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Research Article

A Study on Prooxidative and Neurotoxic Effects of Mercury Chloride in Rats

Abhishek Jha[1], Bano Saidullah[1] and Parvesh Bubber[2]*

[1]*Discipline of Life Sciences, School of Sciences, IGNOU, New Delhi, India*

[2]*Discipline of Biochemistry, School of Sciences, IGNOU, New Delhi, India*

***Corresponding Author:** Parvesh Bubber, Discipline of Biochemistry, School of Sciences, IGNOU, New Delhi, India.

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Abstract

Mercury is a well-recognized heavy metal neurotoxin but it is believed to cause toxicity by multiple mechanisms. There is a myriad of studies on neurotoxic effects of mercury in animals and oxidative stress has been proposed as major mechanistic pathway. In the present study we examined the prooxidative and neurotoxicological effect of mercury chloride (inorganic mercury-$HgCl_2$) on the wistar rats. Studies were carried out in two groups: Group 1 (control) and Group 2 (mercury chloride 0.25 mg/kg bwt). Twelve rats with the average weight of (180 - 200) gm were divided equally in these groups. Oxidative stress markers in mercury administered rats showed a significant ($p < 0.001$) decrease as reflected by the mean levels of superoxide dismutase and glutathione-s-transferase. There was significant decrease in the enzymatic activity of glutathione peroxidase and catalase ($p < 0.0001$), and significant ($p < 0.001$) increase in the glutathione reductase levels in comparison to control. There was significant increase in the lipid peroxidation ($p < 0.05$) and significant decrease in reduced glutathione ($p < 0.001$) in mercury treated group. Histological observation of the cerebral cortex showed a normal structural architecture in control group while mercury administered group showed degenerative changes, necrosis, perivascular edema and degeneration of neurons. Our studies reinforce the serious consequences of mercury susceptibility in the brain that may lead to impaired functioning of brain in the affected organisms.

Keywords: *Mercury Chloride; Cerebral Cortex; Oxidative Stress; Neurotoxicity*

Introduction

Mercury (Hg) is a heavy metal and is the second most common cause of heavy metal toxicity. It occurs naturally in the environment and anthropogenic activities cause its release in the environment. Mercury is also used in several fields such as agriculture, industrial, medical and others and hence its usage cannot be avoided [1]. In today's world, populations are exposed to mercury especially via the pesticide in agriculture and through fluorescent bulbs, batteries widely used in homes [2,3]. Mercury causes deleterious health effects which includes renal, respiratory, immune, dermatologic, reproductive disease [4]. There is growing concern regarding the effects of mercury on body and in particular the brain and the nervous system since many heavy metals can cross the blood brain barrier and can accumulate in the brain and can cause severe damage [5].

Mercury exists in three multiple forms: metallic mercury, inorganic mercury and organic mercury and so it's different forms and different levels of exposure to human beings has led to different clinical patterns of mercury neurotoxicity. Moreover multiple mechanisms have been proposed to explain the biological toxicity of mercury. Mercury chloride ($HgCl_2$) has affinity for endogenous biomolecules linked thiol groups [6] and it attaches to SH- containing proteins, small molecular weight peptides (such as glutathione) and amino acids (such as cysteine) [7], leading to disruption of critical metabolic processes [8,9]. Therefore oxidative stress has been strongly suggested as one of the crucial mechanisms in Hg- induced pathological aspects [6,10,11]. These previous reports underline the biochemical toxic-

ity of mercury but their correlation to pathological changes is missing. In this respect investigation of the biochemical fate of mercury and related structural changes in the brain tissues assumes importance to assess its neurotoxicity. The purpose of the current studies is to investigate accumulation of $HgCl_2$ and whether its intoxication alters the activities of antioxidant enzymes in the rat brain along with related pathological changes.

Materials and Methods

Chemicals

Mercuric chloride ($HgCl_2$) and other reagents were of analytical grade from Merck India Ltd, Mumbai.

Animals

Wistar rats (180 - 220 gm) were obtained from the animal house of Indira Gandhi National Open University (IGNOU), Delhi. The care and use of laboratory were in accordance with National Accreditation Board of Testing and Calibration Laboratories (NABL). All experimental protocols were approved by the Institutional Animal Ethical Committee, IGNOU, New Delhi, India and experiments were performed according to the guidelines of the Committee for control and supervision of Experiments on Animals (CPCSEA), (Reg No- 175/GO/RE/S/14), Government of India. During the experimental study period rats were housed at constant room temperature, humidity, and light cycle (12: 12h light - dark), with free access to tap water and were fed with commercial standard chow ad libitum.

Study Groups: Rats were divided into two groups (n = 6 in each group).

Group 1 (Control): Wistar rats were fed normal pellet diet and water for 21 days.

