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Developmental Neurotoxicity of Cadmium on Cholinergic and Bioenergetic Systems - Protective Role of Vitamin-C

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ABSTRACT

Cadmium (Cd) is a potent neurotoxic metal and an environmental toxin, which induces membrane disturbances in nervous system and leads to neurological impairment. Enzymatic activities of Acetylcholine (ACh), Acetylcholine esterase (AChE), Na^+/K^+-ATPase and Mg^{2+}-ATPase in synaptosomal fractions of cerebral cortex, cerebellum and hippocampus of rat brain were studied. Subcutaneous administration of Cd (2mg/kgbw and 5mg/kgbw) to rats daily for a period of 30 days were found to decrease the activity of AChE,Na^+/K^+-ATPase,$Mg2^+$-ATPase and increase was seen in ACh activity. The aim of the study was to examine the ability of Vitamin –C to prevent and recover the cadmium induced neurotoxicity. Our results indicate that Cd induced alterations in these enzymes in dose dependent manner, and vitamin-C can restore the alteration of these enzymes to control levels either partially or totally in Cd exposed rats, which suggest that Vitamin–C act as a neuroprotective agent and protects the brain from toxic effects of Cd.

Keywords: Cadmium, Vitamin - C, Neurotransmitters, Neurotoxicity, ATPases

I. INTRODUCTION

Environmental pollutants play a pivotal role in the manifestation of neurological disorders. Cadmium (Cd) is one of the environmental toxin and heavy metal which induces neurological consequences. Agency for toxic substances and disease registry (ASTSDR) has ranked Cd among the top seven of the 275 most hazardous substances in the environment (ASTSDR, 2001).Cd is a hazardous substance which can disrupt a number of biological systems, usually at low doses that are much lower than most toxic metals and it can cause acute and chronic intoxications by inhalation and ingestion. Main route of cadmium exposure is food and cigarette smoke in general population (1,2).

Cadmium is known as human carcinogen which can cause neurological impairment, and exerts its toxic effects on central nervous system(CNS),renal and hepatic dysfunction, osteomalacia, pancreatic activity changes and also affects protein synthesis, enzyme systems and various structures and metabolic processes, such as nucleic acids, carbohydrates energy metabolism.(3-5).Increasing evidence showed that Cd is a potent neurotoxicant and a possible etiological factor of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (6). Cd neurotoxicity can involve both peripheral and central nervous systems, symptoms such as fatigue, mental irritability, headache and hyposmia were reported in patients or workers acutely or chronically exposed to Cd (7).

Acetylcholinesterase (AChE, EC 3.1.1.7) is a cholinergic enzyme and enables hydrolysis of the neurotransmitter acetylcholine in cholinergic nerves. It also plays a vital role in ACh cycle, including the release of Ach (Acetylcholine)(8). Inhibition of AChE, resulting in over accumulation of acetylcholine and prolonged electrical activity at nerve endings, comprises a key mechanism of toxicity (9). Alterations

in the AChE activity have been demonstrated by (10) in various diseases and poisonings suggesting that AChE could be an important physiological and pathological parameter (11). Na^+,K^+-ATPase (EC 3.6.1.3) is an enzyme involved in neural excitability (12), metabolic energy production (Mata et al.1980), as well as in the uptake and release of catecholamines. Mg^{2+}-ATPase is an enzyme functioning in order to maintain high brain intracellular Mg^{2+} thus possibly controlling the rate of protein synthesis and cell growth (13).

Vitamin C is recognized as essential nutrients for all species of animals, and this vitamin has a protective role against metal induced toxicity (14). It is an excellent source of electron and thus donates electron to free radicals such as hydroxyl radical and superoxide radical and quenches their reactivity (15).Therefore, the present study was designed to evaluate the effect of vitamin C against cadmium induced neurotoxicity in rats.

