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RESEARCH ARTICLE

EFFECT OF CADMIUM ON BRAIN REGIONS OF MALE ALBINO RAT:PROTECTIVE ROLE OF VITAMIN-C

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ABSTRACT

Oxidative stress is defined as the imbalance between the production of free radicals and the ability of the body to detoxify their harmful effects through neutralization by antioxidants. The aim of the study was to investigate the effect of cadmium on oxidative stress enzymes and the reversal effect of vitamin-C. For this purpose rats were exposed to cadmium through subcutaneous injections and post administration of vitamin-C for a period of thirty days. Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Xanthine oxidase (XOD) enzyme activities and Thiobarbituric acid reactive substances (TBARS) were estimated in cortex, cerebellum and hippocampus of rat brain. cadmium administration led to decrease in the levels of oxidative stress enzymes and increase in the levels of TBARS. Vitamin-C an antioxidant prevented cadmium induced alterations in the antioxidant enzyme and oxidative neutralising enzymes partially to the control level.

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INTRODUCTION

Cadmium (Cd) is an environmental carcinogen, not degraded in the environment and a potent toxicant in a number of tissues of rodents. Risk of human exposure to Cd is constantly increasing through its contamination of the food chain which includes basic foods like cereals and vegetables, which means that the exposure is lifelong so it is classified as a human carcinogen (Waalkes2003; ATSDR2005). (Agency for Toxic Subtances and Disease Registry). Cd is more toxic to young and new born rats when compared to adult rats may be due to differences in the BBB (Blood Brain Barrier) integrity. It can increase BBB permeability by penetrating and accumulating in the brain of young and adult rats (Antonio et al., 2003; Méndez-Armenta and Ríos, 2007; Pari and Murugavel, 2007; Gonçalves *et al*., 2010), which leads to brain intracellular accumulation, cellular dysfunction, cerebral edema and it can disturb the degree and balance of excitation, inhibition in antioxidant levels and synaptic neurotransmission in animal brain (Minami *et al*., 2001; Méndez-Armenta and Ríos, 2007).

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Chemicals

Cd chloride and Vitamin-C were selected as test chemicals. The chemicals used in this study were obtained from Sigma, USA.

Animal exposure to Cd and Vitamin-C

The young albino rats (2months) were exposed to Cd and vitamin-C through subcutaneous injections. Rats were randomly divided in to five groups, first group served as a control, Second and third group rats were treated with low dose of Cd(2mg/kg), and high dose of Cd for a period of 4 weeks, where as fourth and fifth groups of rats were treated with both Cd and vitamin-C(50mg/kg). After the period of dosage the animals were sacrificed through cervical dislocation and the tissues were stored at -80^{0}C for the further biochemical analysis.

Biochemical Studies

Preparation of Brain Mitochondrial Fraction

Brain mitochondrial fractions were prepared following Lai and Clark, 1979. Briefly, the tissue was homogenized in 5 volumes

(w/v) of SET buffer (0.25M sucrose, 10mM Tris-HCl, and 1mM EDTA, pH 7.4). The homogenate was first centrifuge at 800 *g* for 10 min at 4°C, and then the supernatant was centrifuged at 10,000 *g* for 20 min at 4°C. Then the pellet of mitochondrial fraction was suspended in SET buffer.

Estimation of Superoxide Dismutase (SOD) activity

SOD activity was determined by using the epinephrine assay of Misra and Fridovich (1972). The reaction mixture in a final volume of 2.0ml contained 1.76ml of 0.05M carbonate buffer (pH 10.2), 0.04ml of 30mM epinephrine (freshly prepared) and 0.2ml of s the enzyme extract. 1-3mM potassium cyanide will inhibit both Cu/Zn SOD and extracellular SOD resulting only Mn SOD activity only. Changes in absorbance were recorded at 480nm, measured at 10sec intervals for 1min in a spectrophotometer. The enzyme activity was expressed as Units/mg protein.

Catalase assay

Catalase activity in the mitochondrial fraction was assayed following the method of Chance and Maehly, 1955. The reaction mixture in a final volume of 2.5ml contained 0.05M phosphate buffer (pH 7.0) and appropriate amount of enzyme protein. The reaction was initiated by the addition of 19mM hydrogen peroxide (H_2O_2). The decomposition of H_2O_2 was followed directly by measuring the decrease in absorbance at 240nm, at 10 sec intervals for 1min in a spectrophotometer. The catalase activity was expressed as n moles of H_2O_2 metabolized/mg protein/min.

Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

The level of lipid peroxidation in the tissues was measured in terms of malondialdehyde (MDA; a product of lipid peroxidation) content and determined by using the thiobarbituric acid (TBA) reagent. The reactivity of TBA is determined with minor modifications of the method adopted by Hiroshi *et al.* (1979). To 2.5ml of homogenate, 0.5ml of saline (0.9%sodium chloride), 1.0 ml of (20%w/v) trichloroacetic acid (TCA) were added. The contents were centrifuged for 20 minutes on a refrigerated centrifuge at 4000 x g. To 1.0 ml of supernatant, 0.25ml of TBA reagent was added and the contents were incubated at 95°C for 1hr.1ml of n-butanol was added to it. After thorough mixing, the contents were centrifuged for 15 minutes at 4000g in a refrigerated centrifuge. The organic layer was transferred into a clear tube and its absorbance was measured at 532nm. The rate of lipid peroxidation was expressed as µ moles of malondialdehyde formed/gm wet wt. of tissue.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity

GPX activity in the mitochondrial fraction of rat brain was assayed as described by Rotruck *et al.* (1973). The reaction mixture contained 0.2 ml of EDTA, 0.2ml of 4mM sodium azide, 0.2ml of glutathione reduced, 0.2ml of H_2O_2, 0.4ml of 0.32M Sodium pyrophosphate buffer (pH-7.0), 0.1ml of enzyme source. Then the reaction mixture was incubated at 37º C for 10 min. Then the reaction was arrested by adding of 0.5ml 10% TCA. Then centrifuged at 2000rpm for 10min. To 0.5ml of supernatant, 3.0ml of 0.3M disodium hydrogen phosphate and 1.0 ml of DTNB were added and the reaction was read at 412nm in spectrophotometer. The enzyme activity was expressed as µmole/min/mg protein.

Glutathione-S-Transferase (GST) (E.C. 2.5.11.8) activity

GST activity in the cytosol fraction of the tissues of rats was assayed by using 1-chloro-2, 4-dinitro benzene (CDNB) (at 340nm) substrate as described by Habig *et al.* (1974).The reaction mixture in a final volume of 3.0ml contained: 150mM phosphate buffer (pH 7.5), 1mM CDNB, 5mM glutathione(GSH) and an appropriate amount of enzyme protein. The reaction was initiated by the addition of GSH and incubated at 37°C. The formation of a thioether by the conjugation of CDNB to GSH was monitored at 340nm in a spectrophotometer. Thioether concentration was determined from the slopes of initial reaction rates. A molar extinction coefficient 9.6 x 10^{-3} cm^{-1} was used in the calculations. The activity was expressed as µ moles of thioether formed/mg protein/min, where one unit of enzyme activity is defined as oneµ mole of thioether formed/mg protein/min.

Glutathione Reductase (GR)

GR activity in the mitochondrial fraction of the tissues of albino rats was assayed by using EDTA (at 340nm) as described by Staal *et al.* (1969). The reaction mixture in a final volume of 3.0ml contained: 150mM phosphate buffer (pH 7.5), EDTA, NADPH and an appropriate amount of enzyme protein. The reaction was initiated by the addition of GSSH and incubated at 37°C and the reaction was read at 340 nm.

Xanthine Oxidase (XO) activity

Xanthine oxidase activity is estimated by the method given in Worthington Manual (2004). The assay mixture contains 1.9ml of phosphate buffer (pH 7.5), 1.0ml of hypoxanthine and 0.1ml of enzyme source. The reaction is initiated by the addition of enzyme source. Blank will have all the assay mixture except 1.0ml reagent grade water as a substitute to the substrate. Increase in absorbance is recorded and ΔA_{290} is determined from the linear curve. The rate is proportional to enzyme concentration within limits of 0.01 to 0.02 units per test. The activity is expressed as µmoles of urate formed/mg protein/min.

RESULTS

Catalase activity

In the present study from fig 1 it was observed that Cd exposure decreases the mitochondrial catalase activity in all the three brain regions, i.e., cerebral cortex followed by cerebellum and hippocampus in both low and high dose groups. However chelation with antioxidant i.e, Vitamin-C to Cd, reversed the inhibitory effect of Cd in all the three brain regions, i.e., cerebral cortex followed by cerebellum and hippocampus.