Group 2 (Mercury treated group): Mercury in the form of $HgCl_2$ (Merck India Ltd., Mumbai) was given orally (0.25 mg/kg) for 21 days by gavage. Plasma biochemical analysis has also been carried out previously to determine the effects of mercury at this dose in wistar rats [11]. Another study on sub chronic toxicity of mercury chloride in rats, the oral LD_{50} was calculated at 75 mg/kg [12] and doses 1/20th of the calculated LD_{50} dose were used in rats. Since exposure to mercury generally occurs in low dose fashion, this dose was selected for our study.

Preparation of post mitochondrial supernatant (PMS)

Wistar rats were sacrificed by cervical dislocation. Brain tissues were removed and cleaned with ice cold saline. 10% tissue homogenate was prepared in 0.05M phosphate buffer. The tissue homogenate was centrifuged for 10 minutes at 4000 rpm and supernatant was collected. The supernatant was recentrifuged again for 20 minutes at 10000 rpm. Post mitochondrial supernatant (PMS) was collected and stored at -20°C for further biochemical analysis.

Estimation of mercury

Levels of mercury were measured in serum samples through Induced Couple-Mass Spectrometry.

Biochemical analysis

Lipid peroxidation (LPO)

Lipid peroxidation level in the brain was measured by the method of Ohkawa., *et al.* [13] as thiobarbituric acid reactive substances (TBARS). The concentration of TBARS was expressed as μmoles of malondialdehyde per mg of tissue. The absorbance was read at 532 nm.

Reduced glutathione (GSH)

GSH content in brain tissue was measured spectrophotometrically by using Elman's Reagent (DTNB) as a coloring reagent, following the method of Elman [14].

Glutathione-S-transferase (GST) (EC 2.5.1.18)

The glutathione-S-transferase activity was determined spectrophotometrically by method of Habig., *et al.* [15]. The specific activity of GST has been expressed as µ moles of GSH-CDNB conjugate formed/min/mg protein using extinction co-efficient of 9.6 $mM^{-1}cm^{-1}$.

Superoxide dismutase (SOD) (EC 1.15.1.1)

Superoxide Dismutase was assayed by the method of Dhindsa., *et al* [16]. A single unit of enzyme is defined as the quantity of superoxide dismutase required to produce 50% of auto-oxidation. The absorbance was read at 420 nm with UV-VIS spectrophotometer.

Catalase (CAT) (EC 1.11.1.6)

It was estimated in post mitochondrial supernatant in a UV-VIS spectrophotometer as described by Caliborne [17]. The specific activity of catalase has been expressed as µmoles of H_2O_2 consumed/min/mg protein. The difference in absorbance at 240 nm per unit time is a measure of catalase activity.

Glutathione peroxidase (GP_x) (EC 1.11.1.9)

It was estimated by the method of Mohanndas., *et al* [18]. GP_x activity has been expressed as µmol of NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22x10^3$ $M^{-1}cm^{-1}$. The reading was taken at 340 nm using spectrophotometer.

Glutathione reductase (GR) (EC 1.8.1.7)

GR activity was determined by the method of Carrlberg and Mannervik [19]. The GR activity has been expressed as nmol of NADPH oxidized min/mg protein using a molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$. The reading was taken at 340 nm.

Protein estimation

Protein estimation was done by Lowry's method at 660 nm.

Histopathological studies

For histopathological studies, incision was made through the skin and muscle of the skull. The skull was opened through a mid-sagittal incision and cerebral cortex region of the brain tissue was removed and post fixed for 24h in 10% formaldehyde and then rinsed and stored in PBS until sectioning. At the time of sectioning, cerebral cortex was fixed in Bouin's fixative and sections were prepared by using microtome at a thickness of 40 µm and stained with haematoxylin and eosin. Pathological changes were examined using a light microscope.

Biostatistics

The data are expressed as mean ± SEM. Student t test was used to compare the groups. The level of significance was chosen as $p < 0.05$.

Rats were exposed to mercury chloride (0.25 mg/kg bwt) by oral dose for 21 days. The mercury exposed group of rats had higher levels of mercury in their serum (Table 1) which shows that the dose of mercury was adequate for accumulation of mercury in the experimental animals.

S. NO	Group Name	Concentration (µg/l)
1.	Control	< 4.72
2.	$HgCl_2$ treated	> 10

Table 1: *Mercury estimation in serum of the experimental animals.*

Physical observations

Group 1 control rats were fully active. However, mercury chloride administered rats in group 2 shows less activity, restlessness with watery faces as compared to control and deformation was found in the hind limb of wistar rats.

