PROTECTIVE EFFECT OF MELATONIN AGAINST IRON OVERLOAD-INDUCED TOXICITY IN RATS

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ABSTRACT

Objective: Iron overload is a serious, potentially fatal disorder characterized by the deposition of excess iron within tissues leading to functional impairment and reduced life expectancy. The present study was designed to evaluate the efficacy of melatonin in protecting rats against iron overload-induced toxicity.

Methods: Twenty-four adult male rats were randomly divided into four groups: the control, iron overload group, melatonin group and iron overload+melatonin group. Rats received ferric hydroxide poly maltose at a dose of 50 mg/kg body weight (three doses per week) for four weeks through intra peritoneal injections and received the melatonin subcutaneously (10 mg/kg/day) for four weeks.

Results: In iron overloaded rats, the iron status markers: serum, hepatic and renal tissues iron, Total Iron Binding Capacity (TIBC), transferrin and transferrin saturation percentage (TS %) were significantly increased, while a marked decrease in Unsaturated Iron Binding Capacity (UIBC) was demonstrated. The oxidative stress marker malondialdehyde (MDA) was significantly increased while a marked decrease in the catalase (CAT) and glutathione peroxidase (GPx) activities as well as in reduced glutathione (GSH) content accompanied with increased levels of metallothionein (MT) in hepatic and renal tissues were demonstrated. In addition, the liver and kidney functions were disturbed. Co-treatment with melatonin, significantly improved iron overload-induced alterations as indicated by the attenuation of the iron status disturbances, the reduction of the indices of liver and kidney functions and lipid peroxidation product and elevation of antioxidants and MT.

Conclusion: The study showed the potential effect of melatonin against iron overload–induced toxicity through its chelating effect on iron, elevation of MT and improvement of antioxidant status.

Keywords: Iron overload, Metallothionein, Melatonin, Antioxidants.
Egyptian Pharmaceutical. Ind. (EEPI), Alexandria, Egypt. Each ampoule of two ml solution contains elemental iron (100 mg). Induction of iron overload was carried out by intraperitoneal injections of ferric hydroxide polymaltose complex at a dose of 50 mg/kg b. w (three doses per week) for 4 w [14]. Melatonin was purchased from Sigma Chemical Company, St. Louis, MO, USA. It dissolved in a minimum volume of ethanol (96%) and then diluted with distilled water (the final concentration of ethanol was less than 0.5 %). Melatonin was daily injected subcutaneously at a dose of 10 mg/kg body weight for 4 weeks [15].

Animals

Twenty-four male albino rats (body weight 150-200 g) were obtained from the Animal House of the National Research Center, Dokki, Giza, Egypt. The rats were housed in plastic cages at an environmentally controlled room (constant temperature 25-27ºC, with 12 h light/dark cycle) for one week prior to starting the experiments, and they were provided with water and standard rat chow. Animal procedures were performed in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals.

Experimental protocol

After acclimatization, the animals were divided into 4 groups, consisting of 6 rats in each group. Group 1 (control): rats received 0.5 ml of 0.01% ethanol subcutaneously (melatonin diluent) for four weeks. Group 2 (iron overload): rats received intra peritoneal injection of ferric hydroxide polymaltose at a dose of 50 mg/kg body weight (three doses per week) for four weeks. Group 3 (melatonin): rats received the melatonin in ethanol subcutaneously (10 mg/kg/day) for four weeks. Group 4 (iron overload+melatonin): rats received ferric hydroxide polymaltose (as in group 2) and melatonin (as in group 3) for four weeks. The injection of melatonin was 30 min before iron administration and was given 2 h before light off.

Sample collection and tissue preparation

At the end of the experimental period, the animals were fasted for 16-18 hrs. before sacrificing. Animals were decapitated and blood was collected from each animal for separation of serum for different biochemical analysis. Portions of liver and kidney were stored at -80 °C for iron assay. Portions of liver and kidney were immediately washed in ice-cold physiological saline and homogenized in 50 mM potassium phosphate (pH 7.4) to render 10% homogenate for different assays.