II. MATERIALS AND METHODS

Procurement and maintenance of experimental animals

Young albino rats (wistar) were purchased from National Institute of Nutrition, Hyderabad and maintained in the animal house of Nirmala College of Pharmacy, Atmakur, Guntur. The animals were housed in clear plastic cages with hardwood bedding in a room maintained at $28^o \pm 2^o$ C and relative humidity 60 ± 10% with a 12 hour light/day cycle. The animals were fed in the laboratory with standard pellet diet supplied by Sri Venkateswara Traders, Bangalore and water ad libitum.

Chemicals

Cadmium (Cd) and Vitamin C (Vit.C) were selected as test chemicals. The chemicals used in this study namely acetylthiocholine iodide, Thiobarbutric acid, Glutathione oxidized, NADPH, DTNB, were obtained from Sigma, USA. The remaining chemicals obtained from Qualigens, India.

Experimental design

Rats were randomly divided in to five groups, each group consist of 6(n=6) animals.

Group I – served as a control and supplemented with saline

Group-II –low dose of Cd (2mg/kg body weight (bw))

Group III-high dose of Cd (5mg/kgbw)

Group IV- low dose of Cd (2mg/kgbw) + Vit-C (50mg/kgbw)

Group-V –high dose of Cd (5mg/kgbw) + Vit-C (50mg/kgbw)

The young albino rats (1 month) were exposed to low dose of Cd (2mg/kgbw) and high dose of Cd (5mg/kgbw) through subcutaneous injection daily for a period of 3weeks and left for a period of one week for supplementation with Vitamin-C at a dose of 50mg/kg body weight through subcutaneous injection. After the period of dosage, the animals were sacrificed through cervical dislocation and the tissues were stored at -80^0C for the further biochemical analysis. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA,1629/PO/a/12) and approved by the Institutional Animal Ethical Committee at Nirmala college of pharmacy of India(017/IAEC/NCPA 2016).

III. BIOCHEMICAL STUDIES

Preparation of Crude Synaptosomal Fraction:

Brain synaptosomes were prepared by homogenizing in 10 volumes (w/v) of 0.32 M sucrose buffer (0.32M sucrose, 10mM Tris-HCl, and 0.5mM EDTA, pH 7.4).The homogenate was first Centrifuge at 1000g for 10 min at 4°C, and then the supernatant was centrifuged at 12,000g for 20 min. The buffy layer of pelleted synaptosomes was suspended in a low K HEPES buffer (125mM NaCl, 5mM KCl, 1.2mM CaCl2,

1.2 mM Na2HPO4, 1.2 mM MgCl2, 5mM NaHCO3, 10 mM HEPES, and 10mM glucose, pH 7.4).

Estimation of Acetylcholine (ACh)

The acetylcholine content was estimated by the method of Metcalf (16) as given by Augustinson (20). The synaptosomal fractions of cortex, hippocampus and cerebellum were placed in boiling water for 5 minutes to terminate the AChE activity and also to release the bound ACh. To the synaptosomal fractions 1ml of alkaline hydroxylamine hydrochloride followed by 1ml of 50% hydrochloric acid were added. The contents were mixed thoroughly and centrifuged. To the supernatant 0.5ml 0.37M ferric chloride solution was added and the brown colour developed was read at 540nm against a reagent blank in a spectrophotometer.

Estimation of Acetylcholinesterase Activity (AChE)

AChE specific activity was determined following the method of Ellman(17). The reaction mixture contained 3.0ml of phosphate buffer (pH 8.0), 20µl of 0.075M acetylthiocholine iodide (substrate) and 100µl of 0.01M DTNB (5,5-Dithiobis-2-Nitrobenzoic acid). The reaction was initiated with the addition of 100µl of synaptosomal fraction. The contents were incubated for 30 min at room temperature and the color absorbance was measured at 412nm in spectrophotometer.The enzyme activity was expressed as µ moles of ACh hydrolyzed/mg protein/hr.

Estimation of Adenosine Triphosphatase (ATPase) activity (EC 3.6.1.3)

Na^+K^+ and Mg^{2+}ATPase activities in the tissues were estimated following the method of Tirri (18).1% homogenates of the tissues were prepared in 0.25M ice cold sucrose solution. Homogenates were divided into two parts. One part was centrifuged at 1400g and the supernatant thus obtained was used as an enzyme source for Mg^{2+}ATPase, while the other part of the homogenate was used for the estimation of the total ATPase.