Biochemical analysis

Mercury chloride treatment to rats raises lipid peroxidation (Figure 1). There was significant increase in the level of TBARS in mercury chloride exposed group ($p < 0.05$) in comparison to the control group. It indicates higher lipid peroxide levels in the same group of rats. The levels of reduced glutathione (GSH) were also found to be decreased ($p < 0.001$) in mercury chloride exposed group in comparison to the control group (Figure 2). Enzymatic activity of antioxidants was also found to be altered in mercury chloride administered group of animals. A significant decline ($p < 0.0001$) in the enzymatic activity of catalase (CAT) was observed in brains of experimental groups (Figure 3). However there was a significant increase (Figure 4) in activity of GR in ($p < 0.001$) in mercury chloride induced toxicity group. The enzymatic activity of glutathione peroxidase (GPx; Figure 5) ($p < 0.0001$) was also found to be significantly decreased in mercury chloride administered group, superoxide dismutase (Figure 6) and glutathione transferase (Figure 7) were also found to be significantly decreased ($p < 0.001$) in mercury chloride administered group of animals in comparison to the control animals.

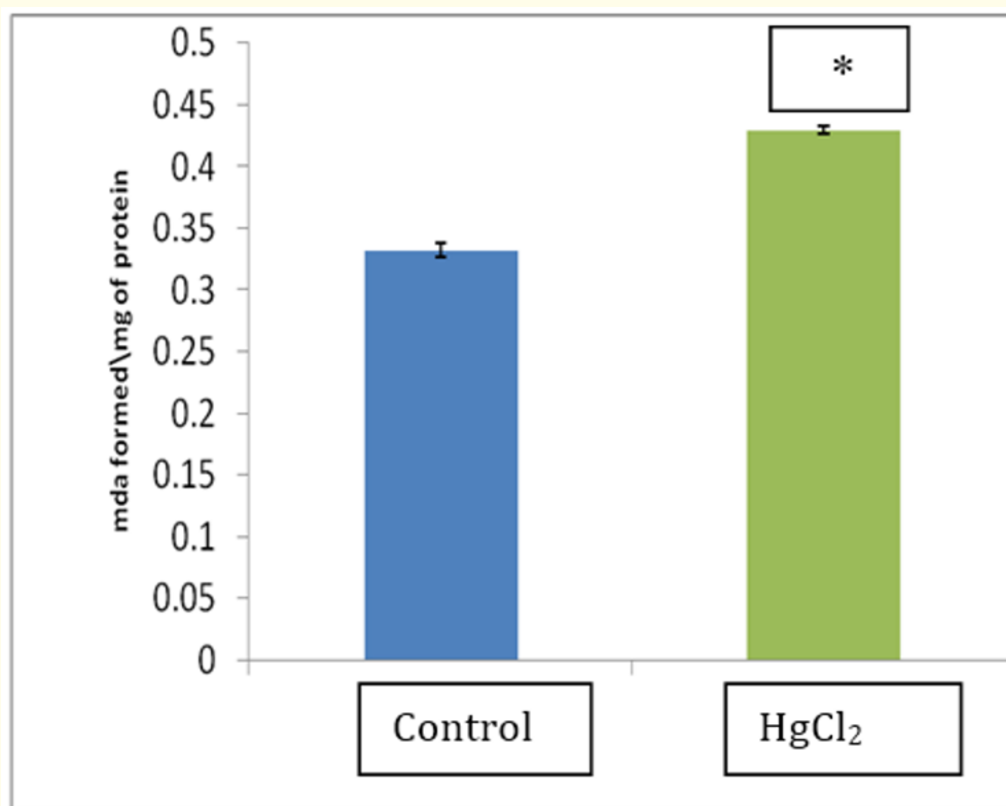


Figure 1: *Level of TBARS in brain of control and mercury administered groups.*
**P < 0.05 control vs HgCl2.*