Biochemical analysis

Blood samples were centrifuged at 3000 rpm for 15 min. to separate serum. Estimation of serum iron and TIBC were carried out according to the methods described by Stookey [16] and Carter [17]. UIBC was calculated as TIBC - iron. The serum T% Fe that indicate the level of iron supply to the tissues was determined as T%= (Serum iron/TIBC) × 100. Serum transferrin was determined by enzyme-linked immunoassay using monoclonal antibodies [18].

Estimation of AST and ALT activities and total bilirubin were carried out using the methods of Reitman and Frankel [19] and Walters and Gerade [20] respectively. T. protein was analyzed in serum using the method of Lowry et al. [21]. Albumin was determined by the method of Doumas et al. [22]. Serum cholesterol and triglycerides were determined according to Alainet et al. [23] &Fossati and Prencipe[24] methods respectively. Urea and creatinine concentrations were determined by the methods of Foster and Hochholzer [25] and Schirmeister et al. [26] respectively. Tissue homogenate was centrifuged at 10,000g at 4 °C for 20 min. and the supernatant was used for analysis of MDA by Ohkawa et al. [27]. CAT by Aebi [28], GSH by Beutler et al. [30] methods. Total iron in liver and kidney tissues was measured using atomic absorption spectroscopy. Briefly, 0.1 g of tissue was weighed and placed in iron free glass tubes (washed with hydrochloric acid). The tissue was dried in an oven at 115 °C for 5 h, 0.3 ml of concentrated sulphuric acid was added and the samples were digested for 2 days. Subsequently, 0.1 ml of concentrated nitric acid and 50 μl of hydrogen peroxide were added and the freed metal salts were determined using atomic absorption spectrophotometer (AAS, Perkin Elmer model A Analyst 100) at 248.3 nm. The results are expressed as μg Fe/g tissue [31]. MT was determined in liver and kidney tissues by cadmium saturation assay according to Onosaka and Cherian[32]. The concentration of MT in each tissue was calculated by assuming that 7 mol of Cd are bound to 1 mol of MT [33] and the levels of MT were expressed as μg MT/g tissue.

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Effect of melatonin on the iron status of iron overloaded rats

The serum iron concentration in iron overloaded rats was significantly (P<0.05) increased by 67% than that in control group. Simultaneously, TIBC, transferrin and T% Fe were increased (P<0.05) by 37%, 36 % and 3% respectively, while UIBC was significantly lower by 22% in iron overload rats than that in control group (table 1). Treatment with melatonin (melatonin+iron overload group) significantly decreased serum iron, TIBC, transferrin and T% Fe by 35%, 22%, 18% and 17% respectively.

At the same time, melatonin elevated serum UIBC by 36% than those in iron overload rats (table 1). The iron significantly (P<0.05) tend to accumulate in the hepatic and renal tissues by 59% and 52%, respectively following iron overload than those in control group, while co-administration of melatonin with iron statistically (P<0.05) decreased iron concentration about 25% in liver and 23% in kidney as compared to iron overloaded rats.

