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Anti-apoptotic effects of melatonin in retinal pigment epithelial cells

Yang Fu[1], Min Tang[1], Ying Fan[1], Haidong Zou[1], Xiaodong Sun[1], Xun Xu[1]

[1]Department of Ophthalmology, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, China, 200080

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1. ABSTRACT

Recent studies have revealed that melatonin exerts strong anti-apoptotic effects. Retina secretes melatonin, and melatonin receptors are distributed in almost all the layers of retina, including the layer of retinal pigment epithelial (RPE) cells. However, it is not known whether melatonin inhibits apoptosis through its anti-oxidant effects and how it works in RPE cells. Here, we show that melatonin decreases H_2O_2-induced apoptosis in RPE cells partially through protection of mitochondria. Melatonin decreased reactive oxygen species in mitochondria and mitochondrial DNA damage, alleviated structural damage and inhibited cytochrome C release.

2. INTRODUCTION

Apoptosis can cause impaired visual function, which is a pathological change that is commonly seen in clinical practice. Because of the specific anatomic and physiological characteristics of retinal pigment epithelial (RPE) cells, apoptosis can be induced in RPE cells by various factors (1-3). Apoptosis of RPE cells occurs in eye diseases including light damage (4) and age-related macular degeneration (5-7). Although the underlying apoptosis mechanism is not yet clear, change in mitochondrial function might play a key role in this process. For instance, various apoptosis signals can be transduced to mitochondria, and subsequently promote or

inhibit apoptosis (8); this phenomenon appears more obvious since mitochondria are abundant in the RPE cytoplasm.

Recent studies have revealed that melatonin, an anti-oxidant, can protect mitochondria and exert strong anti-apoptotic effect (9-13). Retinas can secrete melatonin, and melatonin receptors are distributed in almost all the layers of retina, including the layer of RPE cells (14-16). But it remains unknown whether it inhibits apoptosis via its anti-oxidant effects in mitochondria. The purpose of this study was to investigate the anti-apoptotic effects of melatonin in RPE cells *in vitro*, by focusing on the processes occurring in the mitochondria.

Evidence from our present study demonstrated that melatonin decreased H_2O_2-induced apoptosis in RPE cells, partially by protecting mitochondria. In fact, melatonin decreased reactive oxygen species (ROS) as well as mitochondrial DNA (mtDNA) damage, and alleviated the structural damage and inhibited cytochrome C release though it could not inhibit the release of apoptosis inducing factor (AIF) in the mitochondria. Thus, the process of melatonin-protected mitochondria is incomplete and might be selective.

3. MATERIALS AND METHODS

3.1. Cell culture

RPE cells were purchased from ATCC (American Type Cell Culture, Manassas, VA, USA), thawed as a suspension, and intracellular keratin identification was performed. RPE cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium (1:1) containing 10% fetal bovine serum and were incubated at 37°C with 5% CO_2 until the cells reached 80-85% confluence. Melatonin (M5250, Sigma, St. Louis, MO, USA) was prepared separately in 0.5% ethanol in normal saline buffer.

3.2. TUNEL apoptosis assay

Culture medium was removed, RPE cells were washed twice with PBS, and returned to serum-free DMEM/F12 medium (1:1), containing various concentrations of H_2O_2 (0 or 500μM solutions were prepared from 30% H_2O_2 stock; Sigma, St. Louis, MO, USA) and melatonin (0 or 100μM). After incubation of the cells for 24 or 48 hours, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL Apoptosis Detection Kit; Promega, Madison, WI, USA) assay was performed and cells analyzed by fluorescence microscope (AxioVert S100, Zeiss, exposure time: 1 200ms). Six microscopic visual fields in each sample were examined for quantitative analysis of apoptotic cells.

3.3. Annexin V/PI/Flow cytometry apoptosis assay

Culture medium was removed, RPE cells were washed twice with PBS, and serum-free DMEM/F12 medium (1:1), containing H_2O_2 (0 or 500μM) and melatonin (0 or 100μM) was added. The number of apoptotic cells was determined 24 or 48 hours post-treatment, using Annexin V/Propidium iodide (Molecular Probes, Eugene, OR, USA) flow cytometry (FACScan Becton Dickinson, USA) assay.

3.4. Transmission electron microscopy (TEM) observation

Culture medium was removed, RPE cells were washed twice with PBS, and then cultured in serum-free DMEM/F12 medium (1:1) containing various concentrations of H_2O_2 (0 or 500μM) and melatonin (0 or 100μM). RPE cell ultrastructure was examined 24 or 48 hours post-treatment, using transmission electron microscopy.

3.5. Intracellular ROS measurement

When RPE cells, initially seeded onto slides within culture dishes, reached 80% confluence, culture medium was removed, cells were washed twice with PBS and then cultured in serum-free DMEM/F12 medium (1:1) containing various concentrations of H_2O_2 (0 or 500μM) and melatonin (0 or 100 μM). After incubation for 1 or 2 hours, cells were washed twice with PBS, treated for 1 hour with 2nM 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA, Fluka, St. Paul, MN, USA) and 500nM MitoTracker Red CM-H_2XRos (MTR; Molecular Probes, Eugene, OR, USA), then washed twice with PBS, and then examined by fluorescent microscope (AxioVert S100, Zeiss, Exposure time: 500ms).

