ORIGINAL ARTICLE

The protective properties of melatonin against aluminium–induced neuronal injury

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SUMMARY

Aluminium (Al) toxicity is closely linked to the pathogenesis of Alzheimer's disease (AD). This experimental study investigated the neuroprotective effect of melatonin (Mel; 10 mg/kg bwt) on aluminium chloride ($AlCl_3$; 34 mg/kg bwt) induced neurotoxicity and oxidative stress in rats. Adult male albino Wistar rats were injected with $AlCl_3$ for 7 days. The effect on brain structure, lipid peroxidation (LPO), nitric oxide (NO) levels, glutathione (GSH) content, antioxidant enzymes (SOD, CAT, GPx and GR), apoptotic proteins (Bax and Bcl-2) and an apoptotic enzyme (caspase-3) was investigated. No apparent changes occurred following the injection of melatonin. Melatonin pretreatment of the $AlCl_3$-administered rats reduced brain damage, and the tissues appeared like those of the control rats. Compared to treatment with $AlCl_3$, pretreatment with melatonin decreased LPO and NO levels and increased the GSH content and antioxidant enzyme activity. Moreover, melatonin increased the levels of the anti-apoptotic protein, Bcl-2, decreased the levels of the pro-apoptotic protein, Bax, and inhibited caspase-3 activity. Therefore, our results indicate that melatonin may provide therapeutic value against aluminium-induced oxidative stress and histopathological alternations in the rat brain and that these effects may be related to anti-apoptotic and antioxidant activities.

Keywords

aluminium chloride, anti-apoptotic, brain, melatonin, oxidant/antioxidant, rats

Exposure of human populations to toxic metals can result in damage to a variety of organ systems. One common toxic metal studied is aluminium (Al), which is implicated in many diseases (Fulgenzi *et al.* 2014). Al is the third most prevalent element and represents approximately 8% of the total mineral component in the Earth's crust (Abdel Moneim 2012). The typical adult intake is estimated to be from 3 to 12 mg/day according to dietary aluminium studies conducted in many countries (Abdel Moneim *et al.* 2013). Al is a commonly studied neurotoxin that affects the nervous system, including various regions of the brain (Nehru & Bhalla 2006). Some experts believe that Al crosses the blood–brain barrier and plays a role in the formation of Alzheimer-like neurofibrillary tangles (Sharma *et al.* 2009). Al^{+3} and other metals influence the oligomerization and conformational changes of the β-amyloid protein as a cross-linker in the 'amyloid cascade hypothesis', and therefore, their implications are important in this context. Overall, Al has adverse effects on human memory and causes dementia when it enters the brain (Kawahara & Kato-Negishi 2011).

The mechanism of Al toxicity is poorly understood, but the literature suggests that Al generates reactive oxygen species (ROS) that cause lipid peroxidation (LPO) and oxidative damage to proteins and DNA and decreases intracellular glutathione (GSH) (González *et al.* 2009). To control the level of ROS and to protect cells under stress conditions, mammalian tissues contain several enzymes that scavenge ROS such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and GSH (Dkhil *et al.* 2014). In the brain, low concentrations of the endogenous antioxidant component GSH and the antioxidant enzyme CAT, a high metabolic rate, and a high proportion of polyunsaturated fatty acids make this organ particularly susceptible to oxidative damage (Abdel Moneim 2013). The slow progressive neuronal loss is a common feature of neurodegenerative diseases.

Therefore, therapeutic strategies that prevent oxidative stress constitute a key factor for the treatment of different neurodegenerative diseases.

Antioxidants contribute to the organism's defence through different mechanisms including ROS scavenging, increasing the expression and function of endogenous antioxidants and inhibiting the activity of ROS-generating enzymes. Melatonin (Mel) is a potent free radical scavenger and an antioxidant. Mel can directly detoxify both reactive oxygen and nitrogen species (Reiter *et al.* 2001; Abdel Moneim *et al.* 2014). Melatonin also acts as an indirect antioxidant by regulating the enzymatic activity that promotes the overall antioxidative defence systems in an organism, including GPx, GR, SOD and glucose-6-phosphate dehydrogenase (Reiter *et al.* 1997). In addition, metabolites formed from the interaction between melatonin and free radicals, including N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), are also efficient free radical scavengers (Leon *et al.* 2006).