Table 1: Effect of melatonin on iron status of iron overloaded rats

<table>
<thead>
<tr>
<th>Groups parameters</th>
<th>Control</th>
<th>Iron overload</th>
<th>Melatonin</th>
<th>Iron overload+melatonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum iron(μg/dl)</td>
<td>141.27±7.17</td>
<td>235.36±11.76</td>
<td>138.54±6.58</td>
<td>152.17±6.84</td>
</tr>
<tr>
<td>TIBC(μg/dl)</td>
<td>215.73±4.98</td>
<td>295.03±2.80</td>
<td>209.82±4.68</td>
<td>231.54±4.25</td>
</tr>
<tr>
<td>UIBC(μg/dl)</td>
<td>75.10±2.91</td>
<td>58.94±2.70</td>
<td>72.21±2.42</td>
<td>80.19±4.58</td>
</tr>
<tr>
<td>Transferrin(g/l)</td>
<td>1.80±0.03</td>
<td>2.45±0.04</td>
<td>1.85±0.04</td>
<td>1.94±0.04</td>
</tr>
<tr>
<td>TS (%)</td>
<td>65.18±1.93</td>
<td>80.17±2.19</td>
<td>66.02±1.73</td>
<td>66.32±2.66</td>
</tr>
<tr>
<td>Liver iron(μg/g)</td>
<td>200.32±10.2</td>
<td>318.60±14.5</td>
<td>192.40±9.7</td>
<td>237.90±11.8</td>
</tr>
<tr>
<td>Kidney iron(μg/g)</td>
<td>135.31±6.4</td>
<td>205.27±10.4</td>
<td>130.12±5.5</td>
<td>157.50±6.3</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE (n=6), a: significance difference as compared to control group at P<0.05 and b: significant difference as compared to iron overload group at P<0.05.

Effect of melatonin on MDA and antioxidants in hepatic and renal tissues of iron overloaded rats

As shown in table 2, iron overload significantly (P<0.05) increased the formation of MDA in the hepatic and renal tissues by 73% and 105 % respectively as compared to control group. Co-administration of melatonin resulted in significant (P<0.05) reduction in MDA levels in both liver (30%) and kidney (23%) when compared with iron overload group. The results represented in table 2 also showed that in the iron overload group, there was a significantly (P<0.05)
increase in hepatic and renal CAT activity (by 50 % and 52% respectively) and Gpx activity (by 27% and 39% respectively) as well as GSH content (by 27% and 21% respectively) as compared to control group. Co-treatment with melatonin (iron overload+melatonin group) significantly (P<0.05) elevated the activities of CAT and Gpx and the content of GSH in the hepatic and renal tissues as compared to iron overload group.

Table 2: Effect of melatonin on MDA and antioxidants in hepatic and renal tissues of iron overloaded rats

<table>
<thead>
<tr>
<th>Groups parameters</th>
<th>Control</th>
<th>Iron overload</th>
<th>Melatonin</th>
<th>Iron overload+melatonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA(nmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>47.02±0.57</td>
<td>81.4±0.76a</td>
<td>46.5±0.53b</td>
<td>57.37±0.56b</td>
</tr>
<tr>
<td>Kidney</td>
<td>16.40±0.44</td>
<td>33.58±0.41a</td>
<td>15.27±0.55b</td>
<td>25.73±0.48b</td>
</tr>
<tr>
<td>CAT (U/g)</td>
<td>1.82±0.04</td>
<td>0.90±0.07a</td>
<td>1.77±0.06b</td>
<td>1.16±0.05ab</td>
</tr>
<tr>
<td>Liver</td>
<td>1.74±0.04</td>
<td>0.83±0.07a</td>
<td>1.57±0.06b</td>
<td>1.09±0.07ab</td>
</tr>
<tr>
<td>Kidney</td>
<td>18.09±1.02</td>
<td>14.6±1.06a</td>
<td>19.73±2.53b</td>
<td>16.32±1.08b</td>
</tr>
<tr>
<td>Gpx (U/g)</td>
<td>9.08±0.76</td>
<td>5.58±0.68ab</td>
<td>10.61±0.92b</td>
<td>7.50±0.92ab</td>
</tr>
<tr>
<td>Liver</td>
<td>18.62±1.24</td>
<td>13.52±1.26a</td>
<td>19.12±1.76b</td>
<td>17.58±1.62b</td>
</tr>
<tr>
<td>Kidney</td>
<td>14.47±0.87</td>
<td>11.45±0.57a</td>
<td>14.81±0.95b</td>
<td>13.67±0.96b</td>
</tr>
</tbody>
</table>

Values are expressed as means±SE (n=6), a: significance difference as compared to control group at P<0.05 and b: significant difference as compared to iron overload group at P<0.05.

