Melatonin protects against lead acetate-induced changes in blood corpuscles and lipid profile of male Wistar rats

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ABSTRACT

Treatment of rats with lead acetate at a dose of 15 mg / kg body weight intraperitoneally (i.p) for a period of seven consecutive days caused alterations in the total count of erythrocyte, total count of leukocyte, hemoglobin content, mean corpuscular hemoglobin content, neutrophil count, small lymphocyte count, eosinophil count, erythrocyte sedimentation rate (ESR), total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, Total cholesterol: HDL cholesterol, LDL cholesterol : HDL cholesterol. All these changes were ameliorated when the rats were pre-treated with melatonin at a dose of 10 mg / kg (fed orally) for a similar period of time. The results of the current studies indicate melatonin’s ability to mitigate heavy metal-induced alterations in blood tissue. This is probably brought about through the antioxidant activity of melatonin and may have future therapeutic relevance in humans exposed to lead environmentally or occupationally and in situations where chelation therapy has limited success.

Key words: Melatonin, blood, antioxidant, lead acetate, LDL, HDL, Cholesterol, ESR

INTRODUCTION

Lead is an environmental pollutant, widely distributed, naturally occurring, toxic heavy metal.¹ Lead is also recognized as an occupational hazardous metal² which if enters the body accumulates in soft organs and cannot be metabolized by the body.¹ Lead is one of the few natural substances that have no known use in the human body.¹ At even very low level, lead has been shown to cause health problems.³ The difficulty with lead is that once it is mined from the earth, there is no known way to destroy or make it harmless. Lead reduces the amino levalinic acid synthesis by down-regulating the gene expressing amino levalinic acid synthase and thus lead leads to impairment of erythropoiesis.³

Heavy metals like lead, cadmium etc. have very long half life and are severely toxic at a very low dose². Studies revealed the involvement of oxidative stress as an important mechanism for heavy metal toxicity.² The toxicity of lead (Pb) is well documented and involvement of oxidative stress in lead toxicity is also recognized.³,⁶,⁷ Effect of lead exposure in experimental animals and human has been studied in considerable detail.⁸

Melatonin (N-acetyl-5-methoxytryptamine) was discovered as a potent antioxidant in 1993.⁹ Melatonin’s ability to protect cells and organs from oxidative/nitrosative damage has been confirmed in more than a thousand publications.¹⁰ The indoleamine, melatonin, is produced in all animals and also in plants.¹¹ The antioxidative properties of melatonin are well known. Studies reveal protective action of melatonin against heavy metal induced toxicity and tissue damages.⁶,¹²,¹³ Ameliorative actions of melatonin against a variety of situations involving oxidative stresses are currently intensely being investigated.¹⁴ We have studied the protective effect of melatonin against lead acetate-induced oxidative damage in liver, heart and kidney tissues of male Wistar rats.⁶,¹³

Studies also reveal protective action of melatonin in situations of lead induced hematotoxicities.¹⁵ Melatonin has also been shown to enhance the antioxidant capacity of blood.¹⁶

Melatonin has many functions in organisms, i.e., helping to synchronize circadian rhythms¹⁷, sleep promotion¹⁸, immune stimulation¹⁹, seasonal reproductive regulation²¹, oncosstatic²¹, antidepressive²²
etc. but it seems to be unparallel as an antioxidant for plants to humans. 23, 24, 25

Melatonin has been reported to directly neutralize free radicals.26 Moreover, several of its metabolites have also been shown to scavenge free radicals. 27 There are studies suggesting protective role of melatonin against lead-induced neurotoxicity 28.

Herein, we provide evidence that a low pharmacological dose of melatonin provides protection against lead acetate induced deteriorative alterations in blood tissue of rats. The protection is brought about possibly through the antioxidant activity of melatonin and point toward its therapeutic usefulness as a protective agent against hematotoxic situations arising out of exposure of the humans to lead metal environmentally or occupationally.

MATERIALS AND METHODS
Melatonin and lead acetate were purchased from SRL Chemicals, India. All other chemicals used were of analytical grade and were procured from E. Merck, Germany, India and Sigma Chemicals, USA.

