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Aluminum-induced neurotoxicity and oxidative damage in rabbits: Protective effect of melatonin

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Abstract

OBJECTIVE: The present study was aimed to investigate: (1) the neurotoxic oxidative damage of orally administered aluminum chloride (AlCl3) in rabbits (Biochemical and morphopathological studies). (2) The effect of melatonin as an antioxidant and free radical scavenger on oxidative neuropathic changes.
Methods: Thirty-five male rabbits were divided into 4 groups (A, B, C [10 animals each] and D [5 animals]). Group A received AlCl3 (20 mg/l via drinking water for 3 months). Group B received AlCl3 for 3 months then administered with melatonin (10 mg/kg b.w. sc daily for 15 days). Group C received AlCl3 plus melatonin for 3 months. Group D received the solvent and served as control. Malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) as lipid peroxides as well as superoxide dismutase (SOD) as an antioxidant enzyme were measured. Aluminum residue in the brain tissue was measured spectrophotometerically. The morphopathological changes were also examined by light and electron microscopes.
RESULTS: MDA and 4-HDA were significantly increased in group A versus those of controls while significantly decreased in groups B and C compared with those of A group. SOD run in an opposite manner. Aluminum concentration was significantly increased in groups A, B and C when compared with group D while it significantly decreased in groups B and C when compared with that of group A. The neuropathlogical examination in the animals of group A revealed atrophy and apoptosis of the neurons in cerebral cortex and hippocampus. This was associated with neurofibrillary degeneration as well as argyrophilic inclusion. Schwan cell degeneration and nerve fiber demylination were also encountered. The elaboration of lipid peroxidation products, inhibition of antioxidant enzymes and the morphopathological changes were minimized in the Al/Mel treated groups and markedly improved in Al+Mel treated group
CONCLUSION: Chronic aluminum exposure in rabbits had dramatic encephalopathic morphopathological lesions. It enhances the lipid peroxidation production and inhibits the SOD enzyme. Melatonin had a good prophylactic effect as an antioxidant in aluminum encephalopathy.

ORIGINAL ARTICLE

Introduction

Aluminum (Al) is a well-known toxic agent and represents a severe problem for a variety of medical [1] as well as environmental situations [2]. The evidence implicating Al as a neurotoxin has been continuously mounting. Research with both animals and humans has linked Al with neurocognitive dysfunction and in some cases, death [3,4]. There is a relationship between Al intake and Alzheimer's [5]. The sources of aluminum include air [6], antacids and antiperspirants [7], cosmetics [8], dental preparations, vaccines and allergic extracts [5], food additives [9], tea, water and canned drinks [10–12]. With regard to the absorption of aluminum, the gastrointestinal tract constitutes the main route of entry for this metal into the body. However, the absorption rate is low in normal human subjects [13]. Aluminum hydroxide, administered therapeutically in large quantities as an antacid and phosphate binder, has been suggested to contribute to the aluminum accumulation and toxicity [14–16].

The neurotoxic effect of aluminum has been well documented by a number of human and animal studies [17,18]. Elevated aluminum concentration in the central nervous system have been related to impaired motor function and to a number of cognitive deficits in both human [3,19] and experimental animals [20–22]. De Boni et al. [23] described neurofibrillary tangle formation in pyramidal neurons in the brain stem, cerebral cortex and hippocampus after subcutaneous injection of either aluminum lactate or tartarate in rabbits. Similar findings were reported by Uemura and Ireland [24]. Reusche et. al. [25] reported that light and electron microscopic investigation of the central nervous system (CNS) showed pathognomonic Al-containing intracytoplasmic argyrophilic inclusions in choroid plexus epithelia, neurons and cortical glia, assoccaited with an increase of mean bulk of Al concentration in the cortex and subcortex up to 9.3 mg/g (normal range <2 mg/g). Neuropatholoically, the brain showed nerve cell atrophy and mild loss with stromal spongiosis, proliferation of astrocytes and microglia in the cerebral cortex, basal ganglia and thalamus in a patient with encephalopathy [26]. The mechanisms by which aluminum interacts with the nervous system are only partly understood. Aluminum accumulated both in neurons and astrocytes [27].

