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Neurobehavioral impairments, generation of oxidative stress and release of pro-apoptotic factors after chronic exposure to sulphur mustard in mouse brain

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ABSTRACT

Recent global events have focused attention on the potential threat of international and domestic chemical terrorism, as well as the possibility of chemical warfare proliferation. Sulphur mustard (SM) is one of the potent chemical warfare agents (CWA), which initiates a cascade of events that converge on the redox mechanisms common to brain injury. The present study was designed to examine the effects of chronic SM exposure on neurobehavioral impairments, mitochondrial oxidative stress in male Swiss Albino mice and its role in inducing apoptotic neuronal cell death. The animals were divided into four groups (control, low, medium and high dose) of 5 animals each. Exposure to SM was given percutaneously daily for 12 weeks. The results demonstrated impairment in neurobehavioral indices viz. rota rod, passive avoidance and water maze tests in a dose dependent manner. There was a significant increase in lipid peroxidation and protein carbonyl content whereas, decrease in the activity of manganese superoxide dismutase (MnSOD), glutathione reductase and glutathione peroxidase suggesting impaired antioxidant defense system. Immunoblotting of cytochrome *c*, Bcl-2, Bax and activation of caspase-3 suggest induction of apoptosis in a dose dependent manner. Finally, increased p53 expression suggests that it may target the mitochondrial pathway for inducing apoptosis in response to DNA damage signals. In conclusion, chronic SM exposure may have the potential to generate oxidative stress which may trigger the release of cytochrome *c* as well as caspase-3 activation in neurons leading to cell death by apoptosis in a dose dependent manner which may in the end be responsible for the disruption of cognitive functions in mice.

Introduction

Sulphur mustard (SM), chemically bis[2-chloroethyl]sulfide and commonly known as mustard gas is an alkylating agent that causes serious blisters upon contact with human skin (Smith and Dunn, 1991; Momeni et al., 1992). Due to its simple method of preparation, there is always a potential threat of being used as a chemical warfare agent (CWA) by the terrorist groups, in spite of the successful implication of the Chemical Weapons Convention (Krutzsch and Trapp, 1994). Low dose SM exposure produces a lesion that is mild and characterized by erythema, itching, and sensitivity to touch. With higher doses of SM, erythema develops more rapidly after latency, and is then followed by varying degrees of blistering and necrosis depending on dose. After absorption, SM being an extremely potent alkylating agent, has the potential to damage all cells and all organs (Papiermeister et al., 1991). Absorption and systemic distribution of a significant amount of SM damages the bone marrow (where it destroys the precursor cells, leading to pancytopenia) (Papiermeister et al., 1991). Less commonly, clinical effects are seen in the gastrointestinal tract (usually as a terminal event) and in the central nervous system (CNS) (with ill-defined symptoms such as lethargy and apathy) (Sohrabpour, 1984).

The mechanism of SM-induced cellular injury also involves free radical-mediated oxidative stress (Elsayed et al., 1992), in addition to DNA alkylation, strand breaks, and inhibition of transcription and protein synthesis leading to cell death and acute tissue injury (Sidell et al., 1997). In 2002, Naghii reviewed much of the existing literature connecting SM toxicity and oxidative stress. The work of Vijayaraghavan et al., 1991, found that dermally applied SM induces hepatic lipid peroxidation and GSH depletion in mice. A high concentration of lipid peroxides is toxic to cells (Papiermeister et al., 1991; Naghii, 2002; Elsayed and Omaye, 2004). For biochemical, physiological, and anatomical reasons, the brain is especially vulnerable to oxygen mediated injury (Evans, 1999). SM metabolites form conjugates with glutathione (GSH) by the beta-lyase pathway, and can be found in human urine as specific and useful biomarkers of SM exposure (Black and Read, 1995; Boyer et al., 2004). GSH is a key intracellular antioxidant and its depletion by SM would be expected to increase

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oxidative stress. The proposed causative role of GSH depletion in apoptosis (Ghibelli et al., 1998) comes from data showing that the release of mature cytochrome *c* from mitochondria is a cellular response to GSH depletion, independent of the destiny of the cells, i.e. apoptosis or survival (Ghibelli et al., 1999). Once released from mitochondria, cytochrome *c* triggers activation of downstream caspases, the molecular executors of the apoptotic signaling cascade (Zou et al., 1999). Activation of the apoptotic mitochondrial pathway requires membrane permeabilization, which can be achieved by the opening of the permeability transition pore complex (PTPC), a large proteinaceous complex, regulated by members of the Bcl-2 family (Green and Kroemer, 2004).

SM becomes biologically active after their intramolecular cyclization into sulfonium cations. These cations were demonstrated to interfere with functioning of the cholinergic system and have been found to inhibit the activity of acetylcholinesterase (Vojvodic et al., 1985). Any alteration in the activity of AChE may alter the concentration of acetylcholine in the synaptic cleft. It is well known that the alteration of acetylcholine from its optimal concentration in synapses cause dysfunction of nerve transmission, which is highly related to behavioral activity.