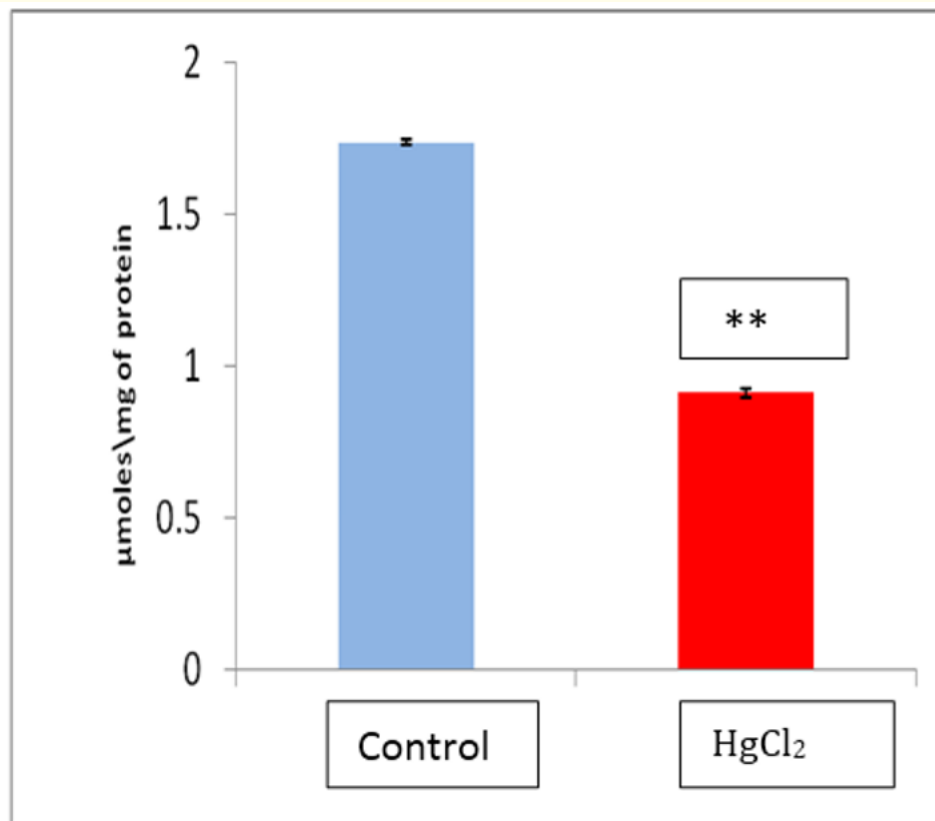


Figure 2: Reduced Glutathione levels in the control and mercury administered groups.
$**P < 0.001$ control vs HgCl2.

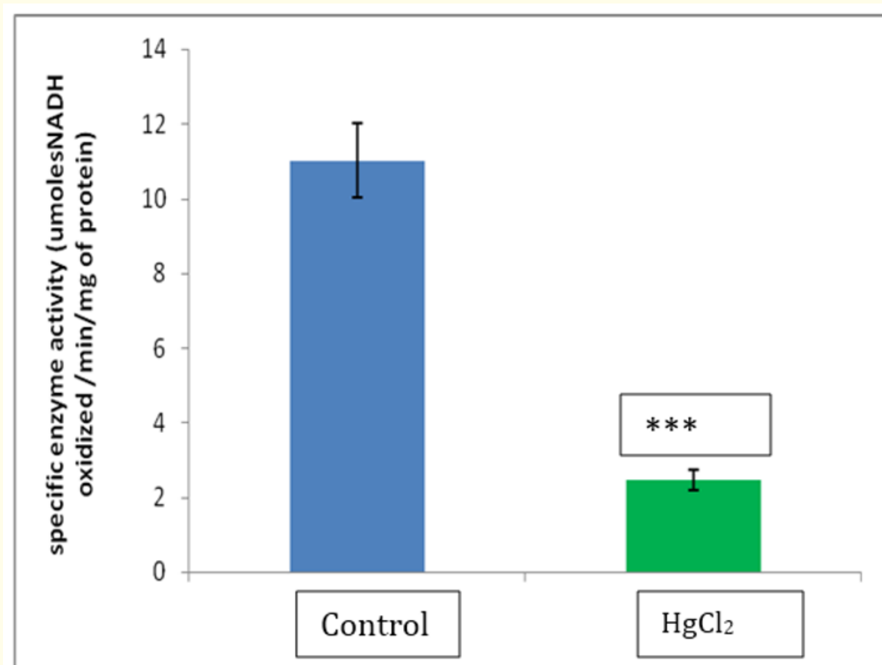


Figure 3: Glutathione Peroxidase activity in control and mercury administered group.
$***P < 0.001$ control vs HgCl2.

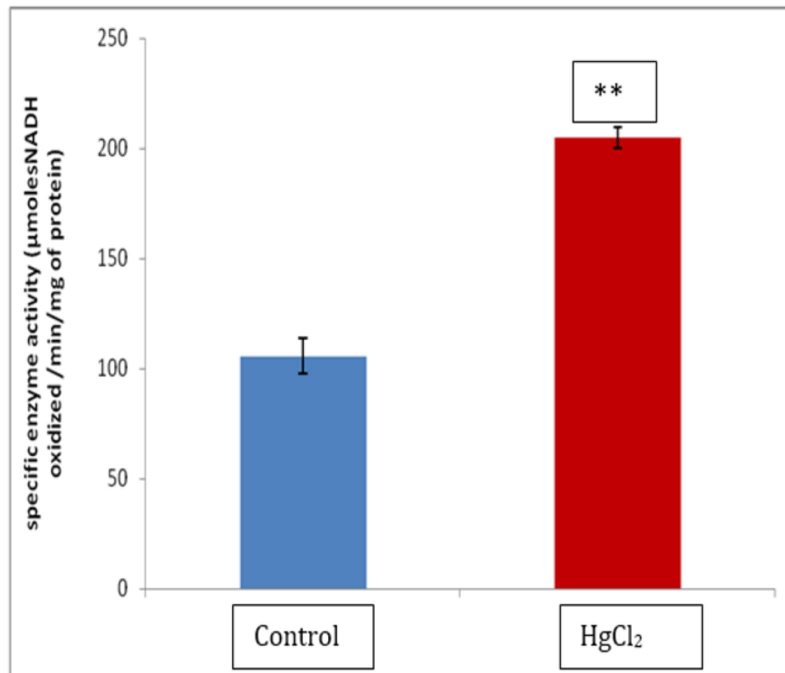


Figure 4: *Glutathione Reductase activity in brain of control and mercury administered groups*
***P < 0.001 control vs HgCl2.*