Melatonin is one of the most powerful scavengers of free radicals. Because it easily penetrates the blood-brain barrier, this antioxidant may, in the future, be used for the treatment of Alzheimer's and Parkinson's diseases, stroke, nitric oxide, neurotoxicity and hyperbaric oxygen exposure [28]. Melatonin, together with other free radical scavengers in the brain, reduces the free-radical damage caused by Al except in the latter stages of the disease process [29].

The present study was aimed to investigate:

(1) the neurotoxic oxidative damage of orally administered aluminum chloride in rabbit (biochemical and morphopathological studies).

(2) the effect of melatonin as an antioxidant and scavenger of free radicals in aluminum-induced oxidative neuropathic changes.

Materials and methods

Chemicals

Melatonin was a gift from Helsinn Chemicals SA (Biasca Switzerland) and dissolved in ethanol before being diluted with saline. Bioxytech LPO-586 assay kit purchased from Cayman Chemical (Ann Arbor, MI, USA) was used for measuring the products of LPO (malondialdehyde [MDA] and 4-hydroxyalkenals [4–HDA]). Aluminum Chloride ($AlCl_3$) was purchased from Sigma Chemical Co. (St Louis. USA). All other chemicals were of the highest quality available.

Animals and treatments

Thirty-five male rabbit (1000–1100 g body weight) were used in the current study. All animals were conditioned at room temperature and natural photoperiod for one week before the start of the experiment. A commercial balanced diet and tap water ad libtum were provided. Animals were divided into four groups: Group A ($AlCl_3$-treated group) 10 animals given $AlCl_3$ in drinking water (20 mg /liter) for three months. Group B ($AlCl_3$/melatonin-treated group) 10 animals given $AlCl_3$ in drinking water (20 mg /liter) for three months, then daily subcutaenouslly administered with melatonin (10 mg/kg b.w.) for 15 days. Group C ($AlCl_3$ + melatonin-treated group) 10 animals given $AlCl_3$ in the drinking water (20 mg/liter) plus daily s.c. administration of melatonin for three months. Group D (control group) 5 animals given only the solvent in the drinking water. Water intake was monitored at weekly intervals. Water intake ranged from 250–300 ml/day/rabbit (roughly 5–6.6 mg $AlCl_3$/day/rabbit)

Melatonin was administrated for rabbits two hours before lights off. After sacrification of the animals, brain samples were taken. One half of the brain hemisphere was taken, stored at –60 °C for biochemical and residual determination. The other half was fixed either in neutral buffer formalin for routine pathological examination or in gluteraldehyde for electron microscopy [30].

Measurement of lipid peroxidation (LPO)

Malondialdehyde (MDA) and 4-hydroxyalkenal (4-HDA) concentrations are considered to be an index of the peroxidation of membrane lipid [31]. The colorimetric kit mentioned above was used to determine the levels of oxidized lipid. At the time of assay, brains from each group were homogenized in ice-cold 50 mM Tris buffer (pH 7.4, 10% w/v) using ultra-Turrax T25b homogenizer and the supernatant prepared by centrifugation at 10 000 g for 10 min. Protein concentrations were measured by the method of Bradford [32] using bovine albumin as standard.

Suproxide dismutase activity

Superoxide dismutase (SOD) activity was estimated based on its ability to inhibit the autoxidation of epi-

nephrine in an alkaline medium (pH 10.2), according to Misra and Fridovich [33].

Histopathological examination

Specimens from the brain were taken from all rabbit groups directly after sacrification. They were fixed in 10% neutral buffer formalin, embedded in paraffin, sectioned at 5 microns and stained with hematoxylin and eosin stain (H&E). The sections were examined using the light microscope.

Preparation of semithin sections

Other specimens were fixed in gluteraldehyde (5%) and approximately 1×1×1-mm blocks were prepared. These blocks were washed in cacodylate buffer (0.1M, pH 7.2) for 1–3 hr and then post fixed in 1% osmium tetraoxide for 2 hr. After repeated washing in cacodylate buffer (4×30 min) and dehydration in ascending grades of ethyl alcohol up to 100% (30 min for every concentration), the specimens were first placed in propylene oxide for 60 min, then in pure epon 812 and incubated in a special polymerization incubator (one day at 35°C, second day at 45°C and three days at 60°C). The blocks were trimmed with LKB ultratom. Semithin section were obtained and stained with toluidine blue (T.B.) for 2 min at 80°C and examined by light microscope.

