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EFFECT OF CADMIUM EXPOSURE ON CHOLINERGIC SYSTEM AND ENERGY METABOLISM OF RAT BRAIN: REVERSAL EFFECT OF α-TOCOPHEROL

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Keywords:

Cadmium (Cd), α-tocopherol, AChE, ACh, Mg^{2+}-ATPase and Na^{+}/K^{+}-ATPase

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ABSTRACT: Cadmium (Cd) is a toxic heavy metal in the environment. The aim of this study was to investigate the potential effect of Cd on cholinergic and bioenergetic systems in three brain regions: cerebral cortex, cerebellum and hippocampus. The young albino rats (3 months) were exposed to low dose of Cd (1.5 mg/kg body weight) and high dose of Cd (3 mg/kg body weight) through intraperitoneal injection daily for a period of 3 weeks. A separate batch of rats were given α-tocopherol at a dose for a period of one week along with low and high dose of Cd. In this study, we observed that the AChE activity and ACh content in synaptosomal fractions and Mg^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities in mitochondrial fractions. We found the decrease in AChE, Mg^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities and increase in ACh content compared to control. The high dose of Cd treated rats showed maximal decrease in the AChE, Mg^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities and ACh content compared to low dose of Cd treated rats. The maximal alterations of these enzymes observed in hippocampus among three brain regions. Whereas, the rats supplemented with α-tocopherol along with Cd showed reversal effect with gradual increase in enzyme activities and decrease in ACh content. In addition to this, we found decrease in body weights in dose dependent manner upon Cd exposure. The above findings suggest Cd toxicity was reversed with α-tocopherol co-administration which could thus be considered for future applications as a neuroprotective agent against chronic exposure to Cd.

INTRODUCTION: However, the AChE activity was increased and ACh content was decreased in the animals supplemented with α-tocopherol along with Cd- exposure. Cadmium (Cd), a widely studied environmental contaminant, has been shown to be toxic for the central nervous system (CNS) [1, 2].

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This metal ion affects the structure of nucleic acids (DNA, RNA), the activity of certain enzymes, the uptake of catecholamines [3, 4] and the levels of various neurotransmitters [5]. The potency of Cd as a neurotoxin has been demonstrated in both *in-vitro* and *in-vivo* studies [6, 7].

In fact, cadmium is shown to block synaptic transmission at peripheral cholinergic and adrenergic synapses *in-vitro* [8]. Recent reports suggest formation of a relatively stable chelator-metal complex by various chelating agents e.g. thiol reagents, dithiothreitol or L-cysteine resulting in a decrease of tissue Cd concentration. Thiol reagents seem to protect brain and kidney enzymes

(*e.g*. Na^+, K^+-ATPase) against Cd-inhibition [7, 9]. Metal-induced generation of reactive oxygen species and in particular peroxides may play a crucial role in Cd- induced carcinogenesis [10].

Acetylcholinesterase (AChE, EC 3.1.1.7) is a cholinergic enzyme, the role of which is very important in the ACh cycle, including the release of Ach [11]. In addition, it was found that AChE is co-released from the dopaminergic neurons, implying an interaction between these two molecules which is important for the dopaminergic function [12]. Na^+ /K^+-ATPase (EC 3.6.1.3) is an enzyme implicated in neural excitability [13], metabolic energy production [14], as well as in the uptake and release of catecholamines [15, 16] and serotonin [17]. Moreover, the role of Mg^{2+}-ATPase is to maintain high brain intracellular Mg^{2+}, changes of which can control rates of protein synthesis and cell growth [18].

The aim of this work was to investigate and confirm the short-term *in-vivo* effects of Cd on brain ACh, AChE, Na^+/K^+-ATPase and Mg^{2+}-ATPase activities in young rats and the effect of the antioxidant α-tocopherol on the above enzymes.

MATERIALS AND METHODS:

Chemicals: Cadmium (Cd) and α-tocopherol were selected as test chemicals. The chemicals used in this study namelyalkaline hydroxylamine hydro-chloride, acetylthiocholine iodide and DTNB (5-Dithiobis-2-Nitrobenzoic acid) were obtained from Sigma, USA. The remaining chemicals obtained from Qualigens, India.

Procurement and Maintenance of Experimental Animals: The young and adult albino rats (3 months old) (wistar) were purchased from Sri Venkateswara traders, Bangalore and maintained in the animal house of Watson Life Sciences, Tirupati. The animals were housed in transparent plastic cages with hardwood bedding in a room maintained at 28 ± 2 ^oC 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.* The protocol and animal use were approved by Y.V. University, India.

