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### PROPHYLACTIC EFFECT OF MELATONIN IN REDUCING LEAD-INDUCED NEUROTOXICITY IN THE RAT

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**Abstract:** Oxidative stress is a likely molecular mechanism in lead neurotoxicity. Considering the antioxidant properties of melatonin, this study investigated the neuroprotective potential of melatonin in the hippocampus and corpus striatum of rats treated with lead. Three groups of male rats (control, lead acetate-treated [100 mg/kg], and lead acetate plus melatonin [10 mg/kg] for 21 consecutive days) were used. Levels of products of lipid peroxidation (LPO), glutathione (GSH) and superoxide dismutase (SOD) activity were measured in brain homogenates. Histological changes in the pyramidal cells of the hippocampus and the putamen of the corpus striatum were examined. The results documented increased LPO and decreased GSH and SOD activity in the brain homogenates of lead-treated rats. Histological observations revealed severe damage and a reduction in neuronal density in the hippocampus and corpus striatum. When melatonin was given to lead-treated rats, it almost completely attenuated the increase in LPO products and restored GSH levels and SOD activity. Also, the morphological damage was reduced and neuronal density was restored by melatonin. Considering the ease with which melatonin enters the brain, these results, along with previous observations, suggest that melatonin may be useful in combating free radical-induced neuronal injury that is a result of lead toxicity.

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Abbreviations used: ALA - aminolevulinic acid; ANOVA - analysis of variance; GSH - glutathione; 4-HAD - malondialdehyde; 4-HAD - 4-hydroxy nonenal; LPO - lipid peroxidation; MDA - malondialdehyde; NF- $\kappa$ B - nuclear factor  $\kappa$ B; NO - nitric oxide; <sup>1</sup>O<sub>2</sub> singlet oxygen -  $\bullet$ OH, hydroxyl radical; ONOO<sup>-</sup> - peroxy nitrite anion; SOD - superoxide dismutase.

**Key Words:** Lead Acetate, Lipid Peroxidation, Superoxide Dismutase, Glutathione, Hippocampus, Corpus Striatum, Melatonin

## INTRODUCTION

Lead neurotoxicity results in behavioral and neurochemical alterations in neurons as a result of changes and disruption of main structural components of the blood-brain barrier, through primary injury to astrocytes and to secondary damage of the endothelial microvasculature [1]. Subsequent morphological changes include neuronal degeneration and pericapillary space dilatation [2].

Lead treatment results in a significant accumulation of lead in all brain regions with maximal levels occurring in the hippocampus. It was also noted that changes in glutathione, lipid peroxidation products, intracellular calcium and membrane fluidity were consequences of lead toxicity. Neurotoxicity associated with lead exposure are believed to be the result of a series of perturbations in brain metabolism, and in particular, a consequence of oxidative stress [3]. That lead-induced enhancement on lipid peroxidation is a major mechanism for some of the toxic effects of lead has certainly been suggested earlier [4]. This supposition is supported by the rise in serum lipid peroxidation levels in workers with occupational exposure to lead and in the reduction in the activity of the antioxidant enzyme superoxide dismutase.

Numerous reports have documented protective actions of melatonin in various models of oxidative stress [5-8]. This is due to its high efficacy as a free radical scavenger and indirect antioxidant [9-11]. Melatonin is an effective hydroxyl radical (OH) scavenger [12]. Additionally, this indole detoxifies other reactive oxygen and nitrogen species including single oxygen [ $^1\text{O}_2$ ] [13], nitric oxide [NO] [14], the peroxynitrite anion (ONOO<sup>-</sup>) as well as its metabolite peroxynitrous acid [15] and hydrogen peroxide [16]. Finally, melatonin stimulates the activities of enzymes that metabolize reactive species [17] and maintains cell membrane fluidity at an optimal level [18].

Melatonin is readily absorbed when it is administered via any route; it crosses all morphophysiological barriers (e.g., blood-brain barrier and placenta) with ease, it seems to enter all parts of every cell where it prevents oxidative damage and preserves mitochondrial function [19]. Considering this, the aim of this work was to investigate the prophylactic role of melatonin in reducing oxidative stress and histological changes in the putamen of the corpus striatum and granular layer of the hippocampus of lead acetate-treated rats.