Mg^{2+}ATPase

The reaction mixture for Mg^{2+}ATPase assay contained 0.5ml of tris buffer (0.13 M; pH 7.4), 0.4ml of substrate ATP, 0.5ml of Magnesium chloride (0.05M $MgCl_2$) and 0.2ml of crude homogenate/ mitochondrial fraction(enzyme source). The contents were incubated at 37°C for 15 minutes and the reaction was stopped by the addition of 10%TCA. Zero time controls were maintained by adding TCA prior to the addition of homogenate/mitochondrial fraction. The contents were centrifuged at 1000g for 15 minutes and the inorganic phosphate was estimated in the supernatant fraction following the method of Fiske and Subbarow (19).

Na^+K^+ATPase

1 %(W/V) homogenate already set apart was used for the total ATPase assay. The reaction mixture in a final volume of 2.6ml contained, 0.5ml of Tris buffer(0.13M; pH 7.4), 0.4ml of substrate ATP, 0.5ml $MgCl_2$(0.05M), 0.5ml potassium chloride(KCl, 0.05M), 0.5ml of sodium chloride(NaCl, 0.05M) and 0.2ml of crude homogenate/mitochondrial fraction(enzyme source). The contents were incubated at 37°C for 15 minutes and the reaction was arrested by the addition of 1.5ml of 10%TCA prior to the addition of homogenate. The contents were centrifuged and the inorganic phosphate was estimated in the supernatant fraction.

Na^+K^+ATPase = Total ATPase - Mg^{2+}ATPase

Estimation of inorganic phosphate

The inorganic phosphate in the supernatant fraction was estimated by the method of Fiske and Subbarow(19). To 1.0ml of the supernatant, 1.0ml of ammonium molybdate solution (2.5 gms in 100ml of $10NH_2SO_4$) was added followed by 0.4ml of ANSA(1-Amino, 2-Napthol, 4-Sulphonic Acid) (2.5mg of ANSA, 97.5ml of 15%sodium bisulphate and 2.5ml of 20%sodium sulphate) and allowed to react for 5 minutes. The blue color formed was measured at 660nm in a spectrophotometer against the reagent blank. The blank contained 2ml of TCA, 1.0ml of

ammonium molybdate and 0.4ml of ANSA. The enzyme activity was expressed as µmoles of inorganic phosphate formed/mg protein/hour.

Statistical treatment of the data

The data were presented as mean ± S.D and the differences were considered to be significant at All P<0.05-0.001 by Two way Annova. All statistical tests were performed using Statistical Package for Social Sciences.

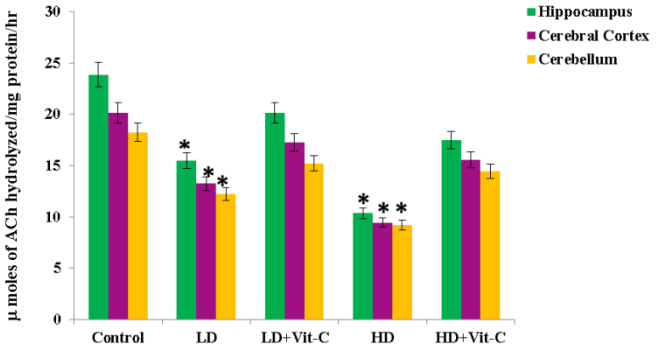

Figure 1. AChE activity

Figure 1 Effect of cadmium on AchE activity in brain regions of Hippocampus, Cerebral Cortex and Cerebellum of control and rats after exposure with cadmium at low dose(LD) (2mg) and high dose(HD) (5mg) through subcutaneous injections. The recovery of cadmium toxicity in separate group of albino rats exposed to cadmium subcutaneously at low and high dose treated with vitamin-C (50mg)(LDV and HDV). All values are mean of values of six albino rats and values marked with(*) are significant at P<0.05-0.001.