Animal treatment
Male Wistar rats of body weight 160-180 gm were used throughout the experiments. The animals were handled as per the guidelines of Institutional Animal Ethics Committee (IAEC) [IAEC/PROPOSAL/DB-2/2010, APPROVAL DATE:16/11/2011 ] in accordance with the committee for the purpose of control and supervision of experiment on animal (CPSEA), Ministry of Environment and Forest, Government of India. All the experimental protocols had the approval of IAEC of the Department of Physiology, University of Calcutta. Prof. P. K. Samanta, M. Sc. (Vet.), Ph. D., Professor and Veterinary Surgeon and CPCSEA Nominee to Department of Physiology, University of Calcutta, acted as the advisor for animal care and handling.

After acclimatization to laboratory conditions, the rats of the melatonin and the melatonin + lead acetate group were fed melatonin dissolved in normal drinking water, at a dose of 10 mg kg\(^{-1}\) body weight for 7 consecutive days. An hour after melatonin was fed, the animals of the lead acetate and the melatonin + lead acetate treated groups were injected with lead acetate solution, intraperitoneally, at a dose of 15 mg kg\(^{-1}\) body weight (LD\(_{50}\) is 150 mg/kg bw) for the 7 consecutive days. The animals of the control group received the vehicle only. Each day the body weight of the animals were measured and recorded.

Collection of blood and preparation of the serum
At the end of the treatment period, the animals of each group were kept fasted overnight. The animals were sacrificed through cervical dislocation after subjecting them to mild ether anaesthesia. Blood was carefully collected from each rat through cardiac puncture in microfuge tube and was allowed to clot for serum to separate out and then centrifuged at 2500 rpm for 15 minutes. Serum, thus obtained, was collected carefully in individual microfuge tube and stored at – 20°C. Serum was separated immediately and used for the analysis of total cholesterol (TC), triglyceride (TG), high and low density lipoprotein (HDL and LDL).

Estimation of hemoglobin content
Hemoglobin content of the blood was estimated quantitatively using Drabkin’s method. In this method, haemoglobin is oxidized to methemoglobin by potassium ferricyanide, which reacts with cyanide ions of potassium cyanide to form cyanmethemoglobin. The absorbance was recorded at 530 nm using a UV / VIS spectrophotometer (Biorad Smartspec Plus).

Estimation of RBC total count
A total count of red blood cell (RBC) was estimated using RBC counting chamber. 29 Blood was drawn into RBC pipette up to mark 1, the extra blood on the outer surface of the pipette was wiped and the RBC fluid (Hayem’s fluid): composition; Na\(_2\)SO\(_4\) 5 g, NaCl 1 g, HgCl\(_2\) 0.5 g dissolved in 200 ml distilled water] was drawn immediately up to mark 101. The contents were mixed well and the clear fluid was expelled from the stem of the pipette which did not contain any blood. Then, a drop of the diluted blood was put at each edge of cover glass placed over the counting chamber. Over flow of fluid and any air bubble inside the chamber was avoided. The central medium sized square was focused under high power and the RBC’s at the four groups of 16 smaller squares at the 4 corners and the 16 smaller squares at the centre portion were counted.

Estimation of white blood cell (WBC) total count
WBC total count was estimated using a WBC counting chamber and using WBC fluid (composition; 0.5-1% glacial acetic acid with methyl blue; acetic acid ruptures RBC and methylene blue stains WBC nuclei) 29. The same method as used in case of enumeration of RBC was followed, using WBC pipette. The WBC in the Neubauer Chamber is counted in large 4 squares.

Determination of hemoglobin content per red blood cell (MCH)
Hemoglobin content per red blood cell (MCH) was determined using the equation 29: MCH = Hgb / RBC count.

Estimation of lymphocytes and neutrophil count
Numbers of small lymphocytes, large lymphocytes and neutrophil were counted from the dry blood film using routine method of differential count 29.

Estimation of monocyte and the eosinophil counts
Monocyte and the eosinophil were also counted from the dry blood film using routine method of differential count 29-31.

Estimation of Erythrocyte Sedimentation Rate (ESR)
Erythrocyte Sedimentation Rate (ESR) was determined in all the samples using The Westergren method. 32

Lipid profile measurement
Total cholesterol was estimated by enzymatic colorimetric method. 33 Triglyceride concentration was measured by the method of Fletcher. 34
HDL cholesterol was estimated by the homogenous enzymatic method and LDL cholesterol was measured by the method of Friedewald et al. Total cholesterol: HDL cholesterol, LDL cholesterol. HDL cholesterol were also evaluated.

