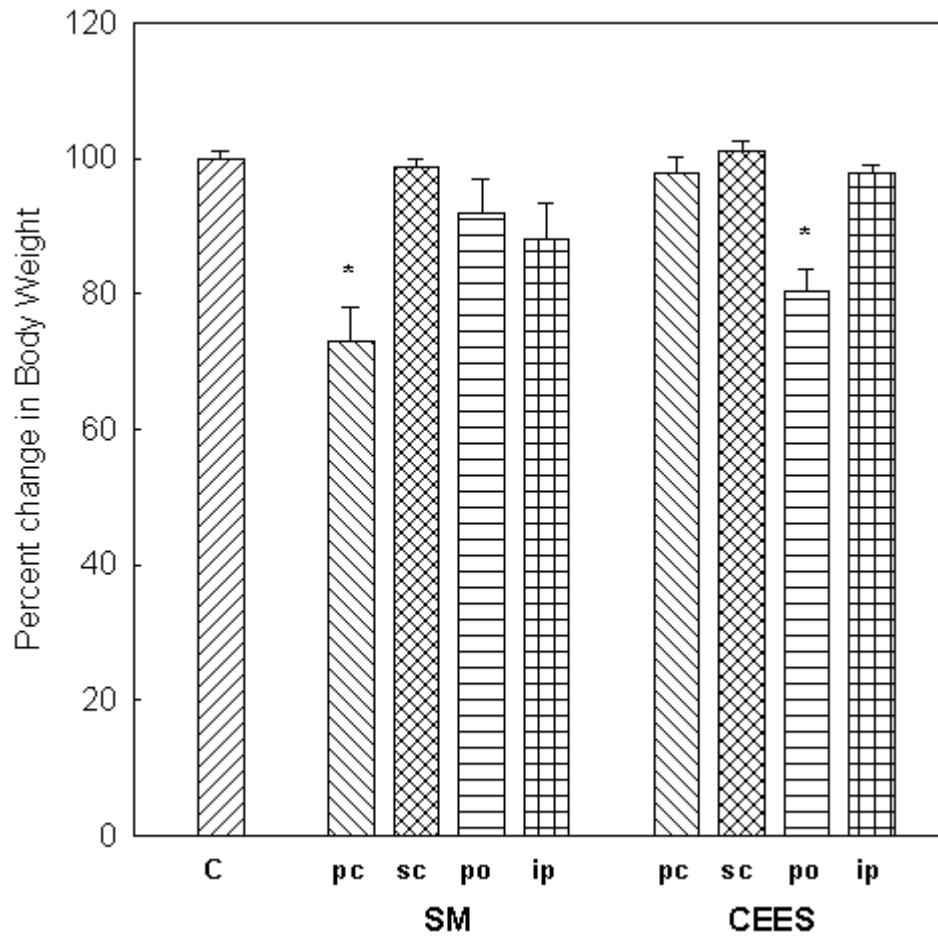


Figure 1 : Effect of SM and CEES (0.5 LD₅₀) by various routes on body weight of mice, 7 days after administration. *Significant from control.

