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Protective effects of melatonin on mitochondrial biogenesis and mitochondrial structure and function in the HEK293-APPswe cell model of Alzheimer's disease

C.-F. WANG[1], C.-Y. SONG[1], X. WANG[1], L.-Y. HUANG[4], M. DING[1], H. YANG[1], P. WANG[1], L.-L. XU[1], Z.-H. XIE[1], J.-Z. BI[1,2,3]

[1]Department of Neural Medicine, Second Hospital of Shandong University, Jinan, China
[2]Institute of Neurology, Shandong University, Jinan, China
[3]Key Laboratory of Translational Medicine on Neurological Degenerative Disease, Shandong University, Jinan, China
[4]Department of Neural Medicine, Qilu Hospital of Shandong University, Jinan, China

Abstract. – OBJECTIVE: The effects and mechanisms of melatonin on Alzheimer's disease (AD) are still not researched thoroughly. 20E2 cells (HEK293-APPswe cells) are a cellular model of AD. The modulation effects of melatonin on the structure and function of mitochondria in 20E2 cells need to be studied.

MATERIALS AND METHODS: The Alzheimer's disease (AD) cell model was assessed for cell viability, expression levels of mitochondrial biogenesis factors (peroxisome proliferator-activated receptor gamma coactivator 1-alpha [PGC-1α], nuclear respiratory factor 1 [NRF1], nuclear respiratory factor 2 [NRF2], mitochondrial transcription factor A [TFAM]), mitochondrial membrane potential, Na^+-K^+-adenosine triphosphatase (ATPase) and cytochrome C oxidase activity, adenosine triphosphate (ATP) level, mitochondrial DNA/nuclear DNA (mtDNA/nDNA) ratio, and mitochondrial structure with and without melatonin.

RESULTS: Melatonin improved 20E2 cell viability, expression of mitochondrial biogenesis factors (PGC-1α, NRF1, NRF2, TFAM), mitochondrial membrane potential, Na^+-K^+-ATPase, and cytochrome C oxidase activity, ATP level, mtDNA/nDNA ratio, mitochondrial structure, and decreased amyloidogenic amyloid precursor protein processing.

CONCLUSIONS: Mitochondrial biogenesis disorder is associated with the pathogenesis of AD through PGC-1α-NRF-TFAM pathway, and melatonin improves the mitochondrial structure and function by enhancing mitochondrial biogenesis and decreasing amyloidogenic APP processing in Alzheimer's disease.

Key Words
Alzheimer's disease, Mitochondrial biogenesis, Mitochondrial structure and function, Melatonin, HEK293-APPswe cells.

Abbreviations

AD, Alzheimer's disease; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; TFAM, mitochondrial transcription factor A; ATP, adenosine triphosphate; mtDNA/nDNA, mitochondrial DNA/nuclear DNA; MT, melatonin; Aβ, β-amyloid protein; APP, amyloid precursor protein; HEK293 cells, Human embryonic kidney 293 cells; 20E2 cells, HEK293-APPswe cells; CTF-β, C-terminal fragment β.

Introduction

Alzheimer's disease (AD), characterized by the accumulation of extracellular senile plaques and intracellular neurofibrillary tangles in several brain regions[1], is the most common neurodegenerative disease[2]. β-myeloid protein (Aβ) is the main component of senile plaque in AD patients. It is produced from an amyloid precursor protein (APP) hydrolyzed by β-secretase and γ-secretase. Therefore, 20E2 cells (HEK293-APPswe cells, stably expressing APP) can be a cell model of AD[3,4]. The brain has especially high energy requirements, consuming about 20% of the body's total basal oxygen[5]. This energy is mainly in the form of adenosine triphosphate (ATP) molecules produced by mitochondria.

Mitochondria play a central role in energy production, which is necessary for virtually all cellular activities. Their role is even more important in neurons that need a large amount of ATP for the synthesis and secretion of neurotransmitters, to

Corresponding Author: Zhaohong Xie, MD; e-mail: xie_zhaohong@126.com
Jianzhong Bi, MD; e-mail: bjz@sdu.edu.cn

enhance synaptic plasticity and to maintain neuronal membrane potential[6]. Therefore, impaired mitochondrial function leads to a pathological state, ranging from subtle alterations in neuronal function to cell death and neurodegeneration. Mitochondrial dysfunction is one of the earliest and most prominent features of AD, and some researches support the involvement of mitochondrial-dependent mechanisms in the pathogenesis of AD[7,8]. Mitochondrial biogenesis can be defined as the growth and division of pre-existing mitochondria. Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in the AD brain and AD cell model[9].

