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Original Article

Melatonin enhances hydrogen peroxide-induced apoptosis in human dental pulp cells

Qianyi Deng [a,d], Qin Liu [b,d], Huini Zhang [a,d], Wenguo Fan [c,d], Jingzhou Li [a,d], Jun Kang [a,d], Hongwen He [c,d**], Fang Huang [a,d*]

[a] Department of Pediatric Dentistry, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangzhou, China
[b] Department of Oral Medicine, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangzhou, China
[c] Department of Oral Anatomy and Physiology, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangzhou, China
[d] Guangdong Provincial Key Laboratory of Stomatology, Guangzhou, China

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Abstract *Background/purpose:* Melatonin, at physiological concentrations, was previously found to inhibit proliferation and promote odontogenic differentiation in human dental pulp cells (hDPCs), but its effect on apoptosis is unclear. Our study aimed to investigate the effect of melatonin on the H_2O_2-mediated viability reduction and apoptosis in hDPCs.
Materials and methods: hDPCs were treated with H_2O_2 (0, 250, 500, 1000 μmol/L), melatonin (0, 10^{-12}, 10^{-10}, 10^{-8} mol/L), and melatonin with H_2O_2 for 24 h. CCK-8 assays were performed to evaluate cell viability. Apoptosis was measured by DAPI and Annexin V/propidium iodide staining. Intracellular reactive oxygen species (ROS) were measured by CellROX® staining and mitochondrial membrane potential ($\Delta\Psi m$) was examined by JC-1 staining.
Results: H_2O_2 obviously decreased the viability of hDPCs in a concentration-dependent manner and melatonin alone also reduced viability by 16–20%. Melatonin was also found to enhance H_2O_2-induced toxicity in a concentration-dependent manner, and the highest physiological concentration of melatonin (10^{-8} mol/L) had the most obvious effect ($P < 0.001$). Treating H_2O_2-exposed hDPCs with melatonin significantly increased the ratio of apoptotic cells with condensed and deformed nuclei ($P < 0.001$), as well as the percentage of Annexin V-positive cells ($P < 0.01$). Furthermore, melatonin significantly increased intracellular ROS levels and induced the loss of $\Delta\Psi m$ in H_2O_2-exposed cells ($P < 0.05$).
Conclusion: Our results indicate that melatonin, at physiological concentrations, can enhance H_2O_2-induced apoptosis in hDPCs and increase H_2O_2-mediated ROS production and $\Delta\Psi m$ loss.

* Corresponding author. Department of Pediatric Dentistry, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, 56 Ling Yuan Road West, Guangzhou 510055, China.
** Corresponding author. Department of Oral Anatomy and Physiology, Guanghua School of Stomatology, Sun Yat-sen University, 74 Zhongshan Road 2, Guangzhou 510080, China. Fax: +86 20 87330582.
E-mail addresses: hehw@mail.sysu.edu.cn (H. He), hfang@mail.sysu.edu.cn (F. Huang).

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Further studies are needed to investigate whether melatonin targets the mitochondrial death pathway during the process.

Introduction

The dental pulp consists of various cells, matrices, nerves, vessels, and collagen fibers, which are important to maintain the form and function of teeth.[1] Dental pulp cells (DPCs), comprising the major cell lineage of dental pulp tissue, have multipotent differentiation characteristics.[2] Throughout life, they remain active and function to repair impaired dentine by generating odontoblasts.[3]

Apoptosis, also known as programmed cell death, is important to control cell numbers and tissue size, and can protect the organism from rogue cells that threaten homeostasis.[4] This occurs in healthy dental pulp, and apoptotic cells are more numerous in the crown, mostly in a sub-odontoblastic locations, contributing to regulation of the pulp cell population.[5] Apoptosis of DPCs was reported to be induced by the resin monomer Bis-GMA,[6] lipopolysaccharides,[7] or some capping agents during pulp wound healing after cavity preparation.[8,9] Hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), with a greater lifetime than other intracellular ROS, can easily diffuse across membranes and was proposed to be the predominant ROS involved in cell functions.[10] Further, low concentrations of H_2O_2 were found to reduce cell viability and induce apoptosis in human DPCs (hDPCs).[11]

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone mainly produced by the pineal gland. It is involved in many important biological processes like circadian regulation, immune modulation, anti-inflammation, neuroprotection, and tumor suppression.[12–15] It also regulates multiple cellular processes such as the proliferation and differentiation of various cell types.[16–18] Moreover, accumulating evidence suggests that melatonin also regulates apoptosis in different cell types. For most tumor cells, it enhances apoptosis and could function with some chemotherapeutics to suppress tumor growth.[13] Moreover, melatonin can also induce apoptosis in normal human platelets.[19] In contrast, for most normal cells, apoptosis is reduced by melatonin. For example, melatonin was found to protect bone marrow mesenchymal stem cells and adipose-derived stem cells from H_2O_2-induced apoptosis.[20,21]

Our previous studies show that physiological concentrations of melatonin can inhibit proliferation and promote odontogenic differentiation of hDPCs,[18] but its effect on apoptosis remains unknown. Here, we used H_2O_2 to induce apoptosis in hDPCs, aiming to explore the effect of physiological concentrations of melatonin on H_2O_2-induced toxicity.