Animal Exposure to Cd and α-tocopherol: The young albino rats (3 months) were exposed to Cd (low dose: 1.5 mg/kg/body weight and high dose: 3mg/kg/body weight). A separate batch of low dose and high dose of Cd exposed rats received α-tocopherol (5 mg/kg/body weight) intraperitoneally for a week. The control animals received only deionized water without Cd. After the period of dosage, (at 4 months) the animals were weighed and body weights were noted. Later animals were sacrificed through cervical dislocation and the tissues were stored at -80 ^oC for the further biochemical analysis.

Biochemical Studies:

Preparation of Brain Mitochondrial Fraction: Brain mitochondrial fractions were prepared [19]. The tissue was homogenized in 5 volumes (w/v) of SET buffer (0.25 M sucrose, 10 mM Tris-HCl, and 1 mM 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.

Preparation of Synaptosomal Fraction: Brain synaptosomes were prepared by homogenizing in 10 volumes (w/v) of 0.32 M sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, and 0.5 mM EDTA, pH 7.4).The homogenate was first centrifuge at 1000*g* for 10 min at 4 °C, and then the supernatant was centrifuged at 12,000*g* for 20 min.

The buffy layer of pelleted synaptosomes was suspended in a low HEPES buffer (125 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM Na_2HPO_4, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES and 10 mM glucose, pH 7.4).

Estimation of Acetylcholine (ACh): The acetylcholine content was estimated [20] by the method of (Metcalf, 1951) as given by (Augustinson, 1963). The synaptosomal fractions of cortex, hippocampus and cerebellum were placed in boiling water for 5 minutes to terminate the AChE activity and to release the bound ACh. To the synaptosomal fractions 1ml of alkaline hydroxy-lamine hydrochloride followed by 1 ml of 50% hydrochloric acid were added. The contents were mixed thoroughly and centrifuged. To the supernatant 0.5 ml 0.37 M ferric chloride solution was added and the brown colour developed was read at 540 nm against a reagent blank in a spectrophotometer.

Estimation of Acetylcholinesterase Activity (AChE): AChE specific activity was determined [21]. The reaction mixture contained 3.0 ml of phosphate buffer (pH 8.0), 20 µl of 0.075 M acetylthiocholine iodide (substrate) and 100 µl of 0.01 M DTNB (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 412 nm in spectrophotometer (Hitachi, Model U-2000).

For the determination of pseudo-cholinesterase activity, the substrate butyrylthiocholine iodide was added instead of acetylthiocholine iodide and the activity of butyrylcholinesterase (BuChE) was estimated and subtracted from the total cholinesterase activity to obtain the specific activity of AChE. 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 [22]. 1% homogenates of the mitochondrial fractions were prepared in 0.25 M 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 0.5 ml of tris buffer (0.13 M; pH 7.4), 0.4 ml of substrate ATP, 0.5 ml of Magnesium chloride (0.05 M $MgCl_2$) and 0.2 ml of 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 mitochondrial fraction. The contents were centrifuged at 1000 g for 15 minutes and the inorganic phosphate was estimated in the supernatant fraction [23].

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

$$Na^+/K^+\text{-ATPase} = \text{Total ATPase} - Mg^{2+}\text{-ATPase}$$

Statistical treatment of the Data: The mean and standard deviation (SD), analysis of variance (ANOVA) was calculated using standard statistical software package.

RESULT:

Body Weights: From the results, in **Table 1**, it was observed that the low and high dose Cd exposure rats showed decrease in their body weights as compared to control. Decrease in body weights were higher in high dose exposure compared to low dose. However, partial recovery in body weights was observed in α-tocopherol supplemented rats at both high dose and low dose Cd exposure.

TABLE 1: LOW AND HIGH DOSE Cd EXPOSURE RATS

Treatment	Body weights (g)
Control	105.5 ± 35.2
Low dose	55.6 ± 23.8
Low dose + α-tocopherol	71.5 ± 32.3
High dose	42.5 ± 18.2
High dose + α-tocopherol	64.5 ± 29.3

Effect of α-tocopherol supplementation on low dose of Cd (1.5 mg/kg body weight) and high dose of Cd (3 mg/kg body weight) induced alterations in the body weight of control and experimental rats. Values are mean ± SD of 6 observations.