The hormone melatonin, also known as N-acetyl-5-methoxy tryptamine, is mainly produced by the pineal gland[10]. The primary function is the regulation of day-night cycles. Melatonin is also an antioxidant that can easily cross cell membranes and the blood-brain barrier[11]. In addition, melatonin can pass through several biological membranes and accumulate in subcellular organelles, particularly mitochondria[12]. Melatonin has beneficial roles in many neurodegenerative disorders, including AD[13-16]. Mitochondria are able to incorporate and accumulate a significant amount of melatonin[17]; indeed melatonin is synthesized and accumulated at high levels in mitochondria[18].

In this study, we used 20E2 (HEK293-APPswe) cells as a cell model of AD. We observed the effects of melatonin on mitochondrial biogenesis, the structure and function of mitochondria, and its possible mechanism.

Materials and Methods

Cell Culture and Treatment

HEK293 (Human embryonic kidney 293) cells and 20E2 (HEK293-APPswe) cells donated by Professor Xiu-Lian Sun (Qilu Hospital of Shandong University, Jinan, China) were cultured in DMEM (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum in 5% CO_2 at 37°C and passaged every 2-3 days. After washing with phosphate buffered saline (PBS, Gibco, Grand Island, NY, USA) twice and treating with 0.25% trypsin (HyClone, South Logan, UT, USA), cells were observed under a microscope.

Melatonin was initially dissolved in double-distilled water to 1 μM. The solution was divided into aliquots and stored at -20°C. Before the use, the solution was diluted in medium to the indicated concentration.

Cell Viability

Cell viability was tested by using a cell counting kit (CCK-8; MedChem Express, Monmouth Junction, NJ, USA). In 96-well plates, 100 μL cell suspension was added to each well for culture in 5% CO_2 at 37°C. When cells reached approximately 50% to 70% confluence, they were treated with concentrations of melatonin (Sigma-Aldrich, St. Louis, MO, USA). After 24 h, 10 μl CCK-8 was added to each well. The absorbance was measured at 570 nm.

ELISA

In the supernatant of HEK293 cells alone and 20E2 cell cultures of the same magnitude, $A\beta_{1-40}$ was detected by using an ELISA kit (Invitrogen, Carlsbad, CA, USA). The optical density of samples was detected by using a microplate reader, and the concentration of $A\beta_{1-40}$ was calculated according to the standard curve.

Western Blot Analysis

After 20E2 cells were treated with melatonin for 24 h, the culture medium was removed, and cells were washed 3 times by PBS. The protein samples were homogenized in ice-cold RIPA lysing buffer (Beyotime, Shanghai, China). Homogenized samples were centrifuged at 12,000 g for 15 min at 4°C. Protein levels in the supernatant were tested by the bicinchoninic acid method. Proteins were separated on SDS-PAGE (140 V) and transferred to polyvinylidene difluoride (PVDF) membranes (180 mA, constant-current, 1.5 h), which were blocked with 5% nonfat dry milk in TBST for 1 h and incubated with the following primary antibodies overnight at 4°C: amyloid precursor protein (APP; 1:1000); nuclear respiratory factor 1 (NRF1; 1:3000); NRF2 (1:3000); mitochondrial transcription factor A (TFAM, 1:5000); peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; all rabbit IgG, Abcam, Cambridge, MA, USA); and β-actin (mouse IgG, 1:1000, Golden Bridge International, Beijing, China). Membranes were blocked with secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (both 1:5000, Golden Bridge International, Beijing, China). The intensity of the bands was quantified by using Image J (US National Institutes of Health, Bethesda, MD, USA).

Mitochondrial Membrane Potential (MMP)

JC-1 dye is a sensitive marker of inner MMP[18]. After treatment with melatonin for 24 h, 20E2 cells were incubated with JC-1 for 15 min in 24-

well plates at 37°C, and red fluorescence (excitation 543 nm, emission 600 nm) and green fluorescence (excitation 488 nm, emission 535 nm) were detected.