Effect of melatonin on liver and kidney functions in serum of iron overloaded rats

As shown in table 3, significantly (P<0.05) increased levels of serum enzymes, ALT by 129% and AST by 49 % and total bilirubin by 118 % in iron overload group, clearly signifies iron induced liver damage. Meanwhile, the levels of T. protein and albumin were significantly decreased by 36% and 35% respectively as compared to control group. The co-treatment of melatonin with iron displayed significant attenuation in these markers by reduction of ALT (76%), AST (20%) and bilirubin (36%) and elevation of T. protein and albumin by 42% and 50% respectively as compared to iron overload group returning them towards normal values. In addition, iron treatment (iron overload group) caused a significant increase (P<0.05) in the levels of cholesterol (42%) and triglycerides (50%) as compared to those of the control group. Co-treatment with melatonin led to significant (P<0.05) decrease in cholesterol and triglycerides levels by 22% and 24% respectively as regard to iron overload group. Regarding renal functions, iron treatment produced about 112% and 73% enhancement in the values of serum creatinine and urea respectively, as compared with control group. Co-treatment with melatonin resulted in 43% and 37% reduction in the values of serum creatinine and urea respectively as compared with iron-treated group.

Table 3: Effect of melatonin on liver and kidney functions in serum of iron overloaded rats

<table>
<thead>
<tr>
<th>Group parameter</th>
<th>Control</th>
<th>Iron overload</th>
<th>Melatonin</th>
<th>Iron overload+melatonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT(U/ml)</td>
<td>20.32±1.80</td>
<td>46.71±4.20a</td>
<td>21.83±1.49b</td>
<td>35.85±2.54ab</td>
</tr>
<tr>
<td>AST(U/ml)</td>
<td>65.80±3.54</td>
<td>98.17±4.15a</td>
<td>63.22±2.73b</td>
<td>78.15±3.04b</td>
</tr>
<tr>
<td>Bilirubin(mg/dl)</td>
<td>1.15±0.05</td>
<td>2.51±0.56a</td>
<td>1.14±0.03b</td>
<td>1.59±0.13b</td>
</tr>
<tr>
<td>T. protein(mg/dl)</td>
<td>6.85±0.47</td>
<td>4.35±0.21a</td>
<td>6.51±0.43b</td>
<td>6.19±0.40b</td>
</tr>
<tr>
<td>Albumin(g/dl)</td>
<td>3.62±0.21</td>
<td>2.34±0.14a</td>
<td>3.65±0.22b</td>
<td>3.52±0.23</td>
</tr>
<tr>
<td>Cholesterol(mg/dl)</td>
<td>13.02±4.21</td>
<td>19.37±6.31a</td>
<td>13.2±6.12b</td>
<td>15.2±5.72ab</td>
</tr>
<tr>
<td>Triglycerides(mg/dl)</td>
<td>115.8±5.71</td>
<td>174.19±6.69a</td>
<td>180.79±3.40b</td>
<td>192.5±4.69a</td>
</tr>
<tr>
<td>Creatinin(mg/dl)</td>
<td>1.18±0.07</td>
<td>2.49±0.04a</td>
<td>1.17±0.05b</td>
<td>1.61±0.03b</td>
</tr>
<tr>
<td>Urea(mg/dl)</td>
<td>35.68±1.07</td>
<td>61.83±2.50a</td>
<td>34.96±1.04b</td>
<td>40.79±1.16ab</td>
</tr>
</tbody>
</table>

Values are expressed as means±SE (n=6), a: significance difference as compared to control group at P<0.05 and b: significant difference as compared to iron overload group at P<0.05.

Effect of melatonin on MT in hepatic and renal tissues of iron overloaded rats

Our results indicated that administration of iron alone resulted in the accumulation of MT in tissues; the level of MT was significantly (P<0.05) elevated by 117% and 158% in liver and kidney respectively as compared to control (Fig.1). On its own, melatonin significantly (p<0.05) increased the MT values in both liver and kidney by 70 % and 68% respectively. Co-administration of iron with melatonin resulted in an increase (P<0.05) in MT level in liver by 43% and kidney by 54% as compared to iron overload group while these values were increased by 212% and 297% in liver in kidney respectively as regard to control group.

