Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 01, pp.11378-11385, January, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

EFFECT OF MANGANESE EXPOSURE ON CHOLINERGIC SYSTEM OF RAT BRAIN: REVERSAL EFFECT OF ALPHA – TOCOPHEROL

[1]Bhuvaneswari Devi, C., [2]Indravathi, G. and [3,*]Kiran Kumari, K.

[1]Department of Biotechnology, SVIMS University, Tirupati, Andhra Pradesh, India
[2,3]Department of Zoology, S.P.W. Degree and PG College, Affiliated to Sri Venkateswara University, Tirupati, Andhra Pradesh, India

ARTICLE INFO

Article History:
Received 17th October, 2014
Received in revised form
14th November, 2014
Accepted 10th December, 2014
Published online 23rd January, 2015

Key words:

Acetylcholinesterase,
Acetylcholine,
Histochemistry,
Brain Regions,
Manganese,
Alpha-Tocopherol,
Eserine.

ABSTRACT

Manganese (Mn) is an essential metallo enzyme component that in high doses can exert serious oxidative and neurotoxic effects. The aim of this study was to investigate the potential effect of acetylcholinesterase (AChE) activity, ACh content and Histochemical Studies in both low dose (2.5 mg/kg bw) and high dose (5 mg/kg bw) of Manganese (Mn) treated young and adult (2 months and 4 months) rat brain with reversal effect of alpha- tocopherol. In this study, it was observed that the AChE activity and ACh content in synaptosomal fraction of low dose and high dose of Mn-exposed rats showed decrease in AChE activity and increase in ACh content at both the ages (2 months and 4 months) when compared to control. However, the AChE activity was increased and ACh content was decreased in the animals supplemented with α-tocopherol along with Mn- exposure. In this study, we have also examined the histochemical studies in adult rat brain i.e, Cerebral Cortex, Hippocampus and Cerebellum. Histochemical staining of AChE in cerebral cortex, hippocampus and cerebellum of control adult rats showed increase of AChE staining and the animals treated with Mn showed loss of AChE staining in three brain regions. However, the administration of Alpha-tocopherol along with Mn showed increase of AChE staining. Among the three brain regions studied, Hippocampus showed maximum activity followed by Cerebral Cortex, and then Cerebellum. The above findings suggest that short-term Mn *in vivo* administration causes a statistically significant decrease in AChE activity. The Mn toxicity was reversed with Alpha-tocopherol co-administration which could thus be considered for future applications as a neuroprotective agent against chronic exposure to Mn and the treatment of manganism.

Copyright © 2015 Bhuvaneswari Devi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Most trace metals influence on the central nervous system function in a complex and dose-dependent manner (International Life Sciences Institute, 1994). Manganese (Mn) is an essential metalloenzyme component (Keen *et al*., 1999) that falls within this rule and can exert serious neurotoxic effects on both human beings and experimental animals at higher concentrations (Dobson *et al*., 2004). Manganism is a serious central nervous system disease that is caused by exposure to high concentrations of Mn oxides, often diagnosed in miners or workers engaged in the ferromanganese-alloy industry and the manufacturing of dry cell batteries (Inoue, 2007). Manganism is usually accompanied by *locura manganica* (Mn madness), thus leading to the development of a neuropsychiatric syndrome: patients in the early stages may complain of anorexia, lassitude, excessive tiredness, apathy, joint pains and muscular cramps, but later develop symptoms of organic psychosis (disorientation, impairment of memory and judgment, acute anxiety, emotional disability, compulsive behavior, flight of ideas, hallucinations, illusions and delusions) followed by psychomotor slowing, cognitive decline and manifestations of an extra pyramidal syndrome that clinically resembles Parkinson's disease (Archibald *et al*., 1987; McMillan *et al*., 1999; Finkelstein *et al*., 2007). This Mn-induced neuropsychiatric syndrome is usually the outcome of low-level long-term occupational exposure to Mn (Finkelstein *et al.,* 2007). The cellular, intracellular and molecular mechanisms underlying the Mn-induced neurotoxicity are both dose and time-dependent (Dobson *et al.* 2004; Villalobos and Castro *et al*., 1994) while they are also thought to be numerous and not well understood. Most mechanisms involve the Mn-induced free radical production (Villalobos and Suarez *et al*., 2001) and changes in the function of nearly all systems of neurotransmission (Bonilla *et al*., 1974; Mustafa *et al*., 1971; Lai and Guest *et al*., 1980; Eriksson and Gillberg *et al*., 1992). In view of the above, high-level short-term exposure to Mn might provide a suitable

Corresponding author: Bhuvaneswari Devi, K.
Department of Biotechnology, SVIMS Universit, Tirupati, Andhra Pradesh, India.

experimental basis for the evaluation of the possible primary targets of Mn neurotoxicity, and contribute to the uncovering of the mechanisms involved in the induction of the clinical neuropsychiatric manifestations. The aim of this study was to shed more light on the effects of short-term Mn administration on: (i) the activities of acetylcholinesterase (ii) ACh content and (iii) Histochemical studies in rat brain.

MATERIALS AND METHODS

Chemicals

Manganese and Vitamin-E was selected as test chemical. The chemicals used in this study namely Thiobarbutric acid, Glutathione oxidized, NADPH, DTNB, Reduced glutathione, Epinephrine were obtained from Sigma, USA. The remaining chemicals obtained from Qualigens, India.

