

Effects of melatonin on expressions of β -amyloid protein and S100 β in rats with senile dementia

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Abstract. – **OBJECTIVE:** To explore the effects of melatonin (MT) on expressions of β -amyloid protein (β -AP) and S100 β in rats with senile dementia.

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly divided into Sham group, Model group and MT group, with 12 rats in each group. Senile dementia models were established in each group except Sham group. After modeling, rats in Model group were given tail vein injection with 0.9% sodium chloride once per day. Rats in MT group were given tail vein injection with MT once per day. Materials were collected at 40 d after the intervention. Hematoxylin-Eosin (HE) staining was adopted to observe histomorphology of hippocampal area, Western blotting to detect expressions of β -AP and S100 β protein, and quantitative polymerase chain reaction (qPCR) to detect expressions of β -AP mRNA and S100 β mRNA.

RESULTS: Histomorphology in hippocampal area of both Model group and MT group was changed compared with that in Sham group. Histomorphology data showed that the damage in the hippocampal area in MT group was improved compared with that in Model group. Western blotting detection showed that expressions of β -AP and S100 β in Model group and MT group were significantly increased compared with those in Sham group ($p < 0.05$). Expressions of β -AP and S100 β protein in MT group were significantly decreased compared with those in Model group ($p < 0.05$). Results of qPCR revealed that expressions of β -AP mRNA and S100 β mRNA in Model group and MT group were also significantly increased compared with those in Sham group, and there were statistically significant differences ($p < 0.05$). Expressions of β -AP mRNA and S100 β mRNA in MT group were significantly decreased compared with those in Model group ($p < 0.05$).

CONCLUSIONS: MT can inhibit expressions of β -AP and S100 β protein in the hippocampal area of model rats with senile dementia, which provides leads for the future treatment of senile dementia.

Key Words:

Senile dementia, Melatonin, β -amyloid protein, S100 β protein, Cell proliferation.

Introduction

Senile dementia, namely incorrectly Alzheimer's disease, is a kind of senile disease with a high clinical incidence rate. This disease is featured by progressive degeneration of the central nervous system, which often causes hypophrenia in the elderly and affects their living quality severely. According to statistics, there are inadequate medical and health-care measures for patients with senile dementia, and less than 50% of patients with senile dementia are diagnosed and treated even in developed countries¹. It is estimated that there will be 80 million people suffering from senile dementia worldwide by 2040². The pathogenesis of senile dementia is characterized also by deposition of amyloid protein (AP), senile plaques and neurofibrillary tangles, leading to neuronal degeneration and necrosis³. This lesion will finally result in memory loss, cognition impairment and loss of speech function of patients. Therefore, senile dementia has currently become a research focus in the field of central nervous system injury⁴. However, there is still no ideal drug for treating senile dementia. Melatonin (MT), as a hormone

that has effects on anti-aging, anti-brain function degeneration, and anti-oxidation, is considered as a potentially effective therapeutic drug for senile dementia⁵. This study aims to observe the effects of MT on expressions of β -AP and S100 β in rats with senile dementia and discuss whether MT can be the effective drug for treating senile dementia.

Materials and Methods

Experimental Animals and Grouping

A total of 36 Sprague-Dawley rats (half males and half females) weighing (220 $\pm$ 20) g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), with license No. SCXK (Hu) 2014-0003. These 36 rats were randomly divided into Sham group, Model group and MT group with 12 rats in each group. All operations and protocols on animals were approved by the Laboratory Animal Ethics Committee of our hospital.

Experimental Reagents and Instruments

Primary antibodies: anti- β -AP antibody and anti-S100 β antibody were purchased from Abcam (Cambridge, MA, USA). Immunohistochemistry kit was bought from Maxim (Fuzhou, Fujian, China). AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix kit and HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit were from Vazyme (Nanjing, Jiangsu, China). β -AP was provided by Sigma-Aldrich (St. Louis, MO, USA). Optical microscope (Leica DMI 4000B/DFC425C) was bought from Leica (Wetzlar, Germany). Stereotaxic apparatus and fluorescence qPCR instrument were offered by Thermo Fisher Scientific (Waltham, MA, USA), Image-lab image analysis system and Image-Pro image analysis system (Bio-Rad, Hercules, CA, USA).

