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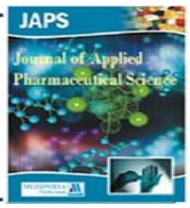

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Neuroprotective role of Melatonin against aluminum-induced oxidative stress in the hippocampus of mouse brain

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ABSTRACT

We evaluated the effect of melatonin (Mel) in male albino mice which received aluminum acetate (Al) for 6 weeks (3.5 mg/kg body weight) (b.w.) i.p. five times per week. Moreover mice received Mel (7mg/kg b.w.i.p. 5 days/week) for 6 weeks. At the end of the treatment hippocampus was removed and processed to examine the oxidative stress markers. Following Al exposure oxidative stress increased significantly, estimated by increased thiobarbituric acid reactive substances (TBARS) and decrease in the activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), reduced glutathione (GSH),glutathione peroxidase (GPx) and glutathione-s-transferase (GST). Al+Mel treatment significantly prevented the aluminum induced decrease in antioxidant enzymes as well as decrease in TBARS. Histopathological evidence in the hippocampi revealed the protective effect of melatonin against Al induced damage in light and transmission electron microscopic (TEM) studies. These results supported that melatonin suppresses the oxidative stress. This may result from the higher efficacy of melatonin in scavenging various free radicals and also because of its ability in stimulating the anti oxidant enzymes.

Keywords: Aluminum, Hippocampus, Oxidative stress, Melatonin.

INTRODUCTION

Aluminum (Al) is the third most abundant metal in the earth's crust. Exposure to Al is almost inevitable, since it is present in air, soil and water (Yang et al., 2008). It is a highly neurotoxic element that may be involved in neuronal degeneration in human and experimental animal brains (Yumoto et al., 2001). It is well established that it induces the production of free radicals in brain. Accumulation of free radicals may cause degenerative events of aging such as alzheimer's disease(AD) (Gracia et al.,2010).This metal may produce adverse physiological effects, however there is no known physiological role for aluminum within the body (Kumar and Gill, 2009). Various epidemiological studies have suggested that, Al play a pathogenic role in AD and known to cross–link hyperphosphorylated proteins, may play role in the pathogenesis of critical neuropathologic leisions in AD (Kawahara 2005; Perl and Moalem, 2006; Shcherbatykh and Carpenter 2007). Treatment with different aluminum compounds to induce neuropathology have yielded several interesting observations, the neuropathologies associated with high brain aluminum levels, including structural, biochemical and neurobehavioural changes (Nayak, 2002) and tend to reduce nerve synapses and conduction, promoting neuro toxicity (Yokel 2000; Yokel et al.,2001). Several investigators have proposed the possible involvement of ROS generation in the onset of aluminum neurotoxicity (Ohyashiki et al., 2002; Savory et al., 2003; Exley, 2004; Sushma et al., 2006). The exact mechanism of aluminum toxicity is not known but accumulating

evidence suggest that the metal can potentiate oxidative and inflammatory events eventually leading to tissue damage (Becaria et al., 2002). Melatonin (Mel) is a very potent antioxidant (Gracia et al., 2010) and has been shown to be effective in arresting neurodegenerative phenomenon (Srinivasan et al., 2005). *In vivo* and *in vitro*, Mel has been known as a radical scavenger with the ability to remove reactive oxidant species (ROS) and reactive nitrogen species (RNS). The melatonin interaction with ROS/RNS is a prolonged process that involves many of its metabolites. This reaction is a novel property of melatonin and explains how it differs from other conventional anti oxidants (Mahieu et al., 2009). Mel and its metabolites have been found to protect tissues against oxidative damage generated by a variety of toxic agents and metabolic processes (Tan et al., 2007; Manda et al., 2007; Peyrot and Ducrocq, 2008). It is able to prevent oxidative stress both through its free radicals scavenging effect and by directly increasing anti oxidant activity (Reiter and Tan, 2003). Melatonin posses the electron rich aromatic indole ring and functions as an electron donor there by reducing and repairing electrophilic radicals (Martinez et al., 2005). Different studies have demonstrated its protective role against Oxidative damage induced by drugs/toxins and different diseases (Al arcon deta lastra et al., 1999; Leon et al., 2004; Munoz Casares et al., 2006). Melatonin possesses several unique advantages. First, its solubility in both lipids and water allows melatonin to be easily distributed into the cell, secondly, its ability to cross the blood brain barrier (BBB) allows melatonin to enter the cental nervous system (Reiter et al., 1999). The melatonin binds aluminum such a binding may shed light into the role of this element in the etiology of AD (Lack et al., 2001). The role of melatonin as a metal chelator has attracted the interest of some investigators (Limson et al: 1998, Gulcin et al., 2003).

