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Melatonin abrogates cadmium induced oxidative stress related neurotoxicity in rats

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Abstract. – *Background:* **Cadmium is a potent neurotoxic heavy metal, which induces oxidative stress and membrane disturbances in brain. Melatonin is an effective antioxidant and free radical scavenger against oxidative stress. The present study was designed to investigate the neuroprotective efficacy of melatonin in protecting the Cd induced changes in the activity of acetylcholinesterase (AChE), levels of lipid peroxidation, protein carbonyls, non-enzymatic antioxidant, enzymatic antioxidant status, membrane bound ATPases and histopathology in the brain of rats.**

Materials and Methods: **Twenty four male albino rats were used. Cadmium induced oxidative neurotoxicity was induced by oral administration of Cd for four weeks. Melatonin was pretreated along with Cd for four weeks to assess its neuroprotective activity against Cd intoxication. Rats treated with vehicles alone were used as controls.**

Results: **Rats intoxicated with cadmium (5 mg/kg/day) for 4 weeks significantly ($p<0.05$) reduced the AChE levels in the plasma and brain, elevated the levels of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and protein carbonyls along with the significant ($p<0.05$) decrease in the levels of non-enzymatic antioxidants (GSH, TSH and vitamins C and E), enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and membrane bound ATPases in the brain tissue. Administration of melatonin (10 mg/kg/day) for 4 weeks in cadmium intoxicated rats significantly ($p<0.05$) diminished the levels of oxidative stress markers, lipid peroxidation and protein carbonyls in brain and significantly ($p<0.05$) elevated the levels of non-enzymatic and enzymatic antioxidants, brain and the activities of AChE, enzymatic antioxidants and ATPases in brain. The histopathological studies in the brain of rats also supported that melatonin markedly reduced the Cd induced pathological changes and preserved the normal histological architecture of the brain tissue.**

Conclusions: **The results of the present study suggest that melatonin may be beneficial in combating the cadmium induced oxidative neurotoxicity in the brain of rats.**

Key Words:

Cadmium, Melatonin, Brain, Antioxidants, Oxidative stress, Rats.

Introduction

Cadmium (Cd) is one of the most toxic pollutants which widely distributed in the environment. Since Cd is not degraded in the environment, the risk of human exposure to Cd is constantly increasing through its contamination of the food chain[1]. The main source of exposure to the toxic and carcinogenic metal cadmium is basic foods like cereals and vegetables, which means that the exposure is lifelong. The increase in Cd pollution has become an important public health concern worldwide[2]. It is well known that long-term exposure to Cd causes various toxic effects in various organ systems such as cardiovascular, kidneys, liver, brain, lung, bones, immune/haemopoietic, endocrine and reproductive systems[3,4].

Cd is able to induce neurotoxicity with a wide spectrum of clinical entities including neurological disturbances, changes in the normal neurochemistry of the brain[5]. Cd is known to induce lipid peroxidation (LPO) by stimulating the production of superoxide anions[6]. Cd induced toxicity is implicated in generation of reactive oxygen species (ROS) and exhaustion of antioxidants[7]. The ability of Cd to induce oxidative stress in brain cells has been reported as the induction of ROS, after the interaction of Cd with mitochon-

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drial sites, leading to the breakdown of the mitochondrial potentials, a consequent reduction of intracellular glutathione levels[8].

The brain tissue is highly susceptible to lipid peroxidation (LPO) because of its high rate of oxygen utilization, an abundant supply of polyunsaturated fatty acids, a deficient antioxidant defense and a high content of transition metals like copper and iron in several regions [9].The enhanced susceptibility of membranes to LPO can lead to loss of membrane bound ATPases activities and modulates the cell functions [10]. ATPases are the membrane bound lipid dependent enzymes, which are involved in active transport, maintenance of cellular homeostasis and also involved in neurotransmission process.

The oxidative effect of cadmium is indirect and is based mainly on the inactivation of thiol groups in critical molecules, inhibiting antioxidant defenses and DNA repair mechanisms[11]. Cadmium alters the activities of oxidative stress neutralizing enzymes, which leads to the disturbances in brain metabolism and also contributes to the neurotoxic effect of cadmium. Cadmium enhances the production of free radicals in the brain and interferes with the antioxidant defense system which in turn leads to a cadmium induced alterations in the structural integrity of lipids and secondarily affects the membrane bound enzymes[12].

Currently, an extensive research has been done to evaluate several natural antioxidants regarding their chemo preventive effects in heavy metal induced toxicities[13,14]. Antioxidants are becoming very popular in combating oxidative stress related diseases and as potential therapeutic agents in various ailments. Antioxidants can counteract the decrease in ATPases activity and the increase in oxidative stress that are induced by cadmium[15].

One of the recently most studied antioxidant agents is melatonin (N-acetyl-5-methoxytryptamine), the main secretory product of the pineal gland, participates in many physiological functions due to its efficacy as a free radical scavenger and indirect antioxidant[16]. Due to its small size and lipophilicity, melatonin crosses biological membranes easily, thus reaching all compartments of the cell. Melatonin has also been shown to be an efficient protector of DNA[17], proteins and lipids in cellular membranes as well as antagonists of a number of endogenous and exogenous free radicals attack during cellular processes[18]. In spite of several studies on the protective effect of melatonin against various xenobiotics, the reports are scanty on the effect of melatonin on cadmium induced oxidative stress related neurotoxicity in the brain of rats.