3.6. Mitochondrial DNA damage measurement

3.6.1. Melatonin treatment groups

Culture medium was removed. RPE cells were washed twice with PBS, and then cultured in DMEM/F12 medium, containing 200 μM H_2O_2 and various concentrations of melatonin (0.1, 1, 10, or 100μM). Control cells were treated with DMEM/F12 medium containing 200μM H_2O_2 Only. Another control solvent cell group was treated with DMEM/F12 medium, 200 μM H_2O_2 and 0.5% ethanol in normal saline buffer. After incubation for 1 hour, culture medium was removed and RPE cells were washed twice with PBS.

3.6.2. Melatonin pretreatment groups

RPE cells of different densities were seeded onto 6-well plates. DMEM/F12 medium with 10% fetal bovine serum (2.5 ml), containing various concentrations of melatonin (0.1, 1, 10, or 100μM) was added to each well. Two cell control groups were also prepared in the same manner. The medium was changed every 24 hours with addition of melatonin; this melatonin pretreatment was stopped after 1, 3, 5, 7 or 14 days (all the cells reached about 80% confluence). After the culture medium was discarded, RPE cells were washed twice with PBS and cultured with serum-free DMEM/F12 medium, containing 200μM H2O2 for 1 hour. The culture medium was discarded and RPE cells were washed twice with PBS.

The cell mtDNA was extracted from melatonin treatment and pretreatment cell groups according to the manufacturer's instructions (Mitochondrial DNA Isolation Kit, K280-50, Biovision, USA). PCR was performed aiming at a reference gene, a deleted fragment gene, and a

Table 1. Primers used in the study

	Nucleotide sequences	Locations in mtDNA	Length
Reference fragment	5′-CTCATATGAAGTCACCCTAG-3′ 5′-GGCAGGAGTAATCAGAGGTG-3′	3 726–3 745 bp 3 836–3 817 bp	111 bp
Deleted fragment	5′-CCGGGGGTATACTACGGTCA-3′ 5′-GGGGAAGCGAGGTTGACCTG-3′	8 150–8 169 bp 13 650–13 631 bp	524 bp
Reserved fragment	5′-AACAGCTATCCATTGGTCTT-3′ 5′-GTGGGCTATTTAGGCTTTAT-3′	12 279–12 298 bp 16 540–16 521 bp	4 262 bp

reserved fragment gene using the primers shown in Table 1. For deleted fragments, mtDNA sequence between the pair of primers, mtDNA locations 8,150~8,169bp and 13,650~13,631bp, was evaluated. Residual fragment of 524 bp could be PCR-extended if a 4,977-bp deletion existed. For reserved fragments, a sequence of 4,262 bp between mtDNA locations 12,279~12,98bp and 16,540~16,521bp was the target sequence. A 111-bp reference gene was used as a control.

3.7. Detection of cytochrome C release

RPE cells were cultured in serum-free DMEM/F12 medium (1:1) with various concentrations of H_2O_2 (0 or 500μM) and melatonin (0or 100 μM) for 1 or 2 hours. Mitochondrial and cytosolic protein lysates were extracted according to the manufacturer's instructions (Mitochondria/Cytosol Fractionation Kit, Biovision, USA). Western blot analysis was performed to evaluate cytochrome C levels using mouse monoclonal anti-cytochrome C antibody (ab13575, Abcam, Cambridge, MA, USA).

3.8. Detection of apoptosis inducing factor release

RPE cells were cultured in serum-free DMEM/F12 medium (1:1) with various concentrations of H_2O_2 (0 or 500μM) and melatonin (0or 100μM) for 3 or 6 hours. Mitochondrial and cytosolic protein lysates were isolated as described above. Western blot analysis was performed to evaluate AIF levels using monoclonal anti-AIF antibody (Cell Signaling Technology, USA).

3.9. Statistical analysis

Statistical analysis system 6.12 software (SAS Institute Inc., Cary, NC, USA) was used for data analysis. When $P<0.05$ diferences were considered significant.

4. RESULTS

4.1. Melatonin inhibited apoptosis

In TUNEL assay, red PI fluorescence identifies dead cells and green fluorescence identifies TUNEL-positive apoptotic cells. Using TUNEL assay, our results showed that most of the dead cells exhibited both red and green fluorescence, meaning that cell death was mainly due to apoptosis. More interestingly, melatonin treatment (100 μM) decreased the percentage of positive staining cells in each group (Figure 1).

Using Annexin V/PI/Flow cytometry apoptosis assay, the percentage of apoptotic cells increased when the duration and the concentration of H2O2 increased. However, the percentage of apoptotic cells decreased when melatonin (100 μM) was added to culture medium (Figure 2).

