Melatonin reverses type 2 diabetes-induced cognitive deficits via attenuation of oxidative/nitrosative stress and NF-κB-mediated neuroinflammation in rat hippocampus

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Abstract

Purpose: To evaluate the protective effect of melatonin on diabetes-induced cognitive dysfunction.

Methods: Rats were fed a high-fat diet + streptozotocin (HFD + STZ) for 15 weeks to induce type 2 diabetes (HFD + STZ group). At the end of the 15-week induction of diabetes, cognitive function in the diabetic rats was estimated using a Morris water maze and an object recognition task. Next, the diabetic rats were treated with melatonin (10 mg/kg, po) for 3 weeks. Thereafter, cognitive function was re-evaluated in the melatonin-treated diabetic rats (melatonin group).

Results: There was a significant (p < 0.01) decrease in the serum glucose and insulin in melatonin-treated diabetes type 2 rats compared with that of diabetes type 2 rats exposed to only HFD + STZ. Treatment with melatonin (10 mg/kg, po) for 3 weeks in diabetic type 2 rats also caused a significant increase (p < 0.01) in the time spent in the target quadrant and preference index in diabetic rats compared with the HFD + STZ group. There were significant decreases in reactive oxygen species (ROS), oxido-nitrosative stress markers, including thiobarbituric acid reactive substances (TBARS), nitrite, and depleted glutathione (GSH) level in the hippocampus of melatonin-treated group, compared with the HFD + STZ-treated group. Moreover, the melatonin-treated group showed significant inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and reduction in the levels of proinflammatory cytokines.

Conclusion: The results demonstrate that melatonin prevents cognitive dysfunction in type 2 diabetic rats by attenuating oxido-nitrosative stress and NF-κB-mediated neuroinflammation. This effect suggests that melatonin may be useful for the management of cognitive dysfunction in patients suffering from diabetes.

Keywords: Cognitive dysfunction, Melatonin, Neuroinflammation, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Oxido-nitrosative stress, Type 2 diabetes

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INTRODUCTION

Diabetes is a metabolic disorder that results from inadequate action and secretion of insulin [1]. Complications in several body systems, such as cranial and peripheral nerves, large vessels, eyes, kidney, and cardiovascular system, are associated with diabetes [2,3]. Cognitive deficits have also been observed in both type 1 and type 2 diabetes patients.

Previous studies suggest that hyperglycemia is associated with the cognitive decline seen in both types of diabetic patients [4]. Hyperglycemia alters multiple mechanisms, causing activation of protein kinase C activity and the polyol pathway, accumulation of sorbitol and advanced glycation end products, disturbances in Ca2+ homeostasis, and augmentation of reactive oxygen species (ROS) levels. Increased levels of ROS and free radical generation disturb oxidative metabolism balance, which leads to detrimental effects on cellular function [5]. The detrimental changes induced by ROS in the cell include depletion of endogenous antioxidants, energy insufficiency, mitochondrial dysfunction, protein and DNA damage, and inflammation.

Furthermore, oxidative stress causes activation of the pleiotropic transcription factor known as nuclear factor kappa B-light-chain-enhancer of activated B cells (NF-κB). NF-κB activation further increases the levels of proinflammatory mediators like proinflammatory cytokines [cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and interleukin (IL)-6]. Several studies have reported the involvement of NF-κB activation in peripheral and central nervous system complications of diabetes [6,7].

Melatonin (N-acetyl-5-methoxytryptamine), a pineal hormone, is one of the well-studied pharmacological interventions that exert protective effects primarily by ROS scavenging activity. In addition, melatonin augments the antioxidant defense system and down-regulates inflammatory mediators [8]. Several studies have demonstrated the neuroprotective action of melatonin in neurodegenerative disorders, such as amyotrophic lateral sclerosis, Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis [9,10]. In a previous study, melatonin was found to have a neuroprotective effect against streptozotocin (STZ)-induced neurobehavioral and neurochemical deficits [11]. The aim of the present study was to investigate the protective effect of melatonin against cognitive dysfunction induced by type 2 diabetes.

EXPERIMENTAL

Animals

Male Sprague-Dawley rats (120 - 140 g) were used in this study. The animal study was approved by Institutional animal ethical committee of Tongji Medical College, China (ref. no. TMC/2015/03) and performed in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use [12].