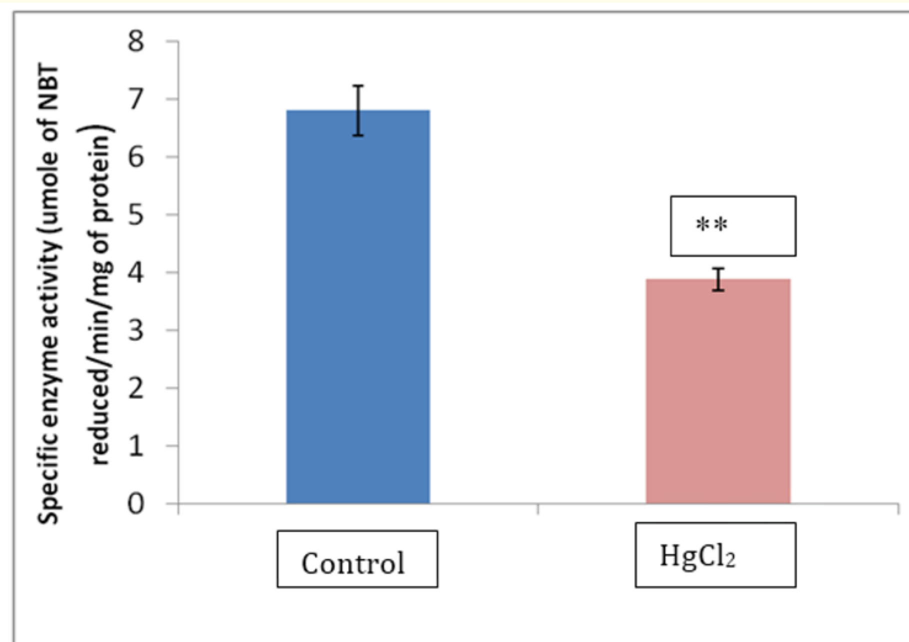


Figure 5: *SOD activity in brain of control and mercury administered groups.*
***P < 0.001 control vs HgCl2.*

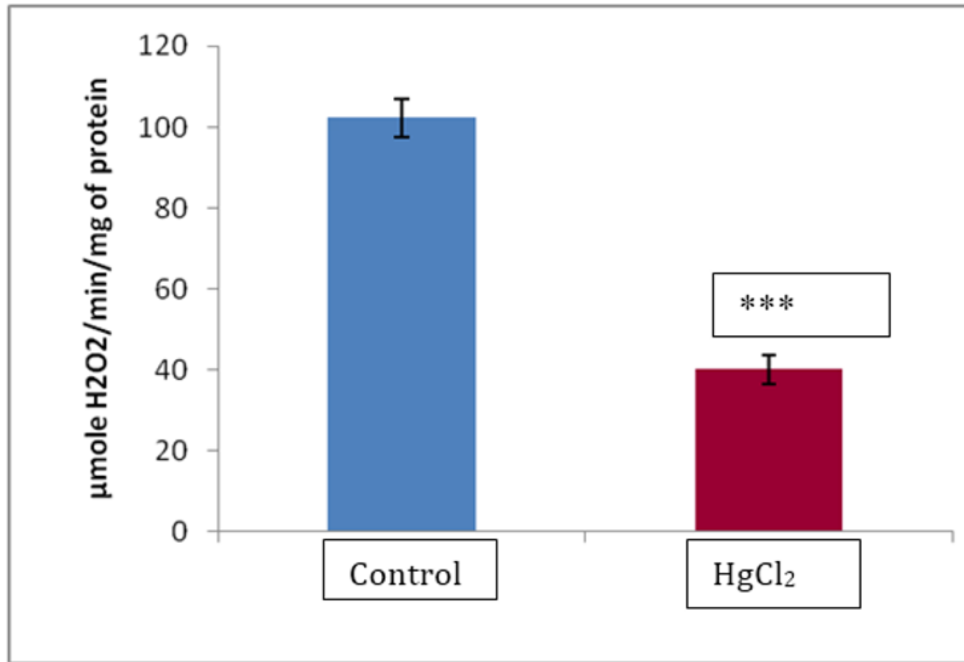


Figure 6: *Enzyme activity of Catalase in control and mercury administered groups.*
****P < 0.0001 control vs HgCl2.*