4.2. Melatonin alleviated the structural damage of RPE cells

In vitro cultured human RPE cells,shuttle-like shaped, were attached to the plate, and grew in a single layer. Intracellular keratin identification showed positive results. Compared to cells treated with H2O2 only, the percentage of dark cells (early apoptosis) and dead cells significantly decreased when RPE cells were cultured with both H2O2 and melatonin (100 μM). TEM analysis showed lower mitochondrial density, few vacuoles, few lipid droplets, and normal mitochondrial structure in melatonin-treated cells (Figure 3).

4.4. Melatonin reduced intracellular ROS

Compared to control cells, melatonin treatment (100μM) significantly decreased intracellular ROS levels induced by H2O2 (50 0μM) in RPE cells (Figure 4).

4.5. Melatonin reduced mtDNA damage

The amount of mtDNA deleted fragments and reserved fragments were not affected by any dose of concurrent melatonin treatment, when melatonin was added within 5days prior to H2O2 treatment. However, when cells were pretreated with high concentrations of melatonin (10μM or 100μM) for 7 days prior to H2O2 treatment, mtDNA deletion decreased while the reserved fragments were increased; the effects became more obvious when the pretreatment duration was extended to 14 days. The incidence of deleted mtDNA fragments decreased and reserved fragments increased, with a dose-dependent relationship to melatonin concentration (Figure 5). Thus, long melatonin pretreatment periods (14 days) can inhibit oxidative damage to mtDNA caused by H2O2 in RPE cells.

4.6. Melatonin inhibited cytochrome C release

In the control group, cytochrome C was seen only in the mitochondrial fraction of RPE cells. However, cytochrome C released from the mitochondria to the cytosol following H2O2 treatment (500 μM, 1 or 2 hours) was inhibited by melatonin treatment (100 μM) (Figure 6).

4.7. Melatonin did not inhibit AIF release

In the control group, AIF was seen in the RPE mitochondrial fraction only. However, melatonin treatment (100 μM) did not inhibit H2O2 (500 μM, 3 or 6 hours)-induced AIF release (Figure 7).

5. DISCUSSION

Melatonin, an indole hormone, was recently shown to exert powerful anti-apoptotic and anti-oxidant effects (17). Melatonin alleviated ischemia-induced apoptosis in RPE cells (17). Our results confirmed the anti-apoptotic effect of melatonin, which inhibited H2O2-induced apoptosis in RPE cells in vitro.

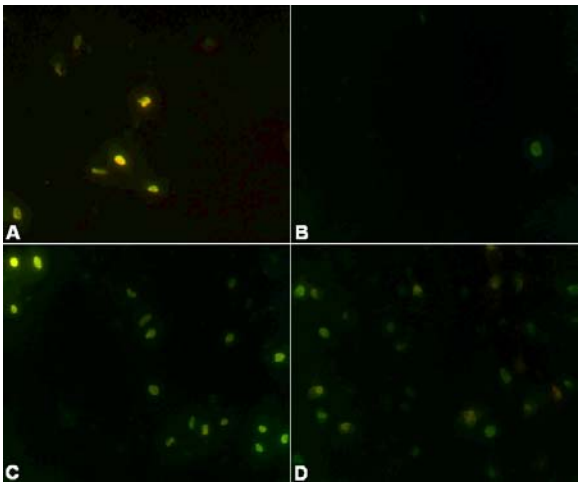

Figure 1. Melatonin inhibits apoptosis examined by TUNEL assay. The different treatment cell groups were as follows: A- For 500 μM H_2O_2 + 0 μM melatonin (24 hours) treatment, apoptotic rate was 6.3 ± 1.3 %; B- For 500 μM H_2O_2 + 100 μM melatonin (24 hours) treatment, apoptotic rate was 1.3 ± 0.5 %; C- For 500 μM H_2O_2 + 0 μM melatonin (48 hours) treatment, apoptotic rate was 10.3 ± 1.6 %; D- For 500 μM H_2O_2 + 100 μM melatonin (48 hours) treatment, apoptotic rate was 4.0 ± 0.8 %. RPE cells were washed twice with PBS and then cultured again in serum-free DMEM/F12 medium (1:1) containing various concentrations of H_2O_2 (500 μM) and melatonin (0 or 100 μM). After incubation for 24 or 48 hours, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling was performed and cells were examined under fluorescent microscope. Red PI fluorescence identifies dead cells while green fluorescence identifies TUNEL-positive apoptotic cells.

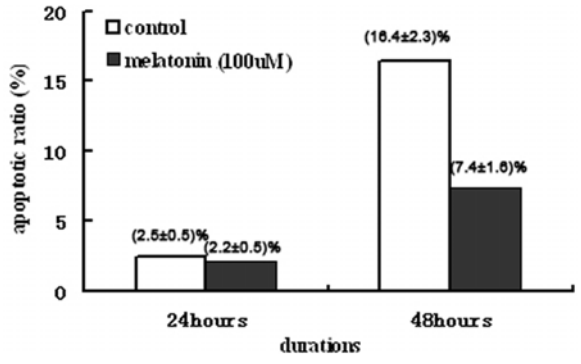

Figure 2. Melatonin inhibits apoptosis, examined by Annexin V/PI/Flow cytometry apoptosis assay. RPE cells were washed twice with PBS. Serum-free DMEM/F12 medium (1:1) containing H_2O_2 (500μM) and melatonin (0 or 100μM) was added. Apoptotic cells were counted after 24 or 48 hours. Annexin V/Propidium iodide flow cytometry analysis was carried out.

