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Induction of oxidative stress by non-lethal dose of mercury in rat liver: Possible relationships between apoptosis and necrosis

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Abstract: *Sprague Dawley strain of male rats weighing 200 ± 10.0 g, were exposed intramuscularly to non-lethal dose of mercury for short acute duration of 24 and 48 hr. Mercury treatment increased thio-barbituric acid reactive substance (TBARS) and conjugated diene (CD) content with increase in duration when compared with control. This reflects possible increase in lipid peroxidation, revealing that sufficient intoxication was generated by non-lethal dose of mercury. Furthermore, mercury treatment decreased tissue glutathione (GSH) content to 2.07 and 1.49 µg GSH mg protein^{-1} with concomitant decrease in glutathione-S-transferase (GST) activity by 26.06 and 36.40% after 24 and 48hr of exposure respectively. The elevations of aspartate transaminase (AST) and alanine transaminase (ALT) levels measured exhibited increase of 287.5 and 214.5% after 48 hr of exposure respectively which were found to be highly significant compared with control. Western blot analysis indicated upregulation of caspase-9 and upsurge in effector caspase-3 activity leading to apoptosis. The concluded findings of the present investigation suggests possible role of early mercury exposure in inducing oxidative stress mediated apoptosis in mammalian model systems as an indicator component of environmental toxicology.*

Keywords: *Mercury, Oxidative stress, Lipid peroxidation, Glutathione, Apoptosis*
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Introduction

Toxic metals, as a class of agents are concern of the highest priority. Metals have vast array of remarkably adverse effects, including those of neurotoxicity, immunotoxicity and carcinogenicity (Loganathan *et al*., 2006; Soni *et al.,* 2008; Yoon *et al.,* 2008; Cavusoglu *et al.,* 2009). Metals are also non-biodegradable and persist in the environment. Anthropogenic use has led to global dispersion of metals in the environment. Defining the mechanisms of metal toxicity has been problematic because of the intricate nature of interactions of metals with living systems.

In the environment, mercury can exist in the elemental form, as inorganic monovalent and divalent salts, such as organomercurials like methyl mercury. Mercury interferes with a large number of cellular processes including the formation of complexes with free thiols and protein thiol groups that can lead to oxidative stress (Stacey and Kappus, 1982). But there is scarcity of data describing its effects on isolated organs or whole animals. Similarly, clinical studies have also not reported a hepatotoxic action for this metal and the liver is therefore not considered as a target organ for its toxicity.

The present study was designed to address the following objectives pertaining to mercury-induced toxicity:

i) Involvement of oxidative stress and antioxidant defense system in a mammalian model during early days of mercury exposure.
ii) Specific cause of induction of oxidative stress by mercury in liver of rats.
iii) Possible relationships or links with apoptotic/necrotic cellular signaling pathways.

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Materials and Methods

Sprague Dawley strain of male rats (200±10.0 g) was used for the study and 48 hr 1/10 LD_{50} (1.2 mg ml^{-1}) of mercuric chloride was selected as the experimental dose. Liver perfusion was done using Phosphate buffered saline (PBS) and necessary aliquots were maintained for enzyme assays, liver function tests and apoptotic analysis.

Assay of lipid peroxidation: Microsomes were isolated from tissue homogenates by 8mM $CaCl_2$ precipitation followed by differential centrifugation (Schenkman and Cinti, 1978). Thiobarbituric acid reactive substance (TBARS) and Conjugated diene (CD) were estimated spectrophotometrically according to the method of Buege and Aust (1978).

Determination of reduced glutathione and GST: Reduced glutathione was determined according to the protocol of Sedlak and Lindsay (1968) and expressed as µg GSH mg protein^{-1}. GST assay was performed according to the protocol of Habig *et al*. (1974) and expressed as nmol DNPG produced min^{-1} mg protein^{-1}.

Liver function tests: Aspartate transminase (AST) and Alanine transaminase (ALT) were determined by the method of Reitman and Frankel (1957).