Representative fields of semithin sections were selected. Ultrathin sections (70 nm) were cut with diamond knife using a Reichert OMVs ultramicrotome. They were mounted in copper grids and stained with uranyl acetate lead citrate stain. The ultrastructural investigation was carried out with Transmission electron microscope (TEM) (Joel Cx II) [34].

Estimation of aluminum in the brain tissues

Aluminum residue in the brain tissues was measured spectrophotometerically according to the method of Jayman and Sivasubramaniam [35].

Statistical analysis

The data are presented as the arithmetic means ± SEM. Statistical analyses were performed using an ANOVA followed by the Student-Newman-Keuls t-test. P<0.05 was considered to be significant. Inhibition or stimulation % in the mean values of LPO and SOD was estimated according to El-Sokkary [36] as follows:

$$\text{Inhibition or stimulation (\%)} = \frac{\text{Mean control value} - \text{Mean treated value}}{\text{Mean control value}} \times 100$$

Results

Lipid peroxidation levels and superoxide dismutase activity

Table 1, revealed that there was a highly significant stimulation (P<0.01) of lipid peroxidation products levels in the $AlCl_3$-treated group (A) versus those of control animals (D). There was a highly significant inhibition (P<0.01) of $AlCl_3$ plus melatonin treated group (C) and the group treated with melatonin after aluminum exposure (B) when compared to $AlCl_3$-treated animals (A). There was no statistical differences between the different melatonin-treated groups (B,C).

SOD activity showed a highly significant inhibition (P<0.01) in $AlCl_3$-treated group (A) when compared to those of controls. Also, there was highly significant stimulation (P<0.01) of SOD activity in the animals given $AlCl_3$ plus melatonin (group C) and significantly stimulation (P<0.05) in the rabbits treated with melatonin after aluminum exposure (group B) versus those of group A.

Aluminum concentration in the brain tissue

Table 2, showed that aluminum concentration was significantly increased in groups A, B and C when compared with group D while it significantly decreased in groups B and C when compared with that in group A.

Morphopathological changes

Group A: Most of the neurons in the cerebral cortex and hippocampus were atrophied manifested by its shrinkage, their nuclei become smaller and darkly stained basophilic with bluish taint cytoplasm (*Figs. 1a,b*). In semithin sections there were perineuronal (*Fig. 1c*) as well as perivascular edema and adventitial

Table 1: Mean values ± S.E of lipid peroxidation products (MDA and 4-HDA), superoxide dismutase (SOD) and inhibition (I) and/or stimulation (S)% in the brain homogenates of control and different treated groups of rabbits.

Measurements / Groups	MDA and 4-HDA (nmol/mg protein)	SOD (Units/mg protein)
Control (Group D)	3.84 ± 0.21	1.33 ± 0.15
Aluminum (Group A)	5.54 ± 0.31**	0.53 ± 0.08**
I or S% vs controls	S=30.7%	I=60.4%
Aluminum/Mel (Group B)	4.48 ± 0.19**	0.86 ± 0.08*
I or S% vs group A	I=19.1%	S=38.5%
Aluminum+Mel (Group C)	4.10 ± 0.23**	0.94 ± 0.06**
I or S% vs group A	I=25.9%	S=43.9%

** (P<0.01) * (P<0.05) S.E: standard error of the mean

Table 2: Aluminum concentration (ppm) in the brain of control and different treated groups of rabbits.

Animal Groups	Group A	Group B	Group C	Group D (Control)
Mean ± S.E	4.211 ± 0.021a	2.231 ± 0.013a,b	1.076 ± 0.008a,b	0.334 ± 0.003

a: Significantly different from group D at P<0.001.
b: Significantly different from group A at P<0.001.

proliferation (*Fig. 1d*). TEM revealed presence of neuronal chromatolysis and degeneration of mitochondrial cristae (*Fig. 1e*). Some of neuronal nuclei undergo apoptosis, in which the nuclei become smaller and had an electron dense, cart wheal appearance (*Fig. 2a*) or very smaller in size with condensation of its chromatin (*Fig. 2b*). Some neurons showed neurofibrillar structure in their cytoplasm (*Fig. 2c*). In the schwan cells of the nerve fiber, there was swollen of the mitochondria with destruction of their cristae (*Fig. 2d*). Vacuolation of myelin sheath was encountered in different degrees (*Fig. 2e*). Argyrophilic inclusions were frequently encountered in the choroids plexus epithelial cells (*Fig. 3c*).