How melatonin inhibits apoptosis was the main question to address in this study. The concentration of melatonin in mitochondria is higher than in cytosol (12), and specific binding sites for melatonin might exist on mitochondria. In brain astrocytes, melatonin decreased mitochondrial production of ROS, alleviated calcium overload, improved mitochondrial permeability transition pore (MPTP) function, reduced cytochrome C release, and inhibited apoptosis (11). Our study confirmed that melatonin has protective effects on mitochondria. Indeed, melatonin decreased mitochondrial ROS and mtDNA damage, alleviated the structural damage, and inhibited cytochrome C release.

Moreover, this study was designed to investigate two more aspects related melatonin protection of mitochondria, by focusing on direct effects of melatonin. Melatonin can bind free radicals (such as the hydroxyl free radical) to form cyclic 3-hydroxymelatonin, then N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK), which are also strong anti-oxidants (18). AMK can eliminate not only ROS, but also reactive nitrogen species (19). The direct effects of melatonin were demonstrated in the present study, as it effectively decreased mitochondrial oxidative stress by down-regulating intracellular ROS levels; this phenomenon appeared mainly in mitochondria, the main producers of ROS. Oxidative damage is one of the causing factors of the MPTP dysfunction (8), which can lead to the release of intracellular active components (cytochrome C and AIF) and subsequently apoptosis (20, 21). In the present study, while melatonin effectively decreased cytochrome C release, it had no effect on AIF release. Whether the protective effect of melatonin on MPTP is selective and/or the mechanisms regulating cytochrome C release and AIF are different remain to be studied.

The second interesting aspect was the indirect effects of melatonin, which can bolster mitochondrial protection by affecting intracellular components. In the present study, the indirect effects of melatonin were assessed by measuring the damage of mtDNA, which is more sensitive to oxidative stress than nuclear DNA (nDNA) (22). Detectable fragments are found in the mtDNA sequence, usually between 7,900 to 16,500 bp, with 4,977 bp being the most commonly deleted fragment; this deletion is positively correlated with age in humans (23). In fact, genes that code for ATPase and NADH dehydrogenase are located within the 4,977 bp fragment. These enzymes are necessary for oxidative phosphorylation, and deletion of these genes can impact mitochondrial function directly and lead to enhanced ROS production (24). In addition to deleted fragments, other markers of mtDNA damage include mtDNA breaks as well as nucleotide mutations (deletion, insertion, or substitution) which can disrupt normal reading by DNA polymerase, and subsequently affect gene translation and transcription. Only relatively normal DNA templates can be extended (25). Theoretically, longer DNA templates have less probability of being extended completely, as long sequences are more susceptible to mutations. More deleted fragments or fewer reserved fragments are considered as an indication of serious mtDNA damage, and *vice-versa*. Thus, our findings indicate that melatonin protects mitochondria by decreasing mtDNA damage.