Na^+,K^+-Adenosine Triphosphatase (ATPase) Activity

Cells were homogenized by ultrasonography on the ice after trypsinization. Na^+,K^+-ATPase activity was assayed by measuring the amount of inorganic phosphate (Pi) released from ATP and detected according to instructions of the detection kit. The definition of 1 unit was 1 μmol released from ATP by 1 mg protein in 1 h. The wavelength of absorbance was 640 nm. Enzyme activity was expressed as units per mg protein (U/mgprot).

Cytochrome C Oxidase and ATP Activity

20E2 cells were treated with melatonin for 24 h. ATP levels were measured by using the ATP Determination Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Cytochrome C oxidase activity was determined by using the cytochrome C oxidase activity kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as instructed.

Real-Time PCR to Detect Mitochondrial DNA (mtDNA) Copy Number

20E2 cells were treated with melatonin for 24 h. MtDNA copy number was measured by real-time PCR with the Step one plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and the SYBR Green detection method. Total intracellular DNA was extracted from cells by using the QIAamp DNA mini kit (QIAGEN, Germantown, MD, USA) following the instructions. Each real-time PCR reaction (20 μl total volume) contained 3 μl template DNA (50 μg), 10 μl of 2 × SYBR Green Real-time PCR Master, 1 μl each forward and reverse primers and 5 μl ultrapure water. The primers for a subunit of human electron transport chain used for mtDNA amplification were forward, 5'-CAAACCTACGCCAAAA TCCA-3' and reverse, 5'-GAAATGAATGAGCCTACAGA-3'[20]. The mtDNA primers (Human Complex II, fragment length 164 bp) were designed to minimize amplification of the mtDNA pseudogenes embedded in the nuclear DNA (nDNA). Primers for human nuclear 18S, as an internal control, were forward, 5'-ACGGACCAGAGCGAAAGCA-3' and reverse, 5'-GACATCTAAGGGCATCA CAGAC-3'. Relative mtDNA and nDNA copy numbers were compared. The ddCt (mtDNA to 18S) represents the mtDNA copy number in a cell[21].

Transmission Electron Microscopy

20E2 cells were treated with melatonin for 24 h, then quickly fixed in 4% glutaraldehyde. After a wash with PBS, cells were fixed with osmic acid (OsO4) and embedded in EPON812 resin (Sigma-Aldrich, St. Louis, MO, USA), then cut successively in 0.06 mm chips. Uranyl and lead citrate were used for staining. Then, transmission electron microscopy was used to observe mitochondrial structure.

Statistical Analysis

Data are presented as mean ± standard deviation (x ± SEM) and were analyzed using SPSS17.0 (SPSS Inc., SPSS Statistics for Windows, Chicago, IL, USA). The data were analyzed by analysis of variance (ANOVA). Two variables used two-factor variance analysis. The comparison among groups was analyzed by single factor variance analysis. The comparison of mean used the Least Significant Difference (LSD) post analysis. $p<0.05$ was considered statistically significant.

Results

The Cell Model of AD

The expression of $A\beta_{1\text{-}40}$ and APP was higher in 20E2 than HEK293 cells (Figure 1). This result showed that 20E2 cells were an appropriate cell model of AD.

Effect of Melatonin on Cell Viability

The effects of melatonin on the viability of 20E2 cells were detected by CCK-8 assay. Cell viability was significantly increased after incubation with 0.1, 1 and 10 μM melatonin for 24 h, whereas small doses (0.01 μM) did not increase cell viability significantly (Figure 2).

Effect of Melatonin on Protein Expression Levels of TFAM, NRF1, NRF2, and PGC-1α

The levels of TFAM, NRF1, NRF2, and PGC-1α were lower in 20E2 than HEK293 cells (Figure 3A), but melatonin treatment for 24 h significantly increased the protein levels which were indicated as dose-dependent (Figure 3B). These data suggested that melatonin promoted mitochondrial biogenesis.