ACh Content: From the results, from **Fig. 1**, it was cleared that ACh content in synaptosomal fraction of brain regions of Cd-exposed (both low and high dose) rats was found to be increased when compared to control tissues. However, the effect was highly pronounced in high dose exposed animals compared to the low dose exposed animals tissues. ACh activity was found to be decreased in the animals supplemented with α-tocopherol along with low and high dose Cd-exposed animals. Among the different brain regions CC was found to be more susceptible tissue towards cadmium induced toxicity compared to the other two brain regions.

AChE Activity: From the results, in the **Fig. 2**, it was observed that the AChE activity was high in

synaptosomal fraction of control rats in the three brain regions. The AChE activity of Cd-exposed (both low and high dose) rats was found to be decreased when compared to control tissues. However, the effect was highly pronounced in high dose exposed animals compared to the low dose exposed animal tissues. AChE activity was found to be increased in the animals supplemented with α-tocopherol along with low and high dose Cd-exposed animals. Among the different brain regions cerebral cortex was found to be more susceptible tissue towards cadmium induced toxicity compared to the other two brain regions.

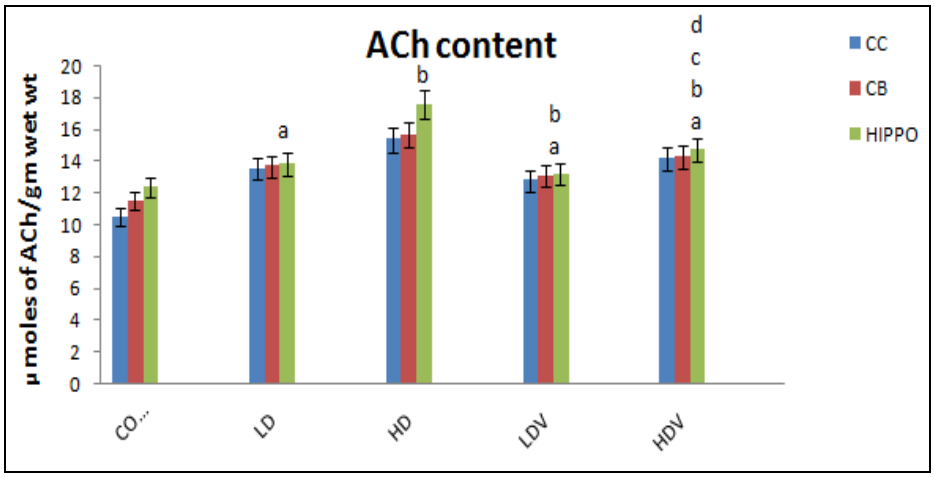

FIG. 1: ACh CONTENT ACTIVITY OF Cd - EXPOSED (BOTH LOW AND HIGH DOSE)

Protective effect of α-tocopherol against low dose Cd (1.5mg/kg body weight) and high dose Cd (3mg/kg body weight) induced alterations in ACh content in brain specific regions; CC (Cerebral Cortex), CBM (Cerebellum) and Hippo (Hippocampus). All values are extremely significant at p<0.0001, expect the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV.