Therefore, the aim of this study was to investigate the protective effect of melatonin against Al-induced neuronal injury. Such investigation will explore the potential therapeutic or preventive approaches that can be developed in future studies, potentially by blocking or minimizing the destructive effects of Al to the brain.

Materials and methods

Chemicals and experimental animals

Anhydrous aluminium chloride ($AlCl_3$; CAS Number 7446-70-0), melatonin (Mel; CAS Number 73-31-4), nitro blue tetrazolium, N-(1–naphthyl) ethylenediamine, thiobarbituric acid (TBA) and Tris–HCl were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of analytical grade. Double-distilled water was used as the solvent.

Adult male Wistar albino rats weighing 150–180 g were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). After an acclimatization period of 1 week, the animals were divided into four groups (seven rats per group) and housed in wire-bottomed cages in a room under standard condition of illumination with a 12-h light/dark cycle at $25 \pm 2°C$. The rats were provided water and a balanced diet *ad libitum*. We followed the European Community Directive (86/609/EEC) and national rules on animal care that was carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals 8th edition.

Experimental protocol

To study the protective effects of melatonin on Al-mediated neuronal injury, the rats were randomly allocated into four groups of seven rats per group. The first group was used as the control, while groups 2 and 4 were orally treated with $AlCl_3$ [34 mg/kg body weight (bwt)] and groups 3 and 4 were intraperitoneally treated with Mel (10 mg/kg bwt). Rats were administered their respective doses every day for 7 days. In group 4, the treatment of Mel was given before $AlCl_3$ within an hour. Mel was intraperitoneally injected at a dose of 10 mg/kg bwt according to Abdel Moneim *et al.* (2014), while $AlCl_3$ was orally administered at an acute neurotoxic dose of 34 mg/kg bwt according to Abdel Moneim (2012).

Twenty-four hours following the last administration, the animals were killed under mild ether anaesthesia. The brains of the rats were carefully removed and washed twice in ice-cold 50 mM Tris–HCl, pH 7.4. Then, each brain was weighed and immediately homogenized to give a 10% (w/v) homogenate in ice-cold medium that contained 50 mM Tris–HCl, pH 7.4. The homogenates were centrifuged at 1200 g for 10 min at 4°C. The supernatants were used for the various biochemical determinations. The total protein content of the homogenized brain was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Oxidative stress

Lipid peroxidation (LPO) in the brain was determined using 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid in a boiling water bath for 30 min. TBARS were determined by the absorbance at 535 nm and expressed as the amount of malondialdehyde (MDA) formed (Ohkawa *et al.* 1979). The nitric oxide level was determined by the optimized acid reduction method in an acidic medium and in the presence of nitrite. The formed nitrous acid diazotise sulphanilamide is coupled with N-(1–naphthyl) ethylenediamine. The resulting azo dye has a bright reddish purple colour that can be measured at 540 nm (Green *et al.* 1982). In addition, the neuronal glutathione (GSH) was determined by the reduction of Elman's reagent [5,5′ dithiobis (2-nitrobenzoic acid); DTNB] with GSH to produce a yellow compound. The reduced chromogen is directly proportional to the GSH concentration, and its absorbance can be measured at 405 nm (Ellman 1959).

Enzymatic antioxidant status

The activities of neuronal antioxidant enzymes, such as superoxide dismutase (SOD), were assayed by measuring the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium (NBT) dye. Brain catalase (CAT) was assayed by adding 50 µl of brain homogenates to 30 mM H_2O_2 in 50 mM of potassium phosphate buffer (pH 7.8), and the consumption of H_2O_2 was measured at 340 nm for 120 s at 20-s intervals. Catalase activity was expressed in units. Glutathione reductase (GR) was assayed indirectly by GR catalysis of the reduction of glutathione in the presence of NADPH, which is oxidized to $NADPH^+$. The decrease in absorbance at 340 nm was measured. Finally, glutathione peroxidase

(GPx) activity in the brain homogenates was measured using the method of Paglia and Valentine (1967). The assay is an indirect measure of the activity of GPx. Oxidized glutathione (GSSG), produced upon reduction of organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm.