Procurement and maintenance of experimental animals

Young albino rats (Wistar) were purchased from IISc, Bangalore and maintained in the animal house of Y.V. University. 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.* The protocol and animal use were approved by Institution Animal Ethical Committee, Y.V. University.

Animal exposure to Mn and Alpha-tocopherol (Vitamin – E)

The young albino rats (both 2 months and 4 months old) were exposed to a low dose of 2.5mg/kg body weight and a high dose of 5mg/kg body weight through intraperitoneal injection for a period of 3 weeks and left for a period of one week for supplementation with Alpha-tocopherol at a dose of 5mg/kg body weight through intraperitoneal injection. 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 by homogenizing 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. Then the pellet of mitochondrial fraction was suspended in SET buffer.

Preparation of Crude 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 K_-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 NaHCO3, 10 mM HEPES, and 10mM glucose, pH 7.4).

Cholinergic System

Estimation of AChE

The specific activity of AChE was determined as described by (Ellman *et al*., 1961). The reaction mixture contained 3.0 ml of 0.1M phosphate buffer (pH 8.0), 20µl of 0.075 M acetylthiocholine iodide and 100 µl of 0.01 M 5, 5-dithiobis 2-nitrobenzoic acid. The reaction was initiated with the addition of 100 µl of crude homogenate. The contents were incubated for 30min at room temperature and the color absorbance was measured at 412nm in spectrophotometer (Hitachi, Model U-2000). The enzyme activity was expressed as µ moles of ACh hydrolyzed /mg protein/h.

Estimation of Acetylcholine (ACh)

The acetylcholine content was estimated 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 also to release the bound ACh. To the synaptosomal fractions 1 ml of alkaline hydroxylamine hydrochloride followed by 1 ml of 50% hydrochloric acid were added. The contents were mixed thoroughly and centrifuged. To the supernatant 0.5 ml 0.37M ferric chloride solution was added and the brown color developed was read at 540nm against a reagent blank in a spectrophotometer.

Histochemistry of AChE

AChE histochemistry was performed as described by (Hedreen *et al*. 1985), the modified method of (Karnovsky and Roots, 1964). Rats were anesthetized with sodium pentobarbital and per fused with 50mM phosphate buffered saline (PBS, Ph 7.4, 4°C) followed by 4% Para formaldehyde (4°C) through cardiac catheter. Brains were removed and post fixed in Para formaldehyde for 2 hours. This was followed by cryoprotection in 10, 20, and 30% sucrose gradients. Eight micron thick sections were cut in a freezing monotome (Bright Instrument Company Limited, England). The sections were rinsed in 0.1 M phosphate buffer (pH 6.0) and incubated in the following media (50 ml): 32.5 ml of 0.1 M phosphate buffer (pH 6.0); 2.0 ml of 0.1 M sodium citrate; 5 ml of 0.03 M cupric sulphate; 1.0 ml of 0.0005 M potassium ferricyanide; 25 mg of acetylthiocholine iodide and 9.5 ml of distilled water. Sections were also incubated with the AChE inhibitor, serine (10^{-4}), in the above media to study the inhibitory effect. The sections were incubated for 20 minutes at room temperature and then dehydrated in ethanol series, cleared in xylene and mounted in permount.

Statistical treatment of the data

The mean and standard deviation (SD), analysis of variance (ANOVA) and test of significance or students 't' test was calculated using standard statistical software package.

RESULTS

AChE activity

From this study, from Fig 1.1, it was observed that the AChE activity in synaptosomal fraction of low dose and high dose of Mn-exposed rats was showed decreased in 2 months when compared to control.