Materials and methods

Cell culture and identification

After approval by the Ethical Review Committee and obtaining signed informed consent from all subjects, hDPCs were derived freshly from carious-free and intact human third molars extracted from male and female patients aged 18 to 25 ($n = 13$). Pulp tissues were isolated aseptically from teeth split with a chisel, minced into pieces, enzymatically digested with collagenase type I, and then cultured in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) and 20% fetal bovine serum (FBS). Through successive subculture, cells were expanded for further experiments (passage 3–5). All reagents for this were purchased from Gibco (Grand Island, NY, USA).

The origin of hDPCs was identified by immunocytochemical staining for the epithelial cell marker cytokeratin (1:100, BOSTER, Wuhan, China) and the mesenchymal cell marker vimentin (1:100, BOSTER). HEK293T cells (cytokeratin-positive epithelial cells from embryonic kidney) were stained for cytokeratin as a positive control, whereas primary antibodies were replaced with phosphate-buffered saline (PBS, Gibco) as a negative control. Photographs were taken with an inverted microscope (Zeiss, Göttingen, Germany).

Cell viability assay

Cell viability of hDPCs was estimated by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates (6×10^3 cells/well) and allowed to adhere overnight before exposure to varying concentrations of H_2O_2 (0, 250, 500, 1000 μmol/L), melatonin (Sigma–Aldrich, St Louis, MO, USA) (0, 10^{-12},10^{-10},10^{-8} mol/L), and melatonin with H_2O_2. After 24 h, cells were gently washed with PBS and media were substituted with 10% CCK-8 in new media; cells were then incubated for 2 h at 37 °C. Absorbance at 450 nm was measured using a spectrophotometer (BioTek, Winooski, VT, USA). This experiment was repeated at least three times.

H_2O_2 and melatonin treatments

To investigate the effect of melatonin on H_2O_2-induced apoptosis, 500 μmol/L H_2O_2 and 10^{-8} mol/L melatonin (based on cell viability results) were used. hDPCs were seeded in 60-mm cell culture dishes (2.3×10^5 cells/dish),

allowed to adhere overnight, and incubated with serum-free DMEM for 24 h. Next, media were substituted with low-serum DMEM (containing 0.5% FBS), and cells were treated with 500 μmol/L H_2O_2, 10^{-8} mol/L melatonin, or both, for 24 h. Cells treated only with low-serum DMEM served as a control.

Morphological analysis of nuclei

The apoptotic morphology of hDPCs was assessed with the chromatin dye 4′,6-diamidino-2-phenylindole (DAPI). After melatonin and (or) H_2O_2 treatments, as indicated, hDPCs were fixed in 4% paraformaldehyde at 4 °C overnight and incubated with DAPI (Roche, Basel, Swiss) for 3 min at room temperature. Stained nuclei were observed under a fluorescence microscopy (Zeiss). Apoptotic bodies, condensed and deformed nuclei, and the total number of DAPI-stained nuclei were counted in nine randomly-selected areas for at least three independent experiments. Greater than 1000 cells from each sample were captured and the ratio of apoptosis was calculated.

Flow cytometric analysis of apoptosis, intracellular ROS levels, and mitochondrial membrane potential

Apoptosis of hDPCs was evaluated using an Annexin V-FITC/propidium iodide (PI) double staining assay (BD, Bergen, NJ, USA). After treatments with melatonin and (or) H_2O_2, hDPCs were collected, rinsed, and incubated with Annexin V-FITC and PI for 15 min at room temperature. Cell samples were then analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA). The proportions of Annexin V-positive cells were recorded as apoptotic rates.

Intracellular ROS levels were measured using CellROX® Green (Invitrogen, Grand Island, NY, USA). Upon H_2O_2 and melatonin treatment, hDPCs were collected and incubated with CellROX® reagent for 1 h at 37 °C, washed with PBS for three times, and analyzed by flow cytometry. Intracellular ROS levels were indicated by the mean intensity of green fluorescence.

A Mitochondrial Membrane Potential Detection Kit (JC-1; Beyotime, Shanghai, China) was employed to analyze mitochondrial membrane potential ($\Delta\Psi m$) in hDPCs. After various treatments, hDPCs were collected and incubated with JC-1 staining solution for 20 min at 37 °C. After rinsing, the cells were resuspended for flow cytometry. The ratio of red (J-aggregates)/green (JC-1 monomer) fluorescence was calculated, which represents $\Delta\Psi m$.