SDS-PAGE and Western blot analysis: 20% tissue homogenate of the liver was prepared in 50 mM Tris-buffer (pH 7.6), containing 0.1 mM Phenylmethylsulfonylfluoride (PMSF) and 1% Triton X-100 and ultracentrifuged at 1,00,000 g for 60 min. The cytosolic supernatants were collected very carefully and the protein content of the sample was measured following the method of Lowry *et al*. (1951). An aliquot of cytosol containing 200 µg protein were run through 10% SDS-PAGE following the method of Laemmli (1970) and blotted on a Polyvinylidenedifluoride (PVDF) membrane (Amersham Biosciences, Hong Kong) following the method of Sambrook *et al*. (1989). The membrane was then incubated with anti-caspase 3 and 9, anti-bcl-2 and anti-bax mouse monoclonal antibody (Santa Cruz Biochemicals, USA) followed by washing and incubating with rabbit anti-mouse IgG linked secondary antibody at a dilution of 1:1000 for 2 hr at room temperature. The bands were developed by incubating the membrane with commercially available 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium solution (BCIP/NBT) (Bangalore Genei, India) diluted with Tris-Hydrochloride buffer (pH 9.5) for 5 min at room temperature.

Statistical analysis: A one-way ANOVA was used to determine the variations between groups and also within each group. The significance of these variations was determined using Duncan's Multiple Range test. All statistical analysis was done using SPSS software.

Results and Discussion

Effects on tissue lipid peroxidation: TBARS content reflected a highly significant ($p<0.001$) increase in liver to 24.06 and 47.86% after 24 and 48 hr of exposure with respect to control. As observed in the hepatic malondialdehyde profile, the CD level also increased after acute exposure of 24 and 48 hr duration. The increase in CD content was found to be lower (13.37%) after 24 hr of exposure but significantly rose to 38.04% after 48 hr of exposure to mercury when compared with control (Table 1).

Thiobarbituric acid reactive substance (TBARS), which includes malondialdehyde (MDA) and other such aldehydes and CDs, are produced by lipid peroxidation and are considered as indicators of oxidative stress. A probable increase in reactive oxygen species, as indicated by an increase in lipid peroxidation revealed that sufficient intoxication was generated by non-lethal dose of mercury.

Effects on GSH-GST detoxification machinery: On treatment with mercury, the GSH content exhibited an overall decrease to 2.07 and 1.49 µg GSH mg^{-1} protein after 24 and 48 hr of exposure. This indicated a fall in level of GSH by 28.62 and 48.62% after 24 and 48 hr of exposure to non-lethal dose of mercury. Concomitantly, GST levels showed a decrease of 26.06 and 36.40% over control after acute exposure of the metal (Table 2). The significant fall noticed after 48 hr of exposure in the GSH-GST detoxification machinery suggested hepatotoxic potential of mercury. GSH depletion to about 20-30% of normal level of glutathione can impair cellular defense against the toxic actions of

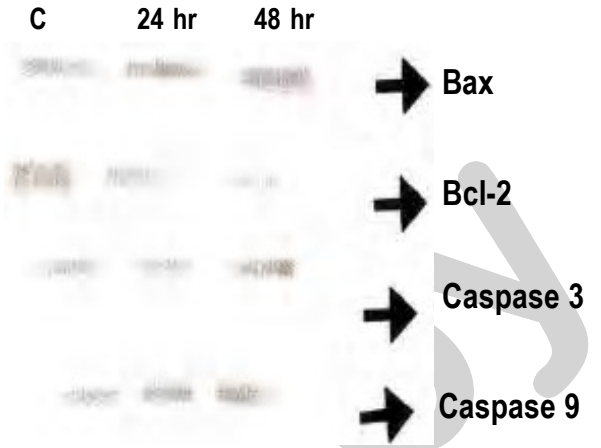

Fig. 1: Western blot analysis of pro-apoptotic Bax, anti-apoptotic Bcl-2, caspase 3 and 9 in rat liver treated with non-lethal dose (1.2 mg ml^{-1}) of mercury

Table - 1: Effect of non-lethal dose (1.2 mg ml^{-1}) of mercury on Thiobarbituric acid reactive substances (TBARS) and conjugated diene (CD) content in rat liver

Duration of exposure	TBARS (nmol MDA mg $protein^{-1}$)	CD (nmol CD mg $protein^{-1}$)
Control	7.98 ± 0.18	13.38 ± 0.59
24 hr	9.90 ± 0.26***	15.17 ± 0.96
% change	24.06	13.37
48 hr	11.80 ± 0.28***	18.47 ± 1.61*
% change	47.86	38.04

Each value represents mean ± SEM. *$p < 0.05$ and ***$p < 0.001$ indicate significant differences from control

Table - 2: Effect of non-lethal dose (1.2 mg ml^{-1}) of mercury on reduced glutathione (GSH) and glutathione-s-transferase (GST) levels in rat liver