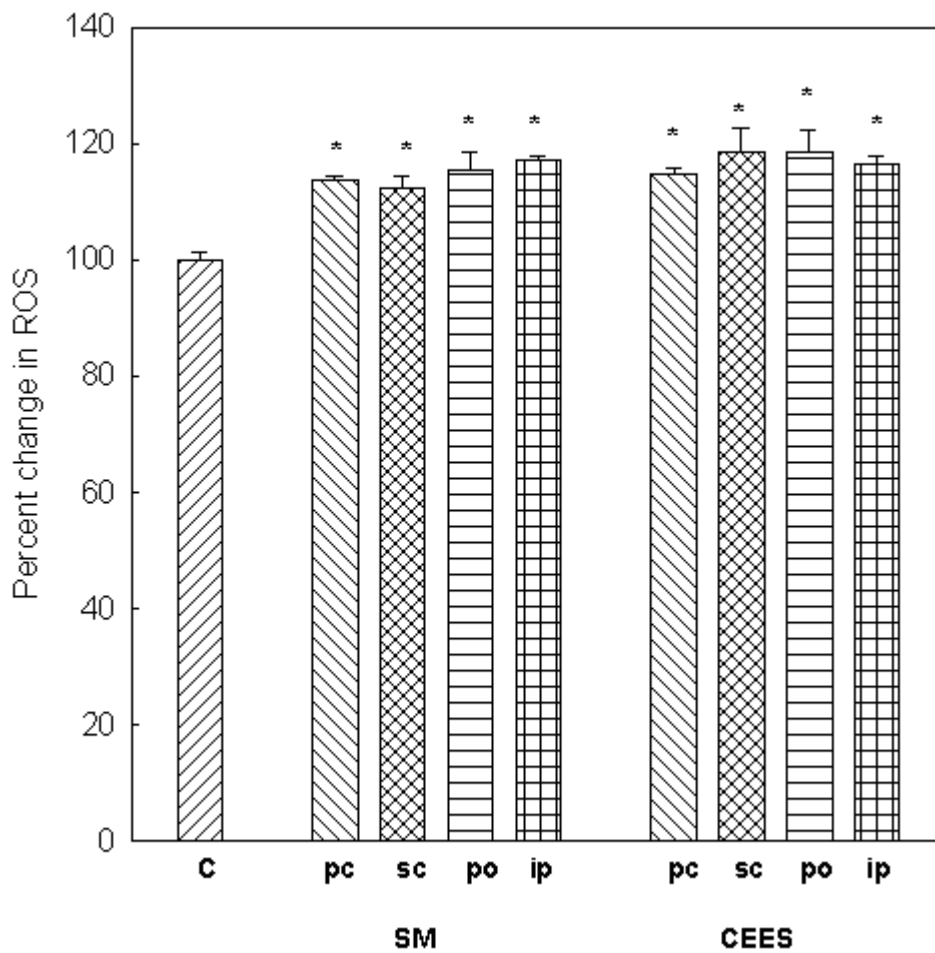
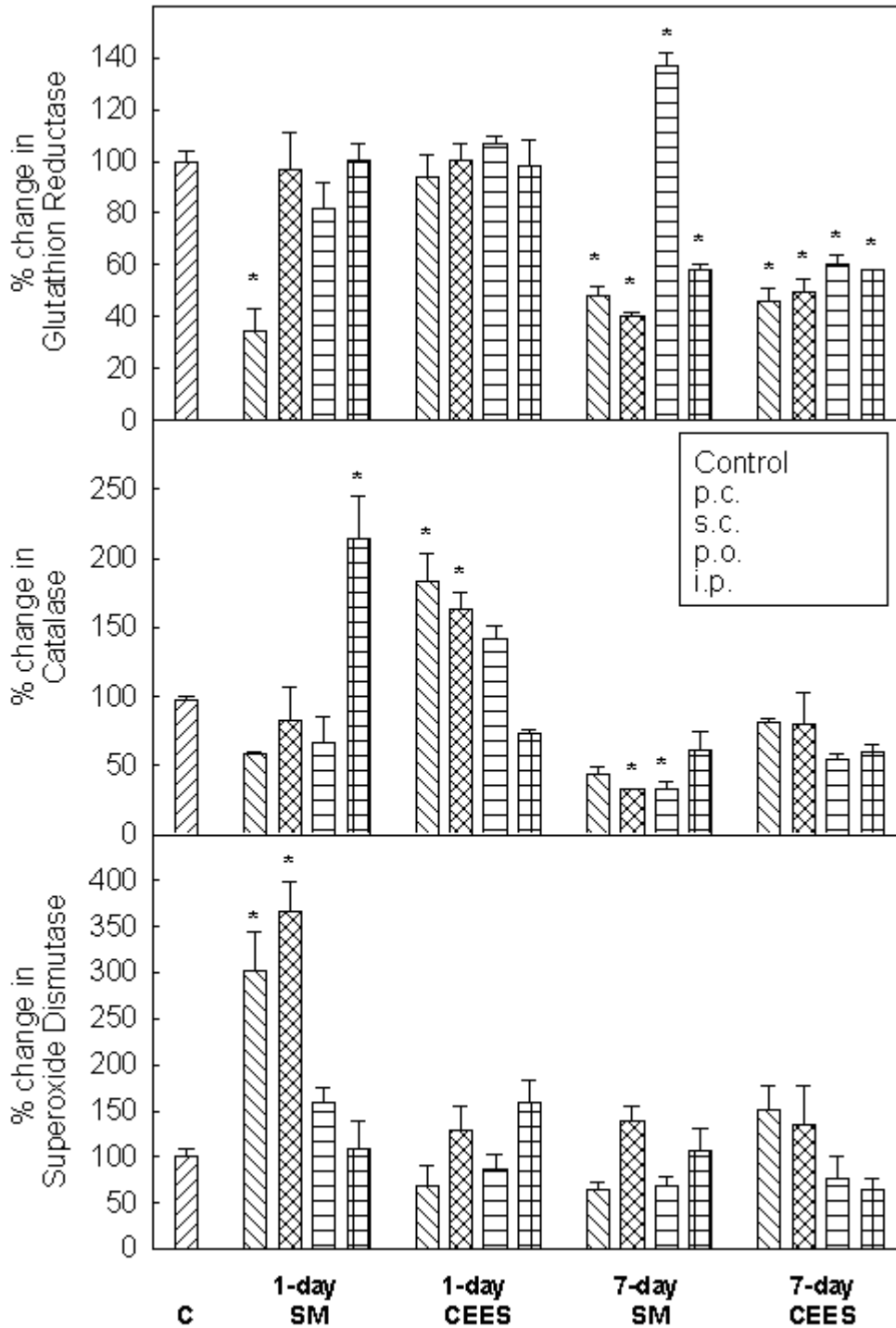


