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Effectiveness of Cytoprotective Agents on Sulfur Mustard Induced Toxicity: The *In vitro* Model

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ABSTRACT

Sulphur mustard (bis (2-chloroethyl) sulfide, SM) is a powerful vesicating chemical warfare agent that causes profound injuries to the eyes, lungs and skin. Despite intensive research following the first use of SM in World War I, there is still no useful pretreatment or therapeutic antidote available. This agent remains a constant chemical threat. A potential approach to combating the debiliting effects of this agent is the use of compounds that can react with this material before it interacts with critical macromolecules. Glutathione (GSH), a tripeptide that exists in high concentrations in cells, reacts with SM and is involved in SM detoxification. Amifostine is a synthetic aminothiol, has been extensively used as a radioprotector. This prompted us to evaluate the protective efficacy of GSH, Amifostine and DRDE-07 (S-2(2-aminoethyl amino) ethyl phenyl sulfide) (synthesized in our lab) against SM toxicity *in vitro* in HeLa cell line. All these compounds are thiol group containing compounds. Pretreatment of HeLa cell with these cytoprotectants led to decrease in cytotoxicity after SM exposure. The protective efficacy of above compounds were evaluated against sulphur mustard using HeLa cells. The above compounds were added to the media 1 hr before the SM exposure and incubated for 24 hrs. cell viability by MTT assay and LDH leakage were measured as end point.

Key words: Sulphur mustard, cytoprotective agents.

1. INTRODUCTION

Vesicants such as sulphur mustard (bis (2-chloro ethyl sulphide), SM) is a class of chemical warfare agents (CWA) that causes blisters at the site of exposure and is a cytotoxic agent.[1] SM forms sulphonium ion in the body which alkylates DNA and several other macromolecules, and induces oxidative stress.[2,3] These cytotoxic effects are manifested in widespread metabolic disturbances whose variable characteristics are observed in enzymatic deficiencies, vesication, abnormal mitotic activity and cell division, bone marrow depression and systemic poisoning.[4] It is an alkylating agent that causes serious blisters upon contact with human skin.[5] SM has been used as Chemical Warfare (CW) agent.[6-10] During the First World War and the Iran-Iraq conflict, SM was used and still remains a threat to both civilians and military personnel.[11-12] The production of SM does not require specialised technology; the danger of terrorist attack against the civilian population is considerable. On the other hand, apart from the purposeful attack, there is a risk of accidental exposure to SM connected with inappropriate disposal of old depots.[13] Biochemical mechanism of action of SM is not clear, therefore, no specific therapy exists.

At the cellular level, SM causes cytostatis, mutation and slow cell death. Eyes, skin and the respiratory tract are the main target organs of SM toxicity.[14] In the recent past, substantial efforts have been made in developing pharmacological strategies against the toxic effects of SM. All these studies were aimed at preventing SM alkylated critical cell targets, improve calcium regulation, protect cell mediated biochemical disruptions or prevent cytotoxicity.[15-16] Several antidotes have been reported for reducing the systemic toxicity of SM in experimental animals.[17-18]

Previously, Dabrowska et al. (1996) reported that SM induces apoptosis in endothelial cells. This observation has been extended to other cell lines.[19] Although some beneficial effects have been observed with some drugs in tissue culture systems, the antidote activity of the test compounds was always too weak to be used as protectants against SM.[20]

A variety of compounds tested to attenuate SM toxicity *in vitro* or *in vivo* include scavengers of SM and SM-induced oxygen radicals,[21-23] inhibitors of cell death and promoters of cell survival, [22] and numerous other pharmacological agents.[24-26]

An effective prophylactic agent against SM is the requirement of the day especially for personnel engaged in the destruction of SM and during inspection by the Organization for the Prohibition of Chemical Weapons.[27-28] There is no effective treatment for SM toxicity and still is a challenge.[16] The study was aimed to evaluate the prophylactic efficacy of GSH, DRDE-07 and Amifostine against SM toxicity in Hela cell line.