In the present study, we aimed to investigate the neuroprotective effect of Melatonin against aluminum-induced oxidative stress changes in the mice hippocampus. Furthermore, we assessed the ability of melatonin to attenuate the aluminum effects on histopathology.

MATERIALS AND METHODS

Animals and treatments

Male adult albino mice weighing between 25 and 30g were used. The animals were housed under conditions of constant temperature (22-24ºC) and humidity (45-50%) in room with a fixed 12h artificial light-dark cycles. Animals were allowed free access to tap water and standard mice chow *adlibitum*. Mice were randomly divided into four groups as follows: group C: control mice; group Al: aluminum acetate treated mice; group Mel: Melatonin treated mice and group Al+Mel: aluminum acetate plus melatonin treated mice.

Al-treated mice received an intraperitoneal injection of aluminum acetate (Sigma Aldrich, USA) during 6 weeks (3.5 mg/kg body weight (b.w), 5 days per week. Aluminum acetate was dissolved in distilled water. Control mice (C) were injected with physiological saline during the same period and frequency. Group Mel received intraperitoneal injections of melatonin at dose of 7mg/kg b.w. and group Al+Mel received both aluminum acetate (3.5 mg/kg b.wt., i.p) and melatonin (7mg/kg/b.wt., i.p) simultaneously during same period and frequency. Body weights were monitored weekly. All experiments were performed in accordance with the National Institutes Health Guidelines for the Care and the Use of Laboratory that were approved by institution's committee

Preparation of tissue homogenates

After the 6 weeks of treatment mice were (n=20 for each group) weighed and anesthetized by intraperitoneal injection of sodium pentobarbital. Subsequently, animals were decapitated and brains were removed. Each brain was placed on ice cold plate and brain region hippocampus was dissected. The dissection procedures required less than 3 min per animal. Twenty animals per group were used for biochemical assays.

Biochemical studies in brain regions

The tissues were homogenized with a polytron homogenizer in different buffers as follows (1) in 50mM phosphate buffer (pH -7.0) containing 0.1Mm EDTA to give 10% homogenate(w/v).(2) in ice cold 50mM tris-HCL buffer(pH-7.4)containing 0.2M sucrose for glutathione -s-transferase(GST).

Measurement of oxidative stress markers

Superoxide dismutase (SOD) was determined according to the method of Misra and Fridovich (1972), Catalase acitivity (CAT) was determined by Aebi 1984. Thio barbituric acid reactive substances (TBARS) was carried out according to the method of Okahawa et al., (1979). Glutathione reductase (GR) activity was assayed according to the modified method of Carlberg and Manervick (1985). Reduced glutathione (GSH) was assayed according to the method of Ellman et al. (1959). Glutathione peroxidase (GPx) activity was determined according to the modified version of flohe and gunzler (1984), glutathione–s-transferase (GST) was assayed according to Habig et al., (1974), and Protein content was determined by Lowry et al., (1951) using bovine serum albumin as standard.

Microscopic studies

Light microscopy

The brain regions were fixed in 10% formalin solution .The tissues were dehydrated through a graded series of alcohols , the tissues were cleared in methyl benzoate, embedded in paraffin wax. Sections were cut up at 6m in thickness and stained with hematoxylin (Harris, 1990) and counter stained with eosin (dissolved in 95% alcohol). After dehydration and clearing, sections were mounted with DPX and observed under microscope.

Transmission electron microscopy (TEM)

The brain regions of different treated mice were fixed in 2.5%-3% glutaraldehyde in 0.1M phosphate buffer (pH-7.2) for 24 hr at 4ºC and post fixed in 2% aqueous Osmium tertroxide in the same buffer for 2hr. Dehydrated in series of graded alcohol, infiltrated and embedded in araldite 6005 resin or spur resin (Spur

1969). Ultra thin (50-70nm) sections were made with a glass knife on ultramicrotome (Leica ultra cut UCT-GA-D/E-1/00). Mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate (Bozzola and Russel, 1998) viewed under TEM (Hitachi, H-7500 from Japan).