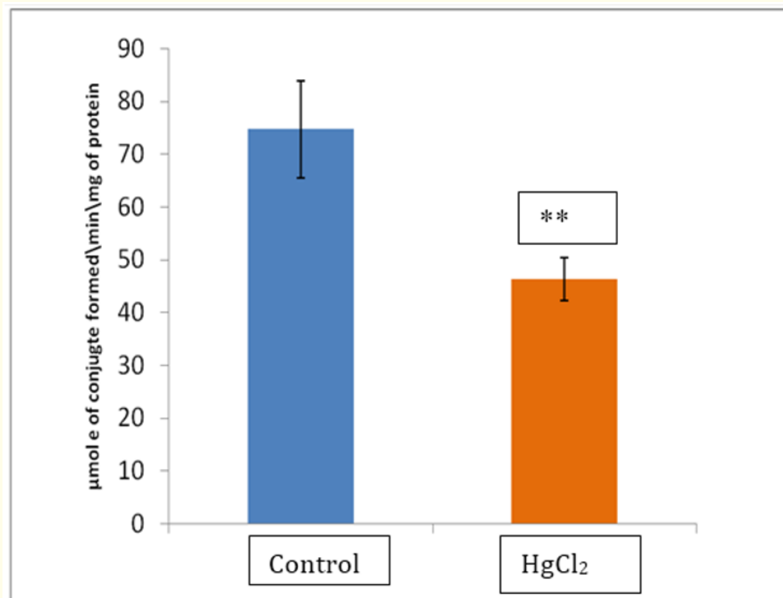
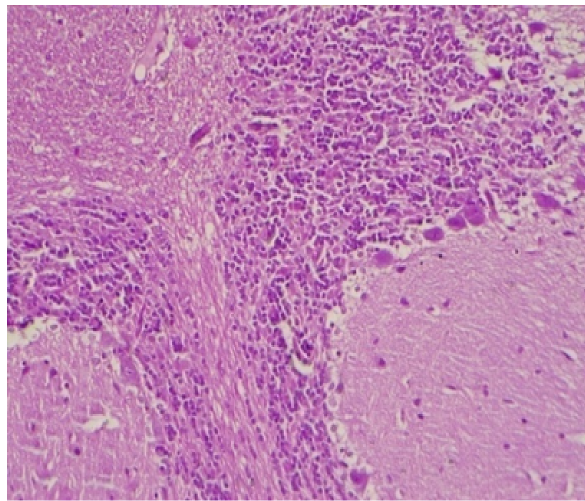


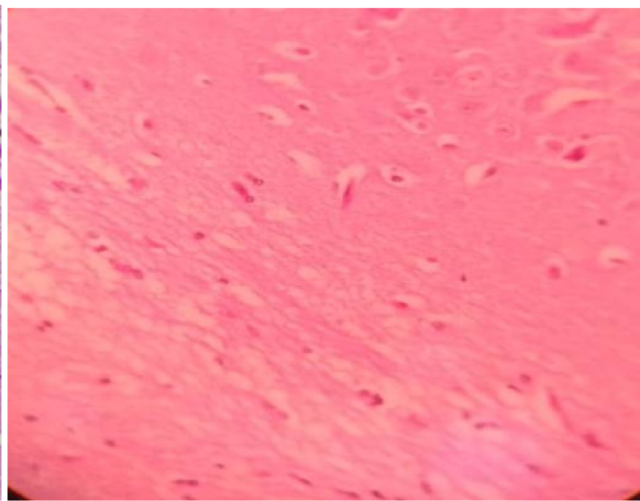
Figure 7: *GST activities in control and mercury administered group.*
***P < 0.001 control vs HgCl2.*

Histopathological observations

There was no sign of perivascular and pericellular edema or any neuron degeneration in the control group (Figure 8a-8c). It shows healthy neurons. Very mild perivascular and pericellular edema was seen in mercury treated group along with degeneration of astrocytes including ischemic neuronal injury (Figure 8d-8f).



a



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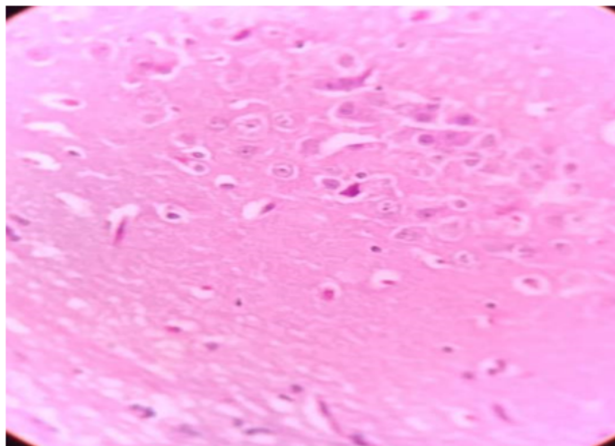