Mn-SOD and Cu/Zn-SOD activity

The activities of mitochondrial Mn-SOD and Cu/Zn-SOD were recorded highest in cerebral cortex followed by cerebellum and hippocampus in the control rats. A decrease in the mitochondrial Mn-SOD and Cu/Zn SOD activity was recorded highest in cereberal cortex in both low and high dose groups. However treatment with Vitamin-C to Cd, reversed the inhibitory of Cd in all the three brain regions.

Glutathione peroxidase activity

The effect of Cd on Glutathione peroxidase (GPx) activity in brain regions in both high and low dose groups is shown in Fig 3. A siginificant decrease of GPx activity was seen in both the groups but the maximum decrease was seen in high dose. However chelation with vitamin-C to Cd reversed the inhibitory effect of Cd in all the three brain regions.

Lipid peroxidation

Mitochondrial lipid peroxidation was calculated in terms of MDA levels. From the study fig 4 it was observed that Lipid peroxidation activity was increased in both low and high dose groups when compared to control. Increase in MDA levels was seen more in high dose groups. However the LPx activity was decreased in the animals supplemented with Vitamin-C along with Cd-exposure. Among all the three brain regions studied, cereberal cortex shows the maximum activity followed by cerebellum and hippocampus.

Glutathione-S-transferase activity and Glutathione reductase activity

The enzymatic activities of antioxidants such as Glutathione-S-transferase (GST) and glutathione(GR) reductase were represented in fig 5 and 5.1, A siginificant decrease was seen in GRT and a significant increase in GST in all the three brain regions when compared to the control rats. Treatment with vitamin-c in Cd intoxicated rats showed significant alterations in the activities of antioxidant enzymes when compared to the Cd treated rats.

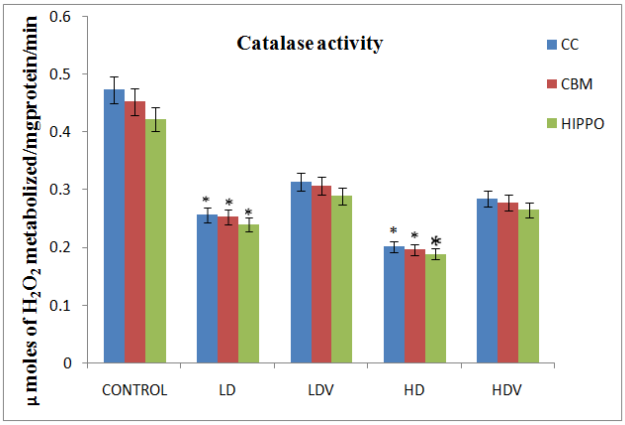

Fig. 1.

Fig 1-Effect of cadmium on catalase activity in three different brain regions Cerebral Cortex(CC), Cerebellum(CBM) and Hippocampus(Hippo) and it reversal by Vitaman-C(Vit-C). Fig 1 represents catalase activity. Control rats were treated with normal saline, remaining rats were treated with Cd at Low dose (LD,2mg/kg/bw) and Highdose (HD,5mg/kg/BW) through subcutaneous injections for a period of 3 weeks and chelated with the Vitamin-C(50mg/kg subcutaneously) for last one week. Each bar represents mean ± SD (n = 6). All values are mean values of six albino rats and values marked with (*) are significant at P<0.05-0.001

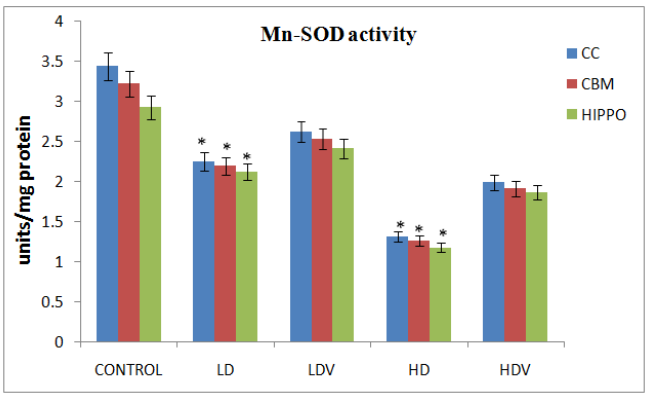

Fig. 2.