Group B: The evidence of apoptosis and neuronal degeneration were noticed in few neurons either in the cerebral cortex (*Fig. 3a*). or hippocampus (*Fig. 3b*). TEM revealed, the presence of argyrophilic inclusion in

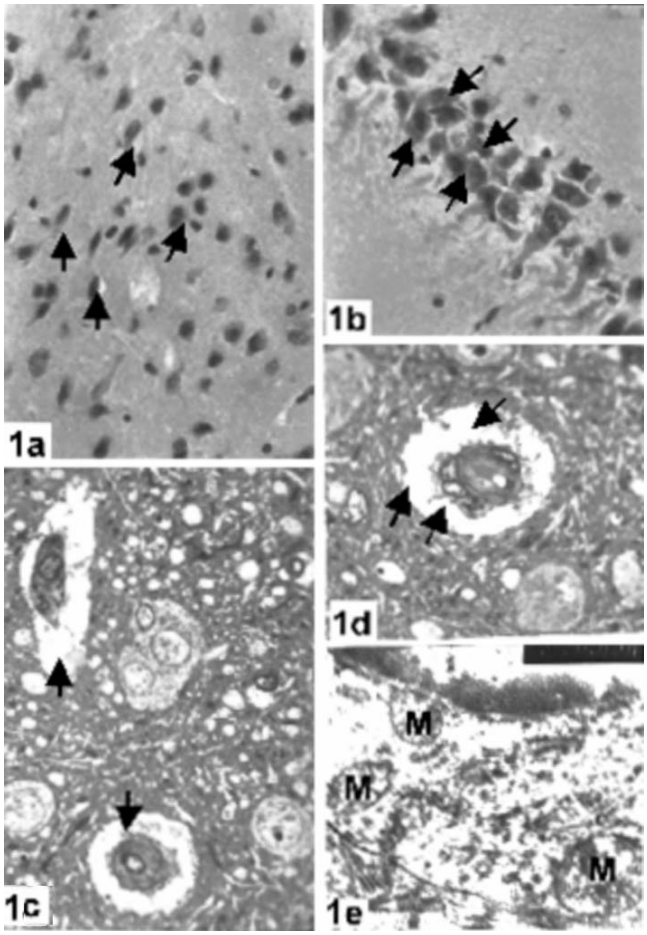

Figure 1. Sections of the brain of rabbits from group A ($AlCl_3$-treated group) showing:
(a) Shrinkage and deeply stained neurons in the cerebral cortex (arrows). H&E. X 400; **(b)** Shrinkage and deeply stained neurons in the hippocampus (arrows). H&E. X 400; **(c)** Perineuronal edema associated with shrinkage and deeply stained neurons (arrows). Semithin section, toulidin blue. X 1000; **(d)** Perivascular edema associated with proliferation of adventitial cells (arrows). Semithin section, toulidin blue. X 1000 and **(e)** Electron micrograph of neuron with chromatolysis associated with swollen mitochondria and destruction of their cristae (M). X 20 000.

