Protective effects of oral melatonin against cadmium-induced neurotoxicity in Wistar rats

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[Received in December 2020; Similarity Check in December 2020; Accepted in June 2021]

The aim of this study was to investigate the effects of oral melatonin on oxidative/antioxidative parameters and histopathological changes in the hippocampal tissue of Cd-exposed Wistar rats, including malondialdehyde (MDA), glutathione (GSH), tumour necrosis factor-alpha (TNF-α), interleukin (IL-6 and IL-10), and gamma-aminobutyric acid (GABA) levels and catalase (CAT), superoxide dismutase (SOD), and acetylcholinesterase (AChE) activities. Thirty-two male Wistar rats were divided randomly into four groups as follows: untreated control (n=8), cadmium (Cd) (n=8), melatonin (Mlt) (n=8), and Cd+Mlt (CdMlt) (n=8). Cd (2 mg/kg) was administered orally by gastric gavage three times a week and Mlt (100 mg/kg) five times a week. The control group received standard feed and water only. After four weeks of treatment, the animals were decapitated and tissue samples taken for biochemical and histopathological evaluations. Mlt caused a significant increase in GSH levels and SOD and CAT activities in the CdMlt group compared to the Cd group. Tissue TNF-α and IL-6 levels were significantly higher in the Cd group than other groups (P<0.05). This effect was significantly countered by Mlt in the CdMlt group (P<0.05). GABA concentrations were significantly higher in the Mlt than other groups (P<0.05). Our findings clearly evidence the protective effects of melatonin against Cd-induced neurotoxicity in rats.

KEY WORDS: AChE; antioxidant; CAT; Cd; cytokine; GABA; GSH; hippocampus; IL-6; IL-10; MDA; oxidative stress; SOD; TNF-α

The main sources of occupational exposure to cadmium (Cd) are metal industries (mining, smelting, processing, product formulations, and battery manufacturing), while the sources of non-occupational exposure include contaminated air (smoking included), water, soil, plants, and food. Exposure through skin, however, is rare (1–3).

Although the main target organs of Cd poisoning are the kidney and the liver, in chronic exposure Cd can also accumulate in different parts of the central nervous system (CNS) by damaging and passing the blood-brain barrier (BBB) or through nasal mucosa or olfactory pathways (4, 5). There it causes oxidative stress and through it neurotransmitter dysfunction and hippocampus-dependent learning and memory impairment in humans and animals (6–8). It has also been implicated in the development of Alzheimer’s and Parkinson’s disease (9).

In recent years, research has been focused on how to counter the harmful oxidative effects of Cd and has included a number of substances with antioxidant and metal-binding properties, especially in Cd-exposed animals (10–12). One of them is melatonin (n-acetyl-5-methoxytryptamine), a hormone secreted mainly from the pineal gland with powerful metal-binding (13), antioxidant (14), and free radical scavenging (15) properties due to its small size and lipophilicity. Recent studies have also shown that melatonin plays a neuroprotective role in many central nervous system (CNS) disorders, including Alzheimer’s and Parkinson’s disease and ischemic brain injury. It has also shown beneficial effects on memory, posture control, and balance (16) and was reported to protect against Cd-induced neurotoxicity in rats by reducing lipid peroxidation and restoring antioxidant defence parameters and acetylcholinesterase (AChE) activity in the plasma and brain (17).

Considering the important role of the hippocampus in spatial navigation, emotional behaviour, regulation of hypothalamic functions, learning, memory, limbic system (18–20), and its damage in Alzheimer’s disease (20, 21), we wanted to complement relatively modest literature (14, 22–24) about beneficial effects of oral melatonin in the hippocampus, especially in regard to cytokines and gamma-aminobutyric acid (GABA) levels. To that effect the aim of our study was to compare common oxidative stress, inflammation, and neurotoxicity parameters [malondialdehyde (MDA) (25), interleukins 6 and 10 (IL-6, IL-10), tumour necrosis factor alpha (TNF-α) (9, 26–28),...
GABA (29–36), acetylcholinesterase (AChE) (17, 37, 38) and antioxidant defence parameters [glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) (39)] in the hippocampal tissue of control, Cd-, and melatonin-treated rats. We also wanted to investigate potential neuroprotective effects of melatonin through histopathological changes in the cerebral cortex of rats exposed to Cd.