Histological study

The brains were washed in saline and fixed in 10% neutral formalin for 24 h, dehydrated in ascending concentrations of ethyl alcohol, cleared in xylene and mounted in molten paraplast at 58–62°C. Slices of 4–5 μm were obtained from the prepared blocks and stained with haematoxylin–eosin. The preparations obtained were visualized using a Nikon (Eclipse E200-LED, Tokyo, Japan) microscope at a magnification of 400×.

Determination of apoptotic markers in brain tissue

Brain homogenates were made in lysis buffer and analysed using a colorimetric caspase-3 assay kit (Sigma-Aldrich Co), according to the manufacturer's instructions. Upon caspase activation, DEVD-pNA is cleaved releasing the pNA moiety, which can be measured at 405 nm. Fifty micrograms of protein per 50 μl was used for each assay in the presence of 200 μM caspase substrate in 50 μl reaction buffer. The background absorbance of the supernatants and the buffers were subtracted from the absorbance of samples before calculating the fold increase in caspase-3 activity. The Bcl-2 protein and Bax protein levels were measured in brain tissue lysates by an ELISA kit (R&D Systems Inc. (Minneapolis, MN, USA)), and the procedure was performed according to the manufacturer's instructions. The levels were expressed as ng/mg of tissue protein.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analysed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a post hoc test according to the Statistical Package for the Social Sciences (SPSS version 17.0 (Chicago, USA)).

## Ethical approval statement:

The study protocol was approved (No. 1/3/12754) by Ethical Committee of King Saud University (KSU).

Results

To examine the effect of Al on oxidative stress markers, lipid peroxidation levels in the brain homogenates of rats treated with AlCl$_3$ were measured (Figure 1). AlCl$_3$ administration for 7 days caused a significant ($P < 0.05$) increase in
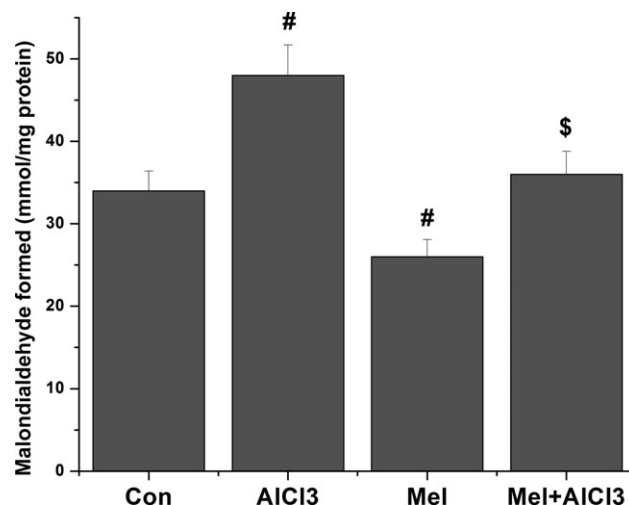
Figure 1 The lipid peroxidation level of malondialdehyde formed in rat brains exposed to melatonin (Mel) and aluminium chloride (AlCl$_3$) for 7 days. Values are the mean ± SEM ($n = 7$). $^\#P < 0.05$, significant change with respect to control; $^\$P < 0.05$, significant change with respect to AlCl$_3$ for Duncan's post hoc test.
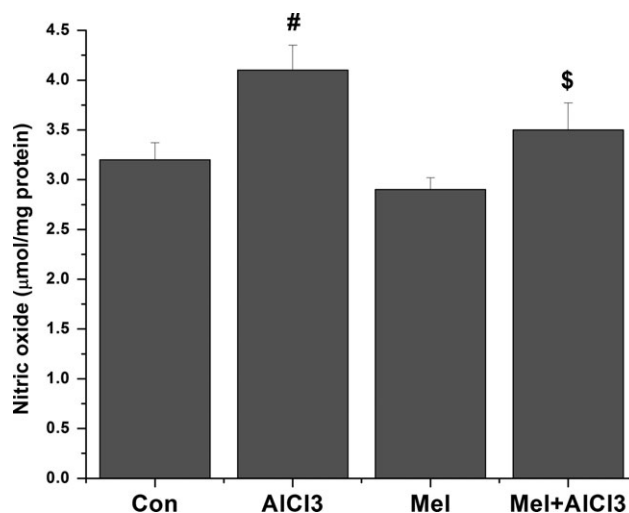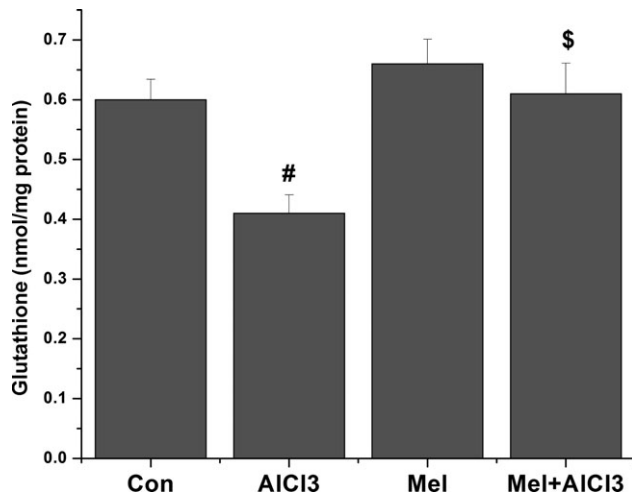