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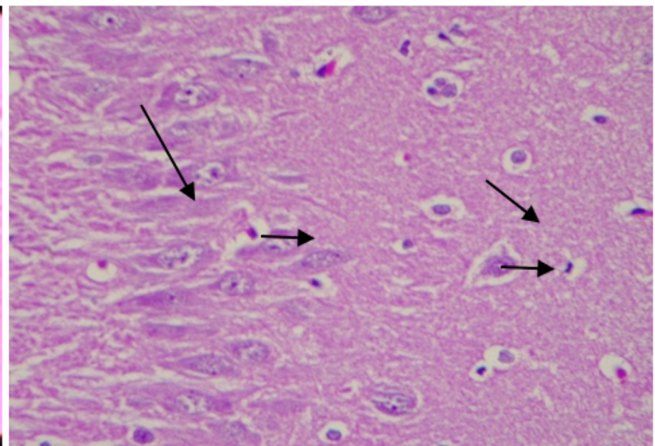

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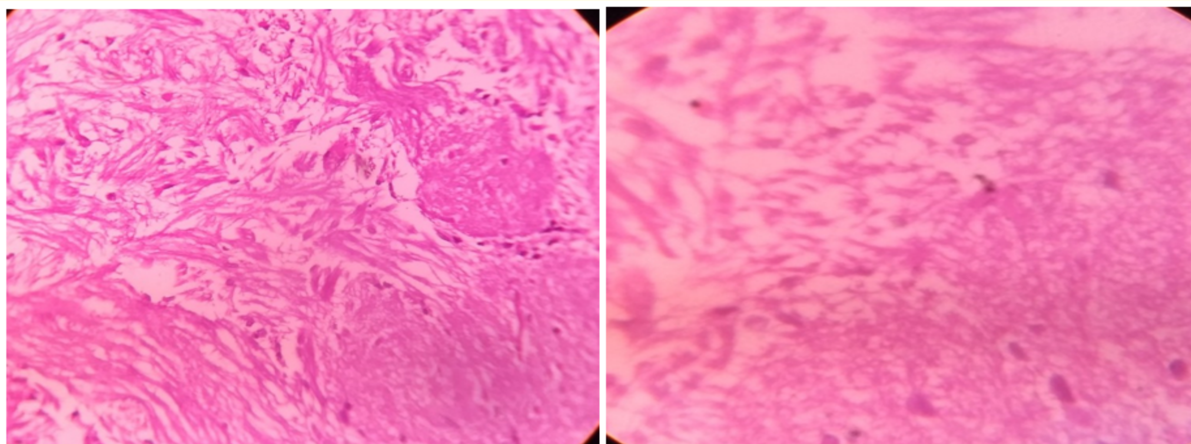

e *f*

Figure 8: *Microscopic evaluation of cerebral cortex of brain of wistar rats (a-c) control, (d-f) Mercury chloride (200x). (a-c) Section of control group showing healthy neurons (d-f) Section of mercury chloride administered group showing perivascular edema, pericellular edema, ischemic neuronal injury and neuron degeneration.*

Discussion

The serious nature of environment mercury toxicity necessitates full understanding of the underlying mechanism involved in producing its harmful effects particularly in the brain. The toxicity of mercury is determined by its chemical form. Differential toxicity patterns depend on the physical and chemical properties of mercury compounds that affect its absorption, distribution, tissue affinities and stability in the affected organism. Mercury chloride ($HgCl_2$) is an inorganic compound used in various fields and its wide occurrence as an environmental pollutant led to its absorption as well as distribution in several tissues. Low fractions of absorbed $HgCl_2$ have been shown to cross the brain-blood barrier and the placenta [12]. Farina., *et al.* [20] has reported induction of permanent abnormalities by sustained, prolonged use and exposure to a wide variety of toxins including heavy metals such as mercury and other metals. These toxins affect adversely to the different regions of brain directly or partly. Several other studies have pointed out that oxidative stress particularly in the mitochondria is a common feature of Fe, Mn and Hg toxicity [21]. Heavy metal mercury undergoes redox cycling reactions and possess the ability to produce reactive radicals such as superoxide anion and nitric oxide in biological systems [22,23]. To understand the precise mechanistic pathway and its correlation with clinical symptoms of heavy metal toxicity - induced human diseases, it is important to use *in vivo* animal models.

In the present study, mercury administration in rats causes an increase in the concentration of mercury much above control within three weeks. A similar report by Oriquat., *et al.* [19] showed much higher levels of mercury in the whole blood of mercury chloride treated rats. Same group also reported distribution of mercury in the brain within range of 4.67 - 3.68 ug/gm. Brain tissues are more susceptible to oxidative damage because of high concentration of unsaturated lipids and high rate of oxidative metabolism [24]. In fact highest MDA levels were found in the brain among the tissues examined in mercury-treated animals [25]. Mercury causes cell membrane damage by lipid peroxidation which leads to the inequality between synthesis and degradation of enzyme protein [26]. The higher levels of reactive oxygen species (ROS) production by mercury can be described by its ability to produce alteration in mitochondria by blocking the per-

meability of transition pore. The exact mechanism for ROS production by mercury is not known till now. It has been proposed that Hg^{2+} binds with thiol groups (-SH) and therefore depletes intracellular thiol, especially glutathione thereby causing cellular oxidative stress or predisposing cells to it [27] leading to the formation of free radicals which may further increase lipid peroxidation. Molecular damage of the cells in mercury toxicity is by the formation of peroxyl radicals which can also be formed in lipid and non-lipid systems such as proteins [28].