MATERIALS AND METHODS

Animals and experimental design

Three weeks old male albino Wistar rats weighing ~200±30 g (n=32), purchased from Balıkesir University Experimental Medicine Research and Application Center (BUEMRAC), were first acclimatised to laboratory conditions for two weeks and then divided randomly into four groups: untreated control, cadmium (Cd), melatonin (Mlt), and Cd+Mlt (CdMlt), each consisting of eight animals. The rats were housed in standard plastic rat cages in an air-conditioned room (at 23±2 °C and 55±10 % humidity) with a 12-hour light/dark cycle. They had free access to standard pellet diet and fresh water.

The animals in the Cd and CdMlt groups received 2 mg/kg of Cd (Cat. No: 1002283736; CdCl₂) by gavage (1 mL/kg bwt) three times a week for four weeks, as described elsewhere (40). Those in the Mlt and CdMlt groups were receiving 100 mg/kg of melatonin (Cat. No: 1002042514) also by gavage five times a week for four weeks, as described elsewhere (41). The control group received only standard feed and fresh water.

The hippocampal tissue samples of the animals were placed in liquid nitrogen and stored at -80 °C until analysis. 200 mg of each tissue sample in 800 mL of isotonic sodium chloride solution was homogenised at 9961 g in an ultrasonic homogeniser (IKA-T25, Merck, Darmstadt, Germany) and centrifuged at 4025 g for 10 min (Sigma18-K, Newtown, Shropshire, UK) to obtain supernatants. MDA and GSH levels and CAT and SOD activities were determined in the supernatants with the enzyme-linked immunosorbent assay (ELISA) using the ELx800 absorbance microplate reader (Biotek Instruments, Winooski, VT, USA) and standard commercial kits (Cat. Nos. 10009055, 703002, 707002, and 706002, respectively; Cayman, Ann Arbor, MI, USA).

Hippocampal tissue cytokine measurements

IL-6, IL-10, and TNF-α were also detected in the obtained supernatants with ELISA (ELx800, Biotek) and commercial kits (Cat. Nos. BMS625, BMS629, and KRC3011, respectively; ThermoFisher Scientific, San Jose, CA, USA) according to the manufacturers’ instructions.

Hippocampal tissue GABA and AChE measurements

GABA concentrations were measured using the double-sandwich ELISA kit (Sunred Biological Technology, Shanghai, China) according to the manufacturer’s instruction. In brief, tissue samples (supernatants) and kit standards (Cat. No: 201-11-0103) were extracted on an extraction plate, derived using an equalising reagent, and subjected to ELISA in GABA pre-coated microtitre strips. The absorbance of the solution in the wells was read at 450 nm within 15 min with a Multiskan FC microplate reader (ThermoFisher Scientific). GABA concentrations were calculated based on optical density using a standard curve.

AChE activities were also determined using a sandwich ELISA kit (Elabscience, Wuhan, China) according to the producer’s instruction. Micro plates provided in this kit were pre-coated with an antibody specific to rat AChE (Cat. No: E-EL-R0355). The absorbance of the solution was immediately read at 450 nm with the above mentioned plate reader.

Cerebral cortex histopathology

Brain tissue samples (cerebral cortex) collected for histopathology were fixed in 10 % buffered formaldehyde solution, embedded in paraffin and cut into 4 µm thick sections, stained with haematoxylin and eosin (H&E), and observed under a light microscope (100x, Nikon, Eclipse Ni, Tokyo, Japan).

In situ TUNEL assay in the cerebral cortex

To identify apoptotic cells in the cerebral cortex we used the DeadEnd™ Colorimetric TUNEL System (Cat No: G7130; Promega, Madison, WI, USA) according to the manufacturer’s instructions.