Figure 2 The nitric oxide level in rat brain exposed to melatonin (Mel) and aluminium chloride (AlCl$_3$) for 7 days. Values are the mean ± SEM ($n = 7$). $^\#P < 0.05$, significant change with respect to control; $^\$P < 0.05$, significant change with respect to AlCl$_3$ for Duncan's post hoc test.

the brain LPO level compared to the control group. However, Mel markedly attenuated LPO in AlCl$_3$-administered rats. AlCl$_3$ induced lipid peroxidation by over 0.4-fold compared to the control rats. Rats treated with melatonin previous to AlCl$_3$ administration show a statistically significant difference ($P < 0.05$) compared to rats only treated with AlCl$_3$.

Moreover, AlCl$_3$ leads to nitrosative stress in the brain of rats (Figure 2). We analysed NO production by measuring

the nitrate and nitrite levels, and we found that $AlCl_3$ induced nitric oxide production by over 0.3-fold compared to the control rats. Rats pretreated with melatonin show a

Figure 3 The glutathione content in rat brain exposed to melatonin (Mel) and aluminium chloride ($AlCl_3$) for 7 days. Values are the mean ± SEM (n = 7). [#]P < 0.05, significant change with respect to control; [$]$P$ < 0.05, significant change with respect to $AlCl_3$ for Duncan's *post hoc* test.

statistically significant difference (P < 0.05) compared to the rats only treated with $AlCl_3$.

The $AlCl_3$-induced neuronal oxidative stress was evident and indicated by a significant reduction (P < 0.05) in the brain GSH content of $AlCl_3$-treated rats compared to the control group. This reduction in the GSH content was attenuated by pretreatment with Mel as shown in Figure (3).

To study the neuroprotective effect of melatonin in rats, modulation of the antioxidant defence system was examined, including the activity of SOD, CAT, GPx and GR enzymes. As shown in Table 1, $AlCl_3$ administration led to the modulation of antioxidant enzymes relative to the control rats. After 7 days of $AlCl_3$ administration, SOD, CAT, GPx and GR activities in the brain homogenates decreased significantly (P < 0.05) compared to the control. However; Mel pretreatment significantly (P < 0.05) elevated the activities of SOD, CAT, GPx and GR compared to the $AlCl_3$ group and even increased the brain SOD activity above its basal value.

To verify the effectiveness of melatonin in protecting the brain from morphological alternations caused by exposure to aluminium, we examined different brain regions (Figure 4). The control group showed regular histological features in the various brain regions. Meanwhile, brain sections of $AlCl_3$-treated rats (group 2) showed neuronal degeneration in the cerebellum and cerebral cortex with

Table 1 Antioxidant enzyme activity in rat brain exposed to melatonin (Mel) and aluminium chloride ($AlCl_3$) for 7 days

Parameter	Con	$AlCl_3$	Mel	Mel + $AlCl_3$
SOD (U/mg protein)	503.17 ± 10.87	438.98 ± 28.45*	568.21 ± 22.58*	541.24 ± 20.06*[,†]
CAT (μmol/mg protein)	0.47 ± 0.02	0.31 ± 0.01*	0.52 ± 0.03*	0.51 ± 0.03[†]
GPx (U/mg protein)	0.37 ± 0.02	0.27 ± 0.02*	0.44 ± 0.03*	0.39 ± 0.02[†]
GR (μmol/mg protein)	7.34 ± 0.41	4.21 ± 0.24*	8.12 ± 0.31*	7.08 ± 0.37[†]