A total of 10,000 cells were harvested from each sample for these flow cytometric analyses. Experiments were repeated at least three times.

Statistical analysis

Data are presented as mean ± standard deviation (SD). One-way ANOVA was used to compare means between multiple groups and least significant difference (LSD) was used for post-hoc analysis. All P values less than 0.05 were considered statistically significant. Statistical analysis was performed using SPSS 20.0 (IBM Corp, Armonk, NY, USA).

Results

Characteristics of cultured hDPCs

Spindle-like hDPCs migrated from the small pieces of dental pulp tissue by day 7 (Fig. 1A (i)), grew to a near confluent state after 14 days (Fig. 1A(ii)), and were subcultured and further purified by distinct digestion in vitro. Cells cultured were positive for the mesenchymal marker vimentin (Fig. 1B(i)) and negative for the epithelial marker cytokeratin (Fig. 1B(ii)).

Viability of hDPCs

From CCK-8 results, H_2O_2 obviously decreased viability of hDPCs in a concentration-dependent manner ($P < 0.05$; Fig. 2A). Further, a reduction in cell viability by 16—20% was observed in hDPCs treated with various concentrations of melatonin (10^{-12}, 10^{-10}, 10^{-8} mol/L) for 24 h ($P < 0.01$; Fig. 2B). Compared to that with H_2O_2 alone, cell viability was also reduced in groups treated with melatonin (10^{-12}, 10^{-10}, 10^{-8} mol/L) and H_2O_2 ($P < 0.05$), which was most obvious with 10^{-8} mol/L melatonin and H_2O_2 ($P < 0.001$, vs. H_2O_2 alone; $P < 0.05$, vs. 10^{-12} mol/L melatonin and H_2O_2; Fig. 2C).

Morphological changes in nuclei and apoptosis of hDPCs

Based on DAPI staining, nuclei of hDPCs in control and melatonin groups were mostly round and homogeneously-stained, whereas in H_2O_2-exposed groups, condensed and deformed nuclei were observed (Fig. 3A). The ratio of apoptotic cells with condensed and deformed nuclei was significantly increased when hDPCs were co-treated with H_2O_2 and melatonin ($P < 0.001$; Fig. 3C).

By Annexin V-FITC/PI staining, the apoptosis rate in each group was calculated as the percentage of Annexin V-positive cells. As shown in Fig. 3B, D, 5.50 ± 0.73% of hDPCs in the control group were apoptotic. In the H_2O_2 group, this rate increased to 7.85 ± 1.46% ($P < 0.01$), whereas in the group co-treated with H_2O_2 and melatonin, this rate was remarkably increased to 10.67 ± 0.88% ($P < 0.01$, compared to H_2O_2 alone). However, with melatonin alone, the apoptotic rate was 5.48 ± 0.99%, and not significantly different than that in the control group.

Altered ROS levels and mitochondrial membrane potential in hDPCs

Based on CellROX® Green staining, intracellular ROS levels were indicated by the mean fluorescence. As in Fig. 4A, B, these levels were significantly elevated by H_2O_2 ($P < 0.001$), and co-treatment with melatonin resulted in an even higher level of ROS ($P < 0.05$).

Mitochondrial membrane potential ($\Delta\Psi m$) was detected by JC-1, a sensitive fluorescent probe. In cells with high $\Delta\Psi m$, JC-1 accumulates in the matrix of mitochondria, forming J-aggregates and showing red fluorescence,

