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Melatonin regulates traumatic optic neuropathy via targeting autophagy

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Abstract. – OBJECTIVE: Traumatic optic neuropathy (TON) usually refers to the indirect damage to the optical nerve, which can cause partial or complete blindness. Melatonin (MT) is a kind of indole hormone, and the retina is one of its natural sites of secretion in the human body. This study aims to explore MT in the retina and optic nerve injuries due to TON.

MATERIALS AND METHODS: Sprague-Dawley (SD) rats were used for TON model in the study. After operation, rats were treated with MT or phosphate buffered saline (PBS) for 4, 7, 14, 21, and 28 days before sacrifice. The changes in retinal ganglion cells (RGCs) were observed via hematoxylin-eosin (HE) staining. Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining was applied to observe apoptosis. Immunofluorescence staining was applied to detect caspase-3 and Western blot was used to detect LC3, cleaved caspase-3 and glyceraldehyde phosphate dehydrogenase (GAPDH).

RESULTS: The number of RGCs in MT group increased compared to the model group. After MT treatment, the increased number of TUNEL positive cells and the increased number of caspase-3 positive cells in the retina of MT group was alleviated. Moreover, Western blot analysis revealed that the LC3-II/LC3-I ratio in the retinal tissue of MT group was further increased, while the increased cleaved caspase-3 protein level in the retina of MT group was alleviated compared to the model group.

CONCLUSIONS: The results of this study revealed that MT therapy affects the apoptosis level of RGCs after TON through alleviating the increased caspase-3 protein level. Its mechanism may be that it further up-regulates the autophagy level of RGCs after TON, ultimately inhibiting the apoptosis of RGCs after TON and playing a neuroprotective role.

Key Words:
Melatonin, Traumatic optic neuropathy, Autophagy, Apoptosis.

Introduction

Traumatic optic neuropathy (TON) usually refers to the indirect damage to the optic nerve caused by the external force transmitted to the optic nerve *via* bone or eyeball movement when the brain suffers from trauma[1], which can cause partial or complete blindness. The clinical manifestations of TON are mainly decreased visual acuity, dyschromatopsia, relative afferent pupillary defect (RAPD) as well as ocular fundus change. The impairment of vision caused by TON is severe and the therapeutic effect and prognosis are poor[2]. Moreover, such an injury accounts for about 0.5-5% in the closed head injury[3]. With the development of scientific medical technology and understanding of nature of disease, there is a consensus about its pathogenesis, damage mechanism and diagnosis, but there are still a lot of controversies about the treatment methods and prognosis. In particular, there are still many problems to be explored and solved in the time of treatment, surgical intervention method, therapeutic regimen and surgical decompression standards, etc.

N-acetyl-5-methoxylamine, melatonin (MT), is a kind of indole hormone, and the retina is one of its natural sites of secretion in the human body[4]. The biosynthesis of MT, besides the pineal gland, is mainly carried out in the retinal photoreceptor cells. With tryptophan as the raw material, 5-hydroxytryptamine is produced *via* hydroxylation and decarboxylation. Then N-acetyl-5-hydroxytryptamine is produced under the action of N-acetyltransferase, and finally MT is formed under the action of oxindole-oxygen-transmethylase *via* methylation. The synthetic amount of MT is limited in the eye, and MT only plays a local role through the paracrine.

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It is always believed that the biological roles of MT are mainly to regulate circadian rhythm and sleep-wake biological rhythm phase conversion, and improve sleep[5]. However, with the deepening of research, it has been found that MT and metabolites have direct and indirect antioxidant property and free radical scavenging function, thereby preventing the cell death or apoptosis, and protecting the intracellular lipid, protein and nucleic acid, etc.

At present, there are many investigations on the role of MT in systemic ischemia reperfusion. Some scholars also apply MT to the research on the retinal optic neuropathy caused by diabetic retinopathy and ocular hypertension. However, there are few and even no researches on MT in the retina and optic nerve injuries due to TON in China. The retinal tissue is very similar to the brain tissue in biological characteristics. The study on the role of MT in TON can provide not only factual and theoretical basis for the clinical application of MT, but also a new way for the investigation of the mechanism of TON and physiological and pharmacological effects of MT.