**Statistical analysis**

Data are presented as means ± S.E.M. Significance of mean values of different parameters between the treatment groups were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

**RESULTS**

**Estimation of hemoglobin content**

Treatment of rats with lead acetate for seven consecutive days caused a significant decrease in the hemoglobin content indicating a state of anemia (33.88% vs control; *P<0.001). Pre-treatment of the rats with melatonin significantly protected the hemoglobin content (53.75% vs lead; **P<0.001). Only melatonin treatment had no effect on the hemoglobin content [Table 1].

**Total Red blood cell (RBC) count**

Total red blood cell (RBC) count was significantly decreased in rats treated with lead acetate for seven consecutive days (15.05% vs Control; *P<0.001). Total RBC count in rats pre-treated with melatonin for the same period of time was observed to be significantly protected from being decreased (17.54% vs lead; **P<0.001). Only melatonin treatment had no effect on the total RBC count [Table 1].

**Erythrocyte Sedimentation Rate (ESR)**

The rate of erythrocyte sedimentation was significantly increased in rats treated with lead acetate for seven consecutive days (60% vs Control; *P<0.001). Pre-treatment with melatonin for the same period of time significantly protected the ESR from being increased (25% vs lead; **P<0.001). Only melatonin treatment had no effect on the ESR [Table 1].

**Table 1: Table shows the content of hemoglobin, red blood cell total count and Erythrocyte Sedimentation Rate (ESR) in lead acetate treated and melatonin protected rats.**

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>Control</th>
<th>Melatonin</th>
<th>Lead</th>
<th>Melatonin+ Lead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g%)</td>
<td>12.10±0.132</td>
<td>12.20±0.146</td>
<td>8.00±0.109*</td>
<td>12.30±0.127**</td>
</tr>
<tr>
<td>Total RBC (X10^6/cm³)</td>
<td>6.71±0.103</td>
<td>6.72±0.104</td>
<td>5.70±0.109*</td>
<td>6.70±0.108**</td>
</tr>
<tr>
<td>Erythrocyte Sedimentation Rate (ESR) (1st hour, mm)</td>
<td>5.00±0.142</td>
<td>5.00±0.121</td>
<td>8.00±0.126*</td>
<td>5.00±0.132*</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P<0.001 compared to lead treated group;

**Mean corpuscular hemoglobin (MCH)**

There was microcytic hypochromic anemia following significant fall in the mean corpuscular hemoglobin (MCH) or Hemoglobin content per red blood cell in lead acetate treated group (39.21% vs control, *P<0.001). MCH was found to be protected from being decreased in the animals pre-treated with melatonin (63.63% vs lead, **P<0.01). Only melatonin had no effect on the MCH [Figure 1].

**Total White blood cell (WBC) count**

Total white blood cell (WBC) count was significantly decreased in rats treated with lead acetate for seven consecutive days (30.51% vs Control; *P<0.001). Total RBC count in rats pre-treated with melatonin for the same period of time was observed to be significantly protected from being decreased (41.46% vs lead; **P<0.001). Only melatonin treatment had no effect on the total WBC count [Table 2].

**White blood cell (WBC) differential count**

**Neutrophil count**

Neutrophil count increased in lead acetate treated group (45.83% vs control *P<0.001) while it was found to be near normal in the animals pre-treated with melatonin (31.42% less vs lead **P<0.01). Only melatonin had no effect on the Neutrophil count [Table 2].
Lymphocyte count
Lymphocyte count decreased in lead acetate treated group (18.36% vs control *P<0.001) while it was protected from being decreased in the animals pre-treated with melatonin (15.00% less vs lead **P<0.01). Only melatonin had no effect on the Neutrophil count [Table 2].

Monocyte, basophil and eosinophil counts
Monocyte, basophil and eosinophil counts were not altered in any of the treated groups [Table 2].

Neutrophil:Lymphocyte
Neutrophil and Lymphocyte ratio increased in lead acetate treated group (79.60% vs control *P<0.001) while it was protected from being altered in the animals pre-treated with melatonin (40.91% vs lead **P<0.01). Only melatonin had no effect on the ratio of neutrophil and lymphocyte [Table 2].

Table 2: Table shows the total count of WBC and differential white blood cell count in lead acetate treated and melatonin protected rats.