2. MATERIALS AND METHODS

2.1 Chemicals

The Sulfur Mustard was synthesized in Defence Research and Development Establishment, Gwalior and found to be above 99% pure by gas chromatographic analysis. The S-2-(2-Aminoethylamino) ethyl phenyl Dihydrochloride (DRDE-07), were synthesized in the Synthetic Chemistry Division of DRDE and were characterized by elemental analysis, IR, ^{1}H NMR, and MS analysis, and the purity was checked by TLC using a mobile phase system of methanol, chloroform and ammonia. A single spot was detected for each compound. Minimum Essential Media Eagle (MEM), Penicillin-Streptomycin-Neomycin solution, DMSO, fetal bovine serum (FBS), 3-[4, 5 dimethylthiazol- 2yl]-2, 5 diphenyl tetrazolium bromide (MTT), and reduced glutathione (GSH) and trypsin were purchased from Sigma-Aldrich, (USA) and other chemicals of highest purity were from E. Merck or Qualigens (India).

2.2 Cell culture and exposure

The HeLa cells were purchased from National Center for Cells Sciences Pune, India. The cells were maintained in MEM supplemented with 10% FBS in CO_2 incubator (New Brunswick Scientific Co., Inc. USA). The CO_2 incubator temperature and humidity was maintained as 37ºC and 95% respectively. The cells were seeded in 25 cm^2 cell culture flask and grown up to 90% confluent for LDH measurement and 24 well plates were used to study the MTT reduction measurement. SM was dissolved in DMSO and stock solution was prepared at the concentration of 10mM. The GSH, amifostine and DRDE-07 was prepared freshly by dissolving them in MilliQ water. For prophylactic study the drugs were added 1 hr before SM exposure after 24 hrs the cells were analysed for MTT.

2.3 Lactate Dehydrogenase (LDH) release

Cell viability was determined by measuring LDH release, an indicator of cytotoxicity, in the growth medium of control and experimental cells. The media was centrifuged to remove dislodged cells, if any. The LDH was measured using commercially available kits from Merck Specialties. Private Limited, Mumbai. This *in vitro* photometric determination of LDH was based on an increase in absorbance at 340 nm caused by the formation of NADH due to the LDH-catalyzed reaction between L-lactate and NAD. The rate of decrease in NADH concentration is determined photometrically and is directly proportional to the LDH activity in the sample material. The results were expressed as % of LDH leakage to extracellular medium compared with control and SM treated cells.

2.4 Cell viability assay

The MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide) assay was performed by a modification of the method described by Mosmann, (1983).[29] Briefly, at the end of each experiment, cultured cells in 24 well plates (with 200 µl of medium per well) were incubated with MTT (20 µl of 5 µg/ml per well prepared in PBS, filtered with 0.45 micron sterile filters) at 37°C for 4 hours in CO_2 incubator. The formazan product was solubilized by addition of 200 µl of dimethyl sulfoxide (DMSO) and 100 µl of 10% SDS in 0.01 M HCl and the OD measured at 570 nm (Biotek µQuant spectrophotometer). The viable cells produced a dark blue formazan product; whereas no such staining was formed in the dead cells. The amount of formazan formation is directly proportional to cell viability.

2.5 Determination of LC_{50} of SM in HeLa cells

HeLa cells were seeded in 24 well plates for LC_{50} determination of SM. The cells were exposed to 0.1, 1, 5, 20, 50, 100, and 250 µmol of freshly prepared SM in dimethylsulfoxide (DMSO). The plates were kept in CO_2 incubator for 24 hours.

2.6 Protective efficacy of DRDE-07, Amifostine and GSH

The prophylactic efficacy of DRDE-07, Amifostine and GSH were evaluated as a 1 hr pretreatment of HeLa cells against SM. 1 µmol of SM was used for the prophylactic efficacy. The GSH, amifostine and DRDE-07 were used at a concentration of 50 µmol and 100 µmol.

2.7 Statistical Analysis

The experimental results were analysed by using one way analysis of variance (ANOVA), followed by Dunnett's test. The significant differences between groups were assumed if the P value is less than 0.05. SigmaStat (SPSS inc, USA) was used for all statistical analysis.