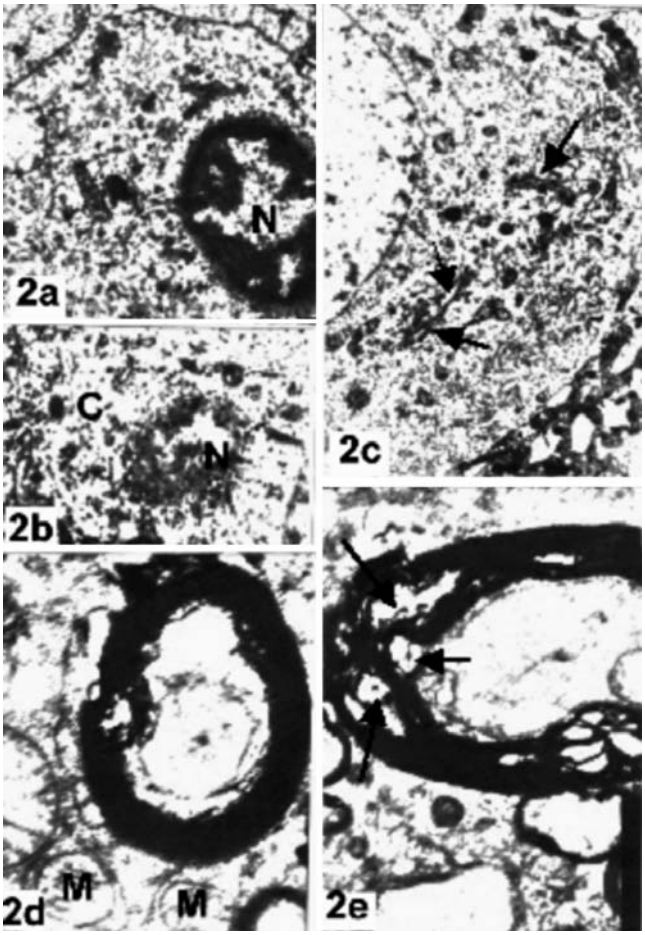

Figure 2. Electron micrographs of neurons from group A ($AlCl_3$-treated group) showing:
(a) Neuron with apoptotic nuclei (N) X 10 000;
(b) Neuron with nuclear pyknosis and cytoplasmolysis (C) X 10 000;
(c) Neuron with neurofibrillary cytoplasmic aggregation (arrows) X 4000; **(d)** Nerve fiber with degenerated mitochondria in Schwan cells (M) X 27 000 and **(e)** Nerve fiber with demyelination of myelin sheath (arrows) X 14 000.

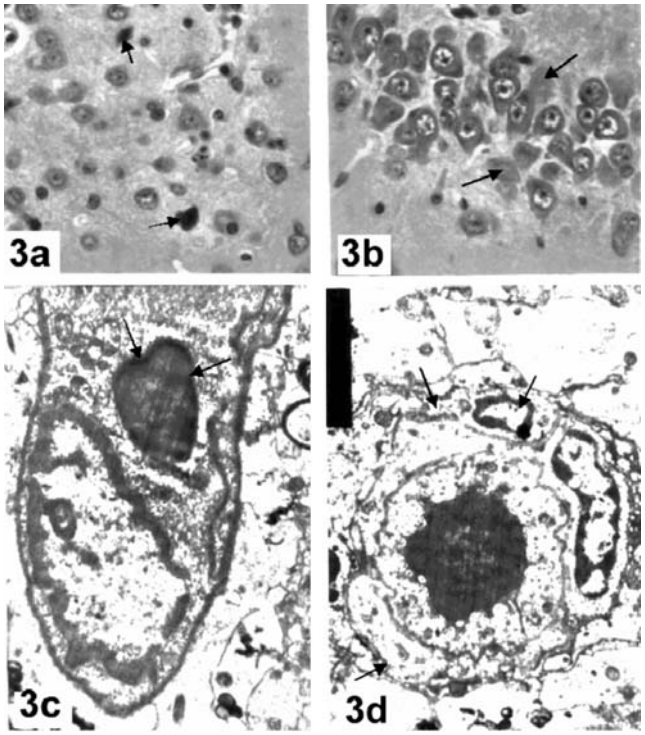

Figure 3. Sections of the brain of rabbits from group B ($AlCl_3$/Mel-treated group) showing:
(a) Few neurons undergo shrinkage and apoptosis in the cerebral cortex (arrows). H&E. X 400; **(b)** Few neurons undergo necrobiosis in the hippocampus (arrows). H&E. X 400; **(c)** Electron micrograph of choroid plexus epithelia with cytoplasmic argyrophilic inclusion (arrows). X 4000 and **(d)** Electron micrograph of blood vessels with adventitial proliferation and perivascular edema (arrows). X 6700.