## MATERIALS AND METHODS

### Animals

Twenty-one adult male Sprague-Dawley rats, weighing  $100 \pm 5$ g, purchased from Assiut University Joint Animal Breeding Unit were used in this study. All animals were kept under the same laboratory conditions of temperature ( $25 \pm 2$  °C) and lighting (14:10 h light:dark cycle) and were given free access to

standard laboratory chow and tap water. The protocol for the experiment was approved by the appropriate animal care committee of Assuit University.

### **Chemicals**

Melatonin was a gift from Helsinn Chemicals SA (Biasca, Switzerland) and dissolved in ethanol before being diluted with saline. The final concentration of alcohol in the injected solution was <0.1%. Bioxytech LPO-586 assay kit, purchased from Cayman Chemical (Ann Arbor, MI), was used for measuring the products of LPO (malondialdehyde [MDA] and 4-hydroxyalkenals [4-HDA]). Lead acetate (dissolved in saline), gallocyanine and chromic potassium sulfate were purchased from Sigma (St. Louis, MO). All other chemicals were of the highest quality available.

### **Experimental protocol**

The rats were divided into three groups. The first group (6 rats) served as controls and received a subcutaneous injection of physiological saline. The second group (7 rats) was given a subcutaneous injection of lead acetate at a dose of 100 mg/kg body weight. The third group (8 rats) was given an injection of lead acetate as well as a subcutaneous melatonin injection at a dose of 10 mg/kg body weight 30 min before lead acetate administration. The injections were repeated daily for 21 days. Twenty-four hours after the last injection, the rats were sacrificed and the brains were removed and divided longitudinally; one-half of each brain was frozen and stored at  $-40^{\circ}\text{C}$  for later biochemical analyses. The other half of the brain was preserved for histological examination.

### **Measurement of lipid peroxidation**

MDA and 4-HDA concentrations are considered to be an index of the peroxidation of membrane lipids. The colorimetric kit mentioned above was used to determine the levels of oxidized lipid. At the time of assay, half brains were homogenized in ice-cold 50 mM Tris buffer (pH 7.4, 10% w/v) using ultra-Turrax T25b homogenizer and supernatants were prepared by centrifugation at 10,000 g for 10 min; these were used to measure MDA and 4-HDA levels. Protein levels were measured using bovine albumin as standard.

### **Superoxide dismutase activity**

Superoxide dismutase activity was estimated according to Misra and Fridovich [20] based on its ability to inhibit the autoxidation of epinephrine in an alkaline medium (pH 10.2).

### **Determination of glutathione**

Total glutathione (GSH) levels were measured using the method of Beutler et al. [21]; this method is based on the development of a stable yellow color when 2-nitrobenzoic acids is added to sulfhydryl compounds.

**Histological examination**

Half of each longitudinally-divided brain was fixed in 10% neutral-buffered formalin, embedded in paraffin, and serially sectioned. The sections were stained with gallocyenin.

**Karyometry and quantitation**

The densities of pyramidal cells in the hippocampus and neurons in the putamen of the corpus striatum were determined by counting these cells in 50 randomly selected fields at high magnification. The volume of the neuronal nuclei was also evaluated. The measurements were performed with the use of an ocular micrometer. Nuclei were selected when they appeared spherical in shape. The diameter of 100 nuclei were measured and the volume was obtained in cubic micrometer by means of the formula of a sphere:  $V = 4/3 \pi r^3$ .

**Statistical analysis**

The data are presented as the arithmetic means  $\pm$  SEM. Statistical analyses were performed using an ANOVA followed by the Student-Newman-Keuls t-test.  $P < 0.05$  was considered to be significant. The present inhibition or stimulation in the mean values of LPO, SOD, GSH and neural density were calculated according to the following formula [9]:

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

**RESULTS**

Administration of lead acetate increased LPO levels (as indicated by the increase in MDA and 4-HDA levels) in brain homogenates versus those of the levels of these constituents in the brain of control rats (Fig. 1 top). The lead-induced increase in LPO was 42%; this increase is significant ( $p < 0.01$ ). When melatonin was given to lead-treated rats it significantly reduced ( $p < 0.05$ ) LPO levels almost to the level of the control rats.