Figure 2 : Effect of SM and CEES (0.5 LD₅₀) through various routes on the level of reactive oxygen species (ROS), 7 days after administration.
 *Significant from control; Control = 355 ± 5 relative fluorescence units/mg of protein.



Control values : Glutathione reductase = 4.9 ± 0.2 mU/mg of protein
 Superoxide dismutase = 5.8 ± 0.9 U/mg of protein
 Catalase = 10.2 ± 0.5 IU/g of tissue

Figure 3 : Effect of SM and CEES ($0.5 LD_{50}$) on glutathione reductase, catalase and superoxide dismutase, 7 days after administration. *Significant from control. Control values : Glutathione reductase = 4.9 ± 0.2

mU/mg of protein; Superoxide dismutase = 5.8 ± 0.9 U/mg of protein;
Catalase = 10.2 ± 0.5 IU/g of tissue.

abcdefghijklmnopqrs

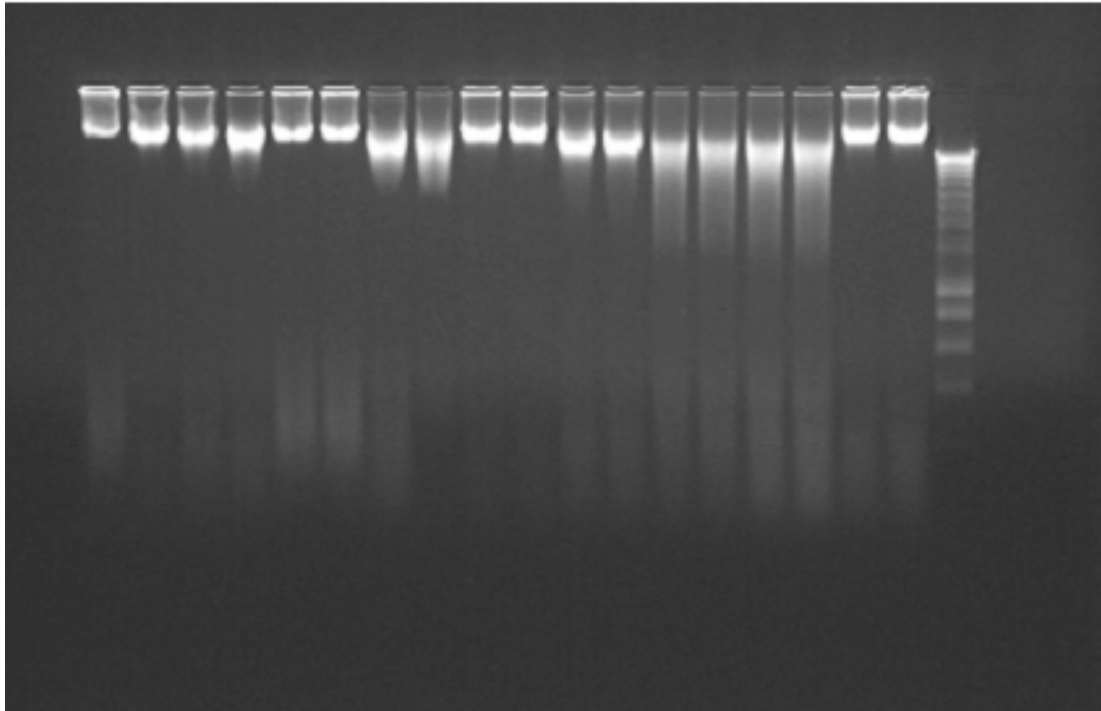


Fig - 4. Agarose gel electrophoresis of genomic DNA isolated from liver of mice, 7 days after SM and CEES administration ($0.5 LD_{50}$). a,b = SM-pc, c,d = SM-sc, e,f = SM-po, g,h = SM-ip, i,j = CEES-pc, k,l = CEES-sc, m,n = CEES-po, o,p = CEES-ip, q, r = Control, s = Marker hyper ladder of 200-10000 base pairs.

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