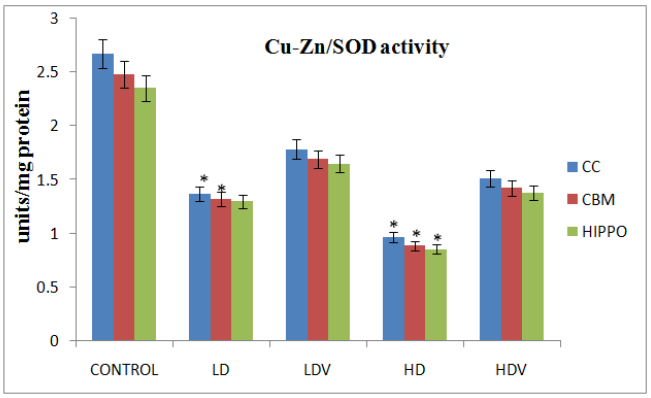

Fig. 2.1.

Fig 2 and 2.1-Effect of cadmium on SOD isoforms (Mn-SOD and Cu/ZnSOD) activities in three different brain regions Cerebral Cortex(CC), Cerebellum (CBM) and Hippocampus (Hippo) and it -reversal by Vitaman-C(Vit-C). Fig 2 and 2.1 represents Mn-SOD and Cu/Zn activity. Control rats were treated with normal saline, remaining rats were treated with Cd at Low dose(LD,2mg/kg/bw) and Highdose(HD,5mg kg/BW) through subcutaneous injections for a period of 3 weeks and chelated with the Vitamin-C(50mg/kg subcutaneously) for last one week. Each bar represents mean ± SD (n = 6). All values are mean values of six albino rats and values marked with (*) are significant at P<0.05-0.001

Xanthine oxidase activity (XOD)

The activity of XOD in brain regions of both control and experimental rats are shown in Fig 6. XOD activity was significantly increased in Cd treated rats when compared with controls. However administration of vitamin-C in Cd exposed rats significantly decreased the activities of XOD partially to the control levels when compared with Cd treated rats.

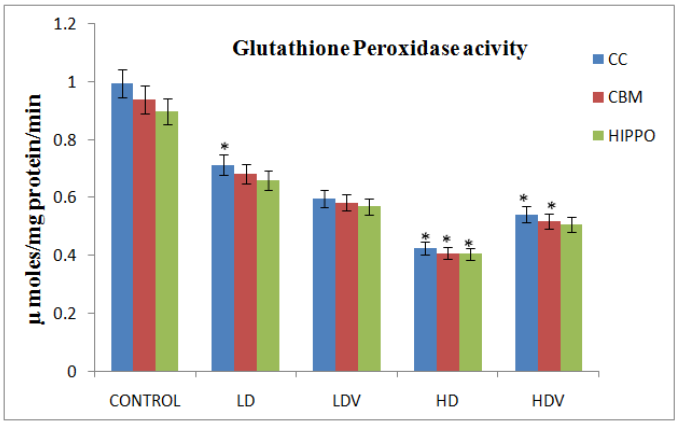

Fig. 3.

Fig 3- Effect of cadmium on Glutathione peroxidase (GPx) activity in three different brain regions Cerebral Cortex (CC), Cerebellum (CBM) and Hippocampus(Hippo) and it reversal by Vitaman-C(Vit-C).

Control rats were treated with normal saline, remaining rats were treated with Cd at Low dose (LD,2mg/kg/bw) and Highdose (HD,5mg kg/BW) through subcutaneous injections for a period of 3 weeks and chelated with the Vitamin-C(50mg/kg subcutaneously) for last one week. Each bar represents mean ± SD (n = 6). All values are mean values of six albino rats and values marked with (*) are significant at P<0.05-0.001.

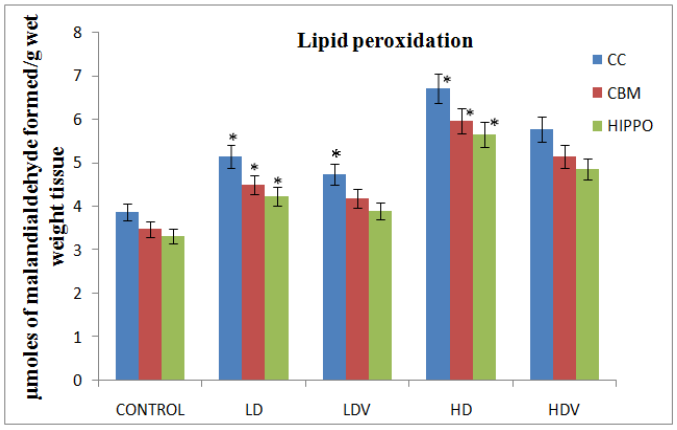

Fig. 4.