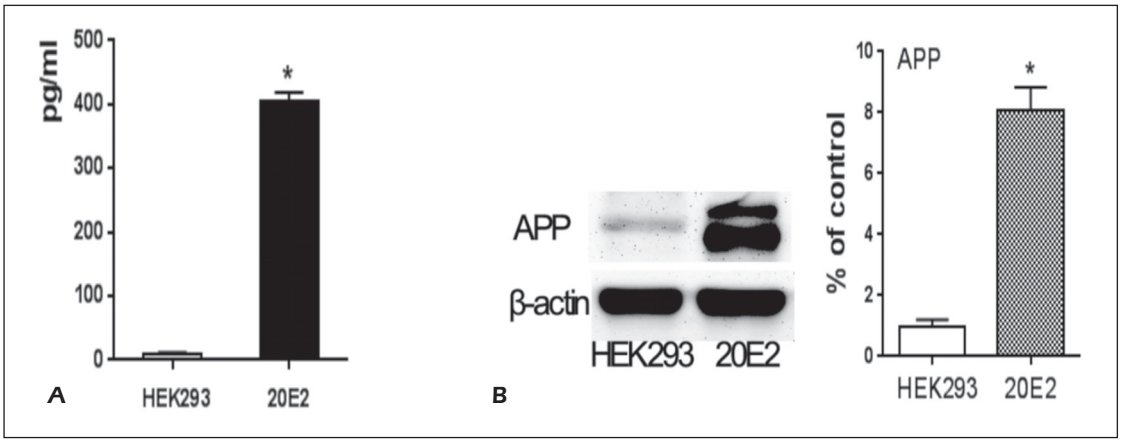

Figure 1. 20E2 cells are an appropriate cell model of Alzheimer's disease. ELISA was used to detect the expression of Aβ1-40 (**A**) and Western blot analysis was used to detect the expression of APP in HEK293 and 20E2 cells (**B**). Data are mean ± SE. **p*<0.05 *vs.* HEK293 cells alone. MT = melatonin.

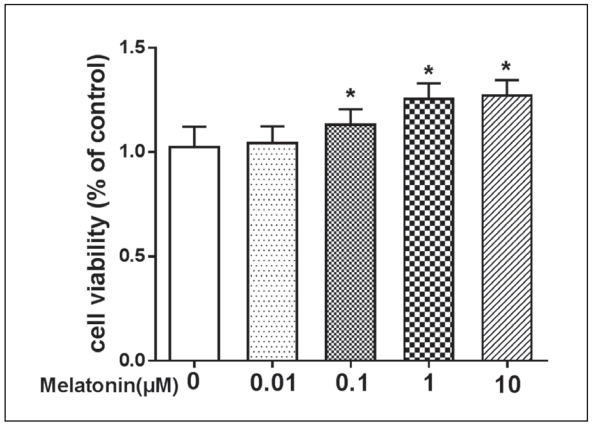

Figure 2. Melatonin increased 20E2 cell viability. 20E2 cells were treated with 0, 0.01, 0.1, 1, 10 μM melatonin for 24 h and cell viability was detected. Data are mean ± SE. **p*<0.05 vs. 0 μM melatonin dose alone. MT = melatonin.