Establishment of Rat Model of Senile Dementia

After successful anesthesia via intraperitoneal injection of 7% chloral hydrate (5 mL/kg) into rats, the hair on the head was removed to expose the skin. After sterilization, rats were fixed on the brain stereotaxic apparatus, and the anterior fontanel was exposed. A bone window was made using a bone drill at 3 mm at the rear of anterior fontanel and 2 mm next to the midline. 4 nmol/L β -AP solution was slowly injected into the hippocampus of the rat using a microsyringe⁶, after the

needle was retained for 5 min. The wound was sutured and dressed using the aseptic dressing. Rats were fed in separate cages.

Treatment in Each Group

In Sham group, the bone window was made only in the hippocampal projection zone without the injection of β -AP. In Model group, the model of an Alzheimer's disease was established using the above methods. After successful modeling, the wound was routinely dressed, and 0.9% sodium chloride solution was intraperitoneally injected once per day after the operation. In MT group, this model of Alzheimer's disease was established using the above methods. After successful modeling, the wound was routinely dressed, and MT (0.15 mg/kg) was intraperitoneally injected once per day after the operation. The material was drawn from each group after intervention for 40 d.

Material Drawing

After successful anesthesia, 6 rats in each group were fixed with paraformaldehyde. The hippocampal brain hippocampal tissues were taken and fixed in 4% paraformaldehyde at 4°C for 48 h. Then, the paraffin-embedded tissue section was prepared for immunohistochemical detection. The hippocampal brain tissues were directly taken from the remaining 6 rats in each group, and placed in an Eppendorf (EP) tube for Western blotting and qPCR.

Immunohistochemistry

After the 5- μ m-thick paraffin-embedded tissue sections were routinely dewaxed and put into water, the citric acid buffer was added, and the mixture was heated in a microwave oven for antigen retrieval. After the sections were rinsed with phosphate-buffered saline (PBS), the endogenous peroxidase blocker was added for 10 min incubation. After the sections were rinsed with PBS again, they were sealed in goat serum for 20 min. Then the serum sealing solution was removed, anti-A β primary antibody (1:200) and anti-S100 β (1:200) were added, and the sections were placed at 4 °C overnight. After the sections were rinsed with PBS, the secondary antibody (1:1000) was added for 10 min incubation. After being rinsed with PBS, the sections were incubated using the streptavidin-peroxidase solution for 10 min, followed by color development via diaminobenzidine (DAB), hematoxylin counterstaining, sealing with neutral gum, observation and photography under a microscope.

Western Blotting

The skin tissues stored at -20°C was added with the lysis solution for an ice bath for 60 min, and centrifuged at 14000 g for 10 min. The protein was quantified using the bicinchoninic acid (BCA) method. The standard curve and absorbance were obtained using a microplate reader, based on which the protein concentration was calculated. After protein denaturation, the sample was separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a corresponding concentration. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane for sealing and washed 3 times with PBS. After sealing using the sealing solution for 1.5 h, anti-A β primary antibody (1:2000) and anti-S100 β primary antibody (1:2000) were added successively, and the membrane was rinsed with Tris-Buffered Saline with Tween-20 (TBST) between every two steps. The secondary antibody was removed via TBST, followed by image development. The membrane was placed in the chemiluminescent reagent for reaction for 1 min, followed by image development in a dark place and analysis using a gel scanning imaging system (Bio-Rad, Hercules, CA, USA).

QPCR

The total ribonucleic acid (RNA) was extracted from the rat hippocampal tissues stored at -20°C using the RNA extraction kit, and reversely transcribed into complementary deoxyribonucleic acid (cDNA) using the reverse transcription kit. The reaction system was 20 μL , and reaction conditions are as follows: reaction at 51°C for 2 min, predenaturation at 96°C for 10 min, denaturation at 96°C for 10 s, annealing at 60°C for 30 s, a total of 40 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an inter-

nal reference, and the relative expression level of messenger RNA (mRNA) was calculated. Primer sequences are shown in Table I.