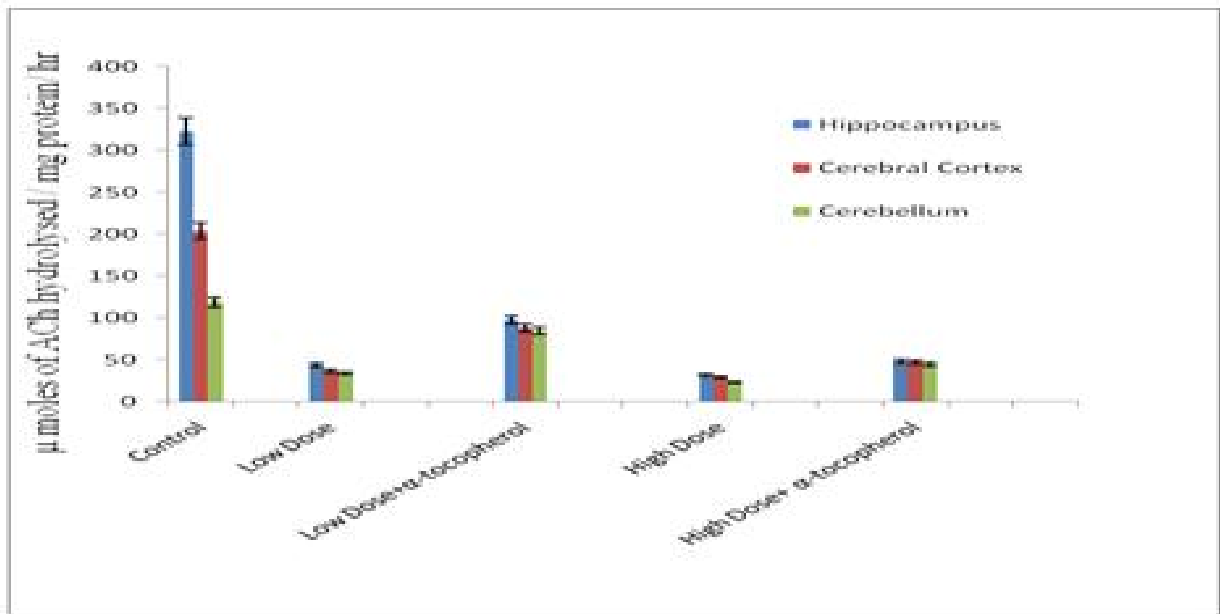


Figure1.1 Effect of Mn exposure on AChE activity in cerebral cortex, hippocampus and cerebellum. Rats were exposed to 2.5 and 5 mg/kg bw Mn and Mn+α-tocopherol supplemented 2 months rats through intraperitonially daily for a period of four weeks. Each bar represents mean±SD (n=6). The values marked with asterisk (*) are significantly different from controls at $p<0.05$

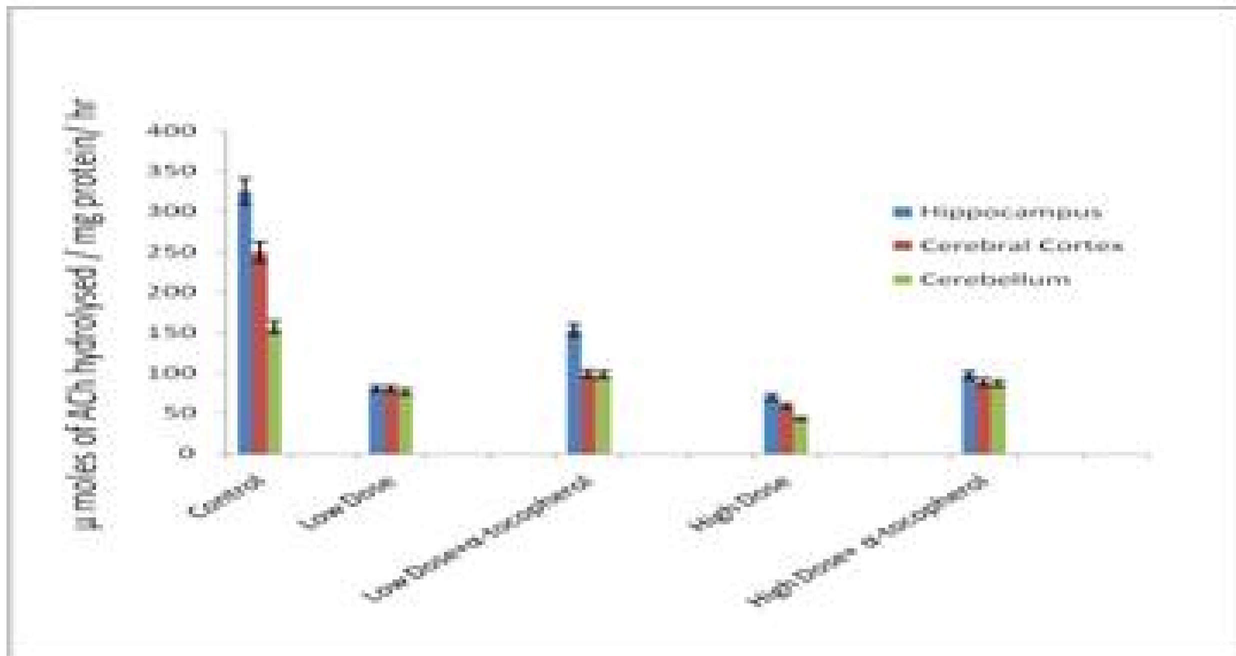


Figure1.2 Effect of Mn exposure on AChE activity in cerebral cortex, hippocampus and cerebellum. Rats were exposed to 2.5 and 5 mg/kg bw Mn and Mn+α-tocopherol supplemented 4 months rats through intraperitonially daily for a period of four weeks. Each bar represents mean±SD (n=6). The values marked with asterisk (*) are significantly different from controls at $p<0.05$