Materials and Methods

Experimental Animals

One hundred and sixty clean Sprague-Dawley (SD) rats aged 3 months in either gender weighing (250 ± 20) g were selected and received the preoperative examination before enrollment. The bilateral eye movement was normal, there was no extra-ocular defect and no deformity of the equal and round pupil, and the light reflex was normal. This study was approved by the Animal Ethics Committee of Yantai Yida Hospital Animal Center.

Experimental Reagents

MT was purchased from Sigma-Aldrich (St. Louis, MO, USA), and it was dissolved by 100 mL absolute ethyl alcohol and normal saline to prepare the 5 g/L solution[6] before application. Primary antibodies as rabbit anti-mouse cleaved caspase-3 monoclonal antibody, rabbit anti-mouse LC3 monoclonal antibody and rabbit anti-mouse GAPDH monoclonal antibody were purchased from CST (Danvers, MA, USA). TUNEL assay kit was purchased from Roche (Basel, Switzerland).

Grouping

The experimental rats were separated into blank control group, sham group, model group and MT group at random using the randomized double-blind controlled method. All the groups were further divided into 5 subgroups: 4 d, 7 d, 14 d, 21 d and 28 d. There were 40 rats in each group and 8 rats in each subgroup. Rats were treated for 4 d, 7 d, 14 d, 21 d and 28 d, and then executed according to the time segment.

Modeling

After the preoperative examination, the TON model was made with the left eye of rats as the surgical eye. The optic nerve sheath was retained according to the optic nerve transverse injury modeling method of Ahmad et al[7]. Criteria of successful modeling: the pupil in the left surgical eye was dilated, the direct light reflex disappeared, there was no surgical eye convex, no bleeding in sclera and conjunctiva, no eyelid closure insufficiency and no postoperative organ failure[3].

Treatment

Rats in MT group were treated with MT gavage (20 mg/kg·d) daily. Rats in blank control group, sham group and model group were treated with the same amount of normal saline, and rats in sham group, model group and each treatment group were treated for 4 d, 7 d, 14 d, 21 d and 28 d, then sacrificed. Rats in blank control group were sacrificed at the same time after treatment.

Materials and Observational Indexes

Before sampling, rats were fasted for 12 h, weighed and anesthetized with 10% chloral hydrate, and the eyeball was quickly removed. Half of the eyeball was put in liquid nitrogen for protein extraction and the other half was used for frozen section making. The changes in retinal ganglion cells (RGCs) were observed *via* HE staining. Count of RGCs: one section was taken from each sample, and 5 non-repetitive high-power fields were randomly selected under the microscope (10 × 40) to count the number of RGCs; then, the average was taken[8].

TUNEL Staining

The section was added with pepsase K for incubation at room temperature for 30 min, and washed with phosphate-buffered solution (PBS) twice; 50 μL TUNEL reaction mixture was added dropwise for incubation in the wet box

at 37°C for 60 min, and then the section was washed with phosphate-buffered saline (PBS) for three times; 50 μL conversion agent-POD was added for incubation in the wet box at 37°C for 30 min, and then the section was washed with PBS for three times; then the color developing agent 3,3′-diaminobenzidine (DAB) was dropped at room temperature for 10 min, and then the section was washed with distilled water fully, followed by hematoxylin re-staining for 1 min, conventional dehydration and transparency, sealing *via* neutral gum and microscopic observation. Motic Med 6.0 pathological image analysis system was used; the retinal tissues were observed and counted in five non-repetitive visual fields, and the TUNEL positive cells in retinal tissues were counted under the microscope (×400). The average was taken as the statistical data (per/HP) for each high power field of samples.

Immunofluorescence Staining

The sections were re-warmed, rinsed, blocked with bovine serum albumin (BSA) and incubated with the primary antibody at 4°C overnight. Then, the sections were washed with PBS, incubated with the secondary antibody at room temperature for 2 h, and rinsed again with PBS. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) for 2 min, and the section was rinsed with PBS, followed by sealing *via* fluorescence anti-quenching agent and microscopic observation. Three eyeball tissue samples were selected from each group. Five visual fields (×400) were randomly selected in each section for photograph. The cells were counted using the Photoshop CS3 software.