Statistical Analysis

In this study, Statistical Product and Service Solutions (SPSS) 20.0 software was used for statistical analysis. Enumeration data were presented as mean $\pm$ standard deviation. The *t*-test was used for data in line with normal distribution and homogeneity of variance, the corrected *t*-test was used for data in line with normal distribution and heterogeneity of variance, and the non-parametric test was used for data not in line with normal distribution and homogeneity of variance. Continuous data among multiple groups were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. Rank sum test was adopted for ranked data, and chi-square test was adopted for enumeration data. $p < 0.05$ were considered statistically significant.

Results

Observation of Hippocampal Morphology Via Hematoxylin-Eosin (HE) Staining

In Sham group, a large amount of hippocampal neurons were in the ovoid shape and arranged evenly, tightly and orderly with clearly stained nuclei and nucleoli. In Model group, the number of hippocampal neurons was reduced. Cells were arranged unevenly, disorderly and loosely. The shape became irregular, and nuclei and nucleoli were stained unclearly. Karyopyknosis occurred in some cells. In MT group, however, the number of hippocampal neurons was relatively increased. Cell appeared to be arranged evenly and orderly, the morphology of which was significantly improved compared with that in Model group (Figure 1).

Table I. Primer sequences.

Name	Primer sequence
β -AP	Forward primer: 5'-ACAGAGACGTTGTGCTGCTTT-3' Reverse primer: 5'-CAGCCTATCTCCCAGGTATCC-3'
S100 β	Forward primer: 5'-CGGAATTCTATGGAAGGGTCTAAGACGTCC-3' Reverse primer: CCTCATTGTGTATAAAATTGTGAGGACA-3'
GAPDH	Forward primer: 5'-ACGGCAAGTTCAACGGCACAG-3' Reverse primer: 5'-GAAGACGCCAGTAGACTCCACGAC-3'

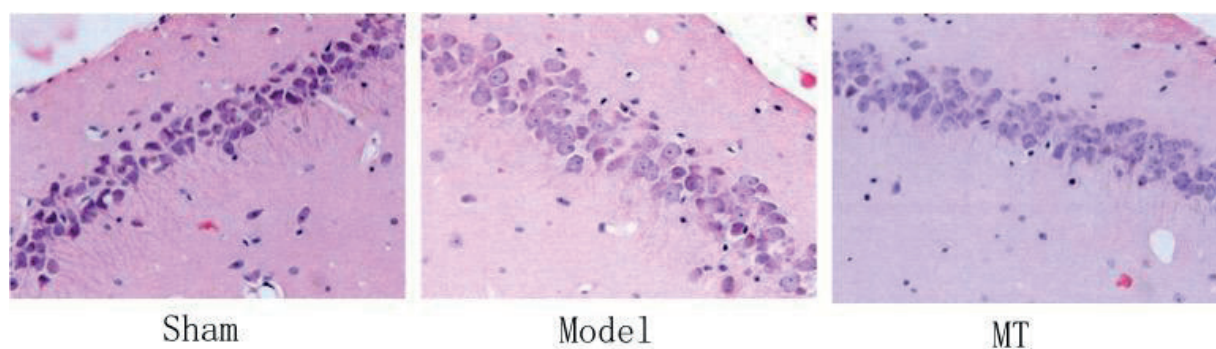


Figure 1. Observation of hippocampal tissue morphology in each group via HE staining ($\times 200$).