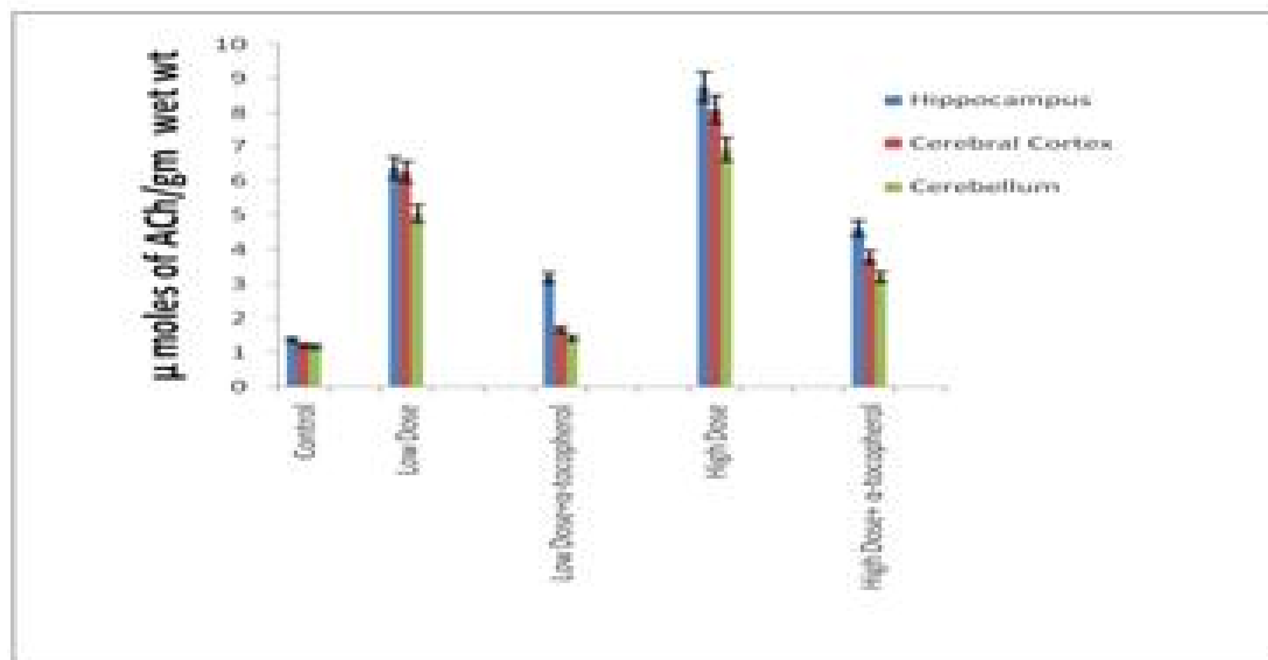


Figure 2.1. Effect of Mn exposure on ACh content in cerebral cortex, hippocampus and cerebellum. Rats were exposed to 2.5 and 5 mg/kg bw Mn and Mn+α-tocopherol supplemented 2 months rats through intraperitonially daily for a period of four weeks. Each bar represents mean±SD (n=6). The values marked with asterisk (*) are significantly different from controls at $p<0.05$

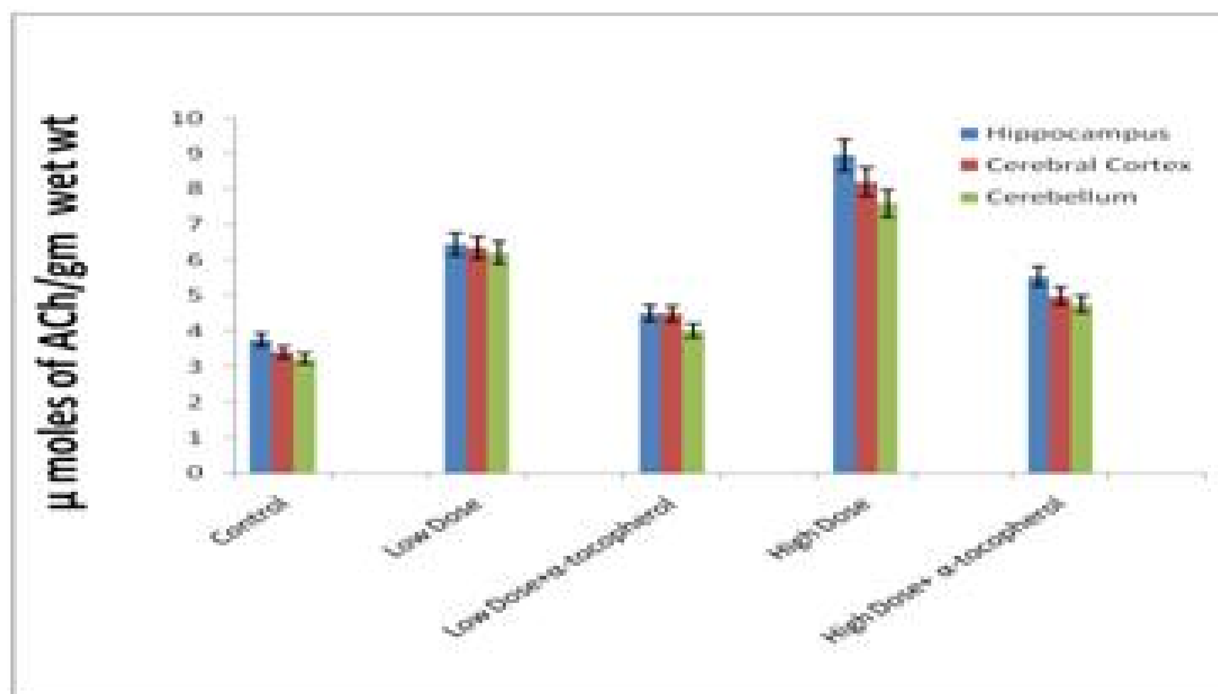


Figure 2.2. Effect of Mn exposure on ACh content in cerebral cortex, hippocampus and cerebellum. Rats were exposed to 2.5 and 5 mg/kg bw Mn and Mn+α-tocopherol supplemented 4 months rats through intraperitonially daily for a period of four weeks. Each bar represents mean±SD (n=6). The values marked with asterisk (*) are significantly different from controls at $p<0.05$

However, the AChE activity was increased in the animals supplemented with α-tocopherol along with Mn- exposure. Unlike the young rats, from Fig 1.2, the 4 months adult Mn treated rats also showed decrease in AChE activity compared to control rats. However, the AChE activity was increased in the animals supplemented with α-tocopherol along with Mn-exposure. However, the adult rats showed maximum AChE activity than young rats. Among the three brain regions studied, Hippocampus showed maximum activity followed by Cerebral Cortex, and then Cerebellum.