Induction of diabetes

All the animals were fed with HFD initially for fourteen days and thereafter single dose of STZ (35 mg/kg, i.p.) was administered to induce diabetes. HFD was provided to the animals throughout the study period. Rats with serum glucose level ≥ 250 mg/dL were considered diabetic animals and were further selected for the study. All the animals were divided into four groups (n = 12) such as control group animals received vehicle (p.o.); HFD group animals were fed HFD and then given single dose of STZ injection. After completion of 15 weeks, vehicle was administrated (p.o.) for subsequent three weeks. HFD + STZ + M 10 mg/kg group of animals were fed HFD and then given single dose of STZ injection. After completion of 15 weeks, melatonin (10 mg/kg) was administered (p.o.) for subsequent three weeks. M 10 mg/kg group of animals were treated with melatonin (10 mg/kg) for three weeks after completion of 15 weeks. Object recognition task and Morris water maze test were performed on 15th and 18th week.

Morris water maze (MWM) test

Cognitive function in all the animals was assessed by MWM test. Circular tank with 60 cm of height and 180 cm of diameter was used in the study. A platform was placed in the tank 2 cm below the water level having the height 38 cm and 12.5 cm diameter which was visible to pups. Tank was divided into four different quadrants and different symbol was placed in the room to differentiate the different quadrants of tank. Training was given to all the pups for 4 days continuously. In each session 4 trials were given at an interval of 30 s in which animal was placed in any one of quadrant. The pups were kept as such for the duration of 20 sec on the platform after it climbs on it. The time taken by the pups to reach the platform was considered as escape latency. However time spent in the target quadrant was estimated by removing the stage.
from the tank and pups were allowed to search the platform.

Object recognition task (ORT)

ORT was performed in a black colored wooden box (50 x 70 x 50 cm³). The animal was initially habituated to the ORT box for 10 min in a day for two consecutive days. On 3rd day, the rat was placed for 5 min in the ORT box and then, two objects of dissimilar appearance (a blue colored truncated cone and a cube-shaped white block) were kept in two opposite corners of the ORT box. Thereafter, the animals were allowed to explore ORT box for another 5-min period and the time spent in exploring the objects was recorded. Thereafter, animals were returned to their respective cage. After of duration of 120 min, the animals were placed in the ORT box for 5 min, and the objects placed back in the ORT box, with the cube-shaped white block used in the previous session changed to a novel object (an ellipsoid-shaped red colored object). The time spent to explore objects was recorded for 5 min period. The animal was considered to be exploring when animal were facing, sniffing, or biting the object. ORT box and the objects were cleaned thoroughly with alcohol to avoid olfactory cues. Cognitive function was evaluated by estimating the time spent in the target quadrant, preference index.

Assessment of glucose and insulin level in rat serum

Blood was collected from the rat in Eppendorf tube and serum was separated out by centrifuging blood at 4 °C for the duration of 5 min at 2000 × g. Level of glucose and insulin in the serum were determined by glucose oxidase method and ELISA kit (Millipore, USA) respectively.

Preparation of brain tissue

At the end of protocol all the animals were sacrificed by cervical dislocation and hippocampus was isolated from the separated brain. 50 mM Tris-HCl of pH 7.4 and 300 mM of sucrose was used to homogenate the brain tissue for 10 min at 10000 rpm. Supernatant was separated out for further study.

Determination of ROS level

ROS level was estimated by adding 50 µl of tissue homogenate in 4.85 ml of potassium phosphate buffer (100 mmol/L and pH 7.4). The mixture was incubated for 15 min with 2’, 7’-dichlorofluorescin diacetate dye at 37 °C. After incubation, mixture was centrifuged at 12,500 g for 10 min at 4 °C. The pellet obtained after centrifugation was mixed with 5 mL of 100 mmol/L PBS on a vortex at 0 °C and subsequently incubated for 60 min at 37 °C. Fluorescence was measured at 488 nm for excitation and 525 nm for emission. ROS level was calculated as picomoles per min per mg of protein.

Assessment of oxido-nitrosative stress

Level of lipid peroxidation (LPO) was determined as per the previously reported method in the brain tissues. Amount of malondialdehyde (MDA) was estimated at 532 nm. Hippocampal GSH content was estimating the absorbance at a wave length of 412 nm and the level of GSH was articulated as micromoles of GSH per milligram of protein. Nitrite level was determined using a commercially available Griess reagent (containing 0.1 % N-(1-naphthyl) ethylene diamine dihydrochloride, 1 % sulfanilamide and 2.5 % phosphoric acid). Equal amount of sample and Griess reagent were mixed and then incubated in a dark place. After 15 min, absorbance was measured at 540 nm.