Statistical analysis

All statistical analyses were run on IBM SPSS v. 25.0 for Windows (IBM Corp., Armonk, NY, USA) starting with the analysis of variance (ANOVA) followed by Duncan’s test. P values ≤0.05 were considered significant.
RESULTS

Table 1 shows hippocampal tissue MDA and GSH levels and SOD and CAT activities. MDA levels were the highest in the Cd group compared to the rest (P<0.05). The oxidative stress was significantly reduced by melatonin in the CdMlt compared to the Cd group (P<0.05). As expected, GSH levels and SOD and CAT activities were the lowest in the Cd group, but melatonin treatment improved them significantly in the CdMlt compared to the Cd group (P<0.05).

Table 2 shows cytokine levels in the hippocampal tissue of our rats. TNF-α and IL-6 levels significantly increased in the Cd group (P<0.05), while IL-10 levels dropped compared to the other groups (P<0.05). Again, melatonin significantly countered these effects of Cd (P<0.05).

Hippocampal tissue GABA concentrations were significantly lower in the Cd group than the other groups (P<0.05), but were increased by melatonin in the Mlt and CdMlt groups compared to the Cd group (P<0.05). However, we found no significant differences in AChE activities between the groups (Table 3).

Histopathological findings in the cerebral cortex

The control group showed normal histomorphology (Figure 1a), while the Cd group had severe multifocal histopathological changes in the cerebral cortex. In this location, most of the pyramidal and granular cells were shrunken and had pericellular halos and dark stained nuclei with lost nucleoli. Many vacuoles of variable sizes were noted in some granule cells and the neuropil tissue. These vacuolar changes were most prominent in the pyramidal layer but were also evident in other layers. Hyperaemia was also noted in some locations (Figure 1b). In the CdMlt group, some pyramidal and granular cells showed signs of cellular degeneration. A vacuolar halo around cells with shrunken nuclei was also seen in this group (Figure 1c). Hyperaemia was not observed. Only a few multifocal areas with these cellular changes were observed compared to the many in the Cd group. No histopathological changes were identified in the Mlt group (Figure 1d).

Figure 2 shows apoptotic granular cells, identified only in the Cd group (Figure 2). No apoptotic (TUNEL-stained) cells were observed in other groups.

Table 1 Hippocampal tissue MDA and GSH levels and SOD and CAT activities by the experimental groups of Wistar rats

<table>
<thead>
<tr>
<th>Pro/antioxidant parameters</th>
<th>Control (n=8) Mean±SD</th>
<th>Mlt (n=8) Mean±SD</th>
<th>CdMlt (n=8) Mean±SD</th>
<th>Cd (n=8) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (ng/mL)</td>
<td>1.717±0.016*</td>
<td>1.738±0.020*</td>
<td>1.785±0.013*</td>
<td>1.877±0.038*</td>
</tr>
<tr>
<td>SOD (ng/mL)</td>
<td>0.434±0.022*</td>
<td>0.432±0.016*</td>
<td>0.420±0.027*</td>
<td>0.390±0.014*</td>
</tr>
<tr>
<td>GSH (µg/mL)</td>
<td>2.170±0.094*</td>
<td>2.177±0.066*</td>
<td>2.013±0.086*</td>
<td>1.136±0.009*</td>
</tr>
<tr>
<td>CAT (U/L)</td>
<td>4.616±0.361*</td>
<td>4.697±0.463*</td>
<td>4.486±0.145*</td>
<td>4.126±0.551*</td>
</tr>
</tbody>
</table>

* Means in the same row with different superscripts differ significantly (P<0.05). Mlt – group receiving melatonin alone; Cd – group receiving cadmium alone; CdMlt – group receiving both melatonin and cadmium. MDA – malondialdehyde; SOD – superoxide dismutase; GSH – glutathione; CAT – catalase; SD – standard deviation

Figure 1 Typical H&E-stained histological images of cerebral cortex sections in Wistar rats by groups (a) normal histological appearance in the control group; (b) pyramidal cells irregular in shape, surrounded by pericellular halos (arrows) and intracellular vacuoles (arrowhead) in the Cd group; (c) more or less normal pyramidal and granular cells and intracellular (arrow) and extracellular vacuoles (arrowhead) in the CdMlt group; (d) histological appearance in the Mlt group
DISCUSSION