In view of the antioxidant and membrane stabilizing properties of melatonin, it is noteworthy to consider that melatonin might bring out beneficial effects on cadmium induced oxidative stress in brain. Therefore, the present study has been designed to evaluate the neuroprotective efficacy of melatonin on cadmium induced oxidative neurotoxicity in the brain of rat.

Materials and Methods

Chemicals

Cadmium chloride, melatonin and other fine chemicals used in this work were purchased from Sigma Chemical Co. (St. Louis, MO, USA). For the estimation of phosphorus, Qualigens diagnostic kit, Mumbai, India was used. Cadmium chloride was dissolved in saline solution (0.9% NaCl). Melatonin was dissolved in ethanol before being diluted with saline. The final concentration of ethanol in the melatonin solution was < 1%.

Animals

The experiments were carried out in adult male Wistar rats (180-200 g) procured from Central Animal House (CAH), Faculty of Medicine and Annamalai University. The animals were housed under the conditions of constant temperature (24 ± 2˚C) and humidity (50-60%), under a 12 h light/12 h dark photoperiod in the CAH of the University. Water and standard laboratory food (Hindustan Lever Ltd., Mumbai, India) were provided *ad libitum*. The experimental protocol used in this study was approved (Reg. No. 450/2007/CPCSEA) by the Institutional Animal Ethical Committee (IAEC) for the purpose of control and supervision on experimental animals of Rajah Muthiah Medical College, Annamalai University, Annamalainagar, India. After acclimatization, the animals were divided into 4 groups, consisting of 6 rats in each group.

Experimental Protocol

Group 1: Control rats received normal saline (Cd diluent) orally and 0.5 ml of 0.01% ethanol subcutaneously (melatonin diluent) for four weeks.

Group 2: Rats orally received Cd as cadmium chloride in saline (5 mg/kg body weight) for four weeks.

Group 3: Rats orally received the cadmium as cadmium chloride (5 mg/kg body weight) in normal saline and injected with melatonin (10 mg/kg body weight) in ethanol subcutaneously for four weeks. The injection of melatonin was 30 min before Cd administration and was given at 4 p.m. (2 h before light off).

Group 4: Rats received the melatonin in ethanol subcutaneously (10 mg/kg/day) for four weeks.

The animals were maintained in their respective groups for four weeks. Food and fluid intake and body weights were measured weekly. At the end of the experimental period, the animals were anesthetized using ether and sacrificed by cervical decapitation. Blood was collected from the jugular vein using heparin as the anticoagulant and centrifuged at 2000 g for 20 min to prepare plasma. Brain tissues were excised washed and homogenized in 0.1 M Tris-HCl-0.001M EDTA buffer (pH 7.4) and centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected and used for the experiments.

Determination of Acetyl Cholinesterase Activity

Acetylcholinesterase (AChE) activity was determined in plasma and brain using acetylcholine iodide as a substrate according to the method of Ellman et al.[19]. In this method AChE in samples hydrolyzes acetylthiocholine iodide into thiocholine and butyric acid. The thiocholine reacts with 5,5′-dithiobis-2-nitrobenzoic acid to form 5-thio-2-nitrobenzoic acid. The yellow colour developed is measured spectrophotometrically at 412 nm (Elico-SL177, Elico LTD. Hyderabad, Andra Pradesh, India).

Determination of DNA Fragmentation

The extent of DNA fragmentation was determined by the method adapted from that of Lin et al.[20]. Brain tissue homogenate was treated with 0.01 M Tris buffer pH 8.0, 1 mM EDTA and 0.5% triton X-100 and centrifuged. Both supernatant and pellet were precipitated with 12.5% TCA. Quantitative analysis of DNA was carried out by diphenylamine reaction. The percentage of fragmentation was calculated from the ratio of DNA in the supernatant to the total DNA.

Estimation of TBARS, LOOH and Protein Carbonyl Content

The concentration of thiobarbituric acid reactive substances (TBARS) were estimated in brain by the method of Niehaus and Samuelsson[21] using 1,1′,3,3′-tetramethoxypropane as the standard. Lipid hydroperoxides in brain homogenates were assayed as described by Jiang et al.[22]. The level of protein carbonyl content was determined by the method of Levine et al.[23].

Determination of Non-Enzymatic Antioxidants

The levels of reduced glutathione (GSH) in brain homogenate were determined by the method of Moron et al.[24] based on the reaction with Ellman's reagent (19.8 mg DTNB in 100 ml of 0.1% sodium citrate). The levels of total sulphydryl groups (TSH) was measured after the reaction with 5,5′-dithiobis-2-nitrobenzoic acid using the method of Ellman[25]. The concentration of ascorbic acid was determined brain by the method of Omaye et al.[26] and vitamin E was measured by the method of Baker and Frank[27]. The values are expressed as g/mg of protein.