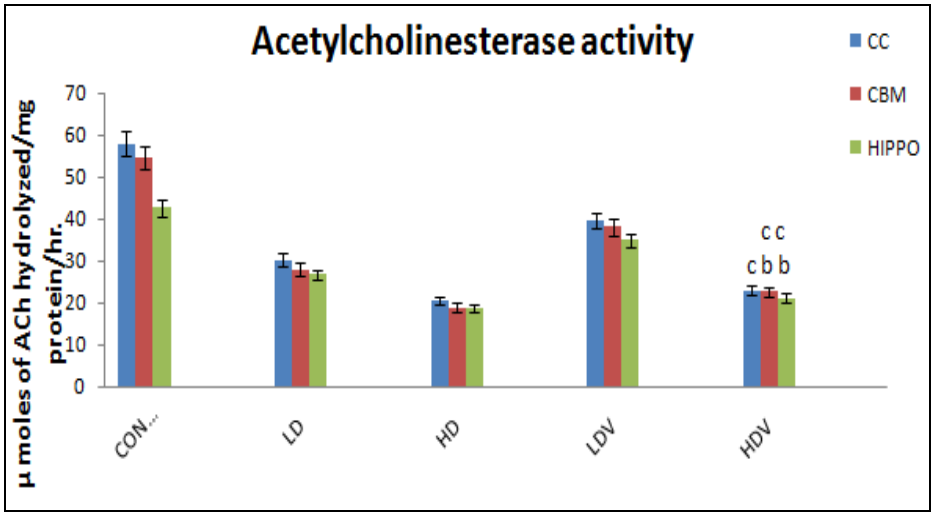

FIG. 2: THE AChE ACTIVITY OF Cd - EXPOSED (BOTH LOW AND HIGH DOSE) RATS

Protective effect of α-tocopherol against low dose Cd (1.5 mg/kg body weight) and high dose Cd (3 mg/kg body weight) induced alterations in AChE activity levels in brain specific regions; CC (Cerebral Cortex), CBM (Cerebellum) and Hippo (Hippocampus). All values are extremely significant at p<0.0001, expect the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV.

Mg^{2+}-ATPase Activity: From the results, in the **Fig. 3**, it was observed that Mg^{2+}-ATPase activity in mitochondrial fraction brain regions of Cd-exposed (both low and high dose) rats were found to be decreased when compared to control tissues. Among the three brain regions the cerebral cortex show high activity followed by cerebellum and hippocampus. The cerebral cortex was found to be more susceptible tissue towards cadmium induced toxicity compared to the other two brain regions. Mg^{2+}-ATPase activity was found to be increased in the animals supplemented with α-tocopherol along

with low and high dose Cd-exposed animals. Among the different brain regions cerebral cortex was found to be more susceptible tissue towards cadmium induced toxicity compared to the other two brain regions.

Na^+/K^+-ATPase Activity: From the results, in the **Fig. 4**, it was observed that Na^+/K^+-ATPase activity was more in all the three brain regions of control animals in mitochondrial fractions. However, the Na^+/K^+-ATPase activity in the brain regions of Cd-exposed (both low and high dose) rats was found to be decreased when compared to control tissues. The effect was highly pronounced in high dose exposed animals compared to the low dose exposed animal tissues Na^+/K^+-ATPase activity was found to be increased in the animals supplemented with α-tocopherol along with low and high dose Cd-exposed animals. Among the different brain regions cerebral cortex was found to be more susceptible tissue towards cadmium induced toxicity compared to the other two brain regions.

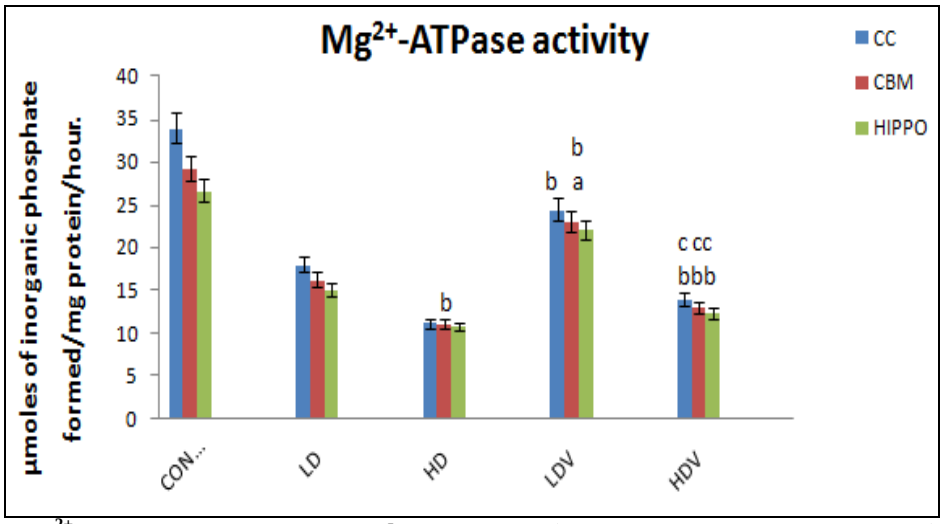

FIG. 3: Mg^{2+}-ATPase ACTIVITY OF Cd - EXPOSED (BOTH LOW AND HIGH DOSE) RATS

Protective effect of α-tocopherol against low dose Cd (1.5 mg/kg body weight) and high dose Cd (3 mg/kg body weight) induced alterations in Mg^{2+}-ATPases activity levels in brain specific regions; CC (Cerebral Cortex), CBM (Cerebellum) and Hippo (Hippocampus). All values are extremely significant at $p<0.0001$, expect the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV.