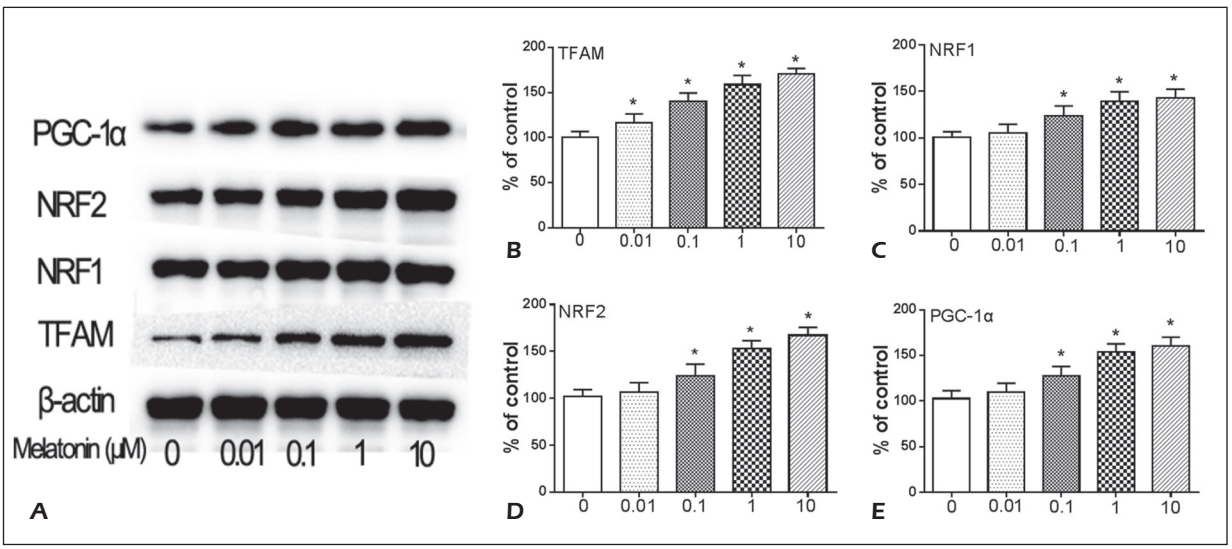

Figure 3. Melatonin improved the expression of mitochondrial biogenesis regulatory factors. Western blot analysis of the protein levels of TFAM, NRF1, NRF2 and PGC-1α in 20E2 cells treated with different doses of melatonin (**A**). Expression was normalized to that of β-actin. Quantified Western blot analysis of protein levels of TFAM (**B**), NRF1 (**C**), NRF2 (**D**) and PGC-1α (**E**). The scanned image of Western blot was analyzed with the software Image J. Data are mean ± SE. **p*<0.05 *vs.* 0 μM melatonin dose alone.

Effect of Melatonin on Mitochondrial Membrane Potential (MMP)

The MMP of cells was reflected by JC-1 staining. With high MMP, JC-1 aggregates in the matrix of mitochondria produced red fluorescence, and with low MMP, JC-1 aggregates produced green fluorescence. After melatonin treatment of 20E2 cells for 24 h, green fluorescence was weakened, and red fluorescence was significantly increased as compared with control treatment (Figure 4).

Effect of Melatonin on Mitochondrial Function

Na^+,K^+-ATPase, and cytochrome C oxidase activity, ATP level, and mtDNA/nDNA ratio were significantly lower in 20E2 than HEK293 cells, and 24 h melatonin treatment increased Na^+-K^+-ATPase, and cytochrome C oxidase activity, ATP level, and mtDNA/nDNA ratio in 20E2 cells (Figure 5).

Effect of Melatonin on Mitochondrial Structure

In 20E2 cells, mitochondrion structure was damaged (mitochondrial swelling, disappeared cristae, vacuoles) (Figure 6). After 1 μM melatonin treatment for 24 h, the damaged mitochondrial structure was improved.

Effect of Melatonin on Amyloidogenic APP Processing

Aβ is derived from an amyloid precursor protein (APP) by β- and γ-secretase cleavage. BACE1 is the predominant endogenous β-secretase and produces the C-terminal fragment β (CTF-β). BACE1 protein level in melatonin-treated 20E2 cells was significantly reduced compared with the control group (Figure 7). To determine the effect of reduced BACE1 level on APP processing, we analyzed the protein levels of APP and CTF-β in 20E2 cells. APP level was not reduced by melatonin. However, CTF-β level was markedly decreased in melatonin-treated 20E2 cells as compared with the control.

Discussion

In this study, we used 20E2 cells as a cellular model of AD and investigated whether the melatonin treatment could improve the mitochondrial biogenesis and enhance the structure and function of mitochondrial in this model. The results showed that the treatment with melatonin increased 20E2 cell viability and the level of mitochondrial membrane potential (MMP) and enhanced the expression of mitochondrial biogenesis factors (peroxisome proliferator-activated recep-