Determination of Enzymatic Antioxidants

Superoxide dismutase (SOD) activity in the brain was assayed by the method of Kakkar et al[28] based on the inhibition of formation of NADH-phenazine methosulphate-nitrobluetetrazolium complex. One unit of SOD corresponds to the amount of enzyme causing 50% reduction of nitro blue tetrazolium/min/mg of protein. Catalase (CAT) was assayed by the method of Sinha[29] by quantitating the H_2O_2 consumed after the enzymatic reaction. Dichromate in acetic acid was used as the coloring agent. The activity of catalase is expressed as μmoles H_2O_2 consumed/min/mg of protein. GPx activity was assayed in brain by the method of Rotruck et al[30]. A known amount of enzyme preparation was allowed to react with hydrogen peroxide (H_2O_2) and reduced glutathione (GSH) for a specified time period. The GSH content remaining after the reaction was measured by reaction with 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB). The GPx activity is expressed as μmoles of GSH oxidized/min/mg of protein. Glutathione S-transferase (GST) in the brain was assayed by the method of Habig[31] by following the increase in absorbance at 340 nm using 1-chloro-2,4-dinitrochlorobenzene (CDNB) as substrate. The GST activity is expressed as mmol of glutathione-chlorodinitrobenzene conjugate formed/min/mg of protein. Protein content in the brain homogenate was estimated by the method of Lowry et al.[32] using bovine serum albumin as a standard.

Determination of ATPases Activities

Total ATPase activity in brain homogenate was measured by the method of Evans[33]. The ATPase activity in 0.1 ml of aliquot of the homogenates were measured in a final volume of 2 ml containing 0.1 ml of 0.1 M Tris-HCl (pH 7.4), 0.1 ml of 0.1 M NaCl, 0.1 ml of 0.1 M $MgCl_2$, 1.5 ml of 0.1 M KCl, 0.1 ml of 1 mM EDTA and 0.1 ml of 0.01 M ATP. The reaction was stopped at 20 min by the addition of 1 ml of 10% TCA and, then, centrifuged (1500 ↔ g for 10 min) and the inorganic phosphorus (Pi) liberated was estimated in the protein-free supernatant. The amount of liberated Pi was estimated according to the method of Fiske and Subbarow (Elico-SL177, Elico LTD. Hyderabad, Andra Pradesh, India)[34].

The activity of Na^+/K^+-dependent ATPase was determined by the method of Bonting[35]. In this assay, 0.2 ml of brain tissue homogenate was added to the mixture containing 1 ml of 184 mM Tris-HCl buffer (pH 7.5), 0.2 ml of 50 mM $MgSO_4$, 0.2 ml of 50 mM KCl, 0.2 ml of 600 mM NaCl, 0.2 ml of 1 mM EDTA and 0.2 ml of 10 mM ATP and incubated for 15 min at 37°C. The reaction was arrested by the addition of 1 ml of ice cold 10% TCA. Then the amount of Pi liberated was estimated in protein free supernatant.

The activity of Ca^{2+}-ATPase was assayed according to the method of Hjertan and Pan[36]. In brief, 0.1 ml of tissue homogenate was added to a mixture containing 0.1 ml of 125 mM Tris-HCl buffer (pH 8), 0.1 ml of 50 mM $CaCl_2$ and 0.1 ml of 10 mM ATP. The contents were incubated at 37°C for 15 min. The reaction was then arrested by the addition of 0.5 ml of ice cold 10% TCA and centrifuged. The amount of Pi liberated was estimated in supernatant.

The activity of Mg^{2+}-ATPase was assayed by the method of Ohinishi et al.[10]. The contents were incubated for 15 min at 37°C and the reaction was arrested by adding 0.5 ml of 10% TCA. The Pi liberated was then estimated in protein free supernatant. The activities of these ATPase enzymes in tissue homogenate were expressed as µg Pi liberated /min/mg protein.

Processing of Tissues for Histopathological Studies

One half of brain was fixed in 10% formalin solution. The fixed tissues were processed, embedded in paraffin and sectioned (5-6 µ). The sections were stained with hematoxylin and eosin (H and E) and observed under microscope. The brain sections were examined for gliosis, pycnosis, spongiosis, inflammatory infiltrate, oedema and meningeal changes.

Statistical Analysis

Values are given as the mean ± S.D. Significant difference between the means of the six groups was statistically analyzed by Duncan's Multiple Range Test (DMRT). The significance levels was set at $p<0.05$ for all the tests. Statistical analysis was performed using SPSS 11.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Body Weight, Organ Weight, and Relative Organ Weight Food and Water Intake

Table I depicts the effects of Cd and melatonin on body weight gain, food and water intake and relative brain weight in control and experimental rats. In Cd treated rats, water and pellet diet consumption significantly ($p<0.05$) decreased with a decrease in body weight. A significant ($p<0.05$) decrease in relative brain weight was recorded in Cd treated rats when compared with control rats. Treatment with melatonin effectively attenuated the Cd-induced alterations in food and water intake, body weight and relative brain weight, when compared with Cd treated rats. Administration of melatonin alone to rats did not show any alterations in these parameters and did not differ significantly from that of the normal control group.

Plasma and Brain AChE

The activity of AChE in plasma and brain and of control and experimental rats are shown in Figure 1. The activities of AChE in brain and plasma was significantly ($p<0.05$) decreased in cadmium treated rats when compared with control rats whereas the administration of melatonin in cadmium intoxicated rats significantly ($p<0.05$) increased the activities of AChE to near normal levels when compared with cadmium treated rats.

Oxidative Stress Markers in Brain

The data presented in Table II shows the changes in the levels of brain lipid peroxidation indices thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and protein carbonyl con-

Table I. Effect of melatonin and cadmium on body weight, organ weight, relative organ weight, food and water intake of control and experimental rats.