Values are the mean ± SEM (n = 7). *P < 0.05, significant change with respect to control; [†]P < 0.05, significant change with respect to $AlCl_3$ for Duncan's *post hoc* test.
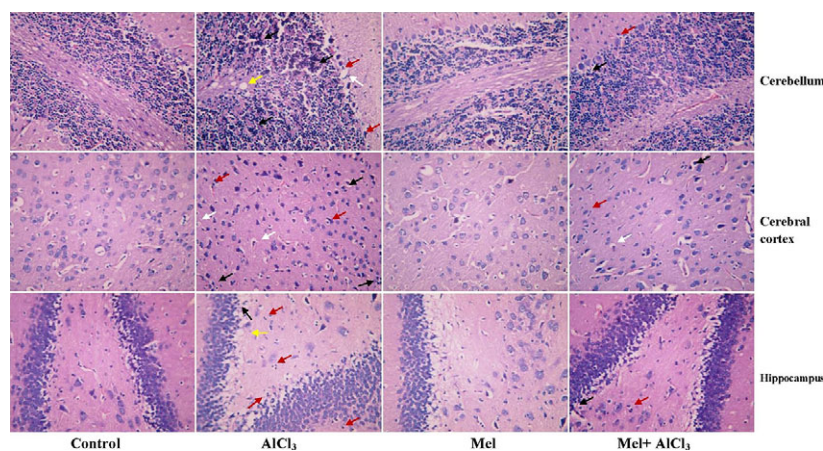

Figure 4 Normal histological features of various brain regions from the control group with well-formed neurons. Meanwhile, brain sections from $AlCl_3$-treated rats show damage in the cerebellum, cerebral cortex and hippocampus, including vacuolated and degenerated neurons (yellow and white arrows respectively) and apoptotic and necrotic neurons (red and black arrows respectively). Brain sections from rats pretreated with Mel showed better recovery and well-formed nuclei without vacuolation and irregular features (400×).

Table 2 Anti-apoptotic/pro-apoptotic protein level in rat brain exposed to melatonin (Mel) and aluminium chloride (AlCl$_3$) for 7 days

Parameter	Con	AlCl$_3$	Mel	Mel + AlCl$_3$
Bcl-2 (ng/mg protein)	32.34 ± 0.14	22.36 ± 0.21*	38.64 ± 0.24*	35.27 ± 0.13[†]
Bax (ng/mg protein)	4.87 ± 0.24	11.08 ± 0.12*	4.03 ± 0.25	5.19 ± 0.37[†]
Bax/Bcl-2 ratio	0.15 ± 0.02	0.49 ± 0.03*	0.18 ± 0.02	0.15 ± 0.01[†]

Values are the mean ± SEM ($n = 7$). *$P < 0.05$, significant change with respect to control; [†]$P < 0.05$, significant change with respect to AlCl$_3$ for Duncan's *post hoc* test.
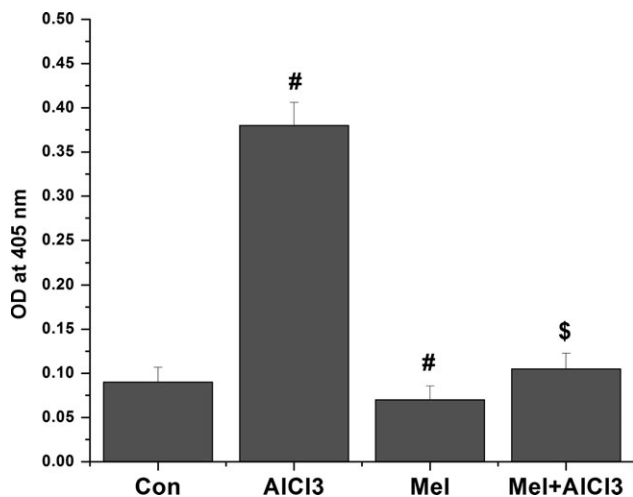
Figure 5 Caspase-3 activity in rat brain exposed to melatonin (Mel) and aluminium chloride (AlCl$_3$) for 7 days. Values are the mean ± SEM ($n = 7$). [#]$P < 0.05$, significant change with respect to control; [$]$P < 0.05$, significant change with respect to AlCl$_3$ for Duncan's *post hoc* test.

vacuolation of neurons, as well as necrotic and apoptotic neurons in the cerebellum, cerebral cortex and hippocampus. However, the brains from rats treated with AlCl$_3$ plus melatonin revealed that most of the histological alterations induced in the AlCl$_3$-treated rats were markedly reduced, although some necrotic and apoptotic neurons were still present (Figure 4).