It is well known that the brain tissue is susceptible to lipid peroxidation due to high use of oxygen and polyunsaturated fatty acids. Our study confirms numerous findings of adverse Cd effects in this respect, showing significant increases in MDA (22, 24, 42, 43) levels and MDA lowering effects in the hippocampal tissue thanks to melatonin administration (42). Several studies have also reported these effects of melatonin in the hippocampal and brain tissue against arsenic (As) and aluminium (Al) poisoning (15, 23, 44).

The same is true for antioxidant response, which is severely affected by Cd (17, 39, 43, 45). Oral administration of melatonin in our study significantly increased and nearly restored GSH, SOD, and CAT, which is in line with earlier findings (17, 42, 44), evidencing its antioxidant action.

Of particular interest is the anti-inflammatory action of melatonin found in our study. Melatonin treatment reversed the elevated levels of TNF-α and IL-6 in the hippocampal tissue of animals receiving Cd, which adds new, even though expected, knowledge, as little has been reported about melatonin effects on IL-6 and TNF-α levels in the hippocampus of rats exposed to Cd. Other studies found beneficial effects of melatonin in rats with induced breast cancer (46) or against aluminium (23), arsenic (44), and fluoride (47). As for IL-10, a regulatory cytokine which has both immunosuppressive and immunostimulatory properties, our findings confirm earlier reports of inhibitory effects of Cd in rat brain (9, 48) and beneficial effects of melatonin (49), which highlight the immunomodulatory potential of melatonin against chronic Cd poisoning (50).

In line with earlier reports on the GABA-lowering effects of Cd (35, 36), our study found the lowest GABA

| Table 2 Hippocampal tissue cytokine levels by the experimental groups of Wistar rats |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Cytokines          | Control | Mlt | CdMlt | Cd |
|                   | (n=8)   | (n=8) | (n=8) | (n=8) |
|                   | Mean±SD | Mean±SD | Mean±SD | Mean±SD |
| TNF-α (pg/mL)      | 0.070±0.001c | 0.070±0.001c | 0.081±0.002b | 0.095±0.001a |
| IL-6 (pg/mL)       | 0.058±0.003b | 0.116±0.171ab | 0.125±0.191ab | 0.217±0.107ab |
| IL-10 (pg/mL)      | 0.256±0.004a | 0.259±0.019a | 0.252±0.005a | 0.207±0.003b |

Means in the same row with different superscripts differ significantly (P<0.05). Mlt – group receiving melatonin alone; Cd – group receiving cadmium alone; CdMlt – group receiving both melatonin and cadmium. TNF-α – tumour necrosis factor alpha; IL-6 – interleukin 6; IL-10 – interleukin 10; SD – standard deviation

| Table 3 Hippocampal tissue GABA concentrations and AChE activities by the experimental groups of Wistar rats |
|-------------------------------------------------|----------------|----------------|----------------|----------------|
| Neurotoxicity parameters | Control | Mlt | CdMlt | Cd |
|                          | (n=8)   | (n=8) | (n=8) | (n=8) |
|                          | Mean±SD | Mean±SD | Mean±SD | Mean±SD |
| GABA (nmol/L)            | 8.124±0.008b | 9.610±0.107a | 7.174±0.082c | 6.252±0.012a |
| AChE (ng/mL)             | 0.781±0.001 | 0.781±0.001 | 0.780±0.001 | 0.780±0.001 |

Means in the same row with different superscripts differ significantly (P<0.05). Mlt – group receiving melatonin alone; Cd – group receiving cadmium alone; CdMlt – group receiving both melatonin and cadmium. AChE – acetylcholinesterase; GABA – gamma-aminobutyric acid; SD – standard deviation

Figure 2 Section showing apoptotic cells (marked with arrows) in the granular layer of a Cd-treated rat (TUNEL method)
hippocampal tissue concentrations in the Cd group. Similar was reported for the hypothalamus of rats treated with Cd (35, 51). As expected, melatonin significantly increased hippocampus GABA concentrations in the CdMlt group in our study, but it is interesting to note that the highest concentration was observed in the Mlt group. It seems that melatonin increases GABA accumulation in different parts of the brain tissue (52).