Groups	Control	Cadmium	Cd + melatonin	Melatonin
Body weight (g)				
Initial	184.3 ± 1.9	237.5 ± 5.1	183.7 ± 2.7	184.0 ± 2.4
Final	224.7 ± 4.5	182.5 ± 2.9	235.2 ± 4.1	237.8 ± 4.9
Organ weight (g)				
Brain	1.72 ± 0.03[a]	1.59 ± 0.02[b]	1.67 ± 0.03[c]	1.70 ± 0.04[a]
Organ-body weight ratio (%)				
Brain	0.72 ± 0.005[a]	0.69 ± 0.004[b]	0.71 ± 0.007[c]	0.71 ± 0.008[a]
Food intake (g/100g body wt/day)	12.08 ± 0.92[a]	8.15 ± 0.67[b]	10.59 ± 0.81[c]	11.96 ± 0.86[a]
Water intake (ml/rat/day)	18.30 ± 2.40[a]	13.20 ± 1.20[b]	16.20 ± 1.50[c]	18.40 ± 2.30[a]

Values are mean ± SD for six rats in each group. Values not sharing a common superscript letters (a, b and c) differ significantly at $p<0.05$ (DMRT).

tent (PCC) in control and experimental rats. The rats treated with Cd the levels of plasma TBARS, LOOH and PCC were increased ($p<0.05$) significantly when compared with control rats. Administration of melatonin in cadmium intoxicated rats significantly ($p<0.05$) decreased the level of these oxidative stress markers in brain when compared with cadmium treated rats.

DNA Fragmentation

Figure 2 represents the DNA fragmentation. In Figure 2A, a smear on agarose gel has been observed in cadmium treated group, indicating random DNA degradation. Melatonin pretreatment was found to be effective to prevent the toxin induced smear formation. In addition, quantitative measurement of DNA fragmentation (by the colorimetric diphenylamine reaction) has also been represented by Figure 2B. In agreement with the above findings, cadmium intoxication increased the extent of DNA fragmentation ($p<0.05$) and that could be prevented by the pretreatment with melatonin.

Non-Enzymatic Antioxidants in Brain

The level of non-enzymatic antioxidants (GSH, TSH and vitamins C and E) in the brain of control and experimental rats were shown in Table III. The levels of GSH, TSH, vitamins C and E were significantly ($p<0.05$) decreased in the brain tissues of cadmium intoxicated rats when compared to control rats. Administration of melatonin in cadmium treated rats significantly ($p<0.05$) protected the depleted levels of GSH, TSH, vitamins C and E contents in brain when compared with cadmium treated rats.

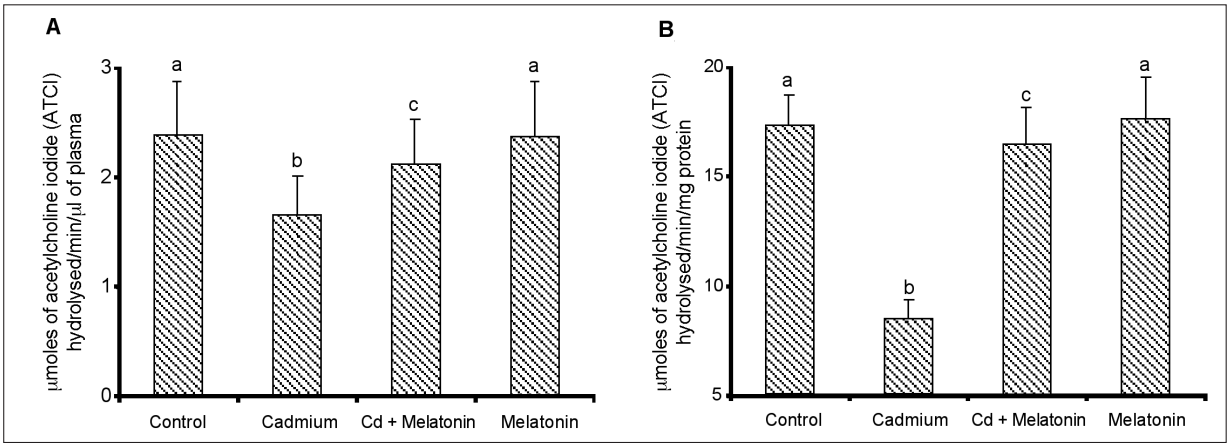

Figure 1. Changes in the activities of acetylcholinesterase (AChE) in A, plasma and B, brain of control and experimental rats. ATCI: acetyl thiocholine iodide; Values are mean ± SD for six rats in each group. Values not sharing a common superscript letters (a, b and c) differ significantly at $p<0.05$ (DMRT).

Table II. Effect of melatonin and cadmium on the levels of TBARS, lipid hydroperoxides and protein carbonyl contents in the brain of control and experimental rats.

Groups	Control	Cadmium	Cd + melatonin	Melatonin
TBARS (nmol/mg protein)	1.76 ± 0.049^{a}	2.37 ± 0.068^{b}	1.89 ± 0.051^{c}	1.74 ± 0.038^{a}
Lipid hydroperoxides (nmol/mg protein)	1.72 ± 0.031^{a}	2.34 ± 0.052^{b}	1.84 ± 0.039^{c}	1.68 ± 0.027^{a}
Protein carbonyl content (nmol/mg protein)	3.59 ± 0.41^{a}	7.67 ± 0.81^{b}	5.16 ± 0.63^{c}	4.71 ± 0.71^{ac}

Values are mean ± SD for six rats in each group. Values not sharing a common superscript letters (a, b and c) differ significantly at $p<0.05$ (DMRT).