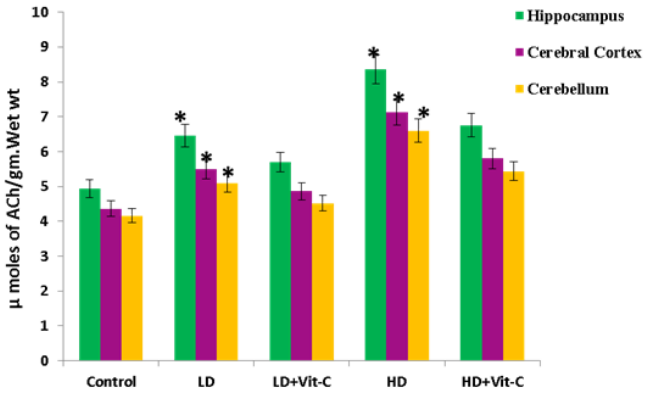

Figure 2. ACh content

Figure 2 Effect of cadmium on ACh activity in brain regions of Hippocampus, Cerebral Cortex and Cerebellum and it reversal by Vitaman-C(Vit-C) of control and rats after exposure with cadmium at low dose (2mg) and high dose (5mg) through subcutaneous injections. The recovery of cadmium toxicity in separate group of albino rats exposed to cadmium subcutaneously at low and high dose treated with vitamin-C (50mg). All values are mean of values of six albino rats and values marked with(*) are significant at P<0.05-0.001

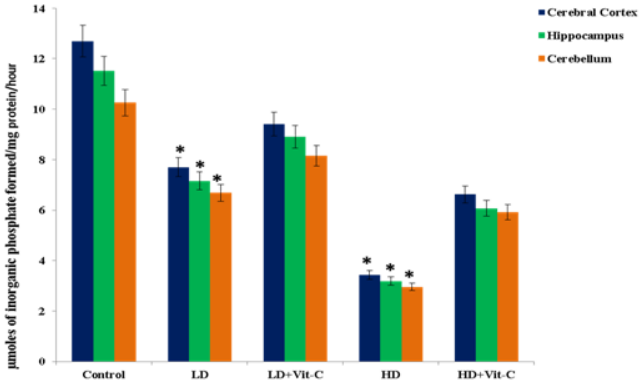

Figure 3. Na^+K^+ATPase activity

Figure 3 Effect of cadmium on Na^+K^+ATPase activity in brain regions of cerebral cortex, cerebellum, and hippocampus of control and rats after exposure with cadmium at low dose (2mg) and high dose (5mg) through subcutaneous injections. The recovery of cadmium toxicity in separate group of albino rats exposed to cadmium subcutaneously at low and high dose treated with vitamin-C (50mg). All values are mean of values of six albino rats and values marked with (*) are significant at P<0.05-0.001.

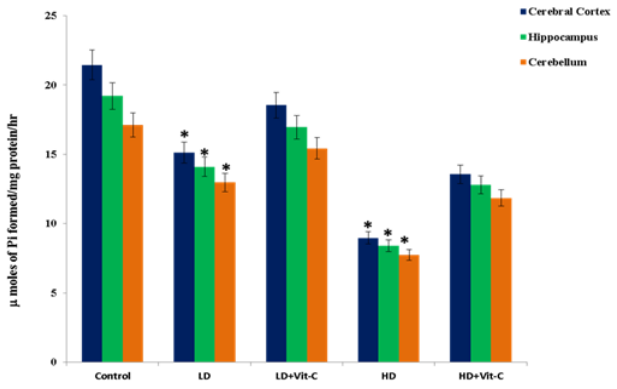

Figure 5. Mg^{2+}ATPase activity

Figure 5 Effect of cadmium on Mg^{2+}ATPase activity in brain regions of cerebral cortex, cerebellum, and hippocampus of control and rats after exposure with cadmium at low dose (2mg) and high dose (5mg) through subcutaneous injections. The recovery of cadmium toxicity in separate group of albino rats exposed to cadmium subcutaneously at low and high dose treated with vitamin-C (50mg). All values are mean of values of six albino rats and values marked with(*) are significant at P<0.05-0.001.