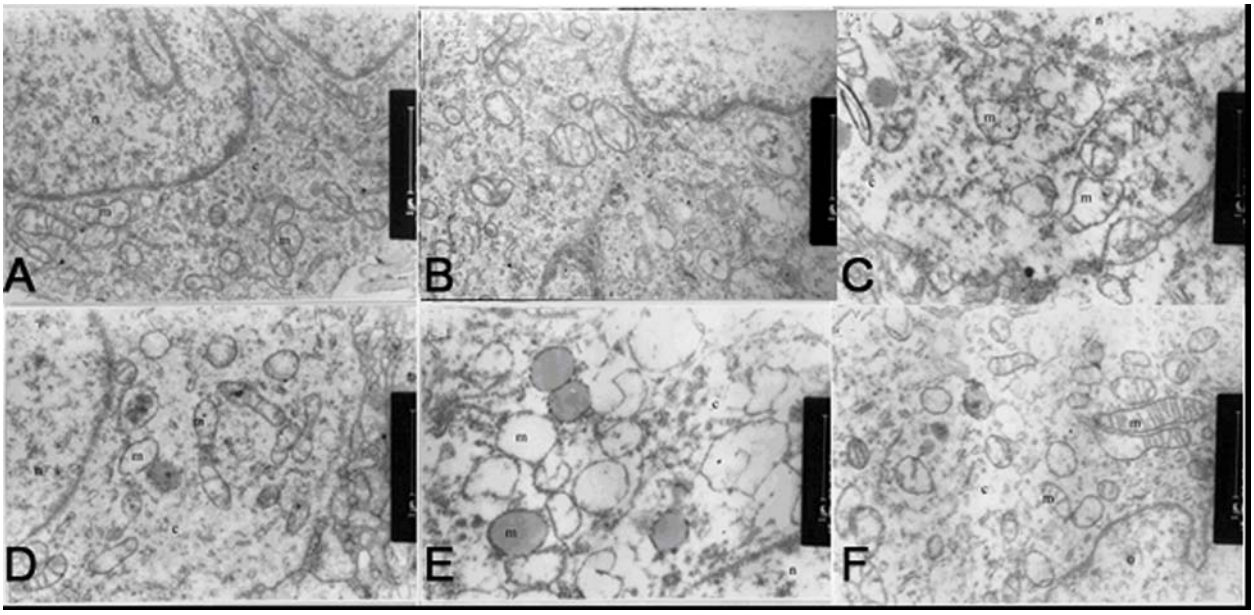

Figure 3. Melatonin alleviated the structural damage of RPE cells. A- 0μM H_2O_2 + 0μM melatonin (24 hours); B- 0μM H_2O_2 + 0μM melatonin (48 hours); C- 500μM H_2O_2 + 0μM melatonin (24 hours); D- 500μM H_2O_2 + 100μM melatonin (24 hours); E- 500μM H_2O_2 + 0μM melatonin (48 hours); and F- 500μM H_2O_2 + 100μM melatonin (48 hours). RPE cells were cultured in serum-free DMEM/F12 medium (1:1), containing various concentrations of H_2O_2 (0 or 500μM) and melatonin (0 or 100μM). The ultrastructure of RPE cells was examined by transmission electron microscopy after incubation of the cells for 24 or 48 hours. Cultured human RPE cells shuttle-like shaped, were attached to the plate, and grew in a single layer. Compared with the cells treated with H_2O_2 only, the percentage of dark cells (early apoptosis) and dead cells significantly decreased when RPE cells were cultured with both H_2O_2 and melatonin (100μM). TEM showed lower mitochondrial electron density, few vacuoles, few lipid droplets, and normal mitochondrial structure in the melatonin treatment groups. n: nuclear, m: mitochondria; and c: cytosol.

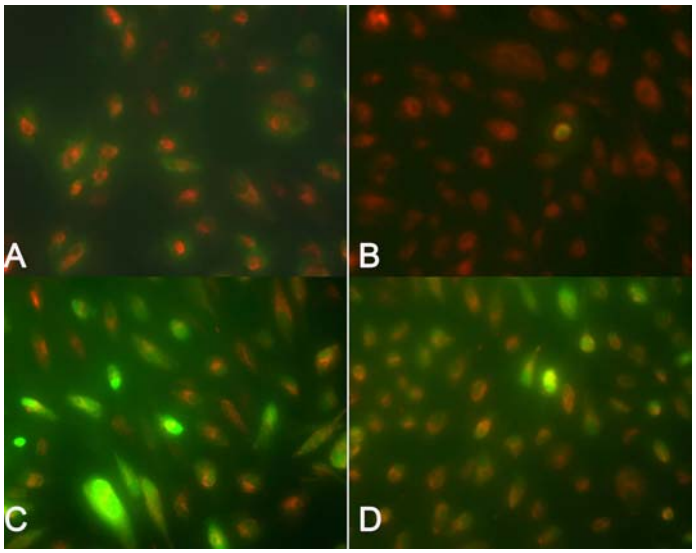

Figure 4. Melatonin decreased intracellular H_2O_2-induced ROS in RPE cells. A- 500μM H_2O_2 + 0μM melatonin (1 hour); B- 500μM H_2O_2 + 100μM melatonin (1 hour); C- 500μM H_2O_2 + 0μM melatonin (2 hours); and D- 500μM H_2O_2 + 100μM melatonin (2 hours). RPE cells were cultured in serum-free DMEM/F12 medium (1:1), containing various concentrations of H_2O_2 (500μM) and melatonin (0 or 100 μM). After incubation for 1 or 2 hours, cells were washed twice with PBS, then were treated with 2nM 2, 7-dichlorodihydrofluorescein diacetate and 500nM MitoTracker Red CM-H_2XRos for 1 hour, and again washed twice with PBS. Cells were examined by fluorescent microscope, and the green fluorescence within the cells indicated the presence of ROS.