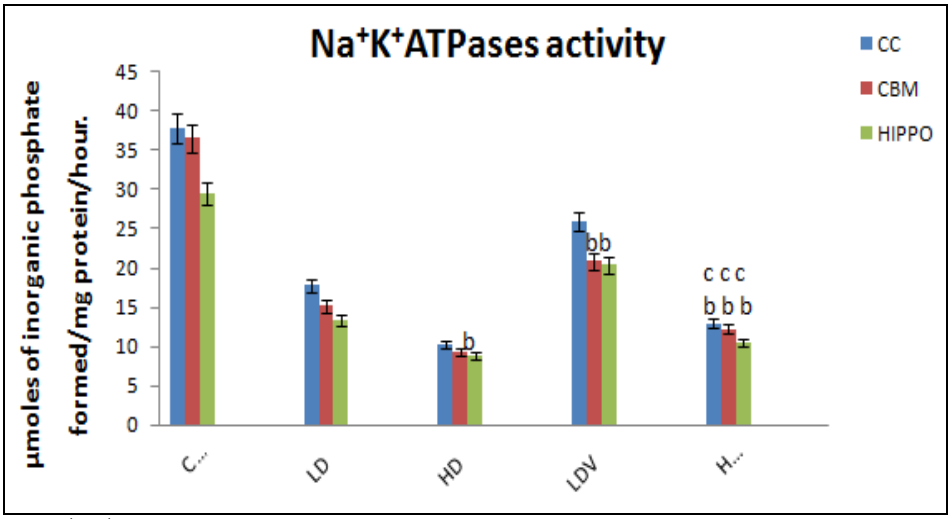

FIG. 4: Na^+/K^+-ATPase ACTIVITY CD-EXPOSED (BOTH LOW AND HIGH DOSE) RATS

Protective effect of α-tocopherol against low dose Cd (1.5 mg/kg body weight) and high dose Cd (3 mg/kg body weight) induced alterations in Na^+K^+ATPases activity levels in brain specific regions; CC (Cerebral Cortex), CBM (Cerebellum) and Hippo (Hippocampus). All values are extremely significant at $p<0.0001$, expect the values (bars) marked with a: compared with control, b: compared with LD, c: compared with HD, d: compared with LDV.

DISCUSSION: Cadmium (Cd) is known to produce a variety of health hazards in humans and experimental animals due to its ability to induce severe alterations in various organs and tissues including the nervous system, following either acute or chronic exposure [24]. In the studies [25], they investigated that the *in-vitro* and *in-vivo* (short- and long-term) cadmium effects on brain AChE, Na^+/K^+-ATPase and Mg^{2+}-ATPase activities. The overall analysis of the data revealed that Cd produced a different pattern of responses on the above enzyme activities. Pure AChE is activated by low Cd concentrations (0.01 mM), while it was inhibited by higher Cd concentrations (>0.1 mM). Brain AChE was found to be inactivated by the high Cd concentrations. Additionally, a dose-response study (1, 2, 5 mg/kg rat short-term treatment of 8 hr exposure) confirmed the AChE dose-dependent inhibition by Cd [25]. In a time-dependent Cd neurotoxicity in rat brain synaptosomal plasma membranes also observed a considerable decrease of AChE activity till 6 hr of Cd exposure (5 mg/Kg rat) followed by a progressive increase up to 24 hr [26].

However, other studies have shown that the immunoreactivity of AChE is decreased in cerebral cortex of rats exposed to Cd during lactation and post weaning, without affecting the ACh levels [27]. In this study, we have also observed that the short-term Cd exposure decreases the levels of AChE and increases the ACh content in brain regions of experimental rats compared to control rats. The results of the present study revealed that the animals exposed to Cd through intraperitoneal injections showed loss in the body weight when compared with control rats (**Table 1**). This supports the observations reported [28, 29] both As and Cd decreased body weight gain and food utilization and that these effects were more pronounced for the mixture. Available reports [30, 31, 32] also indicate that long term exposure to arsenic and cadmium could lead to significant reduction in body weights.

The weight gain has been shown to be influenced by the availability and absorption of nutrients [33]. It was also shown that cadmium decreases nutrient digestion and absorption [34, 35]. It was shown that reduction of body weight can be used as an important indicator for the deterioration of rat general health status. Cd and As inhibitory effect on digestive and absorption enzymes [36, 37] may also account for the decreased in weight of rats as observed [28]. In addition to above findings, in our results we have also find that the administration of α-tocopherol along with Cd shows its reversal effect on the above mentioned brain enzymes and body weights respectively.