IV. DISCUSSION

Brain is the major cadmium siding in the body and has no known mechanism for its elimination (20). In the present study, Cd intoxication brought about remarkable changes in the AChE, ACh. Na^{+},K^{+}-ATPase and Mg2+-ATPase activity in all three brain regions such as Hippocampus(Hippo), Cerebral cortex(CC) and Cerebellum(CB).Generally brain has the highest Acetyl cholinesterase(AChE) activity than the other tissues. AChE plays a key role in terminating neurotransmission at cholinergic synapses and an important brain enzyme which is essential in detecting the neurotoxic effects induced by certain heavy metals. In our present study rats treated with Cd showed decrease in AChE activity and showed increase in ACh activity the possible reason might be due to that this enzyme terminates the signal transmission at the cholinergic synapses of neurons by rapid hydrolysis of the neurotransmitter ACh. Results of our present study indicate that Cd showed an inhibitory effect on AChE activity in a dose dependent manner. Our study also corroborates with the studies of (21, 22), in their study rats treated with Cd also showed in the reduction of AChE and increase in the levels of ACh in brain and plasma.

AChE activity is often considered as a crucial experimental biomarker for the assessment of Cd-induced neurotoxicity (23).The importance of AChE for CNS development and function is paramount, It is a very important enzyme for regulating and terminating cholinergic neurotransmission and it also involves in non-cholinergic CNS functions (24), synaptogenesis as well as in neuronal survival, neurite outgrowth and the regulation of intracellular calcium levels (25). In our study we observed impairment in the cholinergic function in Cd treated animals, alterations in ACh turnover rate and AChE activity. Our results clearly indicate that AChE activity was inhibited by both low and high Cd concentrations when compared to control. In the experiment conducted by (21), activity of AChE in brain and plasma was significantly decreased in Cd intoxicated rats which are parallel with our results. Our results are also consistent with study of (26),they suggested that possible reason might be $Cd^{2+}$is one of the metal inactivators of the enzyme where as Ca^{2+} is the activator which results in conformational change in the protein and leads to the formation of unreactive enzyme species.

The sensitivity of the brain regions to Cd was further supported by the in vivo studies conducted using different concentrations of Cd by (27), they reported that brain AChE was found to be inactivated by high Cd concentrations. In our study inhibitory effect of higher doses of Cd was found to be high in hippocampus followed by cerebral cortex and cerebellum when compared to low dose. However Vitamin-C showed partial recovery against Cd induced neurotoxicity, from the three regions

hippocampus is the most prone area for Cd toxicity our results are consistent with studies of Gonclaves et al (10) where AChE activity was decreased in hippocampus followed by cerebellum. Inhibition of AChE, results in over accumulation of acetylcholine and prolonged electrical activity at nerve endings, comprises a key mechanism of toxicity and activation of AChE leads to a fast ACh degradation and a subsequent down stimulation of ACh receptors causing undesirable effects on cognitive functions (28).

Recent studies have shown that Cd induces neurotoxicity. In fact, some studies have revealed that Cd induces neurotoxicity in animals with a wide spectrum of clinical entities such as changes in the normal brain neurochemistry as well as neurological and behavioral disturbances (29). Neurotoxic potential of Cd in experimental animals is mainly due to its impairment of the catecholominergic and serotoninergic transmission (30). El-Demerdash (31) also reported that there was a Significant decrease of AChE activity in the Cd-treated rat brains by Oral administration of $CdCl_2$ at 5mg/kg bw, for thirty days in Sprague Dawley rats. Similar to our findings, an impairment of the cholinergic function was also observed in Cd treated rats, including alterations in choline and acetylcholine levels, acetylcholine turnover rates, AChE and choline acetyltransferase activities (32).Inhibition of AChE, resulting in over accumulation of acetylcholine we can evidently say that ACh was inhibited by the coadministration of Cd with Vitamin-C efficient in order to maintain AChE into the control levels. From our previous study decrease in behavioral responses of Cd exposed rat confirms that the alterations in the open-field and water maze behavior may be due to the direct inhibitory effect of Cd on AChE in the developing brain. Thus, Cd exposure may affect AChE activity in brain areas controlling learning and cognitive behavior.