ACh Content

In the present study, from Fig. 2.1, it was observed that the ACh content in synaptosomal fraction of low dose and high dose of Mn-exposed rats was increased in 2 months when compared to control. However, the ACh content was decreased in the animals supplemented with α -tocopherol along with Mn- exposure. Unlike the young rats, from Fig 2.2, the 4 months adult Mn treated rats also showed increase in ACh activity compared to control rats. However, the ACh activity was decreased in the animals supplemented with α-tocopherol along with Mn- exposure. However, the adult rats showed maximum ACh activity than young rats. Among the three brain regions studied, Hippocampus showed maximum activity followed by Cerebral Cortex, and then Cerebellum.

Histochemical studies

From the Figures 3.1 to 3.4, Histochemical studies of AChE showed significant alterations in CA1, CA2 and CA3 regions and dentate gyrus of hippocampus, molecular layer of cerebellum and cortical layers in Mn-exposed adult rats. 5 mg/kg bw Mn exposure decreased the staining markedly in CA3 region and dentate gyrus of hippocampus. It was further observed that the effect of Mn decreased AChE staining in molecular and granular cell layers of cerebellum and cortical cell layer. Eserine treated brain sections showed almost no staining of AChE confirming that the above stain was specific for AChE. However, the administration of α-tocopherol along with Mn showed increase of AChE staining. Among the three brain regions studied, Hippocampus showed maximum activity followed by Cerebral Cortex, and then Cerebellum.

DISCUSSION

Mn is an essential element, exposure to excessive levels of Mn can cause a variety of neurotoxic effects that involve (among others) alterations in cholinergic system and bioenergetics which finally leads to alterations in oxidative stress biomarkers (Villalobos and Suarez, 2001; Erikson and Dorman, 2007). Our data revealed a statistically significant Mn-induced reduction in AChE activity in three brain regions (Cerebral cortex, cerebellum and hippocampus) with concomitant increase in the ACh content. When an antioxidant, α-tocopherol was administrated post Mn exposure, lead to the reversal in the alterations caused by the Mn in cholinergic system. And this could be due to the chelating properties of α-tocopherol assisting to the biological inactivation and/or excretion of Mn ions. It should be noted that Mn brain concentrations have not been related to the extent of ACh content (Chen and Cheng , 2006) observed in certain animal brain regions after exposure to Mn, and that such alterations might be (in some extent) reversible (Erikson and Dorman, 2006). However, α-tocopherol (at least under the examined experimental conditions) was proved sufficiently efficient to neutralize the Mn-induced neurotoxicity. Manganese acts at presynaptic levels within the striatum by blocking release of the neurotransmitter, thus creating a localized, relative deficit in caudate function (Inoue *et al*., 1975). The injection of manganese into the caudate nucleus of the rat brain resulted in a predominant ipsilateral turning behavior, accompanied at higher doses by an intermittent, alternating and dose-related incidence of contralateral turning and stereotypes. Tegmental serotonergic and intrastriatal cholinergic pathways were involved in the production of the basic postural asymmetry resulting in turning (Inoue *et al*., 1975). The amount of interference with the nigrostriatal and mesolimbic dopaminergic pathways may determine the concurrent inhibition of locomotion (Acquas and Chiara, 2001; Fenu *et al*., 2001). This impairment of postural asymmetry was more pronounced after bilateral injections of manganese into the caudate nucleus (Inoue *et al*., 1975). Most probably, these effects of manganese are partially mediated by intrastriatal cholinergic pathways.

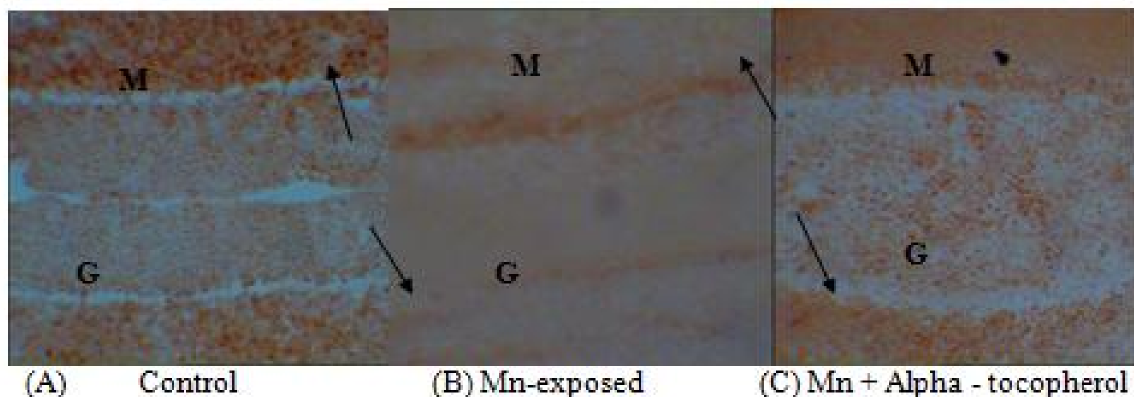


Figure 3.1. Cerebellum – 4 Months

Histochemical staining of AChE in cerebellum of control (A), 5 mg/kg bw Mn-exposed (B) and Mn + Alpha – tocopherol exposed (C) adult (4 months old) rat brain. Cryo-sections were stained for AChE as described in materials and methods section. Areas marked with arrows (↑) Fig (B) show loss of AChE staining. Areas marked with arrows (↑) Fig (C) show increase of AChE staining. M-molecular layer G-granule cell layer; Magnification: 10 X