Another interesting finding was the lack of significant changes in the activity of AChE in the brain tissue in any of the groups in our study. Most importantly, AChE activities were not affected by melatonin treatment. In contrast, other studies reported a rise in the brain tissue of Cd-exposed rats treated with melatonin (17, 42), but this may be due to different dosage regimes.

Our histopathological exam showed that Cd caused severe multifocal changes and apoptotic cells in the cerebral cortex. Apoptosis may have been the result of increased caspase-3/7 activity, as reported by Mahdavi et al. (53). Apoptotic changes induced by Cd in cerebral cortical neurons were also reported by Yuan et al. (54). Melatonin seems to have attenuated these histopathological changes in our study, as we observed but a few multifocal areas and no apoptotic cells in the CdMlt group. This is in line with an earlier report of melatonin (4 mg/kg/day for eight weeks, i.p.) treatment reducing the loss in hippocampal neurons through direct blocking of Cd-induced oxidative stress pathways (14). Shagirtha et al. (17) also reported that oral melatonin pretreatment (10 mg/kg/day for four weeks) mitigated Cd-induced histopathological changes such as spongiform necrosis, nuclear vacuolisation, pycnosis, and lymphocytic inflammation in the rat brain tissue.

CONCLUSION

Our findings show that oral melatonin treatment has a great neuroprotective potential, especially against Cd neurotoxicity in the hippocampus and cerebral cortex and provide useful information for its use against metal poisoning.

Conflict of interests

None to declare.

Acknowledgements

This study was funded as part of the Balikesir University Scientific Research Coordinatiorship Project No. 2019-068.

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Djelotvornost peroralne primjene melatonina u ublažavanju neurotoksičnosti izazvane kadmijem u Wistar štakora

Cilj je ovog istraživanja bio utvrditi djelovanje peroralne primjene melatonina na razine malondialdehida (MDA), glutationa (GSH), čimbenika nekroze tumora alfa (TNF-α), interleukina 6 i 10 (IL-6 i IL-10), enzimske aktivnosti katalaze (CAT), superoksid dismutaze (SOD) i acetilkolinesteraze (AChE) te na koncentraciju gama-aminomaslačne kiseline (GABA) u hipokampusnom tkivu Wistar štakora izloženih kadmiiju (Cd). Također je cilj bio utvrditi histopatološke promjene u cerebralnom korteksu štakora. U tu svrhu istraživanje obuhvatilo 32 mužjaka, nasumce raspoređena u četiri skupine: kontrolnu (n=8), skupinu koja je primala samo Cd (n=8), skupinu koja je primala samo melatonin (Mlt) (n=8) i skupinu koja je istodobno primala Cd + Mlt (n=8). Štakori u Cd i CdMlt skupinama primali su Cd u dozi od 2 mg/kg gavažom na usta triput na tjedan odnosno melatonin u dozi od 100 mg/kg na isti način pet puta na tjedan. Kontrolna je skupina primala samo vodu. Nakon četverotjednog tretmana životinje su dekapitirane te su uzeti uzorci tkiva za biokemijsku i histopatološku analizu. Primjena melatonina dovela je u CdMlt skupini do rasta razina GSH i SOD te aktivnosti katalaze u odnosu na skupinu koja je primala samo Cd. Potonja je pak iskazala značajno više tkivne razine TNF-α i IL-6 od ostalih skupina (P<0,05). Primjena melatonin dovela je do njihova značajnog sniženja u CdMlt skupini (P<0,05). Koncentracije GABA bile su pak značajno više u skupini na melatoninu nego u ostalim skupinama (P<0,05). Naši rezultati potvrđuju da melatonin štiti od neurotoksičnosti izazvane kadmijem u štakora.