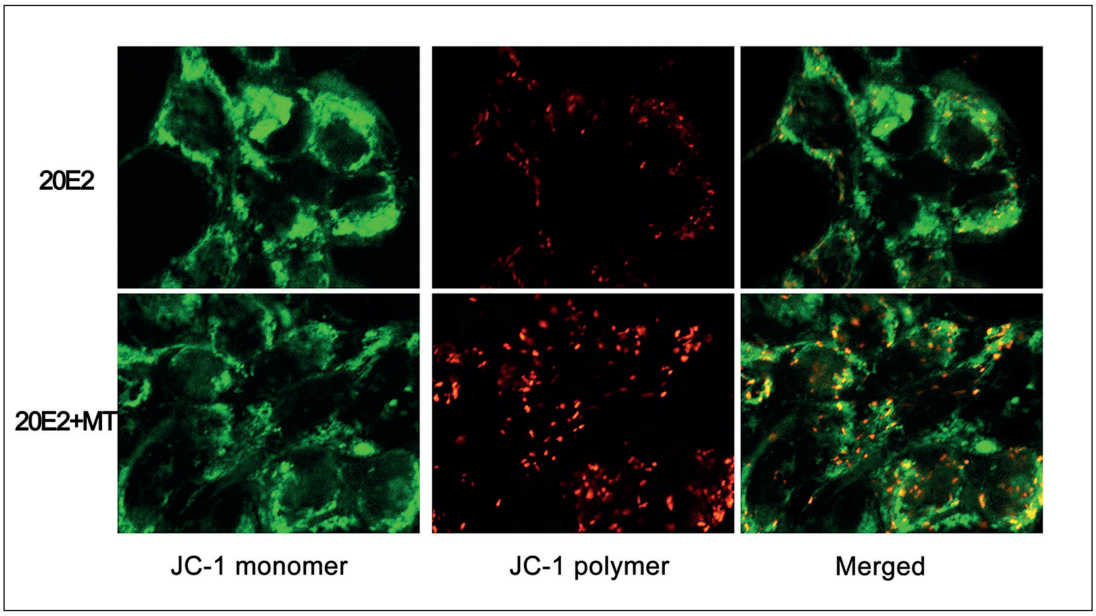

Figure 4. Melatonin enhanced the mitochondrial membrane potential (MMP). 20E2 cells were treated with 1 μM melatonin (MT) for 24 h. Fluorescence confocal microscopy was used to observe MMP detected by JC-1 staining. MT = melatonin. (Magnification 10,000×).

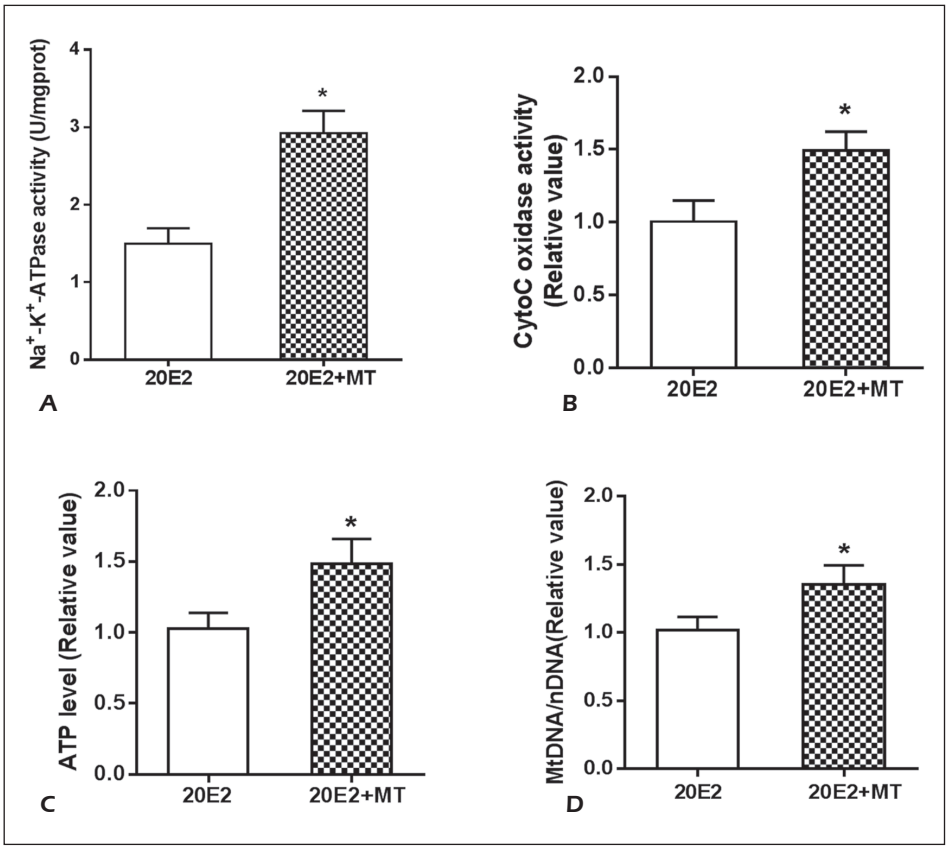

Figure 5. Melatonin improved mitochondrial function. 20E2 cells were treated with 1 μM melatonin for 24 h. Na +, K + -ATPase activity (**A**), cytochrome C oxidase activity (**B**), and ATP level (**C**) were measured. Real-time PCR was used to detect mtDNA/nDNA ratio (**D**). Data are mean ± SE. **p*<0.05 *vs.* 0 μM melatonin dose alone. MT = melatonin.