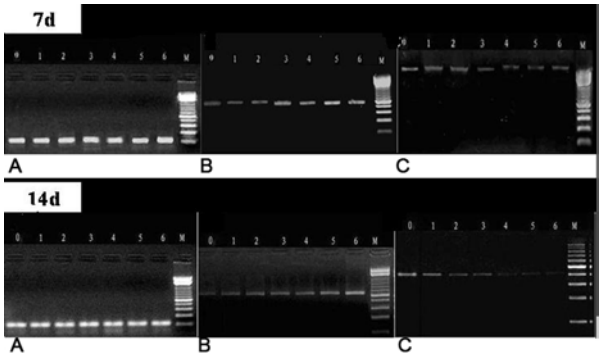

Figure 5. Melatonin decreased deleted mtDNA fragments and increased reserved fragments in a dose-dependent relationship. A- reference fragment group (111bp); B- deleted fragment group (524bp); and C: reserved fragment group (4,262bp). Concentrations of melatonin and H_2O_2 in each group: Kit 0: 0μM H_2O_2 + 0μM melatonin, Kit 1: 200μM H_2O_2 + 100μM melatonin, Kit 2: 200μM H_2O_2 + 10μM melatonin, Kit 3: 200μM H_2O_2 + 1μM melatonin, Kit 4: 200μM H_2O_2 + 0.1μM melatonin, Kit 5: 200μM H_2O_2 + 0μM melatonin, Kit 6: 200μM H_2O_2 + 0μM melatonin (added the solvent of melatonin: 0.5% ethanol in normal saline). RPE cells of different densities were seeded onto 6-well plates. DMEM/F12 medium with 10% fetal bovine serum, containing various concentrations of melatonin was added to each well. The medium was changed every 24 hours with addition of melatonin each time, and this melatonin pretreatment was stopped after 7 or 14 days period. RPE cells were washed twice with PBS and cultured with serum-free DMEM/F12 medium, containing 200μM H_2O_2 for 1 hour. RPE cells were washed twice with PBS, cell mtDNA was extracted, and PCR analysis was performed (primers and reaction conditions are shown above). The incidence of deleted mtDNA fragments decreased and the reserved fragments increased, when high concentrations of melatonin (10 μM or 100 μM) were used for 7 days and with a dose-dependent relationship to the melatonin concentration for the period of 14 days.

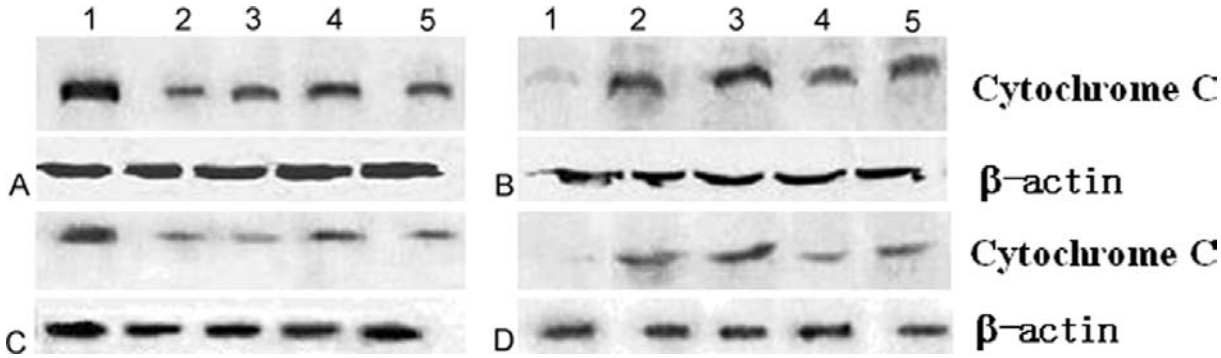

Figure 6. Melatonin inhibits peroxide-induced cytochrome C release. A- cytochrome C in mitochondria (1 hour); B- cytochrome C in cytosol (1 hour); C- cytochrome C in mitochondria (2 hours); and D- cytochrome C in cytosol (2 hours). Cell groups: 1, RPE control; 2 & 3, RPE + 500μM H_2O_2; and 4 & 5, RPE + 500μM H_2O_2 + 100μM melatonin. RPE cells were cultured in serum-free DMEM/F12 medium (1:1) with various concentrations of H_2O_2 (0 or 500μM) and 100μM melatonin for 1 or 2 hours. Mitochondrial and cytosolic proteins were collected, and Western blot analysis was carried out to determine cytochrome C levels using mouse monoclonal anti-cytochrome C antibody. In the control group, cytochrome C was found in the cell mitochondrial fraction only. cytochrome C was released from mitochondria to cytosol after H_2O_2 treatment (500 μM, 1 or 2 hours). Melatonin treatment (100 μM) inhibited peroxide-induced cytochrome C release.

To investigate the indirect effects of melatonin, RPE cells were pretreated with various concentrations of melatonin at different time points, the cells were washed twice with PBS to remove melatonin from the culture medium, so that the final effect depends on intracellular melatonin only. Our results revealed existence of great amount of deleted fragments, but reserved fragments of mtDNA was not affected by any dose of melatonin treatment or by melatonin pretreatment (5 days prior to H_2O_2 treatment). However, the effects of melatonin were apparent when pretreatment duration was extended to 7 or 14 days considering that all cell groups should have similar concentrations of intracellular melatonin. These findings indicate that melatonin exerted indirect effects in our experimental model.

Melatonin is known to up-regulate anti-oxidant enzymes (e.g. glutathione peroxidase, glutathione reductase, superoxide dismutases, and glucose-6-phosphate dehydrogenase) and can down-regulate oxidative enzymes (such as 5- or 12-lipoxygenase and nitric oxide synthase) (26-28). Although the resulting balance from the effects of