Table III. Effect of melatonin and cadmium on the levels of non-enzymatic antioxidants in the brain of control and experimental rats.

Groups	Control	Cadmium	Cd + melatonin	Melatonin
GSH Brain (µg/mg protein)	2.97 ± 0.16^{a}	1.54 ± 0.07^{b}	2.49 ± 0.21^{c}	3.12 ± 0.24^{a}
TSH Brain (µg/mg protein)	7.89 ± 0.47^{a}	4.78 ± 0.34^{b}	6.17 ± 0.51^{c}	8.74 ± 0.59^{ac}
Vitamin C Brain (µg/mg protein)	0.27 ± 0.019^{a}	0.15 ± 0.007^{b}	0.21 ± 0.012^{c}	0.26 ± 0.018^{a}
Vitamin E Brain (µg/mg protein)	0.28 ± 0.024^{a}	0.16 ± 0.018^{b}	0.23 ± 0.021^{c}	0.29 ± 0.039^{a}

Values are mean ± SD for six rats in each group. Values not sharing a common superscript letters (a, b and c) differ significantly at $p<0.05$ (DMRT).

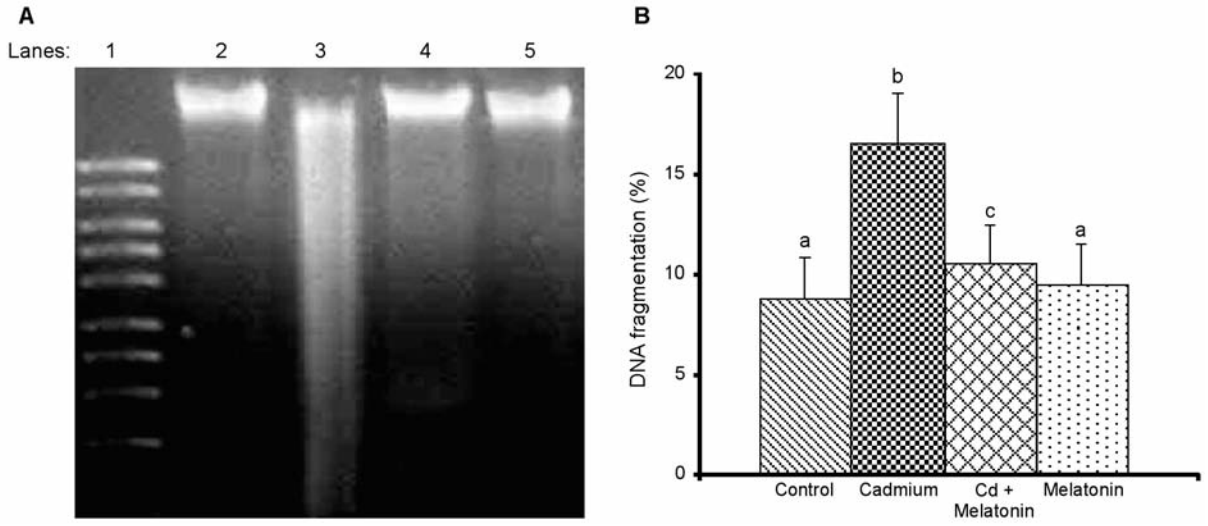

Figure 2. ***A,*** DNA fragmentation on agarose-ethidium bromide gel. DNA isolated from control and experimental brain tissues was loaded into 1% (w/v) agarose gels. Lane 1, marker (1 kb DNA ladder); lane 2, DNA isolated from control rat brain; lane 3, DNA isolated from Cd intoxicated rat brain; lane 4, DNA isolated from melatonin and Cd treated brain. ***B,*** Effect of melatonin on Cd induced extent of DNA fragmentation. Cont: DNA content in normal; Cd: DNA content in Cd intoxicated brain; Cd + Melatonin: DNA content in melatonin and Cd treated brain tissue; Melatonin: DNA content in melatonin treated brain. Each column represents mean ± SD, n = 6. Values not sharing a common superscript letters (a, b and c) differ significantly at $p<0.05$ (DMRT).

Table IV. Effect of melatonin and cadmium on the levels of enzymatic antioxidants in the brain of control and experimental rats.

Groups	Control	Cadmium	Cd + melatonin	Melatonin
SOD (units)	8.29 ± 0.31^{a}	5.81 ± 0.17^{b}	7.24 ± 0.26^{c}	8.21 ± 0.34^{a}
CAT (units)	3.19 ± 0.28^{a}	1.07 ± 0.11^{b}	2.72 ± 0.21^{c}	3.21 ± 0.31^{a}
GPx (units)	3.28 ± 0.31^{a}	1.24 ± 0.14^{b}	2.83 ± 0.24^{c}	3.27 ± 0.38^{a}
GST (units)	5.62 ± 0.14^{a}	4.17 ± 0.08^{b}	5.14 ± 0.18^{c}	5.57 ± 0.11^{a}

Values are mean ± SD for six rats in each group. Values not sharing a common superscript letters (a, b and c) differ significantly at $p<0.05$ (DMRT). SOD units – nitroblue tetrazolium reduction/min/mg protein; CAT units – mmoles H_2O_2 consumed/min/mg protein; GPx units – µg of GSH consumed/min/mg protein; GST units – mmol of GSH-CDNB conjugate formed/ min/mg protein.