3. RESULTS AND DISCUSSION

3.1 LC_{50} determination

The HeLa cells were exposed to different concentration of sulfur mustard showed a dose dependent increasing of LDH leakage in to the medium. The LDH leakage in to the medium is mainly because of membrane damage induced by SM. A linear increase of LDH leakage was observed upto 20 µmol (figure 1). The LC_{50} of sulfur mustard was determined from the graph based on percentage of viability of the cells. The viability of the cells was measured based on the MTT assay and LDH leakage. The metabolic activity of the cells decreased dose dependently. While the LDH leakage in to the medium increased dose dependently upto 20 µmol concentration of sulfur mustard.

3.2 Prophylactic efficacy of GSH, amifostine and DRDE-07

The metabolic integrity of the cells also an important one as far as the development of antidote is concern. The maintenance of metabolic activity of the cells also changed depending upon the nature of the chemicals and duration. The metabolic activity of the cells reduced after sulfur mustard exposure at the dose of 1 µmol. The extra cellular addition of GSH significantly increased the metabolic activity of the cells even at 50 µmol concentration. Amifostine at the dose of 100 µmol and DRDE-07 at 50 µmol provided a significant protection of metabolic activity against sulfur mustard induced toxicity (figure 2,3).

3.3 Discussion

Despite many years of research on these cytotoxic alkylating agents, the mechanism and the initial events leading to cell death are still not fully understood. Few *in vitro* studies showed that, the beneficial effects of antidotes are always very weak and cannot be used as countermeasures against SM, due to the fast reactivity of this compound. Though several antidotes have been screened for their protective efficacy against SM toxicity *in vitro*, they are either ineffective or have not been screened *in vivo.*[24,25,30,31]

Generally the GSH content of confluent cells is about 60% lower than that of dividing cells, yet dividing cells are more susceptible to SM cytotoxicity. In our study depletion of cellular GSH, increases the cells' susceptibility to SM, which may suggest that intracellular GSH does have some protective role against SM toxicity. Adding GSH to the incubation medium, afforded significant protection to the cells against SM. The protection was better when GSH was added one hour prior to SM addition. However, protection was significant, when GSH concentration increased (100 µmol) was added. This finding is supported by the paper of Lindsay *et al*, (1997) [32] showing that monoisopropyl-glutathione ester is required extracellularly to protect A549 cells from SM toxicity.

One speculation about the role of extracellular GSH in protecting the cell against SM toxicity is by its direct reducing effects at the cell membrane. Membrane destabilization after SM exposure has been shown for lysosomal nuclear and cellular membranes. Our findings are that extracellular increase of GSH concentration, afforded protection against SM may have important therapeutically implications.

The cytotoxicity of the alkylating agents such as mustards is believed to act mainly through DNA alkylation.[33] Besides, GSH depletion and subsequent oxidative stress have also been demonstrated in the early phase, prior to cell death.[34] Oxidative stress might be involved in mustard-induced acute toxicity following glutathione depletion.[35,36] In the present study, the depletion of GSH was significantly restored by GSH and DRDE-07 after SM exposure. It is well known that NAC replenish the GSH pool while DRDE-07 may also help in the maintenance of GSH level in the cells and provide the protection against SM.[22]

Amifostine is dephosphorylated to its free thiol molecule (WR-1065) by membrane bound alkaline phosphatase, and the latter enters to the normal cells to give protection against alkylating agents and radiation.[37] Since SM is also an alkylating agent, amifostine was expected to antagonize its toxic effects by similar mechanism. Amifostine is also likely to augment GSH levels by providing a -SH pool and thereby protecting the alkylation of DNA. In the present study amifostine increased the GSH level in HeLa cells, but also offered protection in vivo against SM. The presence of a alkyl or aryl groups in DRDE-07 and its analogues is expected to increase the lipophilicity, but they cannot be metabolized like amifostine to the free thiol by membrane bound alkaline phosphatase. However, the protection given by DRDE-07 analogues was better than amifostine. Therefore, some other mechanism can be attributed to the protective efficacy of DRDE-07. Possibly, the amino group facilitates its entry into the cell to be subsequently used as -SH.