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>Control</th>
<th>Melatonin</th>
<th>Lead</th>
<th>Melatonin+lead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC (X10^9 /cmm)</td>
<td>5.9±0.125</td>
<td>5.9±0.126</td>
<td>4.1±0.129*</td>
<td>5.8±0.127**</td>
</tr>
<tr>
<td>Neutrophils(%)</td>
<td>24.00±1.03</td>
<td>23.00±1.05</td>
<td>35.00±1.09*</td>
<td>24.00±1.08**</td>
</tr>
<tr>
<td>Lymphocytes(%)</td>
<td>49.00±1.23</td>
<td>50.00±1.34</td>
<td>40.00±1.21*</td>
<td>46.00±1.13**</td>
</tr>
<tr>
<td>Basophils(%)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Monocyte(%)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Eosinophil(%)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Neutrophil:Lymphocyte</td>
<td>0.49±0.052</td>
<td>0.46±0.044</td>
<td>0.88±0.046*</td>
<td>0.52±0.044**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P<0.001 compared to lead treated group;

Total Cholesterol:
Total cholesterol level in serum increased in lead acetate treated group (52.00% vs control *P<0.001). The level of cholesterol was found to be protected from being increased in the animals pre-treated with melatonin (25.00% vs lead **P<0.01). Only melatonin had no effect on the serum level of total cholesterol [Table 3].

Triglyceride
Triglyceride level in serum increased in lead acetate treated group (30.43% vs control *P<0.001). The level of triglyceride was found to be protected from being increased in the animals pre-treated with melatonin (10.60% vs lead **P<0.01). Only melatonin had no effect on the serum level of triglyceride [Table 3].

HDL Cholesterol direct
HDL Cholesterol level in serum decreased in lead acetate treated group (18.73% vs control *P<0.001). The level of HDL Cholesterol was found to be protected from being decreased in the animals pre-treated with melatonin (32.24% vs lead **P<0.01). Only melatonin had no effect on the serum level of HDL Cholesterol [Table 3].

LDL Cholesterol direct
The serum level of LDL Cholesterol increased in lead acetate treated group (13.54% vs control *P<0.001). The level of LDL Cholesterol was found to be protected from being increased in the animals pre-treated with melatonin (10.60% vs lead **P<0.01). Only melatonin had no effect on the serum level of LDL Cholesterol [Table 3].

Table 3: Table shows the lipid profile and plasma glucose level of lead acetate treated and melatonin protected rats.

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>Control</th>
<th>Melatonin</th>
<th>Lead</th>
<th>Melatonin+lead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>60.00±1.45</td>
<td>62.00±1.23</td>
<td>77.00±1.34*</td>
<td>66.00±1.22**</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>46.00±1.76</td>
<td>45.00±1.83</td>
<td>60.00±1.65*</td>
<td>46.00±1.58**</td>
</tr>
<tr>
<td>HDL Cholesterol direct (mg/dl)</td>
<td>44.00±1.38</td>
<td>44.24±1.12</td>
<td>35.76±1.44*</td>
<td>47.29±1.57**</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dl)</td>
<td>48.29±2.11</td>
<td>48.37±1.94</td>
<td>54.83±2.01*</td>
<td>49.02±2.87**</td>
</tr>
<tr>
<td>Total Cholesterol:HDL Cholesterol</td>
<td>1.36±0.032</td>
<td>1.40±0.057</td>
<td>2.15±0.043*</td>
<td>1.39±0.056**</td>
</tr>
<tr>
<td>LDL:HDL</td>
<td>1.098±0.012</td>
<td>1.093±0.009</td>
<td>1.533±0.011*</td>
<td>1.037±0.011**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P<0.001 compared to lead treated group;
DISCUSSION

Lead is an environmental toxin, an unnecessary heavy metal in human body. Although the mechanism(s) by which lead induces toxicity has been revealed by several workers to be oxidative stress yet the exact mechanism of lead induced oxidative stress is still under study. Evidences indicate that multiple mechanisms may be involved.  

Lead is dangerous because once it gets into a person’s system, it gets distributed throughout the body through circulation. And lead can cause harm wherever it gets stored in the body. In the bloodstream, for example, it can damage red blood cells and limit their ability to carry oxygen to the organs and tissues that need it, thus causing anemia. Lead adheres to WBC and damages WBC also. Most lead gets stored in the bone marrow, where it causes even more problems. Lead can interfere with the production of blood cells therein. The heme synthesis pathway is adversely affected by lead acetate induced oxidative stress leading to anemia.  

Rats when treated with lead acetate, blood ALAD (Delta-aminolevulinic acid dehydratase) gets inhibited. Melatonin has been reported to have some kind of protective role against lead induced reduction of blood ALAD. 