SOD activity was severely inhibited in the brain homogenates of lead-treated rats compared to those of controls (Fig. 1, middle). The inhibition was 73% with a level of significance of  $p < 0.01$ . Melatonin co-treatment significantly restored ( $p < 0.05$ ) SOD activity in lead treated rats.

Chronic lead administration reduced total GSH levels in brain homogenates compared to those of controls (Fig. 1, bottom). The reduction was 49% and statistically significant ( $p < 0.01$ ). When melatonin was given to lead-treated rats, GSH levels were partially restored ( $p < 0.05$ ) but still significantly lower than control levels.

The neuronal densities of pyramidal cells of the hippocampus (Fig. 1, top) and neurons of the putamen (Fig. 2, bottom) of lead-treated rats were significantly reduced ( $p < 0.01$ ) compared to those of control animals. The reduction was 25% and 38% in the hippocampus and putamen, respectively. When melatonin was given to lead-treated rats, the number of neurons in the hippocampus ( $p < 0.05$ ) and putamen ( $p < 0.01$ ) were increased. With regard to the nuclear volume of the neurons in the hippocampus and putamen, the data revealed no significant changes between any of the three groups (data not show).

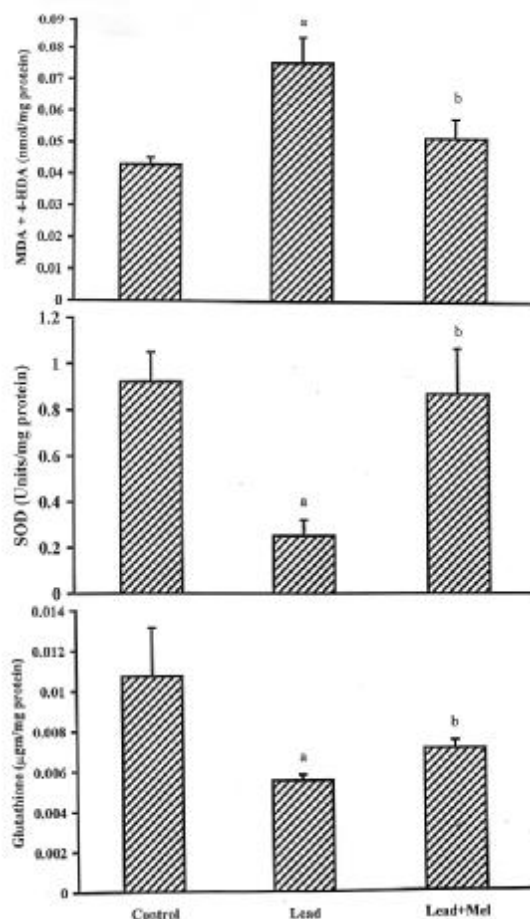


Fig. 1. Changes in levels of lipid peroxidation products [malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA)] (top), superoxide dismutase (SOD) activity (middle) and total glutathione (GSH) levels in the brain of control, lead-treated and lead-treated rats given melatonin (Mel). Data are means  $\pm$  SEM. <sup>a</sup> $p < 0.01$  versus controls; <sup>b</sup> $p < 0.05$  versus lead only.

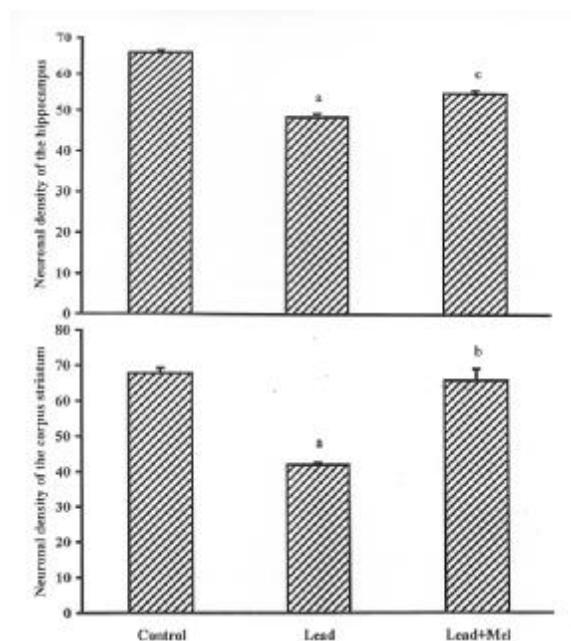


Fig. 2. Neuronal density in the hippocampus (pyramidal neurons) (top) and the putamen of the corpus striatum (bottom) of control, lead-treated and lead-treated rats given melatonin (Mel). Data are means  $\pm$  SEM. <sup>a</sup> $p < 0.01$  versus controls; <sup>b</sup> $p < 0.01$  versus lead only; <sup>c</sup> $p < 0.05$  versus lead only.