Statistical analysis

Results are expressed as mean ±S.D (standard deviation of the mean).Data were analyzed using the one way analysis of variance followed by scheffe contrast. The 0.05 level of probability was used as the criterion of significance in all cases. The statistical analysis was made with statistical package for social sciences (SPSS) program (1995).

RESULTS

Effect on body weight

During the period of treatment, animals in the groups exposed to Al alone or in combination with melatonin had a body weight gain significantly lower than that observed in the control and melatonin only groups (Fig.1).

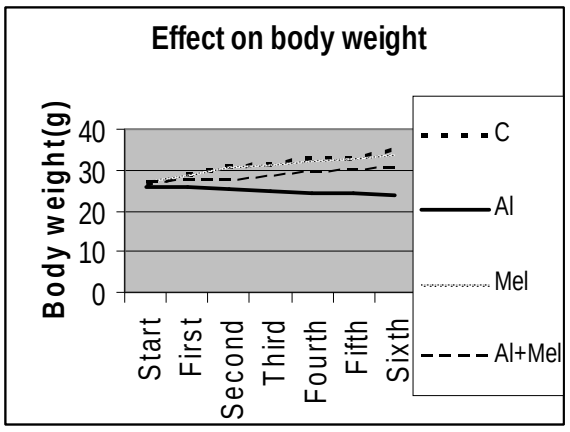

Fig.1 Changes in the body weights during the period of treatment.

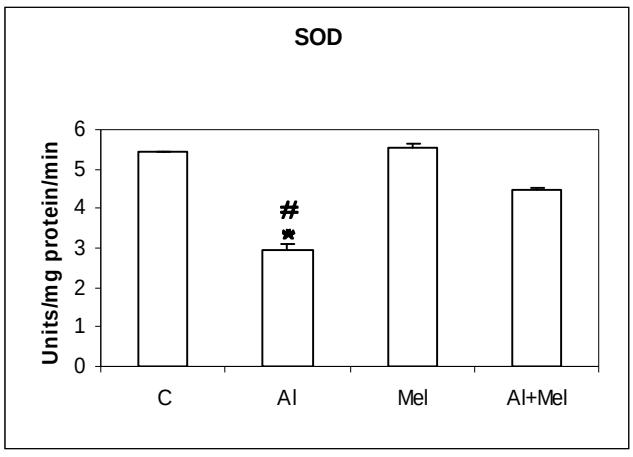

Fig.2 SOD: superoxide dismutase. Data expressed as mean± S.D of six mice in each group.Al:aluminum, Mel:melatonin.Groups-C:Control mice;Mel:mice treated with Mel;Al:mice treated with Al; Al+Mel:mice treated with Al+Mel. *statistically different from control and Mel values ($p<0.05$). # statistically different as compared with Al+Mel values ($p<0.05$).

Oxidative stress markers

Effect on SOD and CAT levels

Al exposure decreased the SOD and CAT activities. Mel treatment induced an increment in SOD (Fig.2) and CAT (Fig.3) activities. SOD and CAT activities were not different from the values obtained in control and Mel treated groups.

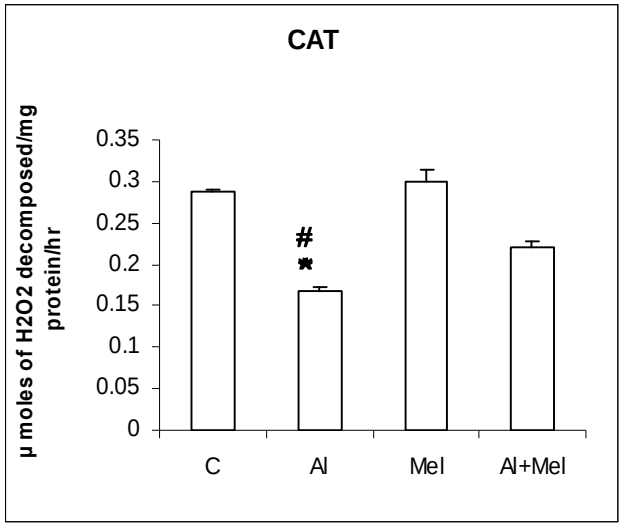

Fig.3 CAT: catalase. Data expressed as mean± S.D of six mice in each group. Al: aluminum, Mel: melatonin. Groups-C: Control mice; Mel: mice treated with Mel; Al: mice treated with Al; Al+Mel: mice treated with Al+Mel. *statistically different from control and Mel values ($p<0.05$). # statistically different as compared with Al+Mel values ($p<0.05$)