Western Blot

The samples were removed from the liquid nitrogen, and the eyeball tissue was weighed and placed in the grinding tube, and the pre-cooled tissue lysis buffer was added for grinding in proportion, followed by cracking on ice for 0.5 h. The lysate was centrifuged at 12000 rpm on the standard centrifuge with the centrifugal radius of 10 cm at 4°C for 10 min. Next, the supernatant was collected and the total protein content was measured using the bicinchoninic acid (BCA) method. A portion of the supernatant was extracted, added with the loading buffer at 4:1 and boiled in the boiling water at 100°C for 10 min, followed by polyacrylamide gel electrophoresis, membrane transfer, sealing with 5% skim milk, incubation of primary antibody, membrane washing, incubation of secondary antibody, membrane washing and ordinary chemical detection via enhanced chemiluminescence (ECL). The film was scanned, saved and analyzed with ImageJ 2.0 software.

Statistical Analysis

Statistical Product and Service Solutions 18.0 (SPSS 18.0, Version X; IBM, Chicago, IL, USA) statistical software were used for the analysis of experimental data, and the data were presented as ($x \pm s$). Analysis of variance (ANOVA) was used for the comparison of means between groups, while one-way ANOVA was used for the comparison among groups. Least significant difference (LSD) was used as its post hoc test. $p < 0.05$ suggested there was significant difference between groups.

Results

Observation of RGCs

The retinal structure of rats in blank control group was clear, the cells were arranged in a single layer, and the layer was clear with larger cell bodies. RGCs of rats in sham group were slightly sparse, and there was no significant difference compared with blank control group. The retinal layers of rats in model group were thinner in varying degrees and the number of cells was decreased compared to the sham group. At treatment for 7 d, the number of RGCs in MT group began to increase significantly. The number of RGCs in MT group was more than that in model group ($p < 0.05$) (Figure 1).

TUNEL Staining

The results of TUNEL staining showed that there were no obvious TUNEL positive cells in sham-operation group at 28 d after modeling compared with blank control group; many TUNEL positive cells were scattered in the retina of model group, and the number of positive cells in model group was significantly increased, and there was a statistically significant difference compared with that in sham-operation group ($p < 0.05$). After MT treatment, the increased number of TUNEL positive cells in the retina of MT group was alleviated, and there was a statistically significant difference compared with that model group ($p < 0.05$) (Figure 2).

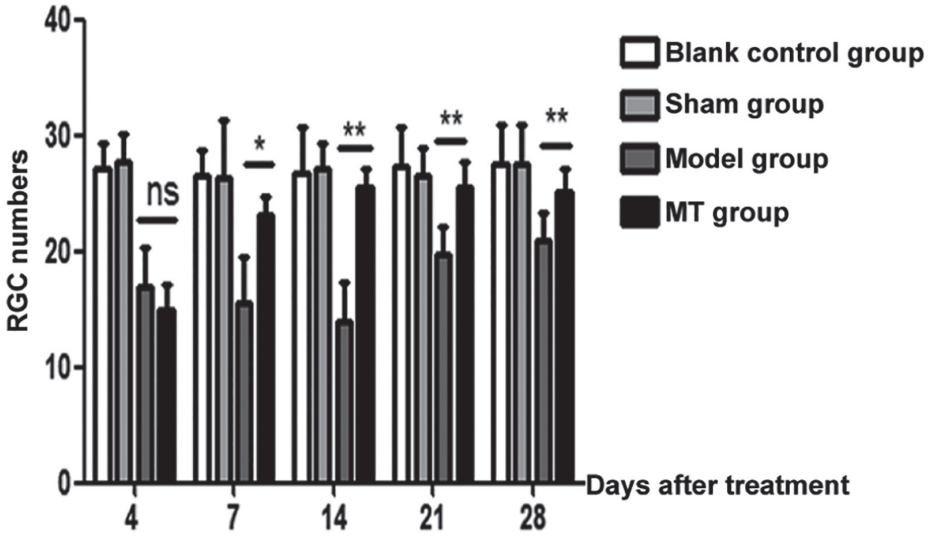

Figure 1. RGC numbers between groups at different times after treatment. Ns, none significant; *, $p < 0.05$; **, $p < 0.01$.