Determination of proinflammatory cytokines and Brain-derived neurotrophic factor (BDNF)

IL-6 and TNF-α level was determined by Rat ELISA kit purchased from Invitrogen Co, Carlsbad, CA, USA. Hippocampal BDNF level was estimated by BDNF Emax® ImmunoAssay System (Promega Corporation, USA). Furthermore, Hippocampus was homogenized and sonicated in 8 volumes of 0.1 M ice-cold PBS buffer (pH 7.4) containing protease inhibitor cocktail. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. The assay was carried out with the help of manufacturer’s kit. The absorbance was measured at 450 nm in ELISA plate reader.

Western blot assay

Hippocampus was homogenized in 500 µL of RIPA buffer containing protease and phosphatase inhibitor cocktail. Thereafter, homogenate was centrifuged at 12,000 g for 20 min. The supernatant was collected and stored at -80 °C for further analysis. The concentration of proteins in the sample was measured using previously described method. Equal amount of protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then separated proteins transferred onto a nitrocellulose membrane. The membrane was blocked by 3 % bovine serum albumin. Thereafter, membranes were incubated with
respective primary antibodies for overnight at 4 °C. Thereafter membranes were washed with TBST and then incubated with a horseradish peroxidase conjugated antibody for 2 h at room temperature. The antigen-antibody-peroxidase complexes were visualized using a colored reaction with BCIP-NBT. The quantitative analysis of bands was carried out with the help of Image J software.

**Statistical analysis**

Data are expressed as mean ± SEM. Student's t test was used to compare the difference between control and diabetic group. One-way analysis of variance (ANOVA) followed by post-hoc Tukey test was used to compare the various groups. Two-way repetitive measure ANOVA followed by post-hoc Bonferroni test was used to find the differences in MWM acquisition data. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Body weight, and serum glucose level and serum insulin levels**

After 15 weeks of diabetes induction, type 2 diabetic rats had significantly increased body weight \( (p < 0.01) \) and showed marked \( (p < 0.01) \) elevation of serum glucose and insulin levels compared with the control group (Table 1). Treatment with melatonin significantly reduced the serum levels of glucose and insulin in type 2 diabetic rats compared with levels in the diabetic control group.

**Type 2 diabetes-induced cognitive deficits**

Cognitive function was assessed using the Morris water maze (MWM) and an object recognition task (ORT) to investigate cognitive impairment in control and diabetic rats at the 15th week of the study protocol. On the first day of the MWM trial, there were no significant differences between mean escape latencies of control and diabetic rats. However, two-way ANOVA revealed longer escape latencies on the second \( (p < 0.01) \), third \( (p < 0.001) \), and fourth \( (p < 0.01) \) days of the acquisition trial by the diabetic group compared with the control group. These results indicate learning deficits in diabetic rats compared with the control group. Furthermore, a probe trial was conducted to assess memory retention in diabetic rats. It was found that diabetic rats spent significantly \( (p < 0.01) \) less time in the target quadrant than control rats, indicating poor memory retention in diabetic rats (Figure 1B).

The effect of diabetes on recognition memory after the 15th week of diabetes induction was assessed using ORT. One-way ANOVA revealed that the diabetic rats failed to discriminate between familiar and unfamiliar objects placed in the ORT box. Consequently, the preference index of diabetic group was significantly \( (p < 0.01) \) higher than that in the control group (Figure 1C). Similar behavioral results were observed after the 18th week of diabetic induction.

**Melatonin treatment ameliorated cognitive deficits**

The melatonin (10 mg/kg)-treatment group displayed a significant reduction in escape latency on the second \( (p < 0.05) \), third \( (p < 0.01) \), and fourth \( (p < 0.05) \) days of the trial compared with the cisplatin-treated group (Figure 2A). Thus, melatonin treatment significantly reversed the type 2 diabetes-induced learning deficits. Furthermore, impairment in spatial learning observed in a probe trial was significantly reversed \( (p < 0.01) \) in diabetic animals after melatonin treatment (Figure 2B). An ORT method was used to verify the effect of melatonin treatment on memory in rats. The melatonin treatment group spent significantly \( (p < 0.01) \) more time exploring the novel object than diabetic rats, indicating improvement in memory function after melatonin treatment (Figure 2C). In addition, melatonin treatment alone in normal rats did not alter the cognitive function.