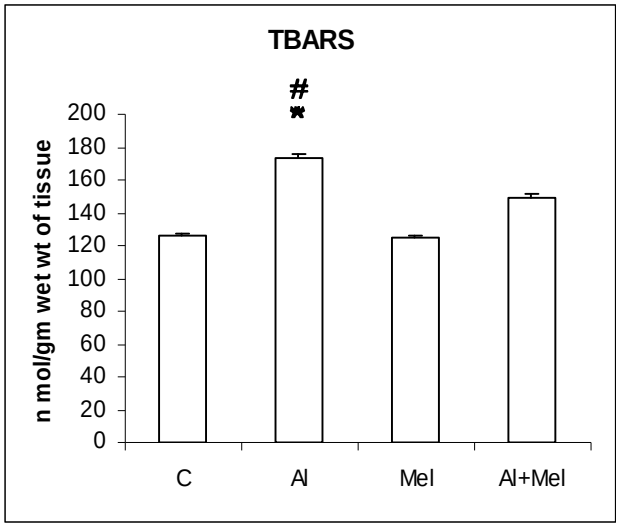

Fig.4 TBARS: thiobarbituric acid reactive substances. Data expressed as mean± S.D of six mice in each group. Al: aluminum, Mel: melatonin. Groups-C: Control mice; Mel: mice treated with Mel; Al: mice treated with Al; Al+Mel: mice treated with Al+Mel. *statistically different from control and Mel values ($p<0.05$). # statistically different as compared with Al+Mel values ($p<0.05$).

Effect on TBARS levels

Brain tissue levels of TBARS, measured as lipid peroxidation end product malondialdehyde (MDA) were higher in Al group than in control mice (Fig.4). TBARS in Al+Mel group and Mel groups were lower than those of Al group.

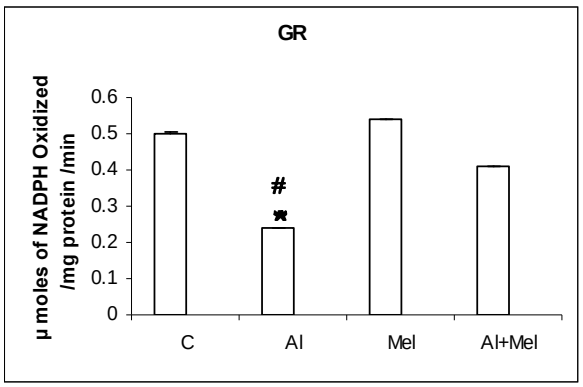

Fig.5 GR: glutathione reductase. Data expressed as mean± S.D of six mice in each group. Al: aluminum, Mel: melatonin. Groups-C: Control mice; Mel: mice treated with Mel; Al: mice treated with Al; Al+Mel: mice treated with Al+Mel. *statistically different from control and Mel values ($p<0.05$). # statistically different as compared with Al+Mel values ($p<0.05$).

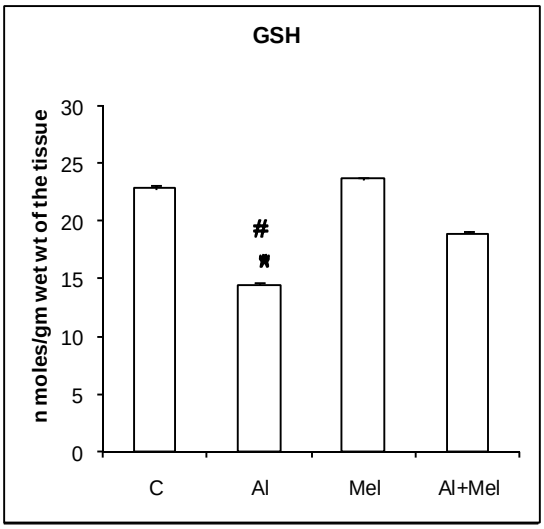

Fig.6 GSH: reduced glutathione. Data expressed as mean± S.D of six mice in each group. Al: aluminum, Mel: melatonin. Groups-C: Control mice; Mel: mice treated with Mel; Al: mice treated with Al; Al+Mel: mice treated with Al+Mel. *statistically different from control and Mel values ($p<0.05$). # statistically different as compared with Al+Mel values ($p<0.05$).