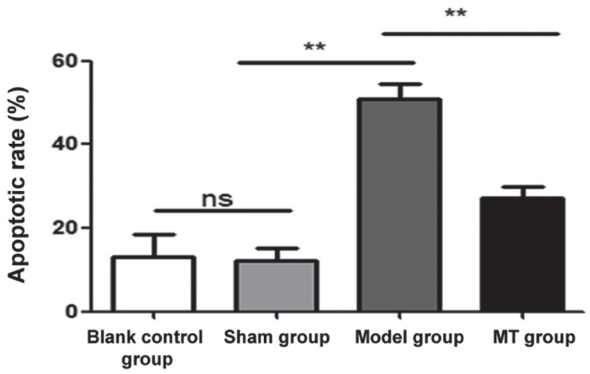

Figure 2. Apoptotic rate represented by the TUNEL staining in the retina between different groups at 28 days after treatment.Ns, none significant; **, $p < 0.01$.

Apoptosis-Related Protein Caspase-3 Fluorescence Staining

The fluorescence staining results revealed that a very small number of caspase-3 positive cells were scattered in the retina of sham-operation group at 28 d after modeling compared with that in blank control group, and there were a number of caspase-3 positive cells in the retina of model group. After MT treatment, the increased number of caspase-3 positive cells in the retina of MT group was alleviated (Figure 3).

LC3-II/LC3-I Ratio

The results of Western-blotting showed that LC3-II/LC3-I ratio in the retinal tissue of mod-

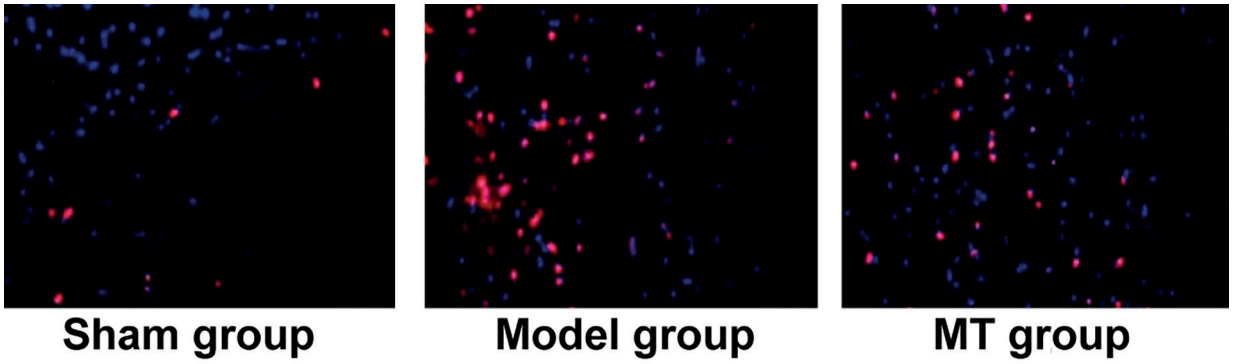

Figure 3. Fluorescence staining of apoptosis-related protein caspase 3between different groups at 28 days after treatment. Blue, DAPI; red, caspase-3 positive cells.

el group at 28 d after modeling was increased significantly, which had a statistically significant difference compared with that in sham-operation group ($p < 0.05$). After MT treatment, the LC3-II/LC3-I ratio in the retinal tissue of MT group was further increased, which had a statistically significant difference compared with that in model group ($p < 0.05$) (Figure 4).

Cleaved Caspase 3 Protein Level

The results of Western-blotting revealed that the cleaved caspase-3 protein level in the retina of model group at 28 d after modeling was increased obviously, and there was a statistically significant difference compared with that in sham-operation group ($p < 0.05$). After MT treatment, the increased cleaved caspase-3 protein level in the retina of MT group was alleviated, and there was a statistically significant difference compared with that in model group ($p < 0.05$) (Figure 4).

Discussion

TON is one of the common and serious complications of traumatic brain injury, showing a gradually increasing trend in recent years[9]. Those with clear indications of operation of TON are mainly treated with surgery. The early surgery is advocated. The timing, indications and complications of surgery are actively assessed, so as to reduce the serious consequences brought about by delaying the best operation time window to the greatest extent. In drug therapy, the full-dose hormones are applied in the early stage, combined with dehydrating agent, Ca^{2+} receptor antagonist, nutritional nerve drugs, vasodilator drugs and hyperbaric oxygen therapy, to realize the symptomatic and supportive treatment and treat the optic nerve injury[10,11]. Transgenic therapy and amino acid receptor antagonists are still in the laboratory research stage.