ATPase's play an imperative role in the maintenance of ionic gradient by coupling ATP hydrolysis with energy processes and itself act as a neurotransmitter, neuromodulator, and may influence the release of other neurotransmitters by acting through its own receptors or by altering the neurotransmitter receptors (33) and also associated with cardiac contractility and other cellular functions such as cell volume regulation and membrane potential (34). The temporal change of the concentration of inorganic orthophosphate, which is produced as a result of the enzyme catalyzed ATP hydrolysis, serves as a measure of enzyme activity. Enzymes such as Na^+K^+ATPases and Mg^{2+}ATPases have a relatively high sensitivity to certain classes of heavy metals and other pollutants and it has been shown that toxicosis from pollutants may develop primarily from ATPase inhibition.

Na^+,K^+-ATPase or sodium pump, is one of the enzyme particularly affected by Cd exposure. Some studies have reported that Cd can inhibit Na^+, K^+-ATPase at high concentrations (35). In addition, This enzyme is one of the major membrane proteins responsible for generating the membrane potential through the active transport of $Na^+$and K^+ ions in the CNS (36), consuming about 40–60% of ATP produced in the brain (37), and its activity is crucial for brain development and function. It is implicated in the metabolic energy production, in the uptake and release of serotonin and catecholamines as well as in the need to maintain cellular volume control and neuronal excitability (38). Thus, disturbances in this enzyme activity could have serious consequences for the CNS functioning and play a relevant role in the learning and memory mechanisms (39).

Cd can leads to alteration of the structural integrity of lipids and affects membrane-bound enzymes such as AChE and Na^+,K^+-ATPase and Mg^{2+} ATPase. Thus, alterations in the lipid membrane by oxidative stress could be a decisive factor in the modification of the conformational state of the AChE and Na^+,K^+-ATPase molecules, which would explain changes in their

activities (40). In our study Cd decreases both Na^+,K^+-ATPase and Mg^{2+} ATPase activities in both low dose and high dose treatments in dose dependent manner. When an antioxidant, Vitamin-C was Coadministrated with Cd exposure, lead to the reversal in the alterations caused by the Cd both in terms of cholinergic and bioenergetics systems and this could be due to the chelating properties of Vitamin-C assisting to the biological inactivation and excretion of Cd ions. However, the effect was highly pronounced in high dose exposed animals when compared to the low dose exposed animals. Both the activities was found to be increased in the animals supplemented with Vitamin-C along with low and high dose Cd exposed animals.

Supplementation with Vit-C reduces the gastrointestinal absorption of Cd and decreases Cd retention. In support of our present study, Vit-C can diminish alterations in Cd intoxicated rats which can be attributed to their antioxidant and metal chelating properties, which significantly reduced the oxidative threat leading to reduction of pathological changes and restoration of its normal physiological function (41, 42). In addition, ascorbic acid plays prophylactic effects on Cd-induced organ toxicity via enhancing cadmium transport and decrease its uptake in rat intestinal segments (43).

V. CONCLUSION

In conclusion, our findings suggest that Cd administration causes a significant decrease in the rat brain AChE and a significant increase in Ach content. Both effects can be, partially or totally, reversed towards control levels by Vit-C coadministration. The activity of Na^+,K^+-ATPase and Mg^{2+}-ATPase activity was significantly inhibited by Cd administration. These findings suggest that Cd may primarily interfere with neuronal excitability, metabolic energy production, as well as with cholinergic systems. It might, however, cause cholinergic dysfunction, oxidative stress and other intracellular deregulating phenomena associated with its chemical resemblance to other essential elements such as Zn, Fe and Ca. Vitamin-C plays an important role to protect vulnerable brain regions from Cd-induced neuronal injury. Since the loss of cellular K^+ is an important factor contributing to induced neuronal damage and supplementation of Vitamin C improved function of Na^+K^+-ATPase could contribute to maintenance of electrical stability of cell membrane and the consequent cell survival.

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