Since brain AChE activity is an important regulator of the behavioral processes, our findings are indicative of Cd toxicity. As concerns calcium (Ca), it should be noted that calmodulin does not differentiate between Ca and Cd, resulting in alteration of cholinergic functioning [38]. Cadmium induced oxidative stress may play a significant role in the mechanism of down-regulation of nAChRs and as such, a marked decrease in hippocampal AChE activity, a key enzyme of cholinergic transmission in the central and peripheral nervous system, has been recorded in cadmium exposed mice. The observations of decreased AChE activity in the brain of rodents exposed to lead and fluoride provide support to our present findings on Cd induced decrease in AChE activity [39, 40, 41].

Thus, it can be presumed that, decrease in AChE activity can severely impair synaptic transmission in the brain and is in accordance with previous report on reduced activities of hippocampal AChE and BuChE on excessive intake of fluoride [41]. Simultaneous administration of α-tocopherol could successfully counteract the deleterious effects study suggests possible loss of cholinergic neurons in cadmium exposed rats. AChE was inhibited by high Cd concentrations and they found that Cd^{2+} is one of the metal in activators of the enzyme (where Ca^{2+} is one of the activators) and can induce a conformational change in the protein, which leads to the formation of an ‘‘unreactive’’ enzyme [42].The observed changes in AChE activity [25]probably indicated that Cd can affect brain cholinergic or dopaminergic mechanism(s) [12].

The activities of the ATPase enzymes are affected by the exposure of Cd indicating the alterations in membrane and neurotransmitter functions. The activities of Na^+/K^+-ATPase and Mg^{2+}-ATPase in brain and uptake of catecholamines have been found to be decreased by chronic *in vivo* Cd administration in Cd pre-incubation in rat brain synaptosomes [43].

In this study, our results showed similar trend. In our study, we have also observed that, the animals supplemented with α-tocopherol along with Cd showed an increase in cholinergic and energy metabolism enzymes respectively. An impairment of the cholinergic function was described in Pb-treated animals, including alterations in acetylcholine turnover rates [44]. The decreased activity of ATPases could also be due to the SH binding nature of Cd or through its oxidative stress in brain [45]. In addition, the changes in Na^+/K^+-ATPase activity could suggest that Cd might modulate brain neuronal excitability [13], metabolic energy production [14] as well as the uptake and release of catecholamines [15, 16] and serotonin. Furthermore, Cd may control rates of protein synthesis and cell growth by altering Mg^{2+}-ATPase activity [17, 18].

Consequently, Cd seems to have an indirect toxic effect on brain Na^+/K^+-ATPase, which is different on pure enzyme. The activities of brain ATPases were found to be decreased higher by two or three times after a single high dose administration, but they were much lower or stable (*e.g.* Mg^{2+}-ATPase) after long-term Cd administration, possibly due to a chronic adaptive mechanism induced by Cd. In contrast, the activities of brain Na^+ K^+ATPase and Mg^{2+}ATPase and uptake of catecholamines have been found to be decreased by chronic *in-vivo* Cd administration in rats and by *in-vitro* Cd preincubation in rat brain synaptosomes [43]. It was also reported that cadmium acetate (10 mg/l) administered to pregnant wistar rats from day 1 to parturition or until weaning (21 day), resulted in inhibited brain AChE and Na^+ K^+ATPase [45].

CONCLUSION: In conclusion, this study demonstrates exposure to cadmium provoked neuronal injury by inducing alteration in enzymes of energy metabolism like Na^+K^+ and Mg^{2+} ATPases and cholinergic AChE which lead to an increase in synaptosomal ACh in brain regions . In dose dependent manner, where high dose exposure showed significant alteration compared to the low dose exposure. Among the brain regions, Hippocampus used found to be more vulnerable towards Cd induced neurotoxicity. However, α-tocopherol treatment have partial ameliorative effects on these disturbances caused by cadmium toxicity by increasing AChE levels and the activities of Na^+K^+ and Mg^{2+}-ATPases. Cd-induced enzyme activity modifications (considering the importance of the above enzymes for the brain function) could change brain cholinergic mechanism(s), neural excitability and metabolic energy production. α-tocopherol could have a protective antioxidant effect on the Cd-induced oxidative stress in the brain. Taking into consideration the gradually increasing Cd concentration in our environment, this work might be of clinical importance.

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CONFLICT OF INTEREST: Nil

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