Apoptosis is a kind of cell death process caused by the intracellular pre-stored death program triggered by *in vitro* and *in vivo* factors, also known as programmed cell death. It is characterized by relatively complete cell membrane and organelle, cell shrinkage and nuclear pyknosis in the morphology[12]. Although various signals can stimulate a variety of intracellular signaling transduction pathways, these pathways will eventually converge to one common pathway, the caspase cascade amplification reaction. Caspase is a kind of apoptotic activating gene, and caspase family

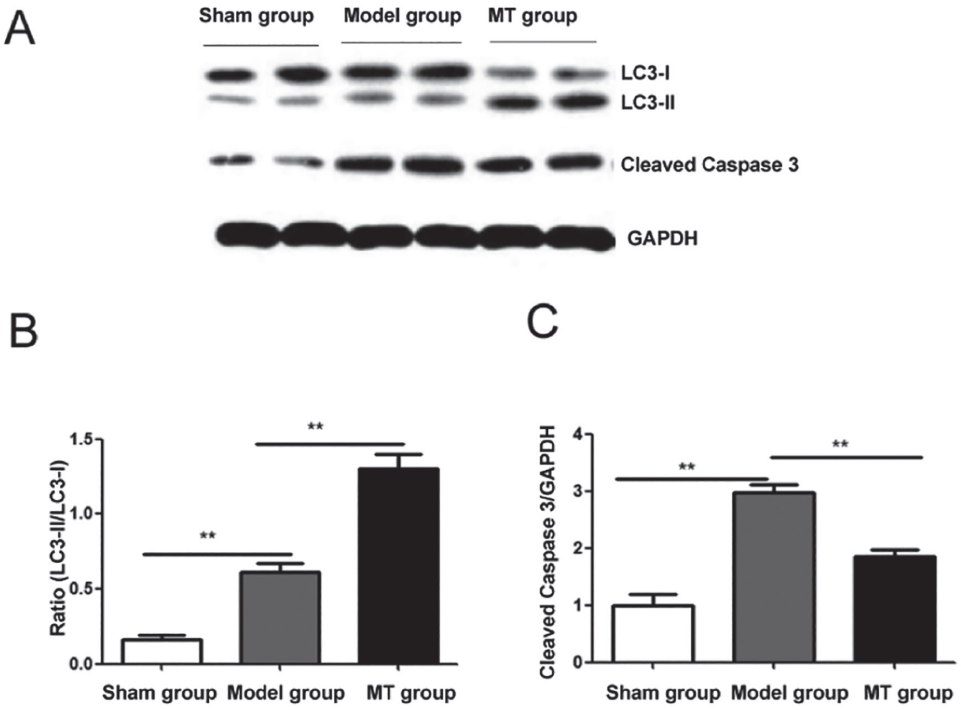

Figure 4. Western blot showed protein levels of LC3 and cleaved caspase 3 between different groups at 28 days after treatment. **, $p < 0.01$.

members are related to the apoptosis and involved in the pathophysiological process of apoptosis. According to different apoptosis signals, a variety of signal stimuli can activate a variety of caspase, and the core member is caspase-3. TUNEL method is widely used in the study on apoptosis because of its sensitivity and reliability. The results of this study showed that TUNEL positive cells and caspase-3 positive cells were increased in the retina after TON, suggesting that the level of apoptosis of RGCs is increased after TON. However, after MT treatment, the number of TUNEL positive cells and caspase-3 positive cells were effectively controlled, indicating that MT fights against apoptosis and plays a neuroprotective effect in RGCs after TON.

Autophagy is closely related to apoptosis, and the morphological features of both autophagy and apoptosis can be even found in the same cell[13,14]. Studies have shown that autophagy can occur prior to apoptosis, and plays an anti-apoptosis and protective effect in the process of apoptosis[15,16]. The results of this work revealed that MT therapy affects the apoptosis level of RGCs after TON through alleviating the increased caspase-3 protein level, and its mechanism may be that it further up-regulates the autophagy level of RGCs after TON, ultimately inhibiting the apoptosis of RGCs after TON and playing a neuroprotective role.

Conclusions

The role of MT in TON was investigated in this paper, which can investigate the mechanism of TON and physiological and pharmacological effects of MT, and provide not only factual and theoretical basis for the clinical application of MT, but also a new way for the clinical treatment of TON.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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