Fig 4- Effect of cadmium on lipid peroxidation (LPx) activity in three different brain regions Cerebral Cortex(CC), Cerebellum (CBM) and Hippocampus (Hippo) and it reversal by Vitaman-C (Vit-C).

Control rats were treated with normal saline, remaining rats were treated with Cd at Low dose (LD,2mg/kg/bw) and Highdose (HD,5mg kg/BW) through subcutaneous injections for a period of 3 weeks and chelated with the Vitamin-C (50mg/kg subcutaneously) for last one week. Each bar represents mean ± SD (n = 6). All values are mean values of six albino rats and values marked with (*) are significant at P<0.05-0.001.

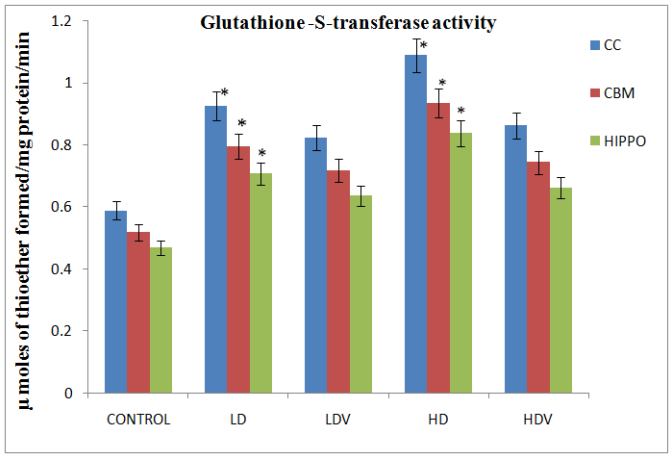

Fig. 5.

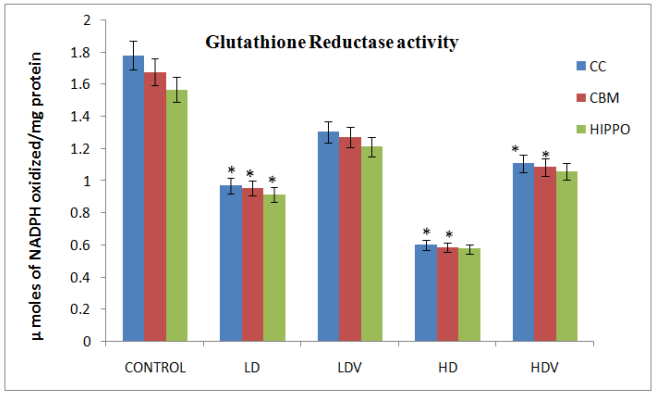

Fig. 5.1.

Fig 5 and 5.1- Effect of cadmium on Glutathione-S-transferase (GST) and Glutathione reductase (GR) activities in three different brain regions Cerebral cortex (CC), Cerebellum (CBM) and Hippocampus (Hippo) and it reversal by Vitaman-C(Vit-C). Fig. 5. and 5.1. represents GST and GRT activity. Control rats were treated with normal saline, remaining rats were treated with Cd at Low dose (LD,2mg/kg/bw) and Highdose (HD,5mg kg/BW) through subcutaneous injections for a period of 3 weeks and chelated with the Vitamin-C(50mg/kg subcutaneously) for last one week. Each bar represents mean ± SD (n = 6). All values are mean values of six albino rats and values marked with (*) are significant at P<0.05-0.001.

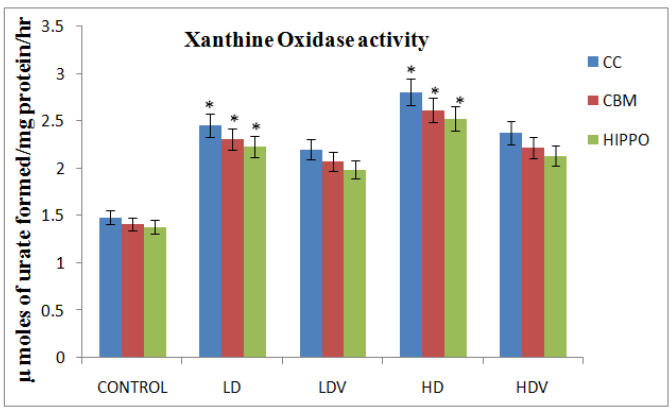

Fig. 6.