To study the anti-apoptotic effects of melatonin treatment in rats, Bax, Bcl-2 and caspase-3 were measured in brain homogenates. As shown in Table 2, the level of Bax in AlCl$_3$-treated rats was higher compared to the control ($P < 0.05$). Pretreatment with Mel significantly decreased the Bax level compared to AlCl$_3$-treated rats. As shown in our data, Bax was minimally present, whereas Bcl-2 was constitutively expressed in the brain tissue. A significant ($P < 0.05$) elevation in the Bcl-2 level was observed in the Mel + AlCl$_3$-treated rats compared to the AlCl$_3$-treated group. In addition, the Bax/Bcl-2 ratio was decreased in the Mel + AlCl$_3$-treated group and restored to control levels (Table 2). Notably, caspase-3 activity showed a similar pattern to Bax (Figure 5). Caspase-3 was significantly elevated ($P < 0.05$) compared to the controls, and Mel pretreatment reduced the caspase-3 activity.

Discussion

Toxic metals are considered major environmental pollutants. Toxic metals are classified as non-biodegradable substances because they are not degraded by cells. They represent a global health risk because of their ability to contribute to a variety of diseases. In this context, Al (which is a highly reactive element and ubiquitous environmental contaminant) has been associated with some diseases (Fulgenzi *et al.* 2014).

While Al is not a transition metal and cannot initiate peroxidation, many studies have looked for a correlation between Al accumulation and oxidative damage in different organs. *In vitro* studies suggest that Al significantly accelerates iron-mediated lipid peroxidation (Ohyashiki *et al.* 1996). AlCl$_3$ intoxicated animals show numerous indicators of oxidative stress, including elevated LPO and NO levels and decreased GSH levels. The results of present study indicate a pattern of AlCl$_3$-induced oxidative stress similar to that found in previous studies, which indicates that Al-induced oxidative stress involves free radical generation (Yousef *et al.* 2005; Abdel Moneim 2012; Romero *et al.* 2014).

Damage to neurons by ROS overproduction is directly related to the pathophysiology of major neurodegenerative diseases including Parkinson's disease and Alzheimer's disease (Abdel Moneim 2013). Increased reactive oxygen species (ROS) were reported in previous studies during Al exposure, which was attributed to electron leakage, enhanced mitochondrial activity and increased electron chain activity. Therefore, one can hypothesize that oxidative stress may be contributing factor towards Al-induced toxicity. LPO is one of the main manifestations of oxidative damage, and it has been found to play an important role in the toxicity of many toxic metals. As shown in Figure 1, we found that AlCl$_3$ induced neuronal LPO. Most *in vitro* studies have attributed Al neurotoxicity to the LPO caused by the interaction between ROS and cell membranes with the latter being the main targets of the oxidant-mediated damage (Kumar & Gill 2014). Therefore, we investigated the effect of Mel as a therapeutic and/or prophylactic agent against AlCl$_3$-induced neuronal injury. We found that Mel pretreatment resulted in a significant reduction in LPO compared to AlCl$_3$-treated rats. Therefore, our findings suggested that melatonin protects against LPO-induced neurotoxicity.

AlCl$_3$ is likely capable of inducing NO production in the microglia and astrocytes of the rat brain (Zaky *et al.* 2013). The increment in NO may be due to the ability of AlCl$_3$ to

enhance the expression of iNOS. The present study shows an elevation in NO production, indicating that Al-induced toxicity may involve changes in NO production. A very large body of evidence indicates that melatonin is a major scavenger of both oxygen- and nitrogen-based reactive molecules, including $ONOO^-$ (Ucar *et al.* 2007). Melatonin has scavenging actions at both physiologic and pharmacologic concentrations. In addition, several of its melatonin's metabolites can detoxify free radicals and their derivatives (Reiter *et al.* 2008). In our experiments, we found that short-term exogenous Mel treatment significantly decreases NO levels in the brain.