## DISCUSSION

Lead is a non-essential, toxic, heavy metal widely distributed in the environment and chronic exposure to low levels of this agent is a matter of public concern in many countries. Lead is frequently found in air, drinking water, soil, and industrial by-products. It is also found in lead-associated work places such as smelting, battery manufacture, stained glass manufacture, and lead-based paint production.

Lead exposure is known to produce adverse effects on the brain [22]. The hippocampal formation is widely considered to be the major site of action of lead in the brain [23]. Learning and memory deficits caused by hippocampal damage are similar to those found in lead-induced neurotoxicity [24]. Neurotoxicity from lead exposure is likely associated with oxidative stress [3]. The current results reveal significant decreases in the neuronal density of the pyramidal cells of the hippocampus and neurons in the putamen of lead-treated rats versus those of controls. In addition, the lipid peroxidation levels were increased while GSH concentrations and SOD activity were decreased relative to those of controls.

Lead readily crosses the blood-brain barrier and causes immediate effects by altering the metabolism and physiology of the brain. These functional alterations result in morphological changes in the brain that can remain even after lead levels have fallen. Thus, there are long-term structural, biochemical, and behavioral effects of lead toxicity [25]. One likely molecular mechanism involved in lead neurotoxicity is the disruption of the prooxidant/antioxidant balance [26, 27] which leads to brain injury via oxidative damage to critical biomolecules such as lipids, proteins, and DNA. Hermes-Lima et al. [26] and Sandhir et al. [27] speculated that lead-induced oxidative damage may result from, a), the inhibition of 5-aminolevulinic acid (ALA) dehydrogenase by lead leading to the accumulation of ALA, a potential endogenous source of free radicals, b), direct interaction of lead with biological membranes, inducing lipid peroxidation, c), an increase of intracellular levels of calcium, impairing mitochondrial function and d), lead-induced decrease in free radical metabolizing enzymes and in GSH levels.

The results of this investigation uncover the neuroprotective potential of melatonin against lead toxicity. In this study melatonin reduced the severity of each change indicative of oxidative damage. Melatonin is a known direct radical scavenger and an indirect antioxidant with high efficacy in the brain [9]. Neuroprotective effects of melatonin have been demonstrated in many models of neuronal cell death in which oxygen free radicals are involved. In models of Parkinson's disease, melatonin completely reversed the increases in lipid peroxidation [28], and it prevented kainate-induced neuronal cell death and reduced lipid peroxidation products in rats and mice [29]. Furthermore, melatonin protects against glutamate-induced cell death in the clonal hippocampal cell line HT22 [30], prevents delayed neuronal death induced by enhanced excitatory transmission in hippocampal pyramidal neurons [31], and rescues neuroblastoma cells exposed to toxic fragments of Alzheimer's  $\beta$ -amyloid [32]. The anticonvulsant capacity of melatonin has been demonstrated against excitotoxin-induced seizures due to quinolinate, kainate, and glutamate in mice and rats, further suggesting neuroprotective actions [33].

In addition to its antioxidant effects, several other mechanisms may be involved in the neuroprotection mediated by melatonin; these include interactions with calmodulin [33] and microtubular components [34], blockade of increases in intracellular  $\text{Ca}^{2+}$  [35], inhibition of activation of NF- $\kappa$ B by cytokines such as tumor necrosis factor  $\alpha$  [36], inhibition of the expression of inducible nitric oxide synthase at the transcriptional level [37], changes in gene expression and activities of antioxidant enzymes [38] and improved efficiency of mitochondrial oxidative phosphorylation [19].

In conclusion, the present data document that lead is severely neurotoxic and that melatonin reduces the resulting damage probably due to its ability to neutralize free radicals that are generated by lead. This is the first study to show the melatonin is protective against heavy metal toxicity.

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