**Melatonin treatment mitigates dixoido-nitrosative stress**

Diabetic rats showed increases in oxidative stress as evidenced by a significant rise in levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Serum glucose (mg/dL)</th>
<th>Serum insulin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>155±11.3</td>
<td>102±4.7</td>
<td>6.2±0.5</td>
</tr>
<tr>
<td>HFD+STZ</td>
<td>242±15.6**</td>
<td>249±17.8**</td>
<td>9.5±0.65**</td>
</tr>
<tr>
<td>HFD+STZ+M 10 mg/kg</td>
<td>231±13.8**</td>
<td>151±9.3**</td>
<td>7.8±0.2*</td>
</tr>
<tr>
<td>M 10 mg/kg</td>
<td>143±9.2</td>
<td>97.5±3.1**</td>
<td>6.6±0.11**</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n = 6). **p < 0.01 compared to control group; *p < 0.01 and **p < 0.01 compared to diabetic control.
Figure 1: Effect of 15-week type 2 diabetes on (a) mean escape latency, (b) percentage time spent in the target quadrant, and (c) recognition memory. Data are expressed as mean ± SEM (n = 6). *p < 0.05, **p < 0.01 compared to control group.

Figure 2: Effect of melatonin treatment on (a) mean escape latency, (b) percentage time spent in the target quadrant, and (c) recognition memory in diabetic rats. Data are expressed as mean ± SEM (n = 6). ##p < 0.01 and ###p < 0.001 compared to control group; *p < 0.05, **p < 0.01, and ***p < 0.001 compared to diabetic group.
of ROS ($p < 0.01$) and malondialdehyde (MDA; $p < 0.01$), and depletion of glutathione (GSH; $p < 0.01$) in the hippocampus region (Figure 3). However, melatonin treatment was associated with significant reduction of oxidative stress by reducing MDA ($p < 0.01$) and ROS ($p < 0.01$) levels in the hippocampus. Moreover, the GSH level was markedly ($p < 0.01$) increased in the hippocampus of melatonin-treated diabetic rats due to mitigation of oxidative stress by melatonin treatment. The nitrite level, an indirect indicator of nitrosative stress that was increased ($p < 0.01$) in the diabetic group, was significantly ($p < 0.05$) reduced by melatonin treatment (Figure 3).

**Melatonin treatment alleviated neuroinflammation and prevented brain-derived neurotrophic factor (BDNF) depletion**

Diabetic rats showed higher levels of hippocampal IL-6 and TNF-α ($p < 0.001$) than normal animals (Figure 4). Treatment with melatonin caused a marked reduction in IL-6 ($p < 0.001$) and TNF-α ($p < 0.05$) levels in diabetic animals. Thus, melatonin treatment significantly alleviated type 2 diabetes-induced neuroinflammation in the hippocampus region. Furthermore, hippocampal BDNF content was significantly ($p < 0.001$) depleted in the diabetic rats compared with control animals. On the other hand, melatonin treatment increased ($p < 0.01$) the BDNF level in the hippocampus compared with that in diabetic rats without melatonin treatment.

**Melatonin treatment inhibited NF-κβ activation**

The results in Figure 5 show that type 2 diabetes significantly ($p < 0.001$) increased the protein expression level of NF-κB and phosphorylated IκB in diabetic animals. Melatonin treatment significantly downregulated the NF-κB protein expression level ($p < 0.001$) and phosphorylation of IκB ($p < 0.05$) in the diabetic rats.
DISCUSSION

This study evaluated the protective effect of melatonin against cognitive dysfunction in type 2 diabetic rats. Previous experimental reports demonstrated that both type 1 and type 2 diabetes cause cognitive dysfunction via alterations in neurovascular and synaptic architecture [13]. In this study, an HFD with a single low-dose injection of STZ was used to induce type 2 diabetes in rats. Cognitive function was evaluated in diabetic animals on the 15th week as evidenced by MWM and ORT results. The results regarding HFD + STZ-induced cognitive deficits are supported by previously reported studies [21]. Chronic treatment with melatonin for 21 days after 15 weeks of inducing diabetes significantly reversed the HFD + STZ-induced cognitive deficits. The observed protective effect of melatonin against cognitive dysfunction is in accordance with previous experimental studies of melatonin [14]. On the 18th week, HFD + STZ diabetic animals were characterized by hyperglycemia and exhibited significant learning and memory impairment.