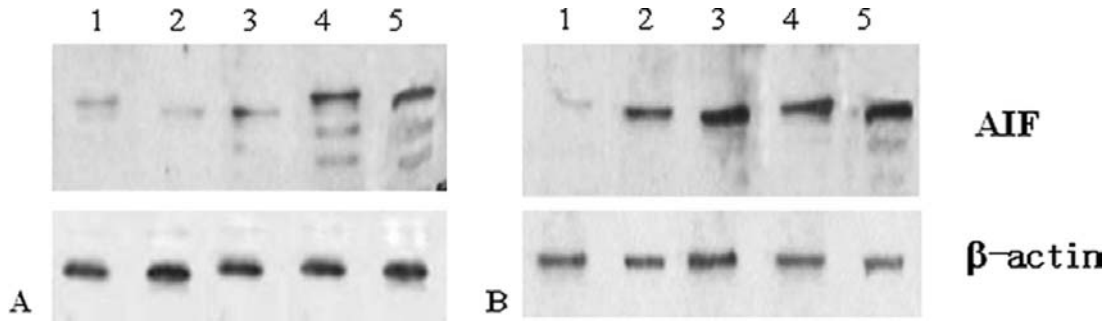

Figure 7. Melatonin (100 μM) did not inhibit peroxide-induced apoptosis inducing factor (AIF) release. A- AIF in mitochondria; B- AIF in cytosol. Cell groups: 1, RPE control; 2, RPE + 500μM H_2O_2 for 3 hours; 3, RPE + 500μM H_2O_2 for 6 hours; 4, RPE + 500μM H_2O_2 + 100μM melatonin for 3 hours; and 5, RPE + 500μM H_2O_2 + 100μM melatonin for 6 hours. RPE cells were cultured in serum-free DMEM/F12 medium (1:1) with various concentrations of H_2O_2 (0 or 500μM) and melatonin (0 or 100μM) for 3 or 6 hours. Mitochondrial and cytosolic proteins were collected and AIF levels were determined by western blot analysis, using specific monoclonal anti-AIF antibody. In the control group, AIF was detected in the RPE mitochondrial fraction only. AIF was released from mitochondria to cytosol after H_2O_2 treatment (500μM, 3 or 6 hours). Melatonin treatment (100 μM) did not inhibit peroxide-induced AIF release.

these enzymes plays a key role, the main enzymes that contributed to the effect of melatonin in the present study were not investigated.

In the present study, mitochondrial protection and anti-apoptotic effects of melatonin were demonstrated, although the protection due to melatonin was incomplete and selective. Many issues are yet to be addressed, including mechanism(s) of melatonin-regulated MPTP, the intracellular components affected by melatonin treatment, other melatonin-induced antiapoptotic pathways besides mitochondrial pathway. Further investigation is needed to provide data required to prove the relevance of melatonin to the retina. The clinical application of the anti-apoptotic effect of melatonin in retinal protection deserves further investigation.

6. CONCLUSIONS

The present study demonstrated that melatonin decreased H_2O_2-induced apoptosis in RPE cells partially through its protection of mitochondria. However, its mitochondrial protection activity is incomplete and selective.

7. ACKNOWLEDGEMENTS

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8. REFERENCES

1. C.K. Dorey, F.C. Delori, K. Akeo: Growth of cultured RPE and endothelial cells is inhibited by blue light but not green or red light. *Curr. Eye Res.* 9, 549-559 (1990)

2. D.M. Snodderly: Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *American J. Clin. Nutrition* 62, 1448S-1461S (1995)

3. A. Barak, L.S. Morse, T. Goldkorn: Ceramide: A Potential Mediator of Apoptosis in Human Retinal Pigment Epithelial Cells. *Invest. Ophthalmol. Visual Sci.* 42, 247-254 (2001)

4. A. Wenzel, C. Grimm, M. Samardzija, C.E. Remé: Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog. Retinal Eye Res.* 24, 275-306 (2005)

5. J.L. Dunaief, T. Dentchev, G.-S: Ying, A.H. Milam: The role of apoptosis in age-related macular degeneration. *Arch. Ophthalmol.* 120, 1435-1442 (2002)

6. X. Ding, M. Patel, C.-C. Chan: Molecular pathology of age-related macular degeneration. *Prog. Retinal Eye Res.* 28, 1-18 (2009)

7. J.W. Miller: Treatment of age-related macular degeneration: Beyond VEGF. *Jap. J. Ophthalmol.* 54, 523-528 (2010)

8. G. Kroemer, N. Zamzami, S.A. Susin: Mitochondrial control of apoptosis. *Immunol. Today* 18, 44-51 (1997)

9. R.J. Reiter, J.M. Guerrero, G. Escames, M.A. Pappolla, D. Acuña-Castroviejo: Prophylactic actions of melatonin in oxidative neurotoxicity. *Ann. N.Y. Acad. Sci.* 825, 70-78 (1997)

10. R.J. Reiter, D.-X. Tan, J. Cabrera, D. D'Arpa, R.M. Sainz, J.C. Mayo, S. Ramos: The oxidant/antioxidant network: role of melatonin. *Biol. Signals and Receptors* 8, 56-63 (1999)

11. M.-J. Jou, T.-I. Peng, R.J. Reiter, S.-B. Jou, H.-Y. Wu, S.-T. Wen: Visualization of the antioxidative effects of melatonin at the mitochondrial level during oxidative stress-induced apoptosis of rat brain astrocytes. *J. Pineal Res.* 37, 55-70 (2004)

12. P.M. Iuvone, G. Tosini, N. Pozdeyev, R. Haque, D.C. Klein, S.S. Chaurasia: Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. *Prog. Retinal Eye Res.* 24, 433-456 (2005)