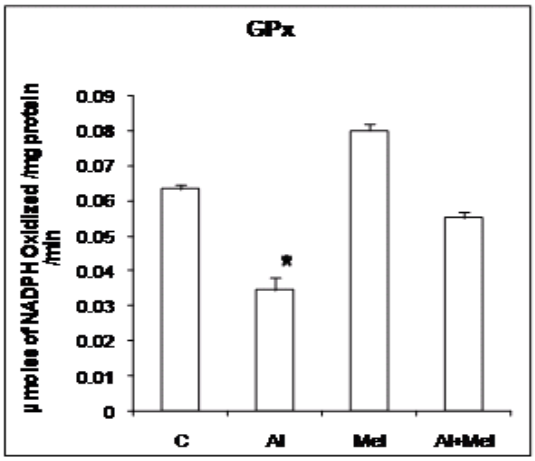

Fig.7 GPx: glutathione peroxidase. Data expressed as mean± S.D of six mice in each group. Al: aluminum, Mel: melatonin. Groups-C: Control mice; Mel: mice treated with Mel; Al: mice treated with Al; Al+Mel: mice treated with Al+Mel. *statistically different from control and Mel values ($p<0.05$). # statistically different as compared with Al+Mel values ($p<0.05$).

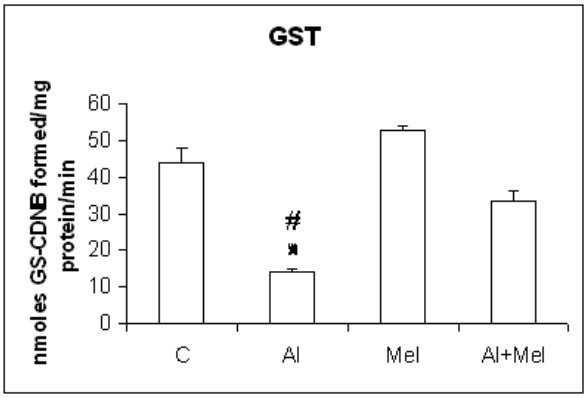

Fig.8 GST: glutathione-s-transferase. Data expressed as mean± S.D of six mice in each group. Al: aluminum, Mel: melatonin. Groups-C: Control mice; Mel: mice treated with Mel; Al: mice treated with Al; Al+Mel: mice treated with Al+Mel. *statistically different from control and Mel values ($p<0.05$). #statistically different as compared with Al+Mel values ($p<0.05$).

Effect on glutathione system

GR activity was found to be decreased in aluminum exposed mice group as compared to control animals (Fig.5). GSH levels were significantly decreased in Al group. Mel treatment normalized the significant decrease of GSH level in Al-treated mice (Fig.6). Al exposure induced a marked reduction in GPx (Fig.7) and GST (Fig.8) activities also and treatment with melatonin reduced the effect of Al.

Pathomorphological changes in hippocampus

Light microscopy

Light microscopy analyses of hippocampus are depicted in figures 3.3.1.Control animals showing normal morphology with intact neurons with nucleus and cytoplasm (figure 3.3.1.A). Aluminum exposure in (figure 3.3.1.B) showed the loss of neurons, vacuolated cells, karyopycnosis of nucleus. However, Mel antagonized this effect (Figure 3.3.1.D).

Electron microscopy

Al administration was found to cause several necrosis like changes in hippocampal CA1 cells such as condensed chromatin, loss of the morphology of the cellular structure (figure 3.3.2 C and D). In Al+ Mel treated animals these profound changes were found to be absent (figure 3.3.2 G and H).

DISCUSSION

Aluminum is the one of the most studied and powerful neurotoxin (Flaten, 2001) whose elevated levels have also been reported in the autopsied brain samples of patients with neurological disorders such as amylotrophic sclerosis (Sinczuk-Walezak, 2001). Al can affect the chemistry and physiology of brain resulting in various clinical manifestations. In the present study, a decrease in body weight in aluminum exposed animals as compared control group. The results are in agreement with Cherrot et al., 1995 who also observed the body weight loss after intraperitoneal administration of aluminum salts. Following melatonin treatment, a marked increase in body weight was observed.

The present work was performed to examine the effect of melatonin, a potent scavenger of reactive oxygen species, in the alterations induced by Al on the oxidative stress parameters. Mel (N-acetyl 5 methoxy tryptamine) is naturally produced by the pineal gland from serotonin by a process catalysed by enzymes: arylalkylamine-N-acetyltransferase and hydroxindazole-O-methyltransferase. The synthesis and release of Mel in normal conditions is stimulated by darkness and inhibited by light in response to signals originated in the suprachaismatic nucleus (Cardinali, 2007).