Enzymatic Antioxidants Brain

The activities of enzymatic antioxidants (SOD, CAT, GPx and GST) status in the brain of control and experimental rats were presented in Table IV. A significant ($p<0.05$) decrease in the activities of SOD, CAT, GPx and GST were observed in cadmium intoxicated rats when compared with control rats. Treatment with melatonin in cadmium intoxicated rats showed a significant ($p<0.05$) increase in the activities of antioxidant enzymes in the brain when compared with cadmium intoxicated rats.

Membrane Bound ATPases in Brain

The data in Table V summarizes the changes in the activities of ATPase enzymes (Na^+K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase) in the brain of control and experimental rats. A significant ($p<0.05$) decrease in the activities of ATPase enzymes were observed in cadmium intoxicated rats as compared with control rats. Treatment with melatonin in cadmium treated rats significantly ($p<0.05$) elevated the activities of ATPase enzymes in brain when compared with cadmium alone treated rats.

Histological Changes in Brain

Figure 3 illustrates the histopathological assessment of brain tissue of control and experimental animals. Cd intoxicated rats exhibited marked gliosis, nuclear pycnosis spongiform necrosis and lymphocytic inflammatory infiltrates as against normal architecture shown by the brain of vehicle and melatonin control rats (Figure 3). Treatment with melatonin prior to the Cd intoxication reduced the incidence of these pathological changes in the brain tissue and showed almost normal architecture similar to those of the untreated control.

Discussion

The brain has a high rate of oxidative metabolism, consuming ~20% of the cardiac output. At the same time, the brain compared to lung, liver and other organs, contains relatively low levels of enzymatic and non enzymatic antioxidants and high amounts of peroxidizable unsaturated lipids, rendering it more vulnerable to oxidative stress compared to other tissues[37]. Increasing evidences suggested that, excessive production free radicals

Table V. Effects of melatonin and cadmium on the activities of membrane bound ATPases in the brain of control and experimental rats.

Groups	Control	Cadmium	Cd + melatonin	Melatonin
Total ATPases	1.42 ± 0.18^{a}	0.98 ± 0.12^{b}	1.37 ± 0.15^{c}	1.43 ± 0.16^{a}
Na^+K^+ ATPases	0.40 ± 0.07^{a}	0.23 ± 0.02^{b}	0.35 ± 0.05^{c}	0.39 ± 0.06^{a}
Ca^{2+} ATPases	0.43 ± 0.06^{a}	0.27 ± 0.03^{b}	0.38 ± 0.04^{c}	0.42 ± 0.05^{a}
Mg^{2+} ATPases	0.32 ± 0.04^{a}	0.19 ± 0.02^{b}	0.29 ± 0.03^{c}	0.31 ± 0.03^{ac}

Values are mean ± SD for six rats in each group. Values not sharing a common superscript letters (a, b and c) differ significantly at $p<0.05$ (DMRT). The activities of ATPase were expressed as µg pi liberated/min/mg protein.

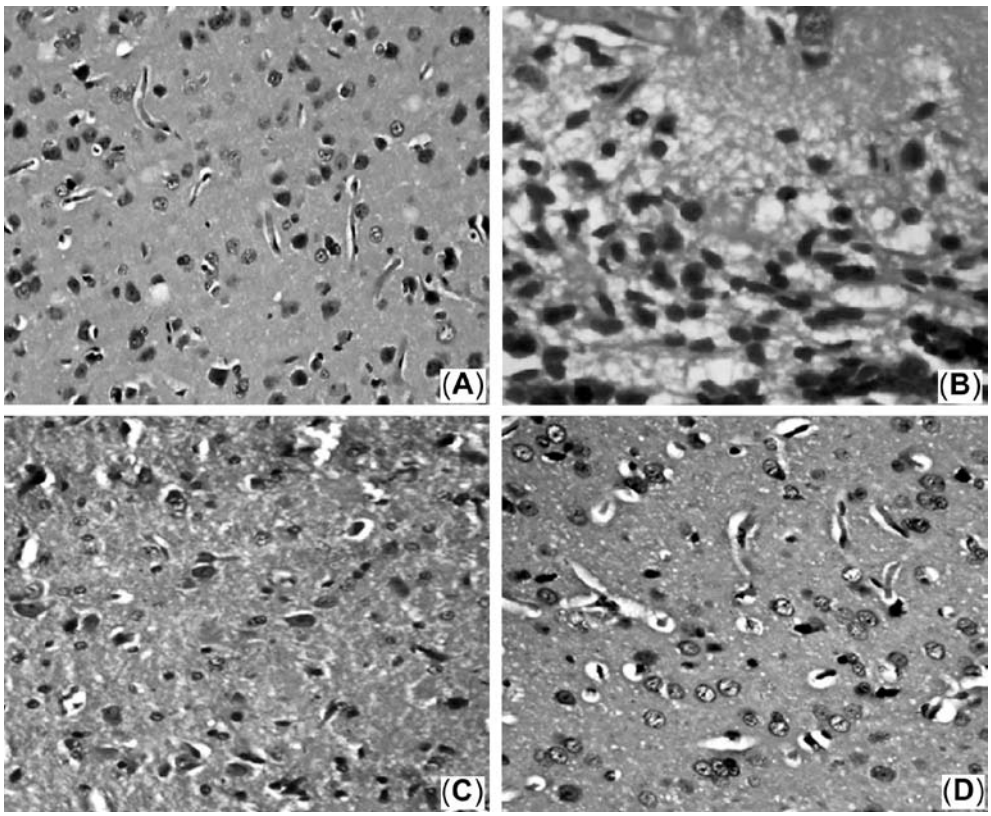

Figure 3. Representative photomicrographs showing histopathological changes in rat brain (H & E , 200X) of control and experimental rats. ***A,*** Control rat brain showing the normal histological architecture. ***B,*** Cadmium intoxicated rats showing spongiform necrosis, nuclear pycnosis and lymphocytic inflammatory changes. ***C,*** Cadmium and melatonin treated rats showing near normal architecture of the brain tissue. ***D,*** Melatonin control rats showing normal histology of brain.