4. CONCLUSION

The above studies show that the sulfur mustard is very toxic to HeLa cells and threat to both civil and defense populations. Despite more than ninety years of research on the development of antidotes none has proved with significant protections. A number of drugs are being screened by both *in vivo* and *in vitro* models for the prophylactic and therapeutic efficacy. DRDE has developed few antidotes (DRDE-07 and its analogues), some of them particularly DRDE-07 is giving very good protection both by *in vivo* and *in vitro* models. In the present studies DRDE-07 was compared with known compounds like GSH and amifostine against sulfur mustard toxicity.

The conclusion from the study is that pretreatment of HeLa cells with GSH (both 50 and 100 µmol) is giving significant protection followed by DRDE-07 and amifostine based the mustard toxicity a combination of different antidotes can be tried. To achieve an optimal and significant protection against sulfur sulfur mustard target the different types of biomolecules depending upon dose and duration of exposure.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper

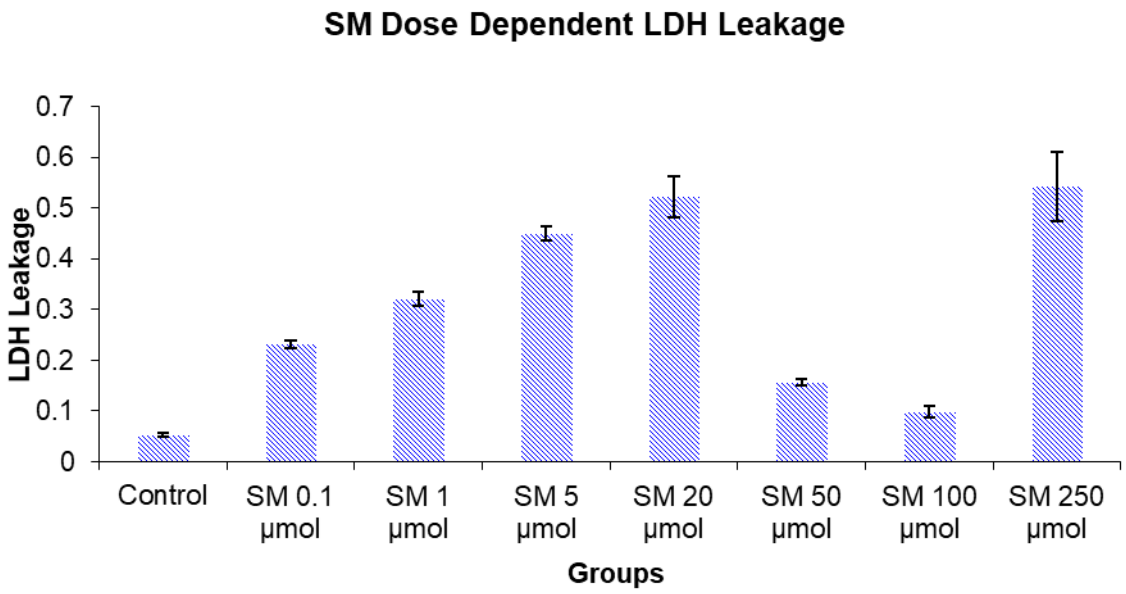

Fig.1: LDH leakage of Sulphur Mustard (SM) dose dependently

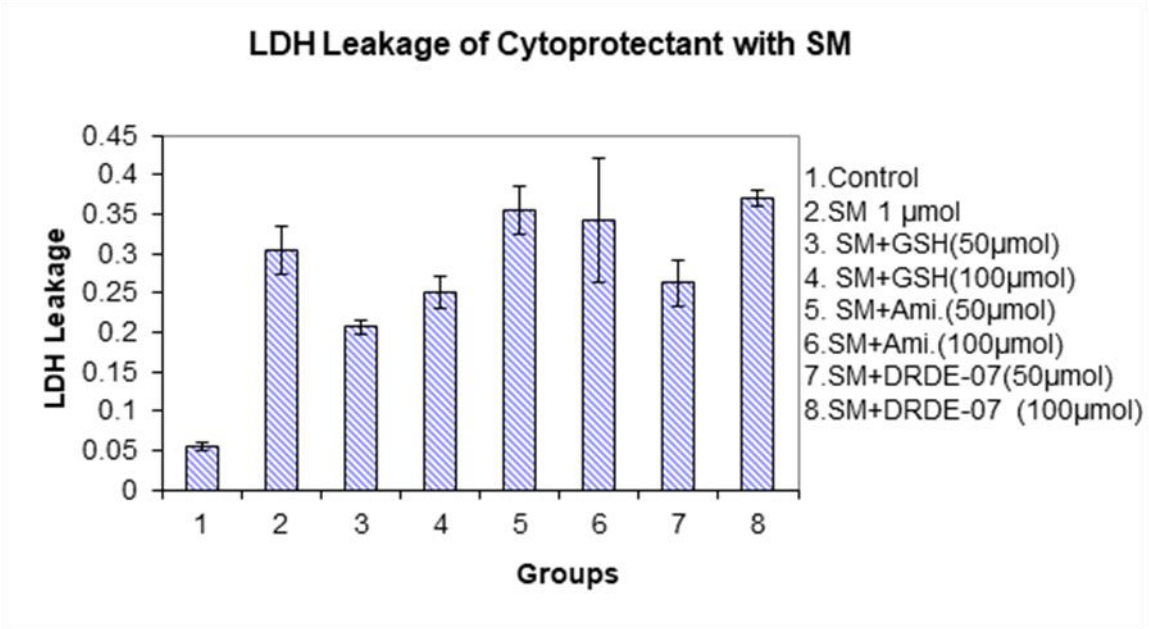

Fig.2: LDH leakage of Cytoprotectants with SM

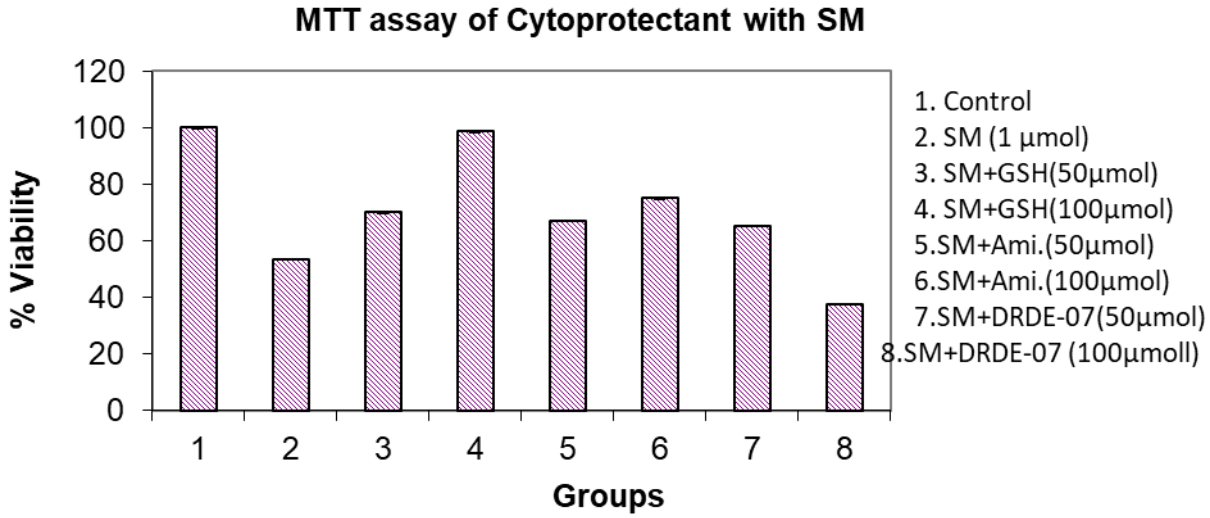

Fig.3: MTT assay of cytoprotectants with SM

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