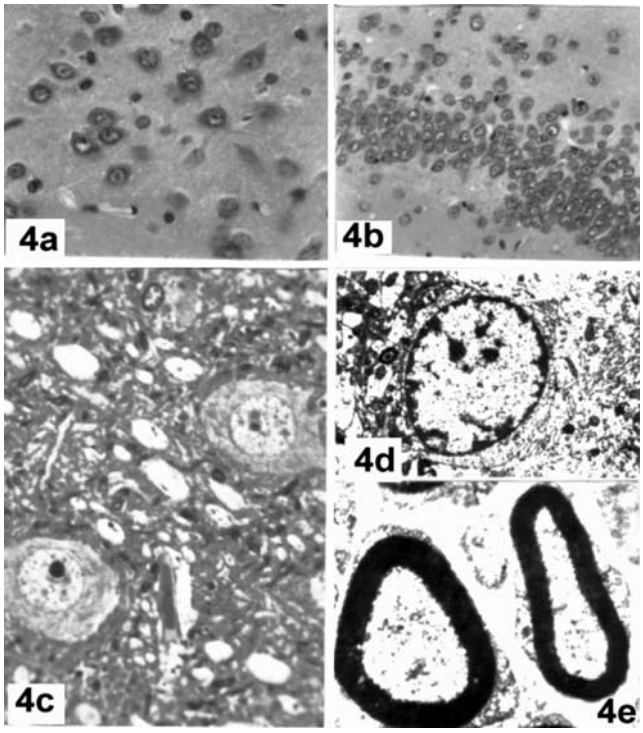

Figure 4. Sections of the brain of rabbit from group C ($AlCl_3$+Mel-treated group) showing:
(a) Normal neurons in the cerebral cortex. H&E. X 400; **(b)** Normal neurons in the hippocampus. H&E. X 400; **(c)** Normal neurons and perineuronal tissue of cerebral cortex. Semithin section, toulidin blue. X 1000; **(d)** Electron micrograph of neuronal cell body with normal nuclear and cytoplasmic appearance. X 4000 and **(e)** Electron micrograph of nerve fiber in the white matter with normal structure. X 10 000.

the choroid plexus epithelia (*Fig. 3c*). The blood vessels still showed perivascular edema (*Fig. 3d*).

Group C: The neurons in the cerebral cortex and hippocampus appeared more or less normal with H& E stain (*Figs. 4a,b*). Semithin sections revealed normal neuronal and perineural structure (*Fig. 4c*). TEM revealed minimal chromatolysis in the neurons (*Fig. 4d*). The nerve fibers showed normal structure of myelin sheath and schwan cells (*Fig. 4e*).

Discussion

Al, a metal without redox capacity in biological systems, potentiates the stimulation of lipid peroxidation. The toxicity of this metal has been the subject of much controversy in the past few decades. It has been generally believed that the metal is innocuous to human health, a causal role for dialysis dementia, osteomalacia, and macrocytic anemia. The exact mechanism of aluminum toxicity is not known, but there are several lines of evidence that show the metal's capacity to exacerbate oxidative events.

In the present study, Al concentration was significantly increased in groups A, B and C when compared with group D while it significantly decreased in groups B and C when compared with that in group A. Following chronic aluminum exposure to rabbits (20 mg/kg body weight daily for three months) there was a significant enhancement in the level of lipid peroxidation in the brain tissue. The antioxidant enzyme, SOD was decreased significantly. Similar results were reported [37]. Jia et al. [38] reported that the concentration of aluminum was increased and also MDA in the hippocampus while SOD was reduced.

Amador et al. [39 suggested that the facilitation of Al accumulation during brain oxidative injury might contribute to Al neurotoxicity and neuronal cell degeneration. Ward et al. [40] found that exposure to Al gluconate resulted in increases in tissue Al in various brain regions, frontal, temporal, parietal cortex and hippocampus which was parallel by elevation of tissue iron in these regions.

A significant enhancement in the levels of lipid peroxidation at the completion of aluminum treatment was reported. The antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase were also seen to decrease following aluminum exposure [37].

The morphopathological changes induced due to chronic aluminum exposure in rabbits for three months involved the neurons, blood vessels and nerve fibers in the examined cerebral cortex and hippocampus. The neurons undergo either degenerative or apoptotic changes. The degenerative changes manifested by

mitochondrial damage, pyknosis of the nuclei, chromatolysis and atrophy. Neurofibrillary degeneration, similar to neurofibrillary tangle in Alzheimer,s disease was also observed in some neurons. These degenerative changes were also reported in experimental animals due to aluminum exposure by many investigators [41–44]. Oxidative damage to the brain cell components may be an important mechanism mediating the neurotoxicity of aluminum [45]. Significant increase in lipid peroxidation products (MDA and 4-HDA) in rabbits of aluminum treated group will confirm this suggestion. Yokel [43] suggested that disruption of the inositol phosphate system, calcium regulation, facilitation of Fe-induced oxidative injury and disruption of basic cell processes may mediate primary molecular mechanisms of Al-induced neurotoxicity. Many additional mechanisms of aluminum neurotoxicity could put in consideration.