This impairment was coupled with marked increases in oxido-nitrosative stress, neuroinflammation, NF-κB activation, and BDNF depletion in the hippocampus region. Treatment with melatonin for 21 days significantly alleviated the cognitive deficits by augmentation of BDNF levels and attenuation of oxido-nitrosative stress, neuroinflammation, and NF-κB in the hippocampus.

Hyperglycemia plays a crucial role in the pathogenesis of diabetes-induced cognitive dysfunction [15]. Hyperglycemia-induced ROS is considered a major culprit in the pathogenesis of diabetes-associated complications [16]. In the brain, ROS-generated oxidative stress disturbs the synaptic function and thereby causes cognitive impairment [17]. In the present study,
Figure 5: Effect of melatonin treatment on NF-κB, IκB, and phospho-IκB expression levels in the hippocampus of diabetic rats. Results are expressed as mean ± SEM (n = 3). ***p < 0.001 compared to control group; **p < 0.01 and ***p < 0.001 compared to diabetic group; $p < 0.05$ and $$$p < 0.001$ compared to IκB

the levels of MDA (a lipid peroxidation marker) and nitrite (an indicator of nitrosative stress) were elevated in the hippocampus region of the diabetic group. Increased levels of oxidative stress deplete enzymatic and non-enzymatic antioxidants levels and disrupt the normal functioning of mitochondria, thereby exaggerating oxidative stress. The results showed that diabetes significantly depleted the GSH level in the hippocampus region. Melatonin and its metabolites are powerful free radical scavengers and augment the expression of several antioxidant enzymes [18-20]. In this study, melatonin treatment significantly alleviated oxidative stress as evidenced by reduction of MDA and nitrite level and augmentation of GSH level in the hippocampus.

The involvement of the NF-κB cascade in the pathogenesis of diabetes-induced complications is well documented in previous experimental studies [21]. The NF-κB signaling pathway has been implicated in a number of neurodegenerative diseases as well and it is activated by oxidative stress and proinflammatory cytokines [22]. Under physiological conditions, NF-κB remains in an inactive condition in the cytoplasm by forming a complex with the inhibitory kappa beta (IκB) family. However, in cases of activation, NF-κBs detach from IκB and translocate to the nucleus where they bind to the κB sequence of DNA. This bond alters the gene expression level of several inflammatory mediator proteins, such as IL-6, TNF-α, iNOS, and COX-2. These NF-κB induced inflammatory mediators eventually cause neuronal cell death and cognitive impairment [23]. Our results showed up-regulation of NF-κB protein in the hippocampus of diabetic rats compared to normal rats. Proinflammatory cytokines (IL-6 and TNF-α) were also significantly increased in the hippocampus of diabetic rats, which indicates profound
neuroinflammation. Numerous studies suggest that the neuroprotective effect of melatonin is mediated by inhibition of the NF-κB cascade [24].

In the present study, similar findings were observed after chronic treatment with melatonin. Diabetes-induced NF-κB and its associated neuroinflammation were significantly reduced after 21 days of melatonin treatment. Moreover, the results revealed an increased level of nitrite in the diabetic animals, which indirectly indicates the up-regulation of iNOS level in the hippocampus. However, melatonin treatment caused marked reduction in the nitrite level of diabetic animals. The reduction of nitrite level by melatonin is attributed to its reactive nitrogen species (RNS) scavenging property and NF-κB inhibition activity.

CONCLUSION

The results of this study demonstrate that melatonin prevents cognitive dysfunction in type 2 diabetic rats by attenuating oxidative-nitrosative stress and NF-κB-mediated neuroinflammation. These results indicate that melatonin can potentially be used for the management of cognitive dysfunction in patients suffering from diabetes. However, further studies are required to confirm this.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this study.

Authors’ contribution

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhang Xuyan performed the experiments and wrote the report. Ye Jing Ping, Wang Zhongjiing, Ding Sheng, Li Li, and Yang Fan analyzed data and gave comments. Mao Hong designed the project, supervised the experiments, and wrote the entire manuscript. All authors read and approved the final manuscript.

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