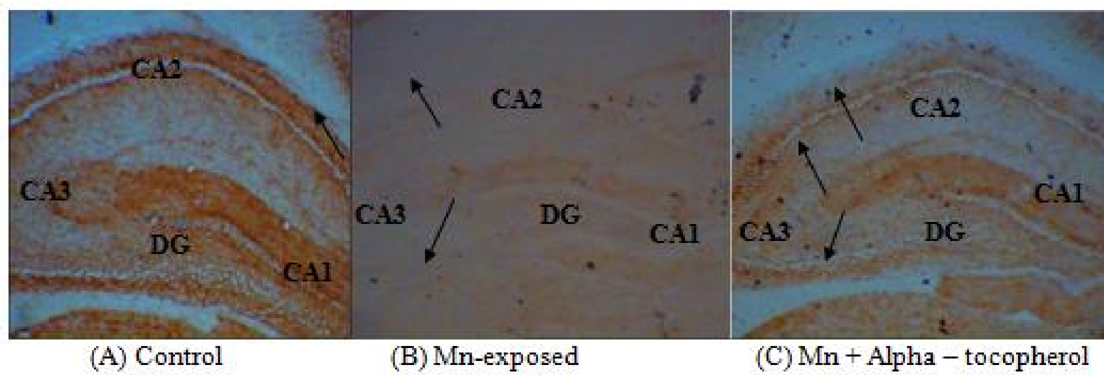


Figure 3.2. Hippocampus – 4 Months

Histochemical staining of AChE in hippocampus of control (A), 5 mg/kg bw Mn-exposed (B) and Mn + Alpha – tocopherol exposed (C) adult (4 months old) rat brain. Cryo-sections were stained for AChE as described in materials and methods section. AChE staining is intense in CA1, CA2, CA3 and DG areas of control brain (A). Loss of AChE staining is conspicuously seen in CA3 and DG areas of Mn-exposed (B) brain. Areas marked with arrows (↑) in Fig (C)show increase of AChE staining is conspicuously seen in CA3 and DG areas of Mn + Alpha – tocopherol exposed brain. Areas marked with arrows (↑) fig (C) show loss of AChE staining. DG-dentate gyrus; Magnification: 40 X

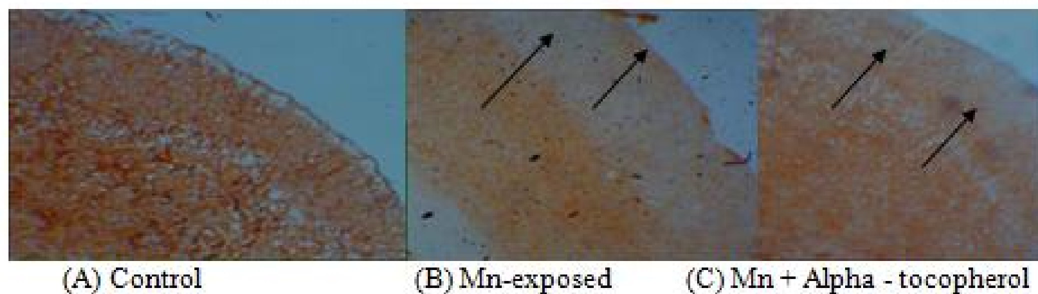
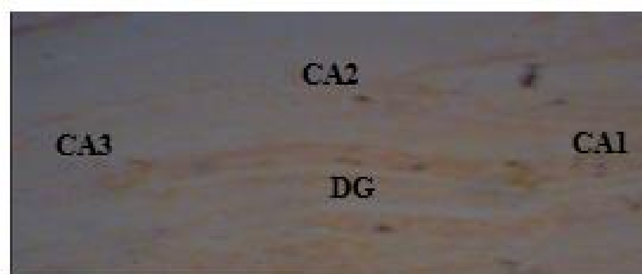


Figure 3.3 Cortex – 4 Months

Histochemical staining of AChE in cerebral cortex of control (A), 5 mg/kg bw Mn-exposed (B) and Mn + Alpha – tocopherol exposed (C) adult (4 months old) rat brain. Cryo-sections were stained for AChE as described in materials and methods section. Areas marked with arrows (↑) Fig (B) show loss of AChE staining. Areas marked with arrows (↑) Fig (C) show increase of AChE staining. Magnification: 10 X



Eserine treated – 4 Months

Histochemical staining of AChE in hippocampus of control adult (4 months old) rat brain incubated with eserine. Cryo-sections were stained for AChE as described in materials and methods section. DG-dentate gyrus; Magnification: 40 X

The effects of Mn^{2+} on quantal ACh release have been studied with conventional microelectrode techniques. Mn^{2+} led to increased miniature end-plate potential (MEPP) frequency at low levels. Stimulation of the motor nerve caused substantial increases in MEPP frequencies, with maximal frequency attained in the presence of Mn^{2+}; further stimulation led to a fall in frequency. Thus, Mn^{2+} is able to enter the nerve terminal through a voltage-gated channel. Once within the terminal, Mn^{2+} may stimulate quantal release by releasing Ca^{2+} within the terminal (Kita *et al*., 1981).