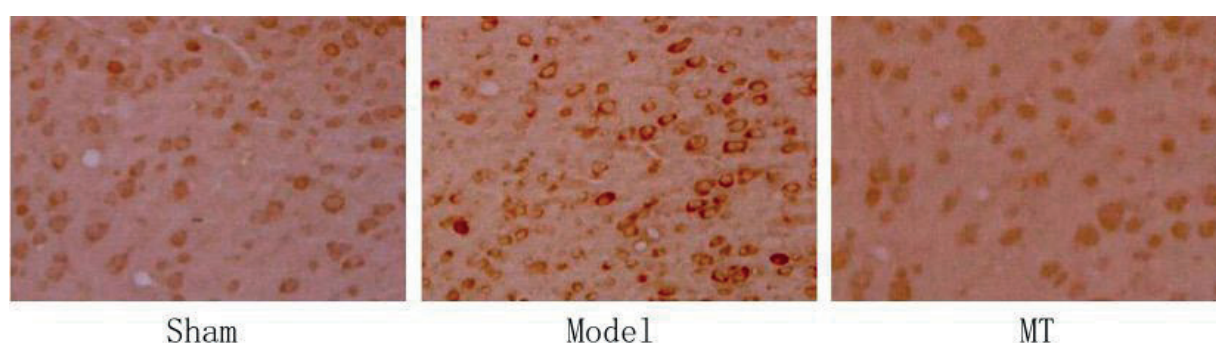


Figure 2. Detection of β -AP and S100 β protein expressions via immunohistochemistry ($\times 200$).

Detection of β -AP and S100 β Expressions Via Immunohistochemistry

The positive expressions of β -AP and S100 β protein showed the dark brown color. The positive levels of β -AP and S100 β protein were higher in Model group and MT group, compared to that in Sham group (Figure 2). The result of mean optical density showed that compared with those in Sham group, positive expressions of β -AP and S100 β protein were significantly increased in Model group and MT group ($p < 0.05$). Compared with those in Model group, positive expressions of β -AP and S100 β protein in MT group were significantly reduced, indicating the effect in Model group was alleviated by MT treatment ($p < 0.05$) (Figure 3).

Detection of β -AP and S100 β Protein Expressions Via Western Blotting

The β -AP and S100 β protein expression levels were up-regulated in Model group and MT group (Figure 4). Moreover, we also found that, compared with those in Model group, the relative expressions of β -AP and S100 β proteins in MT group were statistically decreased ($p < 0.05$) (Figure 5).

Detection of mRNA Expression Levels Via qPCR

We also detected β -AP and S100 β expressions at mRNA level. In a similar context, compared with those in Sham group, the levels of β -AP and

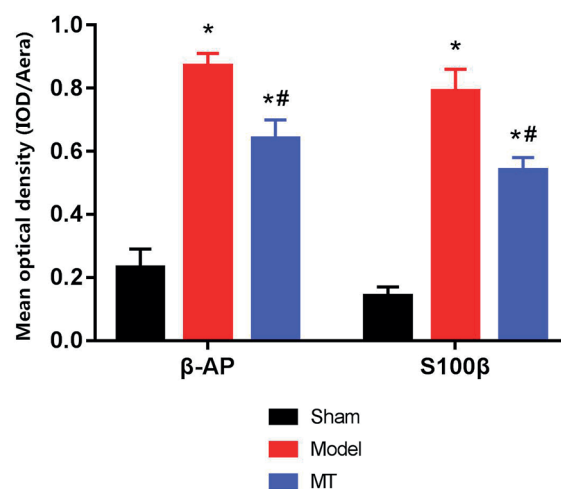


Figure 3. Mean optical density of β -AP and S100 β protein expressions. Note: * $p < 0.05$ vs. Sham group, # $p < 0.05$ vs. Model group.



Figure 4. Detection of relevant protein expressions via Western blotting.

S100 β in Model group were significantly increased ($p<0.05$). However, the levels were significantly diminished by the treatment of MT, although they were still higher than those in Sham group ($p<0.05$) (Figure 6).

Discussion

Senile dementia and Alzheimer's disease represent the manifestation of the decline in neurological brain function of the elderly patients. It is currently believed that the main pathological symptoms of Alzheimer's disease include (1) neuritic plaque, (2) neurofibrillary tangles, (3) β -AP deposition and (4) cholinergic neural necrosis and denaturation⁷. β -AP and S100 β proteins play important roles in the pathogenic process of Alzheimer's disease. It has been

revealed that β -AP deposition is positively correlated with the severity of Alzheimer's disease⁸. The accumulation of β -AP resulted in the decline in cognitive function. It has been proved that the long-term increase of soluble β -AP can cause and aggravate the deterioration of brain neurological function⁹. However, β -AP in a low concentration exerts excellent nutritional, supportive and protective effects on the development and function of brain neurons¹⁰. Excessive deposition of β -AP in the brain can activate oxidative stress, calcium ion, endoplasmic reticulum stress and Tau protein phosphorylation, and block synaptic plasticity¹¹. Additionally, abnormal high level of β -AP protein in the brain can increase the expression of G protein-coupled receptor 2, accelerate the formation of β -AP plaques and aggravate the inflammation, exhibiting a strong neurotoxic effect, which leads