Fig.6- Effect of cadmium on Xanthine Oxidase (XOD) activity in three different brain regions Cerebral cortex(CC), Cerebellum (CBM) and Hippocampus (Hippo) and it reversal by Vitaman-C(Vit-C).Control rats were treated with normal saline, remaining rats were treated with Cd at Low dose (LD,2mg/kg/bw) and Highdose (HD,5mg kg/BW) through subcutaneous injections for a period of 3 weeks and chelated with the Vitamin-C(50mg/kg subcutaneously) for last one week. Each bar represents mean ± SD (n = 6). All values are mean values of six albino rats and values marked with (*) are significant at P<0.05-0.001.

DISCUSSION

The main objective of our present study was to examine whether treatment with Vitamin-C can reverse Cd induced oxidative stress in rat brain regions. We observed a significant positive effect of Vitamin-C on altered oxidative stress markers and antioxidant enzymes. Among all tissues brain tissue is highly vulnerable to LPO (Lipid Peroxidation) due to its high rate of oxygen utilization, a poor antioxidant protection, high content of transition metals like copper and iron in several regions and abundant supply of polyunsaturated fatty acids(Calabrese *et al*., 2000). The present study was carry out to examine Cd induced oxidative stress in rat brain, in our study we observed a significant increase in lipid peroxidation (i.e is increase in MDA (malinoldehyde) levels) in all the three brain regions(cortex, cerebellum, hippocampus). Out of all the three regions cortex shows highest amount of increase in LPO.

Possible reason for increasing LPO in three brain regions might be due to that cd induces lipid peroxidation (LPO) by stimulating the production of superoxide anions(El-Demerdash *et al*., 2004), and an increase in LPO results decrease in the antioxidant activity of SOD(super oxide dismutase). From Fig 4 we can clearly see increase in MDA levels and decrease in SOD levels in all the three brain regions.

SOD has been considered to be an important antioxidant enzyme that protects from the damage caused by superoxide radicals. Cu, Zn-SOD is a dimeric enzyme that dismutates superoxide to molecular oxygen and hydrogen peroxide. From our results we have demonstrated that Cd strongly inhibited the activities of SOD isoforms such as Cu, ZnSOD and Mn-SOD. Some studies have showed that Cd can replace Zn^{2+} to reduce SOD activity (Bauer *et al*., 1980; Kofold *et al.,* 1991). One of the main mechanism involved in cd inducing oxidative stress in brain is by generation of the reactive oxygen species(ROS) by treating the rat brain with Cd. Cd has high affinity for the sulphydril groups in enzymes and proteins and its binding can alter their correct function. Binding of Cd to the sulphydril groups of glutathione could allude in the induction of oxidative stress (Antonio *et al*., 2002).

After treating with Cd it get interact with mitochondrial sites which leads to the breakdown of the mitochondrial potentials which results in subsequent reduction of intracellular glutathione levels (Lopez *et al.,* 2006). Increase in LPO not only induces ROS but also lead to the loss of membrane bound ATPases activities and modulates the cell functions. In our present study Cd alters the antioxidant enzymes such as SOD, CAT (catalase), GPx(glutathione peroxidase). Alteration in these enzymes leads to production of free radicals which results disturbances in brain metabolism, structural integrity of lipids, membrane bound enzymes and also contributes to the neurotoxic effects, in our previous study we observed that Cd induces neuro chemical alterations and decrease in ATPase activities. Our results are also consistent with these results (Shukla *et al*., 1995). Oxidative stress not only alters the antioxidant enzymes but it also alters dopaminergic neurons (Block *et al*., 2007; Liu and Hong, 2003).

According to the studies by Bondy *et al.* brain has a high rate of oxidative metabolism consuming 20% of the cardiac output and when compared with other organs such as lung, liver and other, brain contains relatively low levels of enzymatic and non-enzymatic antioxidants and high amounts of peroxidizable unsaturated lipids, rendering it more exposed to oxidative stress when compared to other tissues. Our results have shown a significant reduction of both glutathione peroxidase (GPx) and glutathione reductase (GR) activity in the three brain regions. A profound decrease was observed in GPx and GR activities as well as the marked increase in MDA and GST levels is observed in cortex of Cd exposed rats. Cd exposure is indicative of the lowered antioxidant capacity of this brain region being possibly related to their vulnerability towards Cd.