13. M.-J. Jou, T.-I. Peng, L.-F. Hsu, S.-B. Jou, R.J. Reiter, C.M. Yang, C.C. Chiao, Y.F. Lin, C.C. Chen: Visualization of melatonin's multiple mitochondrial levels of protection against mitochondrial Ca2+-mediated permeability transition and beyond in rat brain astrocytes. *J. Pineal Res.* 48, 20-38 (2010)

14. S.M. Reppert, D.R. Weaver, V.M. Cassone, C. Godson, L.F. Kolakowski Jr.: Melatonin receptors are for the birds: molecular analysis of two receptor subtypes differentially expressed in the chick brain. *Neuron* 5, 1003-1015 (1995)

15. J. Scher, E. Wankiewicz, G.M. Brown, H. Fujieda: MT1 melatonin receptor in the human retina: Expression and localization. *Invest. Ophthalmol. Visual Sci.* 43, 889-897 (2002)

16. E. Savaskan, R. Jockers, M. Ayoub, D. Angeloni, F. Fraschini, J. Flammer, A. Eckert, F. Müller-Spahn, P. Meyer: The MT2 melatonin receptor subtype is present in human retina and decreases in Alzheimer's disease. *Curr. Alzheimer Res.* 4, 47-51 (2007)

17. N.N. Osborne, M.S. Nash, J.P.M. Wood:.Melatonin counteracts ischemia-induced apoptosis in human retinal pigment epithelial cells. *Invest. Ophthalmol. Visual Sci.* 39, 2374-2383 (1998)

18. B. Claustrat, J. Brun, G. Chazot: The basic physiology and pathophysiology of melatonin. *Sleep Med. Rev.* 9, 11-24 (2005)

19. A.L. Guenther, S.I. Schmidt, H. Laatsch, S. Fotso, H. Ness, A.R. Ressmeyer, B. Poeggeler, R. Hardeland: Reactions of the melatonin metabolite AMK (N-acetyl-5-methoxykynuramine) with reactive nitrogen species: Formation of novel compounds, 3-acetamidomethyl-6-methoxycinnolinone and 3-nitro-AMK. *J. Pineal Res.* 39, 251-260 (2005)

20. B.F. Godley, G.-F. Jin, Y.-S. Guo, J.S. Hurst: Bcl-2 overexpression increases survival in human retinal pigment epithelial cells exposed to H_2O_2. *Exp. Eye Res.* 74, 663-669 (2002)

21. B. Mignotte, J.L. Vayssiere: Mitochondrial control of apoptosis. *Adv. Cell Aging Gerontology* 5, 93-122 (2001)

22. D.E. Sawyer, S.D. Roman, R.J. Aitken: Relative susceptibilities of mitochondrial and nuclear DNA to damage induced by hydrogen peroxide in two mouse germ cell lines. *Redox Rep.* 6, 182-184 (2001)

23. P.-H. Lin, S. H. Lee, C. P. Su, Y.H. Wei: Oxidative damage to mitochondrial DNA in atrial muscle of patients with atrial fibrillation. *Free Radical Biol. Med.* 35, 1310-1318 (2003)

24. Y.-H. Wei, H.C. Lee: Oxidative stress,mitochondrial DNA mutation,and impairment of antioxidant enzymes in aging. *Exp. Biol. Med.* 227, 671-682 (2002)

25. F.M. Yakes, B. Van Houten: Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc. Natl. Acad. Sci. USA* 94, 514-519 (2004)

26. C. Rodriguez, J.C. Mayo, R.M. Sainz, I. Antolín, F. Herrera, V. Martín, R.J. Reiter: Regulation of antioxidant enzymes: a significant role for melatonin. *J. Pineal Res.* 36, 1-9 (2004)

27. H. Zhang, M. Akbar, H.Y. Kim: Melatonin: an endogenous negative modulator of 12-lipoxygenation in the rat pineal gland. *Biochem. J.* 344, 487-493 (1999)

28. M. Storr, P. Koppitz, A. Sibaev, D. Saur, M. Kurjak, H. Franck, V. Schusdziarra, H.-D. Allescher: Melatonin reduces non-adrenergic, non-cholinergic relaxant neurotransmission by inhibition of nitric oxide synthase activity in the gastrointestinal tract of rodents in vitro. *J. Pineal Res.* 33, 101-108 (2002)

Abbreviations: RPE: retinal pigment epithelial, ROS: reactive oxygen species, mtDNA: mitochondrial DNA, AIF: apoptosis inducing factor, DMEM/F12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, TEM: Transmission electron microscopy, MPTP: mitochondrial permeability transition pore, AFMK: N1-acetyl-N2-formyl-5-methoxykynuramine, AMK: N-acetyl-5-methoxykynuramine, nDNA: nuclear DNA

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Send correspondence to: Min Tang, Shanghai Jiaotong University Affiliated First People's Hospital, Ophthalmology, Shanghai, 200080, China, Tel: 86-021-33011075, Fax: 86-021-33011075, E-mail: fuyang9377@sina.com.cn

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