In the earlier studies, significant inhibition AChE activity was observed following lengthy periods of exposure to manganese. Life-long treatment with manganese partially abolished the aging-associated decrease in AChE activities in the hypothalamus, cerebellum and striatum (Lai *et al*., 1981). In the present study, our results showed that, a significant decrease was observed in AChE activities in the Cerebral Cortex, Hippocampus and Cerebellum of Mn treated rats. In another study, rats were chronically treated with manganese chloride from conception onward for a period of over 2 years.

AChE activity was examined in the hypothalamus, cerebellum, pons and medulla, striatum, midbrain and cerebral cortex (which included the hippocampus). Manganese-treated 2-month-old and 24–28-month-old rats and age-matched controls were studied. In the control group, AChE activities decreased in all regions, particularly in the striatum, during aging. Following treatment with manganese chloride, AChE activity was not affected in any of the rat brain regions studied on the first, third and eighth month of treatment. Later on, AChE activity diminished in the caudate nucleus on the eighth month of treatment with manganese chloride but was not altered in any other region throughout the life-long study (Martinez and Bonilla, 1981). The above study was contradictory to our studies. AChE was shown to be associated with increased oxidative and nitrosative stress, alterations in energy metabolism (Milatovic *et al*., 2006) and consequent degeneration by pyramidal neurons from the CA1 hippocampal region of rat brain.

Ultimately, the additive or synergistic mechanisms of cellular disruption caused by manganese and other toxicants may lead to cellular dysfunction and cellular neurodegeneration (Gupta *et al*., 2007). In high manganese-exposed mice, the activity of AChE in the brain was decreased. The immunoreactivity of glial fibrillary acid protein (GFAP) and the average relative density of GFAP-positive products in the hippocampus (area CA3) of both the low and high manganese-exposed groups, especially in the high-dose manganese group, were significantly higher than those of the control group.

The gain of body weight, brain weight and the ratio of brain weight to body weight in the high-dose manganese-exposed group was significantly decreased (Zhang *et al*., 2001). The co-administration of Mn and α-tocopherol was, on the other hand, sufficiently efficient in order to maintain AChE into the control levels.

This finding is of importance, because the observed Mn-induced decrease in AChE activity has been associated with high brain Mn accumulation by a previous chronic study (Lai and Chan, 1992) conducted on rat striatum and cerebellum. Could this finding support the view that α-tocopherol exerts its neuroprotective effect towards Mn through a chelator-metal ion interaction that limits intracellular and extracellular free Mn^{2+} levels in the brain? The answer requires further investigation, because (i) the Mn– α-tocopherol interaction is not of a simple nature (Shen and Dryhurst, 1998) and (ii) Mn has been shown to exert different effects on AChE activity in different stages of the life circle (Finkelstein and Milatovic, 2007).

Conclusion

In conclusion, our findings suggest that Mn 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 α-tocopherol co-administration, which could thus be considered for future applications as a neuroprotective agent against chronic exposure to Mn and the treatment of manganism. These findings suggest that Mn might not primarily interfere with neuronal excitability, as well as with the uptake and release of catecholamines, serotonin and glutamate. It might, however, cause oxidative stress, cholinergic dysfunction and other intracellular deregulating phenomena associated with its chemical resemblance to other essential elements such as Mg^{2+}.

Acknowledgements

The authors acknowledge the financial support from DST No: SR/FT/LS-103/2009 (G)

REFERENCES

Acquas, E., Chiara, G.D. 2001. "Role of dopamine D1 receptors in the control of striatal acetylcholine release by endogenous dopamine", Neurol Sci, Vol. 22, pp. 41–42.

Archibald, F.S. and Tyree, C. 1987. "Manganese poisoning and the attack of trivalent manganese upon catecholamines", *Arch Biochem Biophys,* Vol. 256, pp. 638–650.

Augustinssion, K.B. 1963. "Cholinesterase and anticholinesterase agents", (Ed. G.B. Koelle). Springer Verlag. Berlin, Vol. 5, pp. 89-128

Bonilla, E., Diez-Ewald, M. 1974. "Effect of □-dopa on brain concentration of dopamine and homovanillic acid in rats after chronic manganese chloride administration", *J. Neurochem*, Vol. 22, pp. 297–299.

Chen, M.T., Cheng G.W., Lin C.C., Chen, B.H. and Huang, Y.L. 2006. "Effects of acute manganese chloride exposure on lipid peroxidation and alteration of trace metals in rat brain", *Biol. Trace Elem Res.,* Vol. 110, pp. 163–178.

Dobson, A.W., Erikson, K.M., Aschner, M. 2004. "Manganese neurotoxicity", *Ann N Y Acad Sci*., Vol. 1012, pp. 115–128.

Ellman, G., Courtney, K., Andres, V. and Feather-Stone, R. 1961. "A new and rapid colorimetric determination of acetylcholinesterase activity", Biochem Pharmacol, Vol. 7, pp. 88–95.

Erikson, K.M., Dorman, D.C., Fitsanakis, V., Lash, L.H. and Aschner, M. 2006. "Alterations of oxidative stress biomarkers due to *in utero* and neonatal exposures of airborne manganese", Biol Trace Elem Res, Vol. 111, pp. 199–215.

Erikson, K.M., Dorman, D.C., Lash, L.H. 2007. "Manganeses inhalation by Rhesus monkeys is associated with brain regional changes in biomarkers of neurotoxicity", *Toxicol Sci.,* Vol. 97(2), pp. 459-466.