tor gamma coactivator 1-alpha [PGC-1α], nuclear respiratory factor 1 [NRF1], nuclear respiratory factor 2 [NRF2], mitochondrial transcription factor A [TFAM]). In addition, Na^+,K^+-ATPase and cytochrome C oxidase activity, ATP level and mtDNA/nDNA ratio were increased, mitochondria ultrastructure was improved and the amyloidogenic APP processing was decreased.

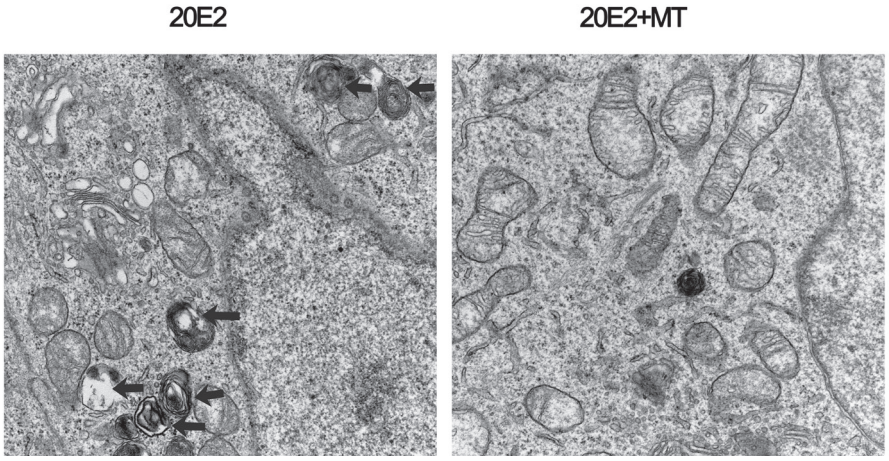

Figure 6. Melatonin improved mitochondrial structure. The morphology of mitochondria observed by transmission electron microscopy. *Arrows* show damaged mitochondrial structure. MT = melatonin. (Magnification 30,000×).