DISCUSSION

The transferrin, TIBC, UIBC, and TF% evaluate the blood's ability to bind and transport iron and are a reflection of iron stores. Evidence for iron toxicity in the current study was exhibited by the remarkable elevation of serum, liver and kidney iron concentrations, serum TIBC, transferrin and T% in iron-overloaded rats, while serum UIBC was significantly decreased. Such increments were associated with exacerbated oxidative stress status and alterations of antioxidants. These results were in agreement with that recorded in several models of iron overload. Administration of ferric nitrilotriacetate was found to induced a significant deposition of iron in rat liver associated with oxidative stress [34], feeding rats with diets supplemented with ferric sulfate resulted in dose related increases in liver non-heme iron and lipid peroxidation [35] and injection of mice with iron dextran for 5 days exhibited an increased in liver iron deposition [36]. Regarding to renal tissue, Nematbakhsh et al. [37] described the deposition of iron in renal tissue of rats received iron dextran for a period of 4 weeks. Additionally, the results of the current study came in accordance with the data of Nahdi et al. [38] who stated that iron overload elicited an elevation.
in serum and liver iron as well as TS%. Such alterations resulted in the presence of non-transferrin binding iron that catalyzing the formation of reactive radicals [39].

In the current study, the treatment of iron overloaded rats with melatonin reduced serum, hepatic and renal tissues iron concentration, serum TIBC, transferrin level and TS % while it elevated serum UIBC, thus supporting its iron chelating potency. Previously, Limson et al. [40] investigated the possible role of melatonin in metal regulation in the central nervous system using an electrophoretic technique. The authors showed that melatonin formed complexes with several heavy metals including iron (III) not iron (II) and suggested that it removes iron (III) unbound to a protein, thus preventing it from reducing back to the form in which it generates free radicals via the Fenton reaction. Additionally, the hydrophilic and lipophilic nature of melatonin allows it freely moves across all cellular barriers facilitating the removal of toxic metals thus favoring the metal binding role of melatonin. However, the efficacy of melatonin is mainly attributed to its high lipophilicity [41]. It is well documented that the lipophilic agents are very effective in reducing toxic effects of heavy metals and their elimination from various organ systems [42, 43].

Moreover, in the present study, treatment of rats with ferric hydroxide poly maltose complex resulted in an elevation of MDA concentrations (as lipid peroxidation product) while GPx and CAT activities and GSH content in mice and rat liver [44, 36]. On the other hand, ferric nitrotriacetate administration induced significant alterations in renal oxidative/antioxidant status [45]. However, iron is a well-known inducer of reactive oxygen species (ROS) and its ability to accelerate lipid peroxidation is well-established [46]. Iron overload can destruct the balance between pro oxidants and antioxidants, leading to severe loss of total antioxidant status level. Antioxidant enzymes such as CAT and GPx operate in concert with non-enzymatic molecules such as GSH to contrast the ROS actions and to avoid oxidative damage [47].

In the current study, administration of melatonin to iron overloaded rats significantly attenuated the increased levels of lipid peroxidation with concomitant improvement in the levels of liver and renal enzymatic and non-enzymatic antioxidant defenses. Melatonin was found to maintain the optimal fluidity of cellular membranes accomplished by reducing the peroxidation of inherent polyunsaturated fatty acids and indirectly reducing increased membrane rigidity [48] by positioning itself within cellular membranes to restrict damage to polyunsaturated fatty acids by toxic reactants [49]. Moreover, melatonin is considered as one of the most effective antioxidants where it exerts direct antioxidant effects via its free radical scavenging properties and/or by inhibiting their generation and exerts indirect antioxidant effects by stimulating antioxidant enzymes and inhibiting the activities of pro-oxidative enzymes [7].