Glutathione has been shown to be a significant factor in heavy metal mobilization and excretion, specifically with application to mercury, cadmium and arsenic. GSH is an important intracellular antioxidant that spontaneously neutralizes several electrophiles and reactive oxygen species, whereas GSH/GSSG ratio maintains the redox status of the cell. The binding of mercury to glutathione leads to successive elimination of intracellular glutathione. The levels of reduced glutathione are decreased in several types of cells on exposure to all forms of mercury [21]. In our studies, glutathione levels were also found to be significantly decreased. Glutathione-mercury complexes decrease intracellular damage by preventing mercury from inflowing into tissue cells and becoming an intracellular toxin.

Cells try to counter oxidative stress using the first line defense system such as radical - scavenging enzymes like SOD, CAT, GST and GPx which are called as antioxidant enzymes. Acute exposure of mercury causes decrease in the activity of anti-oxidative enzymes and LPO [29]. Our results also corroborate these studies, since we also found out higher levels of lipid peroxidation along with histopathological damage and alterations in SOD, GPx and CAT activities in the brain tissues. Formation of reactive oxygen species within the membrane and lipoprotein of peroxyl and alkoxyl radicals affects the brain to a large extent that leads to decrease in the activity of metabolic enzymes as well as an increase in the LPO product [30]. Catalase plays a significant role in protecting cells from oxidative stress. As an antioxidant enzyme, it plays a major role against oxidative stress by catalyzing H_2O_2, which is toxic to the cells [31]. Glutathione peroxidase (GPx) catalyzes the oxidation of GSH to GSSG, this oxidation reaction occurs at the expense of H_2O_2. Therefore it also helps in clearing the toxic compounds present in the cell e.g. hydrogen peroxide. GSH is a substrate of enzyme GST, a phase II enzyme that plays a key role in cellular detoxification of xenobiotics, electrophiles and reactive oxygen species through their conjugation to GSH. Mercury inhibits the activities of free radical quenching enzymes such as catalase, superoxide dismutase and glutathione peroxidase [32]. SOD is family of metallo enzyme, which is considered to be a stress protein which is synthesized in response to oxidative stress. Low levels of SOD might be due to SOD inhibition related to the covalent attachment of mercury ions to SOD active site reactive cysteine residues [33] or to a decreased availability of Cu and Zn as a result of their binding to metallothioneins, which are involved in the detoxification of metals such as mercury [34]. Alternatively, SOD inhibition may also be a consequence of excessive reactive oxygen species generation, which would affect enzyme structure [35]. SOD catalyzes superoxide anion radical dismutation into hydrogen peroxide. Therefore, regardless of the underlying mechanism, SOD inhibition may contribute to the enhanced oxidation observed in mercury-exposed rats. The alteration in the activity of antioxidative enzymes along with SOD results in a number of deleterious effects due to accumulation of superoxide radicals and H_2O_2. The scavenger role of antioxygenic enzymes in removing toxic electrophiles helps the cell to maintain its internal environment to a limited extent at lower concentration of mercury. An increase in the oxidative stress may be due to a decrease in the antioxidant defenses or due to an increase in the processes that produce oxidants [31].

Mercuric chloride induced toxicity in the experimentally mice was found to be associated with chronic neuropathology in brain particularly in cerebral cortex, hypothalamus and cerebellum [36,37] has also reported clumping of cerebral cortical cells, necrosis of cells in animals following administration of different doses of mercury for a period of three weeks. Similarly to these recent reports, in our studies on mercury administered wistar rats, neurodegeneration in the cerebral cortex were characterized by morphological changes such as neuronal loss and vacuolization. The neurodegenerative changes in the cerebral cortex could invariably affect learning, memory and hearing abilities associated with its functions. Moreover, neuropathological changes along with oxidative stress and increased lipid peroxidation can also cause neuronal death.

Conclusion

The mechanism of mercurial damage in CNS is not fully understood. In this study, it is demonstrated that mercuric chloride administration causes significantly increased lipid peroxidation as well as decreased levels of antioxidative enzymes along with severe disruption in the structural integrity of brain region such as cerebral cortex. This indicates that exposure to heavy metal such as mercury causes serious metabolic disturbances and deleterious effects that will ultimately lead to tissue or organ damage. The brain seems to be highly sensitive organ affected by mercury toxicity. The observed changes in the oxidative stress mechanistic pathway suggest that these detrimental changes in heavy metal induced toxicity may perhaps be ameliorated by treatment with effective antioxidants.

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Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for content and writing of the paper.

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