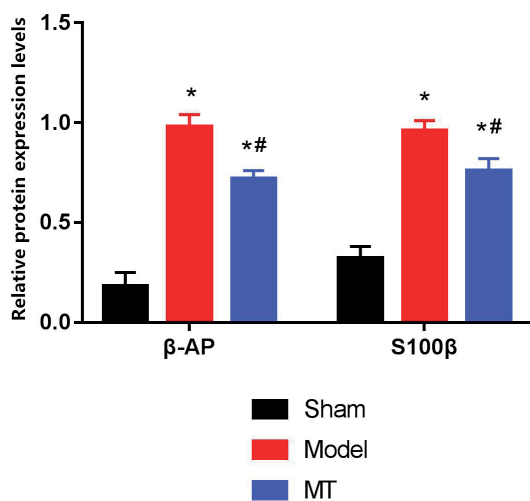


Figure 5. Relative protein expression levels. Note: * $p<0.05$ vs. Sham group, # $p<0.05$ vs. Model group.

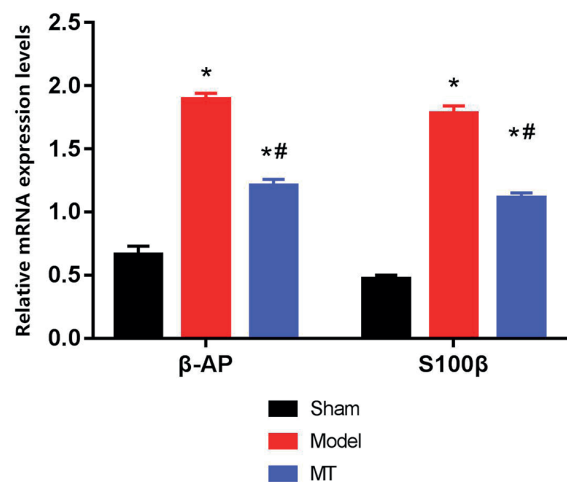


Figure 6. Relative mRNA expression levels. Note: * $p<0.05$ vs. Sham group, # $p<0.05$ vs. Model group.

to neuronal necrosis, apoptosis and synaptic dysfunction^{12,13}. Therefore, β -AP has become one of the important targets in the treatment of Alzheimer's disease. S100 β also plays an important role in the development and function of the nervous system under physiological conditions¹⁴. Clinical evidence demonstrated that the content of S100 β in the elderly patients with Alzheimer's disease was markedly higher than that in the normal elderly¹⁵. The beneficial or adverse effect of S100 β on cells depends on the content of S100 β in the body. S100 β in a low concentration in the body displays the nutritional and supportive effects on the nervous system, whereas S100 β at aberrant high level is harmful to the nervous system, and results in neuronal necrosis, apoptosis, and denaturation through regulating the calcium ion concentration in neurons. It is currently thought that a high concentration of S100 β is associated with degenerative disease of the nervous system¹⁶. MT, as a hormone secreted by the pineal gland, is considered to have a neuroprotective effect¹⁷. According to studies, MT effectively eliminates oxygen free radicals and regulates enzymes correlated with oxidation and anti-oxidation to realize the anti-oxidative effect indirectly¹⁷⁻²⁰. Consistently, our study indicated that expressions of β -AP and S100 β proteins were elevated in the hippocampus of these rats which may be associated with the changes observed in hippocampal tissue morphology of rats with Alzheimer's disease. Recent finding unraveled that Tiapride is more effective for the treatment of senile dementia²¹. Notably, in our study, MT remarkably improved the hippocampal tissue morphology and reduced the expressions of β -AP and S100 β , although the exact mechanism, related to possible signaling pathways, requires further investigation.

Conclusions

We demonstrated that MT alleviates the pathological changes in hippocampal tissue morphology of this model of rats, with down-regulation of β -AP and S100 β , which provides peer insights for the future development of new drugs.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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