in brain and the imbalance between oxidative species and antioxidant defenses are related to the pathogenesis of neurodegenerative diseases[38]. Recent studies have shown that cadmium produces ROS, resulting in an increased lipid peroxidation, depletion of sulphydryls, altered calcium homeostasis, impairment of antioxidant defenses and finally DNA damage[8,39]. Neurotoxic potential of cadmium in experimental animals is mainly due to its impairment of the catecholominergic and seretoninergic transmission[40].

The study of brain enzyme activities, such as AChE is a key enzyme in detecting the neurotoxic effect of certain heavy metals. Numerous studies have suggested that the free radicals production could at least in partly associated with the decreased activity of brain AChE[41]. It has been reported that decreased activity AChE leads to the accumulation of acetylcholine, which causes cholinergic hyperactivity, convulsion and status epilepticus[42]. Alterations in the mechanisms of neurotransmitters release have also been implicated in cadmium neurotoxicity and cadmium may block the influx of Ca^{2+} through membrane channels into the nerve terminal following the action potential. These decreases in calcium influx caused by cadmium would be associated with an altered transmitter release[43].

Since the brain AChE activity is an important regulator of the behavioral processes, the decreased level of AChE in plasma and brain might be one of the indicators for cadmium induced toxic manifestations in brain. Administration of melatonin in cadmium intoxicated rats restored the activity of AChE in plasma and brain. An important aspect of melatonin's role in neuroprotection is by the attenuation of radical formation through its anti excitatory and anti-inflammatory effects. It also efficiently interacts with various reactive oxygen and nitrogen species as well as organic radicals[44]. The efficacy of melatonin in cadmium induced neurotoxicity is mainly attributed to its high lipophilicity[45]. It is well documented that the lipophilic agents are found to be very effective in reducing toxic effects of heavy metals and their elimination from various organ systems[46,47].

The ability of cadmium to induce oxidative stress in brain cells has been reported as the induction of ROS, after the interaction of Cd^{2+} with mitochondrial sites, leading to the breakdown of the mitochondrial potentials, a consequent reduction of intracellular thiols and antioxidants[8]. Monroe and Halvorsen[48] provided the evidence that the neuronal inhibition Jak Kinase activated selectively in neurons by increasing intracellular levels of oxidative stress offers a new mechanisms by which cadmium may exert their neurotoxic effect.

Cadmium can penetrate the blood brain barrier (BBB) and accumulate into the brain which is easily susceptible to cadmium induced lipid peroxidation. The major forms of cellular damage in brain are lipid peroxidation, protein oxidation and thiol depletion. The present study demonstrates the elevated levels of lipid peroxidation, increased formation of lipid hydroperoxides and protein carbonyls with reduced level of TSH in the brain of cadmium treated rats might be due to the over production of free radicals and lipid peroxidative products, which leads to oxidative modifications of proteins. Oxidative damage to proteins as assessed by formation of carbonyl groups is a highly damaging event and may occur even in the absence of lipid peroxidation[49].

Under the present experimental conditions, it has been observed that cadmium toxicity increased the extent of DNA fragmentation which may be regarded as an indicator of increased ROS production during toxin exposure period. Treatment with melatonin along with cadmium, however, effectively inhibited the metal toxicity by decreasing the level of DNA fragmentation via its quenching effect on reactive oxygen species and their toxic intermediates during cadmium intoxication.

Growing evidence suggests the impairment of antioxidant defense system in the brain of cadmium intoxicated rats[12,40]. GSH is the most abundant non-protein thiol that maintains the cellular redox status and providing first line of antioxidant protection against oxidative stress in brain[50]. Thiols are potent chelators capable of mobilizing even intracellularly bound cadmium and also provide an antioxidant defense function by removing cadmium from the site of deleterious oxidant reactions[51]. The diminished levels of GSH and TSH in cadmium intoxication could be due to increased utilization to overwhelm the production of free radicals by Cd and subsequent lipid peroxidation in brain. Vitamin C is considered as the most important antioxidant of plasma and also acts as an anti-stress factor. It scavenges the free radicals produced by Cd[52]. Vitamin E, the lipid soluble chain breaking antioxidant also plays a critical role in detoxifying the Cd toxicity[53]. GSH is an important antioxidant defense, which forms complexes with Cd through the free sulphydryl group and, thereby, alters Cd distribution and excretion[54]. In agreement with this, the depletion of non-enzymic antioxidant levels in plasma may be due to increased utilization to reduce the Cd induced oxidative stress. The direct free radical scavenging ability of melatonin decreased the Cd induced lipid peroxidation, which could reduce the utilization of non-enzymic antioxidants and consequently lead to improvement of GSH, TSH, vitamins C and E levels in brain.