Furthermore, in the current study, iron overload caused many adverse effects including an elevation in the activities of ALT and AST and total bilirubin level as well as decreased contents of T. protein and albumin in indicating liver injury. Such alterations could be attributed to the generation of ROS and oxidative damage by excess hepatic iron that may result in chronic necro-inflammatory diseases [50] and leakage of cellular enzymes into the blood stream due to the altered liver membrane permeability. The reduction of serum T. protein and albumin levels in the present work could be ascribed to changes in protein and free amino acids metabolism and their synthesis in injured liver cells and/or increased protein degradation. These disturbances in liver markers was in agreement with data described by Whittaker and Chanderbh [51], Manjunatha and Srinivasan [52] who reported that iron-induced liver damage had been assessed by leakage of enzymes such as ALT and AST and lactate dehydrogenase into the blood. In addition, the decrease in T. protein and albumin were in accordance with those recorded previously, where the iron accumulation disrupts the cell redox balance and generates chronic oxidative stress, which damages DNA, lipids and protein in hepatocytes leading to both necrosis and apoptosis [53].

Additionally, in the present study, the significant increase of serum cholesterol and triglycerides in iron-overloaded animals are in line with similar finding reported by Whittaker and Chanderbh [51] who found that serum cholesterol concentration increased directly followed iron supplementation and Sylvain et al. [54] who demonstrated a significant increase in triglycerides, free cholesterol and cholesterol ester in iron overloaded rats. These alterations might be attributed to the marked membrane lipid peroxidation that considered with the cytotoxicity as an important factor altering lipid metabolism [51] possibly by their effect on lipogenesis-related genes [55] and key enzymes for cholesterol homeostasis [56].

In the current study, administration of melatonin to iron overloaded rats reduced the serum ALT and ALT activities as well as the total bilirubin, cholesterol and triglycerides concentrations and induced increases in serum T. protein and albumin, indicating its protective effect over liver and improvement in its functional efficiency. The hypcholesterolemic effect of exogenous melatonin might work through the augmentation of endogenous cholesterol clearance mechanisms [57]. Moreover, melatonin was found to have an extraordinary anti lipemic effect [58].

In the present work, elevated levels of urea and creatinine were observed in iron-overloaded rats, which were attenuated by the co-treatment with melatonin. The disturbance of renal functions came similar to previous studies in which iron overload with ferric sulfate [35] or ferric nitrotriacetate [45] exhibited high levels of urea and creatinine in rats. These increments in serum urea and creatinine could be attributed to the destruction and malfunction of kidney cells due to iron deposition.

The current study indicated that iron administration alone resulted in the accumulation of MT in hepatic and renal tissues. These results are similar with that recorded by Yasutake and Hirayama [59] who demonstrated that feeding on the Fe-enriched diet brought about significant alterations of MT levels in both liver and kidney with concomitant Fe accumulation. Some investigators documented induction of hepatic MT after intra peritoneal injections of Fe in rats [60] and suggested that parenteral injection of iron would cause significant stress and, as such, promote MT biosynthesis [61]. In the current study, co-treatment of melatonin resulted in more increases in MT level in liver and kidney, thus giving more chelating effect on iron. The possible mechanisms of MT in protection against oxidative injury are due its capacity to bind heavy metals through the thiol group of its cysteine residues. It was found that MT serves in the management of essential divalent metal cations in many cell types through interfering with the toxic effects of xenobiotic, heavy metals and free radicals [62]. Furthermore, MT, a sulfhydryl–rich metal binding protein, may function in a manner similar to GSH. Where in
MT provides an intracellular ‘nucleophile sink’ to trap free radicals, electrophiles and alkylation agents [63, 64].

CONCLUSION

In the present study, it could be concluded that melatonin is effective in ameliorating the toxic effect of iron by chelating it, improvement of the iron profile, elevated the metal binding protein MT associated with the reduction of oxidative stress in liver and kidneys of male rats.

CONFLICT OF INTERESTS

Declared None

REFERENCES