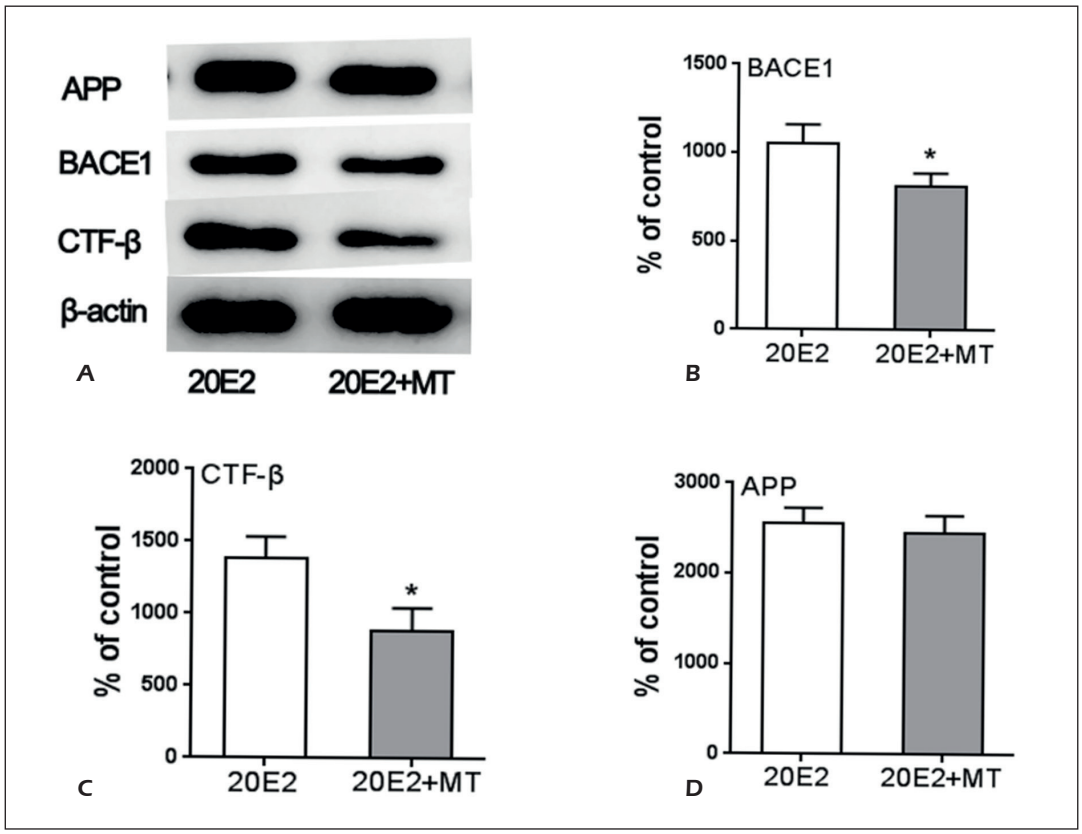

Figure 7. Melatonin significantly decreased BACE1 and CTF-β levels. Expression of BACE1, CTF-β, and APP in 20E2 cells with and without 1-μM melatonin treatment (**A**). Quantitative PCR analysis of BACE1 (**B**), CTF-β (**C**) and APP (**D**) mRNA level with normalization to β-actin level. Data are mean ± SE. *$p<0.05$ *vs.* 0 μM melatonin dose alone. MT = melatonin.

The mitochondrial disorder is involved in the pathological process of neuronal degeneration[22]. Sheng et al[9] showed that impaired mitochondrial biogenesis likely contributes to mitochondrial dysfunction in AD. Mitochondrial biogenesis is regulated by the PGC-1α-NRF-TFAM pathway. The regulatory factors of mitochondrial biogenesis include NRF1 and NRF2, which control the nuclear genes to encode mitochondrial protein and TFAM, which drives the transcription and replication of mtDNA. The expression of NRF1, NRF2, and TFAM is regulated by PGC-1α[23]. According to Sheng et al[9], the expression levels of NRF1, NRF2, TFAM, and PGC-1α was decreased in hippocampal tissues from AD patients and an AD cell model. In addition, with the decrease in these mitochondrial biogenesis factors, mtDNA/nDNA ratio, ATP level, and cytochrome C oxidase activity were also significantly reduced in the AD cell model. Based on their data, impaired mitochondrial biogenesis likely contributes to mitochondrial dysfunction in AD, and enhancing mitochondrial biogenesis may be an approach for treating AD. In our work, melatonin could improve the expression of NRF1, NRF2, TFAM, and PGC-1α. Besides, melatonin can improve cell viability, MMP, and mitochondrial structure and function in 20E2 cells. So, melatonin may improve mitochondrial dysfunction by promoting mitochondrial biogenesis in AD.

Swerdlow et al[24] reported that mitochondria participated in the initiating process of AD, which was associated with Aβ deposition and *tau* protein. Aβ exists in the mitochondrial matrix and the inner and outer membrane. This process will damage mitochondria, including decreased mitochondria number, membrane potential, ATP production, and axoplasmic transport disorder. In addition, melatonin decreased amyloidogenic APP processing by downregulating BACE1 level. BACE1 is the predominant endogenous β-secretase and produces a 99-amino acid C-terminal fragment of APP (CTF-β) during the amyloidogenic processing of APP. We observed a significant reduction of BACE1 and CTF-β levels in 20E2 cells treated with melatonin. But the expres-

sion level of AβPP was not reduced by melatonin. In addition, we found that with the promotion of mitochondrial biogenesis, the amyloidogenic APP processing was decreased. So, we think that mitochondrial biogenesis is involved in the amyloidogenic APP processing. We speculated that melatonin can decrease Aβ deposition by down-regulating BACE1 levels through promoting mitochondrial biogenesis in 20E2 cells.

Conclusions

We found that melatonin may have a protective effect on mitochondrial structure and function, which may be related to its promotion of mitochondrial biogenesis and the involvement of the PGC-1α-NRF-TFAM pathway.

Acknowledgements

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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