Duration of exposure	GSH (µg GSH mg $protein^{-1}$)	GST (nmol DNPG min^{-1} mg $protein^{-1}$)
Control	2.90 ± 0.35	11.51 ± 0.67
24 hr	2.07 ± 0.23	8.51 ± 1.24
% change	-28.62	-26.06
48 hr	1.49 ± 0.16**	7.32 ± 0.72*
% change	-48.62	-36.40

Each value represents mean ± SEM. *$p < 0.05$ and **$p < 0.01$ indicate significant differences from control

Table-3 Effect of non-lethal dose (1.2 mg ml^{-1}) of mercury on Aspartate transaminase (AST) and Alanine transaminase (ALT) levels in rat liver

Duration of exposure	(ALT) (IU/L)	(AST) (IU/L)
Control	4.00 ± 0.57	12.00 ± 0.57
24 hr	9.50 ± 0.28**	41.66 ± 2.02***
% change	137.5	247.16
48 hr	12.58 ± 1.59**	46.50 ± 0.86***
% change	214.5	287.5

Each value represents mean ± SEM. **$p < 0.01$ and ***$p < 0.001$ indicate significant differences from control

both biological reactive intermediates and reactive oxygen species and may lead to cell injury and death (Reed, 1990). In this context the present study summarizes that acute exposure to non-lethal dose of mercury substantiates and significantly hampers the liver function through reactive oxygen species mediated oxidative damage. Also it contradicts earlier reports that chronic or acute exposure to high doses of metals generally leads to depletion of GSH (Maiti and Chatterjee, 2001). The depletion of GSH has been found to promote oxidative stress in various model systems, whereas Ramos *et al*. (1995) and Yeh *et al*. (2002) have not observed such an inverse relationship.

Effects on ALT and AST levels: A highly significant increase in ALT activity of 137.5 and 214.5% with respect to control was noticed after 24 and 48 hr of exposure to mercury. AST levels also exhibited a highly significant increase of 247.16 and 287.5% over control after 24 and 48 hr respectively (Table 3). The hepatic function tests relating to duration dependent upsurge in the serum AST and ALT levels corroborated the phenomenon of oxidative stress observed in the present study. The results are in agreement with the findings of Brahen *et al*. (1988). When there is damage to the cells of liver, the enzyme escapes into the blood and the AST enzymatic activity increases (Sastry, 1985). Increase of ALT level in blood indicates liver cell damage (Ford and Boyd, 1962).

Effects on apoptosis: Western blot analysis was performed with mouse monoclonal antibodies for pro-apoptotic bax, anti-apoptotic bcl-2, initiator caspase-9 and effector caspase-3 in control and mercury treated samples of rat liver to determine whether the amount of proteins varied with duration of exposure (Fig. 1) *i.e.* 24 and 48 hr.

Decreases in GSH and NADH levels and the production of ROS occur in many forms of apoptosis (Susin *et al*., 1998). In addition, developing brain cells exposed to arsenic undergo a loss of glutathione and an increase in cellular ROS leading to apoptosis (Chattopadhyay *et al*., 2002). Mercury is a redox inactive metal that challenge antioxidant defenses by binding to thiols in the cell and induce apoptosis.

In metal-induced apoptosis, it is thought that the mitochondria are most pertinent in mediating apoptosis putatively via metal-induced reactive oxygen species generation (Chen *et al*., 2001). Western blot findings in the present study indicated upregulation of caspase-9 and subsequent significant upsurge in effector caspase-3. The disruption of mitochondrial membrane potential causes the release of Cytochrome-c (Cyt-c) from mitochondria to cytosol. Cytochrome c binds to apoptotic activating factor (Apaf-1) in a 2:1 ratio, forming an oligomeric Apaf-1-Cyt-c complex (apoptosome) in the presence of dATP or ATP (Zou *et al*., 1999). This oligomerized complex then recruits the initiator caspase of this pathway, procaspase 9 and induces its autoactivation. Caspase-9 in turn activates downstream caspases including caspase-3. Increased activation of bax with increase in duration of exposure coupled with down-regulation of anti-apoptotic bcl-2 expression suggesting increased membrane permeability thereby promoting apoptosis.

Induction of apoptosis by diverse stimuli including growth factor withdrawal, radiation, hyperthermia, glucocorticoids and many chemotherapeutic agents is inhibited by bcl-2. Bcl-2 protects against many but not all types of physiological cell death. Bcl-2 may be of significance in protection against free radicals and oxidative stress (Hockenbury *et al*., 1993).

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