As oxidative damage is mediated by free radicals, investigating the status of endogenous antioxidant enzymes under different treatment conditions was necessary. $AlCl_3$ administration caused a significant ($P < 0.05$) decrease in the activity of antioxidant enzymes and GSH compared to the control group (Figure 3), and this is in accordance with a previous report by Abdel Moneim (2012). The decreased enzymatic and non-enzymatic molecules might have contributed to the elevated protein and lipid oxidation level during Al toxicity. Inhibition of the enzymatic activities may also contribute to the Al-induced decline in the mRNA expression of endogenous antioxidants (Gonzalez *et al.* 2007). In the present study, increased lipid peroxidation, associated with decreased antioxidant status in $AlCl_3$-treated rats, can therefore be related to an insufficient antioxidant potential. In rats treated with $AlCl_3$ plus melatonin, we observed a marked increase in GR and GPx activities, which resulted in normalization of the brain GSH level. Therefore, melatonin protects lipids against $AlCl_3$-induced oxidation due to the ROS/RNS-scavenging and antioxidant activity of the indolamine (Acuna Castroviejo *et al.* 2011).

Histological investigations revealed that $AlCl_3$ exposure caused progressive alterations in the brain. The findings corroborate with the previous findings of Bhadauria (2012) and Abdel Moneim (2012) as Al has been implicated in the pathogenesis of several clinical disorders. Melatonin pretreatment could improve, to some extent, the altered brain histopathologies.

To explore the mechanism by which melatonin attenuates $AlCl_3$-induced apoptosis, Bax, Bcl-2 and caspase-3 protein levels were measured in brain homogenates. ROS increase the permeability of the mitochondrial membrane and result in mitochondrial failure (Huang *et al.* 2008). The mitochondrial membrane permeability is dependent upon the mitochondrial permeability transition pore that results in the release of cytochrome *c* from the mitochondria and into the cytosol (Yang *et al.* 2014). Once released, cytochrome *c* binds Apaf-1 in the cytoplasm forming a complex that can activate caspase-9 with subsequent activation of death-inducing caspase-3 (Ghribi *et al.* 2002). In our study, we observed $AlCl_3$ induced apoptosis in the brain of rats through the activation of caspase-3, and the injection of melatonin decreased the activity of caspase-3. These results suggest that melatonin exerts its anti-apoptotic effects, at least in part, by inhibiting caspase activation.

The mitochondria-mediated intrinsic pathway is controlled by the Bcl-2 family of proteins. The Bcl-2 protein family is classified into two subgroups according to structural homology: the anti-apoptotic proteins such as Bcl-2 and Bcl-XL and the pro-apoptotic proteins such as Bax and Bak. The balance between the pro- and anti-apoptotic proteins of the Bcl-2 family is an important determinant of cell survival or death. Bcl-2 may function as a counteracting force to reduce damage by reducing lipid peroxidation triggered by cytotoxic stimuli such as ROS (Akifusa *et al.* 2009). Bcl-2 was also found to prevent the release of cytochrome *c*. In contrast, Bax regulates apoptosis, not only by dimerizing with anti-apoptotic Bcl-2 proteins, but also by regulating cytochrome *c* release and subsequent caspase-3 activation (Cai *et al.* 2011). Our results showed that Mel treatment reversed the altered Bcl-2 and Bax levels induced by $AlCl_3$ and substantially restored the Bcl-2/Bax ratio. In the current study, melatonin administration inhibited all toxic events induced by $AlCl_3$. Melatonin scavenges oxygen and nitrogen-based reactants generated in the mitochondria, stabilizes the mitochondrial membrane and enhances anti-apoptotic signalling.

In conclusion, exposure to aluminium chloride could generate free radicals, which resulted in the elevation of neuronal lipid peroxidation and nitric oxide levels and reduction in the enzymatic and non-enzymatic antioxidant components. However, melatonin could protect against $AlCl_3$-induced neurotoxicity. This protective effect of melatonin may be due to the radical scavenging activity that maintains an adequate level of non-enzymatic and enzymatic antioxidant defence against $AlCl_3$ neurotoxicity. These novel results suggest that melatonin may be an effective treatment to alleviate the devastating effects of aluminium-induced neurotoxicity.

Acknowledgements

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