The most dramatic effect of Al is that of producing intraneuronal perikaryal neuroflamentous aggregates. Possible mechanisms of Al neurotoxicity could be related to cell damage via free radical production, impairment of glucose metabolism and effects on signal transduction [41]. Inoculation of aluminum chloride (AlCl3) to young adult New Zealand white rabbits induces motor neuron degeneration marked by intraneuronal neurofilamentous aggregates similar to that observed in amyotrophic lateral sclerosis (ALS) in the presence of a glial response [42]. The most pronounced changes in brain tissue included spongioform changes in the neurons specially those of hippocampus, nuclear deformity, and neurofibrillary degeneration, similar to neurfibrially tangles in Alzheimer's disease. It is concluded that accumulated aluminum in brain and altered amino acid neurotransmitters are important mechanisms of aluminum neurotoxicity [44].

The current study demonstrated apoptosis in the neuronal cell body of cerebral cortex and hippocampus. He and Strong [42] demonstrated apoptosis in the brain tissue of aluminum exposed animals and suggested that the lake of apoptosis in their study was not related to the losses of the apoptotic neurons in post-mortem tissue. Apoptosis was recognized in rabbit brain due to aluminium-induced oxidative damage [46] and in other investigations [47].

Cerebral edema either perineural or perivascular associated with angiopathic lesions in aluminum treated group. Increase in the levels of lipid peroxidation products due to aluminum exposure will enhance the angiopathic lesions and subsequently edema formation. The presence of argyriophilic inclusion in the choroid plexus epithelia was also recorded in dialysis associated encephalopathy (DAE) which characterized by lysosome-derived intracytoplasmic, Al-containing and pathognomonic [25]. Schwan cell degeneration and demyelination of myelin sheath were prominent finding in aluminum treated groups. Brain spongiosis were encountered in human with aluminum encephalopathy [26].

The stimulation of lipid peroxidation products, inhibition of antioxidant enzymes and the morphopathological change were minimized in the Al/Mel treated group (B) and markedly improved in Al+Mel treated group (C). This will prove the prophylactic effect of melatonin as an antioxidant.

Melatonin is well known for its functional interactions with the neuroendocrine axis and with ciecadian rhythms. It has also been found to be a free radical scavenger and antioxidant [48]. Neuroprotective effects of melatonin have been demonstrated mainly in models of neuronal cell death in which oxygen free radicals or excitotoxins are involved. In the models of Parkinson's disease, melatonin completely reversed the rises in lipid peroxidation [49,50]. Melatonin also prevented kainate-induced neuronal cell death and reduced lipid peroxidation products in rats and mice *in vivo* [51,52]. Furthermore, melatonin protects against glutamate-induced cell death in the clonal hippocampal cell line HT22 [53], prevents delayed neuronal death induced by enhanced excitatory transmission in hippocampal pyramidal neurons in culture [54], protect the hippocampus against lead-induced neurotoxicity [55] and rescues neuroblastoma cells exposed to toxic fragments of Alzheimer's β-amyloid [56]. An anticonvulsant activity of melatonin has been demonstrated against excitotoxin-induced seizures by quinolinate, kainate, and glutamate in mice and rats [57,58]. The occurrence of increased brain damage after stroke or excitotoxic seizures in melatonin-deficient rats is in line with these findings [59].

Besides the antioxidant potential, several other mechanisms are considered to be involved in the neuroprotection mediated by melatonin, including interactions with calmodulin [60] and microtubular components [61], blockade of increases in intracellular Ca^{+2} levels [62], inhibition of activation of NFκB by cytokines such as tumor necrosis factor [63], inhibition of the expression of inducible nitric oxide synthase at the transcriptional level [64] and changes in gene expression of antioxidant enzymes [65].

Reduction of cerebral edema, an early symptom of ischemia, is one of the most important remedies for reducing subsequent chronic neural damage. Melatonin is effective in reducing edema formation in ischemic animals *in vivo*, especially in the cerebral cortex. Melatonin may be highly useful in preventing cortical destructions such as motor, sensory, memory and psychological impairments [66].

In conclusion, chronic aluminum exposure in rabbits had a dramatic encephalopathic morphopathological lesions. It enhances the lipid peroxidation production and inhibits the SOD enzyme. Melatonin had a good prophylactic effect as a neuroantioxidant in aluminum encephalopathy.

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