In the present study, we observed a significant decrease in the hemoglobin and total count of RBC(s), mean corpuscular hemoglobin (MCH) and total WBC count in lead acetate treated animals compared to control. Melatonin alone did not alter any of those, and pre-treatment of rats with melatonin prevented the reduction of hemoglobin, RBC total count, MCH and WBC count indicating that melatonin can protect against lead induced anemic conditions. Lead has been reported to induce immune reactions in human and experimental animals. The mechanism is not yet well studied. Alterations in neutrophil and lymphocyte count in rats following lead exposure has also been reported earlier. We observed an increase in neutrophil count in the lead acetate treated experimental group, which also indicates neutrophilia. There is report of elevation in neutrophil count in humans who got exposed to lead i.e., lead-exposed workers. We found in our study that treatment of rats with lead acetate caused a significant decrease in small lymphocyte count. Pre-treatment of rats with melatonin at a dose of 10 mg/kg bw, fed orally protected the lead induced alterations in neutrophil and lymphocyte counts in the experimental rats. This also reveals that melatonin has the ability to protect against lead acetate induced immune reactions. This is further supported by some other studies which suggest that melatonin has the ability to modulate the immune system and enhance immune surveillance and has an ameliorative action at a higher dose administered intra-gastrically against lead induced hematotoxic alterations in rats. 

The Erythrocyte Sedimentation Rate (ESR) measures the rate by which Red Blood Cells (RBCs) settle in whole blood. The Red Blood Cells sediment because they have density greater than that of the plasma. With an alteration in the distribution of charges on the surface of the RBC (which normally keeps them separate from each other) results in their coming together to form large aggregates known as rouleaux. Changes in red cell shape or numbers may also affect the ESR. The increased sedimentation rate of red blood cells in various diseases is well recognized and hence ESR is used for diagnosis of various pathological states. 

We observed enhanced ESR in rats exposed to lead acetate for seven days and the same was found to be protected from being increased when the animals were pre-treated with melatonin. This shows that melatonin imposes protection against lead induced alteration in ESR indicating protective role of melatonin against lead-induced toxicity. Heavy metals like lead and cadmium are known to affect the lipid profile in children. We observed an increase in the level of total cholesterol, triglyceride and LDL cholesterol in lead acetate treated rats. We also observed an increase in the ratios of total cholesterol:HDL cholesterol and LDL:HDL in the rats treated with lead acetate intraperitoneally for seven consecutive days. Recent studies reveal similar observations i.e., increased level of total cholesterol, triglyceride, LDL cholesterol, total cholesterol: HDL cholesterol and LDL:HDL in battery workers who get exposed to lead occupationally. Our finding is in agreement with the fact that exposure to lead acetate causes altered lipid profile, increased total cholesterol and decreased HDL cholesterol and that lead acetate exposure increases synthesis of cholesterol and transport to peripheral tissues whereas reverse cholesterol transport to the liver is not affected. An abnormality in lipid profile is considered a significant landmark in the pathogenesis and progression of atherosclerosis and cardiovascular diseases. Thus, lead acetate exposure occupationally or environmentally increases the risk of cardiovascular diseases. In our experimental model, pre-treatment of the animals with melatonin at a dose of 10 mg/kg bw, protected against the lead acetate induced increase in the levels of the total cholesterol, triglyceride, LDL cholesterol and as well the Total cholesterol: HDL cholesterol and LDL:HDL ratios. We had seen significant decrease in the level of HDL cholesterol in the serum of the rats treated sub-chronically with lead acetate which was observed to be protected and maintained at normal level on pre-treating the animals with melatonin. Melatonin alone, when fed orally, was found to have no effect on the levels of the above mentioned parameters in the experimental rats. Melatonin, thus, protects against lead acetate induced alterations in the lipid profile and thus it can be used at a pharmacological dose as a potent protector against the risk of lead induced cardio-myopathies in individuals exposed to lead environmentally or occupationally. We have also observed that melatonin has a protective role against lead acetate induced cardiac-tocicity in male Wistar rats through its antioxidant mechanism. Thus, our work reveals that melatonin, at low pharmacological dose has the potential to provide protection against lead acetate induced alteration in hematological parameters and serum lipid profile suggesting that this small indole may be considered as a therapeutic antioxidant for human use where mankind is exposed to lead environmentally or occupationally.

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REFERENCES


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