The detoxification of ROS in brain involves the co-operative action of the intracellular antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase. The decreased activity of these antioxidant enzymes in the brain of cadmium intoxicated rats was also observed in the present study. The diminished activities of these antioxidant enzymes might be due to the binding of cadmium with sulphydryl group of enzymes, replacement of essential metals from their active sites and oxidative modification of amino acid side chains, which alters the enzyme structure and leads to the inactivation or impaired activity of enzymes. Thus, the decreased activity of brain antioxidant system by cadmium leads to the accumulation of free radicals and increased the levels of LPO, which in turn increases the oxidative damage to the brain tissue.

Administration of melatonin in cadmium intoxicated rats significantly attenuated the increased levels of lipid peroxidation and protein carbonyl formation with concomitant improvement in the levels of brain enzymatic and non-enzymatic antioxidant defenses. An effective therapy of cadmium toxicity involves both metal chelating and antioxidant actions. The protective action of melatonin against LPO as a factor modifying membrane organization may due to melatonin's ability to scavenge the LPO initiating agents, which produces during the peroxidation of lipids[16]. Since membrane structure and functions are influenced by proteins in membrane and cadmium is known to damage thiol proteins[15], it is possible that the protective action of melatonin to membrane damage induced by cadmium may be partially related to the ability of the indole to prevent protein damage. Additionally melatonin reportedly stimulates several antioxidant en-

zymes, including glutathione reductase, glutathione peroxidase and superoxide dismutase, promoting quick disposal of H_2O_2 from rat brain cortical cells[55] and also enhances the production of enzymes that are involved in the synthesis of glutathione by inducing the expression of γ-glutamyl cysteine synthetase (γ-GCS), the rate limiting enzyme in glutathione synthesis[56]. From these studies, it is, thus, suggested that melatonin due to its free radical scavenging property it may reduce the consumption of endogenous antioxidants and, thereby, inhibit the cadmium mediated free radicals generation and elevated levels of lipid peroxidation in brain.

Cadmium enhances the production of free radicals in the brain of adult rat and interferes with the antioxidant defense system which in turn leads to a cadmium induced alteration of the structural integrity of membrane lipids and secondarily affect the membrane bound enzymes such as Na^+K^+-ATPase[12]. The saturation of lipid bilayer of several areas of brain regions as a result of LPO in cadmium exposed animals causes disturbances in membrane fluidity and intracellular Ca^{2+} concentrations[39]. In the present study rats treated with cadmium showed a significant inhibition in the activities of membrane bound ATPases which is in consonant with the reports of Pari and Murugavel[13] which showed the decreased levels of membrane bound ATPase in the brain of cadmium intoxicated rats.

Na^+K^+-ATPase is a key enzyme implicated in neural excitability, metabolic energy production, as well as uptake and release of catecholamines[57] and serotonin[58]. The role of Mg^{2+} ATPase is to maintain the high intracellular Mg^{2+} level in brain. Changes of which can control the rate of protein synthesis and cell growth[59]. Ca^{2+} ATPase functions as secondary messenger in central nervous system. The alterations in Ca^{2+} level also leads to severe pathological lesions in brain[60]. The Ca^{2+} overload mediated by cadmium also inhibit the Ca^{2+} ATPase activity in cell membrane and eventually potentiate irreversible cell destruction[61]. The activities of the ATPase enzymes are affected by the exposure of cadmium[40] indicating the alterations in membrane and neurotransmitter functions.

Several studies have shown that cadmium is a potent inhibitor of brain Na^+K^+ ATPase, Mg^{2+}ATPase and it also inhibits the choline transport in synaptosomes. Cadmium interacts either by inhibiting or stimulating the activity of adenylate cyclase, depending upon the concentration of cation, presumably through its interaction with an enzymatic site closely related to the allosteric site of the regulatory unit of the Cd-ATPase complex. The decreased activity of ATPases could also be due to the SH binding nature of cadmium or through its oxidative stress in brain[40]. Administration of melatonin in cadmium intoxicated rats markedly restores the activities of membrane bound ATPases, which denotes that melatonin conserves the integrity of cell membrane and normal physiological functions of brain. The free radical scavenging nature of melatonin along with its ability to preserve the mitochondrial function[44] might contribute to the enhancement of brain ATPases activity in cadmium intoxicated rats following the administration of melatonin.

Histological examination of the brain tissue reveals that cadmium intoxication caused abnormal ultra structural changes in the brain tissue including spongiform necrosis, nuclear vacuolization pycnosis and lymphocytic inflammatory changes. Regarding the histopathological observation, no significant difference has been observed between control and melatonin treated groups. Melatonin pretreated cadmium intoxicated rats the observed pathological impairments by cadmium have been recovered significantly which indicates that melatonin is capable of preventing the neuronal damage induced by cadmium. Therefore, it may be suggested that melatonin might inhibit Cd induced brain damage.

In conclusion, the results of the present study demonstrates that melatonin exhibited a significant protective action against cadmium induced neurotoxicity in rats *via* inhibiting the lipid peroxidation, protein carbonyl formation and increasing the endogenous antioxidant defense systems in plasma and brain with subsequent restoration of AChE, membrane bound ATPase enzymes and normal histoarchitecture of the brain tissue. Further, melatonin also restored the normal histological architecture in the brain of Cd intoxicated rats. Accordingly it may be suggested that melatonin can serve as a potential therapeutic candidate for the brain injury associated with Cd induced oxidative stress in the brain.

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