It has been well documented that the drug is a very potent scavenger of hydroxyl and peroxyl radicals. Melatonin antioxidant properties result mainly from electron donation and unrestricted crossing of morpho-physiological barriers and its easy access to subcellular compartments facilitates the ROS scavenger effect (Tan et al., 2007; Reiter et al.,2008). Melatonin can act as an indirect antioxidant through the activation of the major antioxidant enzymes including superoxide dismutase,catalase, glutathione reductase and glutathione peroxidase (Akubulut et al., 2008; Rodriguez et al.,2008).

SOD is considered important in protecting against oxidative stress, but as its increase accelerates H_2O_2 formation and could enhance oxidant toxicity in aerobes (Scott et al., 1987) it has to be coupled to CAT and other enzyme systems. Consequently our results regarding the enzyme relations showed that Sharma et al. (2007) found induction of oxidative stress in brain tissue and serum after aluminum chloride exposure (175.5mg/kg/day orally for 10 weeks) and reported that glutathione reductase, reduced glutathione, GPx, CAT and SOD levels decreased significantly while concentrations of TBARS and glutathione-s-transferase increased in brain tissue and serum (Moumen et al., 2001). Al induced an increment in lipid peroxidation (LPO) levels with depletion of glutathione and reduction of activity of glutathione peroxidase (GSH-Px), glutathione–S-transferase (GST) and Catalase (CAT) (Mahieu et al., 2005).

The present study showed that melatonin reduces the harmful effects of lipid peroxidation in the brain. This finding is relevant because increased levels of oxidative stress and products of lipid peroxidation in the brain are the major contributing factors in the development of neurodegenerative diseases (Vargas et al., 2004). There is evidence that Al is able to produce free radicals that cause lipid peroxidation, there by damaging neuronal membranes and increasing blood–brain barrier permeability (Srivastava and Jain,2002). Our results indicate that a pattern of Al-induced oxidative stress similar to that found in other studies, suggesting Al-induced oxidative stress involving free radical generation in the brain (TBARS accumulation in hippocampus) (Practico et al.,2002;Gomez et al., 2005; Srivastava and Jain 2002; Flora et al.,2003; Moumen et al., Sushma et al., 2007). Inhibition of TBARS by the administration of melatonin strongly suggests the neuroprotective properties of melatonin. It was also found to counter the aluminum induced structural damage both at microscopic and ultramicroscopic levels. The hippocampus is recognized to be the most sensitive area for aluminum intoxication (Julka et al., 1996). Aluminum-induced the ultrastructural alterations showed coarse and clumpy chromatin, vacuolation(multi-vesicular bodies), pigment accumulation, e.t.c. These observations are similar to the data reported by Deloncle et al. (2001).Melatonin defends cells from a variety of oxidative stress events and it is effective in preventing different types of degenerative disorders in which free radical generation is involved (Benitez-King et al.,2003;Rodriguez et al., 2004; Kilic et al.,2004). Melatonin supplementation significantly reversed the Al induced cell injury in cerebellum.(Sushma et al., 2007). Thus, the antioxidant properties of Mel have been extensively studied, as well as the use of this molecule as a cell protector and as a potential disease preventing agent (Karbownick and Reiter, 2000; Reiter and Tan, 2003; Bandopadyay and Chattopadyay, 2006).

In summary, the present data show melatonin inhibits lipid peroxidation and also reverses aluminum-induced oxidative stress and ultra-structural alterations.

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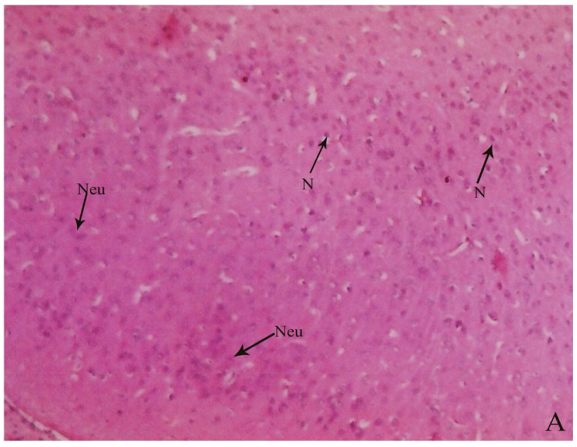

Fig. A: Control mice hippocampus showing Nucleus (N) and Neurons (Neu).