On the contrary, SOD, CAT is significantly reduced, which possibly accounts for the marked increase in lipid hydroperoxides observed in brain region. Current studies revealed that Cd produces ROS, which results increase in MDA levels and decrese in sulphydryls, deficiency in antioxidant defences, differences in calcium homeostasis and finally leads to DNA damage. (Kumar *et al*., 1996; lopez *et al*., 2006). Our results are also consistent with these results which results increase in MDA levels, depletion in GSH, GPx SOD and CAT activities. Recent studies by Chen *et al*., (2008, 2011) elucidated that Cd induced neural toxicity is due to induction of ROS species which leads to oxidative stress. Cd induced oxidative stress is time and dose dependent manner in PC12 and SH-SY5Y cells (Chen *et al*., 2008).

These studies are parallel with our studies that decrease in antioxidant enzymes SOD isoforms, CAT, GPx and GR is more in high dose when compared to low dose and controls, which clearly indicates Cd induces oxidative stress is time and dose dependent manner. Our present findings suggest that the disturbance in oxidant-antioxidant balance after the Cd insult might play a part in rendering brain tissue more vulnerable to free radical induced injuries. Studies by Banerjee *et al*. (1999) revealed that enzymatic scavengers of oxygen free radicals (OFRs) such as SOD, CAT, GPx, GST, GR and G6PDH may protect the system from deleterious effect of OFRs. Siginificant reduction levels of SOD and CAT results in accumulation of superoxides and peroxides.

From our present study we observed a significant reduction in the activities of enzymatic antioxidants in Cd exposed cortex, cerebellum, hippocampus could be interpreted to tilt the balance between antioxidants and prooxidants which results in higher levels of ROS and an increase in MDA levels. One of the main route for transportation of Cd in to brain by

occupational is the olfactory route. It is transported beside the primary olfactory neurons to their terminations in the olfactory bulbs there by passing intact BBB (Bonider *et al*., 2008; Czarnecki *et al*., 2011). Embryos treated with Cd also showed alterations in the brain development particularly in the hind brain region (Chow *et al*., 2008). *Invivo* exposure of Cd cause damage in cerebral micro vessels and it may allied with oxidative stress. Cd exposure showed a lateral decrease in micro vessel enzymes involved in cellular redox reactions, such as SOD, CAT, GPx and a subsquent increase in LPO (Shukla *et al.,* 1996). Cd showed a siginificant increase in levels of LPO in parietal cortex, cerebellum and straitum (Mendzarmentia *et al*., 2003). Patients with Parkinson's disease and neuropsychiatric disorder also showed alterations in the enzyme activities of SOD, CAT and enzymes which involved in free radical damage (Ravikumar *et al*., 2000).

Ingestion of Cd results in exploitation of glutathione (GSH) and protein binding hydroxide, and superoxide anions (Valko *et al*., 2005). Increase in oxidative stress in a biological system may be due to increase in LPO levels in Cd induced rats which may lead to alterations in the antioxidant defence system (Jemai *et al*., 2007; Newairy *et al*., 2007). Antioxidants can neutralize increased oxidative stress induced by Cd(El-Missiry *et al.,* 2000). Vitamin-C is a water-soluble vitamin required by the body to maintain normal metabolic activities, and is synthesized in the body to meet al l physiological and biological requirements (Bardakioúlu *et al*., 2005).

Recent studies revealed that administration of antioxidants such as Vitamin–C can partially prevented the inhibition of oxidative stress marker enzymes. Studies of Ajuwon and Idowu, 2010 treating broilers with Vitamin–C decreased the MDA levels either partially or to the control levels. Studies by Stefanello *et al*., (2011) also showed that the administration of Vitamin-C decreases the Cd induced toxicity. In conclusion findings of our present study submit that the brain regions such as cortex, cerebellum, hippocampus revealed a variable depletion in antioxidant enzyme activities and oxidative stress marker enzymes on exposure to Cd indicating that early exposure of Cd may lead to peroxidative reactions in membrane lipids of the brain which leads to increase in MDA levels and decrease in oxidative neutralising enzymes. Vitamin-C may develop as a useful antioxidant against Cd induced toxicity.

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