Till now, molecular mechanism of SM toxicity has been studied only in few organs like eyes, skin and the respiratory tract while literature is almost silent about its toxic effects in the brain. So the present work was designed to study the chronic effects of different low doses of SM for a long duration on motor skills, memory and learning ability of male Swiss Albino mice and to unravel the molecular mechanism of SM-induced neuronal degeneration in terms of mitochondrial oxidative stress and its role in inducing apoptotic neuronal cell death.

Materials and methods

SM was synthesized at Defence Research and Development Establishment (DRDE), Gwalior, India. Antibodies were obtained from Santa Cruz Biotech. USA. All chemicals used in this study were obtained from Sigma, USA.

Animals and their care. Male Swiss Albino mice in the weight range of 20–25 g were procured from the institute animal house (DRDE) Gwalior, India. The animals were housed in the polypropylene cages, kept in well-ventilated rooms on a bedding of dried husk that was changed weekly, and allowed free access to food and water. A day before percutaneous administration of SM, hair on the back of the animals was closely clipped using a razor. All animal experiments were carried out with the approval of Institutional Animal Ethical Committee.

Experimental design. LD_{50} by percutaneous route in mice was 8.1 mg/kg.

The animals were divided into four groups of 5 animals each:

Control — equal volume of PEG applied cutaneously.
Low dose — 1/80 LD_{50} (0.10125 mg/kg percutaneous).
Medium dose — 1/40 LD_{50} (0.2025 mg/kg percutaneous).
High dose — 1/20 LD_{50} (0.405 mg/kg percutaneous).

Neurobehavioral studies

Motor function test. Rota rod test was carried out to evaluate the muscle strength and coordination movement of the animals (Dunham and Meya, 1957). As a part of the test procedure, the animals were initially trained to maintain themselves on the rotating rod at 15 rpm for a period of more than 3 min. Subsequently after a period of 24 h, the animals were again screened for their ability to remain on the rotating rod for 3 successive trials of 3 min each.

Memory function tests

Passive avoidance test. The experiment was performed by the method of Piala et al. (1959) with slight modifications. In brief, a shuttle box apparatus, consisting of a dark and an illuminated chamber separated by a door was used in the study. The floor of dark chamber consisted of a metal grid wired to deliver shocks of controlled intensities and durations whereas, the floor of the illuminated chamber was properly insulated. On the first day, each animal was placed into the illuminated compartment and allowed to explore both chambers of the apparatus for 5 min. On the second day, each animal was placed into the illuminated chamber of the apparatus. As soon as the mice entered the dark chamber, 0.1 mA, 40 V was applied till the animal crossed the door and came back into illuminated chamber. After the shock, animal was removed and returned to their home cage. On day 3, each animal was placed into the illuminated chamber, and the latency to enter into the dark chamber was measured, which served as measure of retention of avoidance response.

Morris Water Maze test. This test was carried out by the method of Morris (1984) with slight modifications. The animal is trained to escape from water by swimming to a hidden platform. It can find the platform, which is under the water and serves as a 'rescue' from the stress situation, by using visual extra-maze cues. In the navigation tank, the place of the platform is the same on each day but the starting point of the animal varies. This method requires a long-term spatial memory and learning.

Acquisition test. A water tank of 600 mm diameter was filled with water up to 8 cm from the top of the tank. A platform with an 8×8 cm top surface was placed in the middle of one quadrant about 24 cm from the side. The top surface of the platform was submerged about 1 cm below the surface of the water. All animals were given four training trials (acquisition) on days 1 to 4. On each training trial the animal was placed into the water with its nose facing the side of the tank at one of four randomly selected locations corresponding to each quadrant of the maze, and then it was released. On 5th day the number of entries and time spent in the quadrant containing the hidden platform was recorded.

Retrieval test. On the 6th day the platform was removed, and each animal was placed in the center of the water tank and allowed to swim for 90 s. The time spent in the target area (where the platform had been positioned on days 1 to 5) was recorded. The 5th day acquisition test is considered a measure of spatial reference memory, and the retrieval test is considered a measure of the strength of spatial memory.

Biochemical markers of oxidative stress

Isolation of mitochondria. Mice brain mitochondria were isolated using a recently described method of Brown et al. (2004). The dissected brains were placed in glass dounce homogenizer containing five volumes of isolation buffer with 1 mM EGTA, 215 mM mannitol, 75 mM sucrose, 0.1% BSA, and 20 mM HEPES and pH adjusted to 7.2 with KOH. The tissue homogenate was spun at 1300 $\times g$ for 5 min in an Eppendorf microcentrifuge at 4 °C. The resulting pellet was re-suspended in 0.5 ml of isolation buffer and spun again at 13,000 $\times g$ for 10 min in order to pellet the mitochondria. The pellet was washed in the appropriate buffer and containing EGTA and centrifuged at 10,000 $\times g$ for 10 min and re-suspended in the same buffer at a concentration of 10 mg/ml. The integrity of the mitochondria was checked by assessing respiratory control ratio (data not shown).

Lipid peroxidation. Lipid peroxidation was assayed by the method of Wills (1966). A mitochondrial sample of 0.5 ml was diluted to 1.0 ml