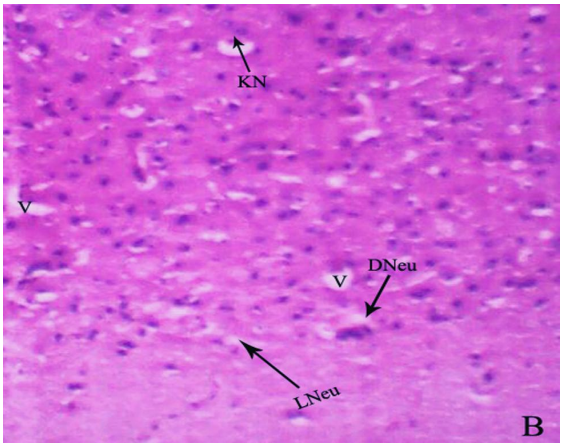

Fig. B: Aluminum acetate treated mice hippocampus showing loss of neurons (LNeu), Degenerated Neurons (DNeu), Karyopyknosis of neuronal cells (KN) and vacuolated cells (V).

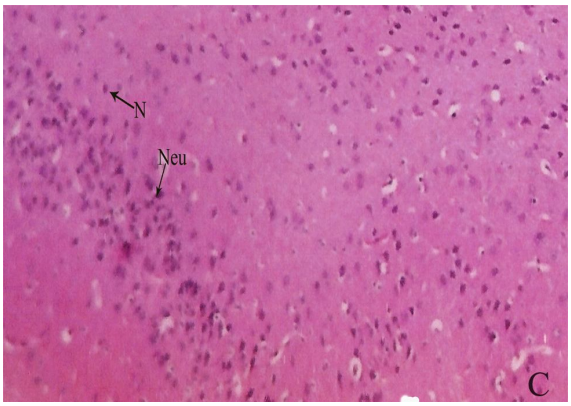

Fig. C: Melatonin treated mice Hippocampus showing neuronal cells (Neu) and cells with clear nucleus (N).

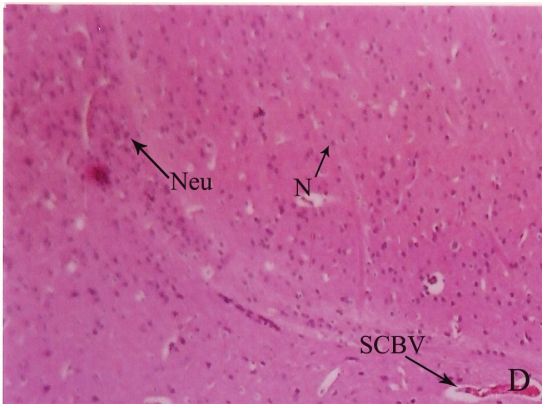

Fig. D: Aluminum acetate + Melatonin treated mice Hippocampus showing cells with clear nucleus (N), neurons (Neu) and slight congestion of blood vessels (SCBC).

Fig 3.3.1.Light microscopy showing protection of hippocampal neurons from aluminum induced damage by melatonin in mice hippocampus (A) Control mice group, (B) Aluminum- treated group (C) melatonin treated group (D).aluminum plus melatonin treated group (L=Loss of neurons, V=Vacuolated, N=cells with clear Nucleus, Neu=Neuron; DNeu=Degenerated neurons, KN =Karyopyknosis of neuronal cells, SCBV=Slight congestion of blood vessels)

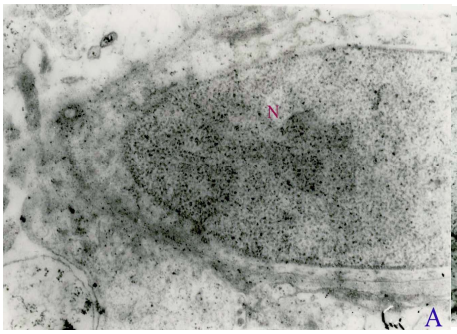

Fig. A: Electron micrograph of control mice hippocampu showing nucleus (N)

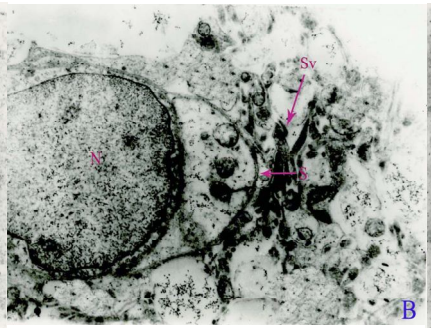

Fig. B: Electron micrograph of control mice hippocampus showing nucleus (N) , synaptic vesicles (Sv), and synapse (S).