Eriksson, H., Gillberg, P.G., Aquilonius, S.M., Hedstrom, K.G., Heilbronn, E. 1992. "Receptor alterations in manganese intoxicated monkeys", *Arch Toxicol,* Vol. 66, pp. 359–64.

Fenu, S., Acquas, E. and Chiara, G.D. 2001. "Role of striatal acetylcholine on dopamine d1receptor agonist-induced turning behavior in 6-hydroxydopamine lesioned rats: a microdialysis-behavioral study", Neurol Sci, Vol. 22, pp. 63–64.

Finkelstein, Y., Milatovic, D. and Aschner, M. 2007. "Modulation of cholinergic systems by manganese", Neurotoxicology, Vol. 28, pp. 1003– 1014.

Gupta, C.R., Milatovic, S., Dettbarn, D.W., Aschner, M. and Milatovic, D. 2007. "Neuronal oxidative injury and dendritic damage induced by carbofuran: Protection by memantine", *Toxicol Appl Pharmacol*, Vol. 219, pp. 97–105.

Hedreen, J.C., Bacon, S.J. and Price, D.L. 1985. "Amodified histochemical technique to visualized acetylcholinesterase containing axons", *J. Histochem. Cytochem,* Vol. 33, pp. 134-140.

Inoue, N. 2007. "Occupational neurotoxicology due to heavy metalsespecially manganese poisoning". *Brain Nerve,* Vol. 59, pp. 581–589.

Inoue, N., Tsukada, Y., Barbeau, A. 1975. "Behavioral effects in rats following intrastriatal microinjection of manganese", *Brain Res,* Vol. 95, pp. 103–124.

International Life Sciences Institute 1994. "Risk Assessment of Essential Elements", International Life Sciences Institute Press, Washington, DC.

Karnovsky, M.J., Roots, L. 1964. "A direct coloring thiocholine method for cholinesterase", *J. Histochem. Cytochem,* Vol. 12, pp. 219-225.

Keen, C.L., Ensunsa, J.L., Watson, M.H., Baly, D.L., Donovan, S.M. and Monaco, M.H. 1999. "Nutritional aspects of manganese from experimental studies", Neurotoxicology, Vol. 20, pp. 213–23.

Kita, H., Narita, K., Van der Kloot, W. 1981. "Tetanic stimulation increase the frequency of miniature end-plate potentials at the frog neuromuscular junction in Mn^{2+}-, Co^{2+}-, and Ni^{2+}-saline solutions", *Brain Res.,* Vol. 205, pp. 111–121.

Lai, J., Leung, T. and Li, L. 1981. "Brain regional distribution of glutamic acid decarboxylase, choline acetyltransferase, and acetylcholinesterase in the rat: effects of chronic manganese chloride administration after two years", J Neurochem, Vol. 36, pp. 1443–1448.

Lai, J.C., Chan, A.W., Leung, T.K., Minski, M.J. and Lim, L. 1992. "Neurochemical changes in rats chronically treated with a high concentration of manganese chloride), *Neurochem Res.*, Vol. 17, pp. 841–847.

Lai, J.C., Guest, J.F., Leung, T.K., Lim, L. and Davison, A.N. 1980. "The effects of cadmium, manganese and aluminium on sodium-potassiumactivated and magnesium-activated adenosine triphosphatase activity and choline uptake in rat brain synaptosomes", Biochem Pharmacol, Vol. 29, pp. 141–146.

Martinez, H. and Bonilla, E. 1981. "Water intake and brain choline-acetyltransferase and acetylcholinesterase activities in manganese treated rats", *Neurobehav Toxicol Teratol,* Vol. 3, pp. 277–280.

McMillan, D.E. 1999. "A brief history of the neurobehavioral toxicity of manganese: some unanswered questions", *Neurotoxicology,* Vol. 20, pp. 499–507.

Metcalf, R.L. 1951. "In Methods of biochemical analysis", Interscience Publishers Inc, Vol 5, pp. 1-57.

Milatovic, D., Gupta, R., Aschner, M. 2006. "Anticholinesterase toxicity and oxidative stress". Sci *World J.*, Vol. 6, pp. 295–310.

Mustafa, S.J. and Chandra, S.V. 1971. "Levels of 5-hydroxytryptamine, dopamine and norepinephrine in whole brain of rabbits in chronic manganese toxicity", *J. Neurochem,* Vol. 18, pp. 931–933.

Shen, X.M., Dryhurst, G. 1998. "Iron- and manganese-catalyzed autoxidation of dopamine in the presence of -cysteine: possible insights into iron- and manganese-mediated dopaminergic neurotoxicity", *Chem Res Toxicol*, Vol. 11, pp. 824–837.

Villalobos, V., Castro, F., Bonilla, E., Estevez, J. and Davila, J.O. 1994. "Manganese toxicity: muscarinic receptor binding in the mouse brain", *J. Toxicol Environ Health,* Vol. 42, pp. 185–191.

Villalobos, V., Suarez, J., Estevez, J., Novo, E. and Bonilla, E. 2001. "Effect of chronic manganese treatment on adenosine tissue levels and adenosine A2 receptor binding in diverse regions of mouse brain", Neurochem Res, Vol. 26, pp. 1157–1161.

Zhang, D., He, X., Huang, S., Li, Y. 2001. "Effect of manganese exposure on brain development in postnatal mice", Wei Sheng Yan Jiu, Vol. 30, pp. 260–262.

****