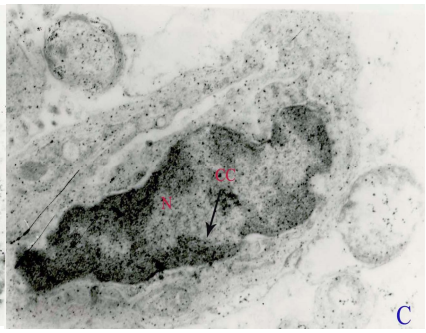

Fig. C: Electron micrograph of aluminum acetate treated mice hippocampus showing condensed chromatin (CC) within the nucleus (N).

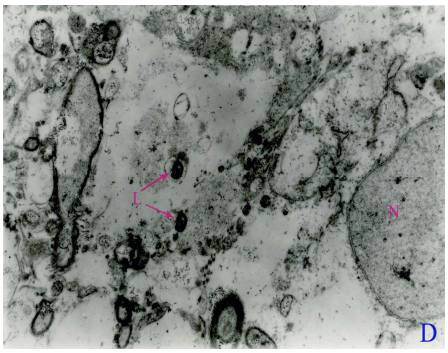

Fig. D: Electron micrograph of aluminum acetate treated mice hippocampus showing lipofuscin pigments (L).

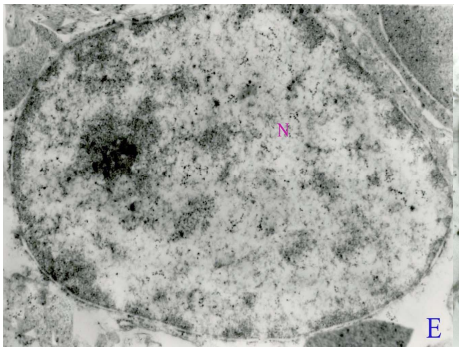

Fig. E: Electron micrograph of melatonin treated mice hippocampus showing nucleus (N).

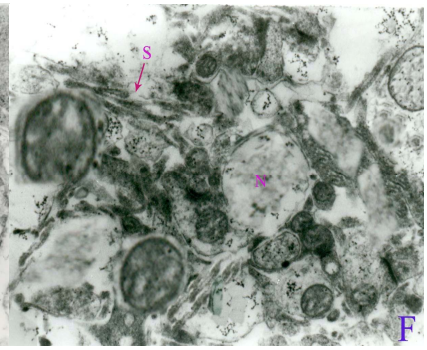

Fig. F: Electron micrograph of melatonin treated mice hippocampus showing

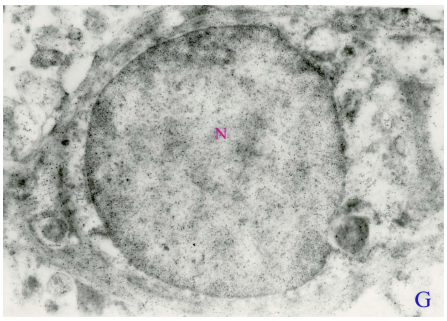

Fig. G: Electron micrograph of aluminum + melatonin Treated mice hippocampus showing nucleus (N).

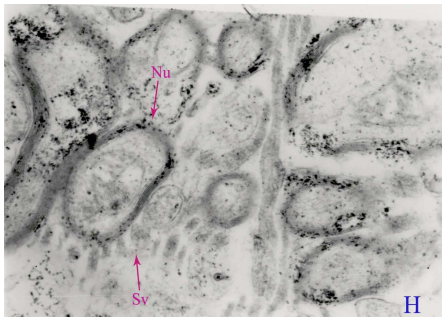

Fig. H: Electron micro photograph of aluminum + melatonin treated mice hippocampus showing cross section of neuron (Nu) and synaptic vesicles (Sv).

Fig 3.3.2. Transmission electron micrographs (TEM) showing changes in different cellular structure of hippocampai cells of CA1 region in different groups (A&B) control groups, (C&D) Al acetate treated groups, (E&F) Al + Mel and (G&H) Mel treated group.(Sv:Synaptic vesicles, N=Nucleus, S=Synapse, Nu=Cross section of neuron, L=Lipofuscin pigments; CC=Condensed chromatin).