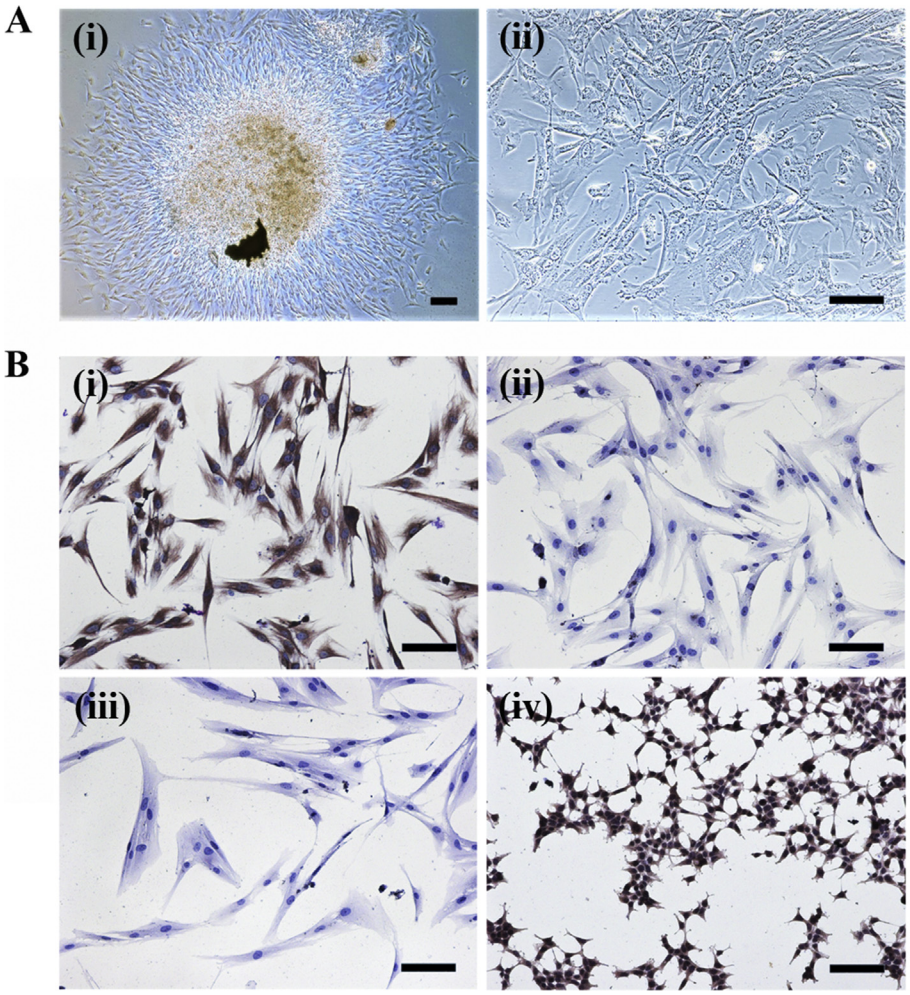

Figure 1 The isolation, culture, and identification of human dental pulp cells (hDPCs). (A) Primary culture: cells migrated from human dental pulp tissue pieces after 7 days of culture (i). hDPCs grew to a near-confluent state after 14 days of incubation (ii). (B) Identification of hDPCs by immunocytochemical staining: hDPCs were positive for vimentin (i) and negative for cytokeratin (ii). The negative control (iii) and positive control for cytokeratin (iv) are shown. Scale bars = 100 μm.

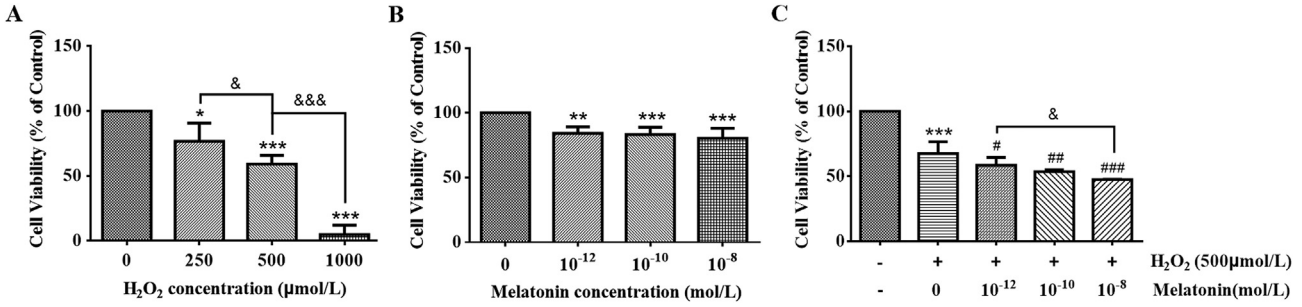

Figure 2 Cell viability of human dental pulp cells (hDPCs), as assessed by CCK-8 assays, exposed to different concentrations of H_2O_2 (0—1000 μmol/L) for 24 h (A), melatonin (0—10^{-8} mol/L) for 24 h (B), or melatonin (10^{-12} — 10^{-8} mol/L) with H_2O_2 (500 μmol/L) for 24 h (C). Data are presented as the mean ± SD ($n = 4$, 4, and 3, respectively). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, vs. H_2O_2 alone; $^{\&}P < 0.05$, $^{\&\&\&}P < 0.001$.

whereas in cells with low ΔΨm, JC-1 exists as a monomer, showing green fluorescence. The ΔΨm is indicated by the ratio of red/green fluorescence. As shown in Fig. 4C, D, H_2O_2 treatment caused ΔΨm loss in hDPCs ($P < 0.001$) and melatonin significantly enhanced this in H_2O_2-exposed cells ($P < 0.05$). However, with melatonin only, there was no significant difference in intracellular ROS levels and ΔΨm compared to those in controls.

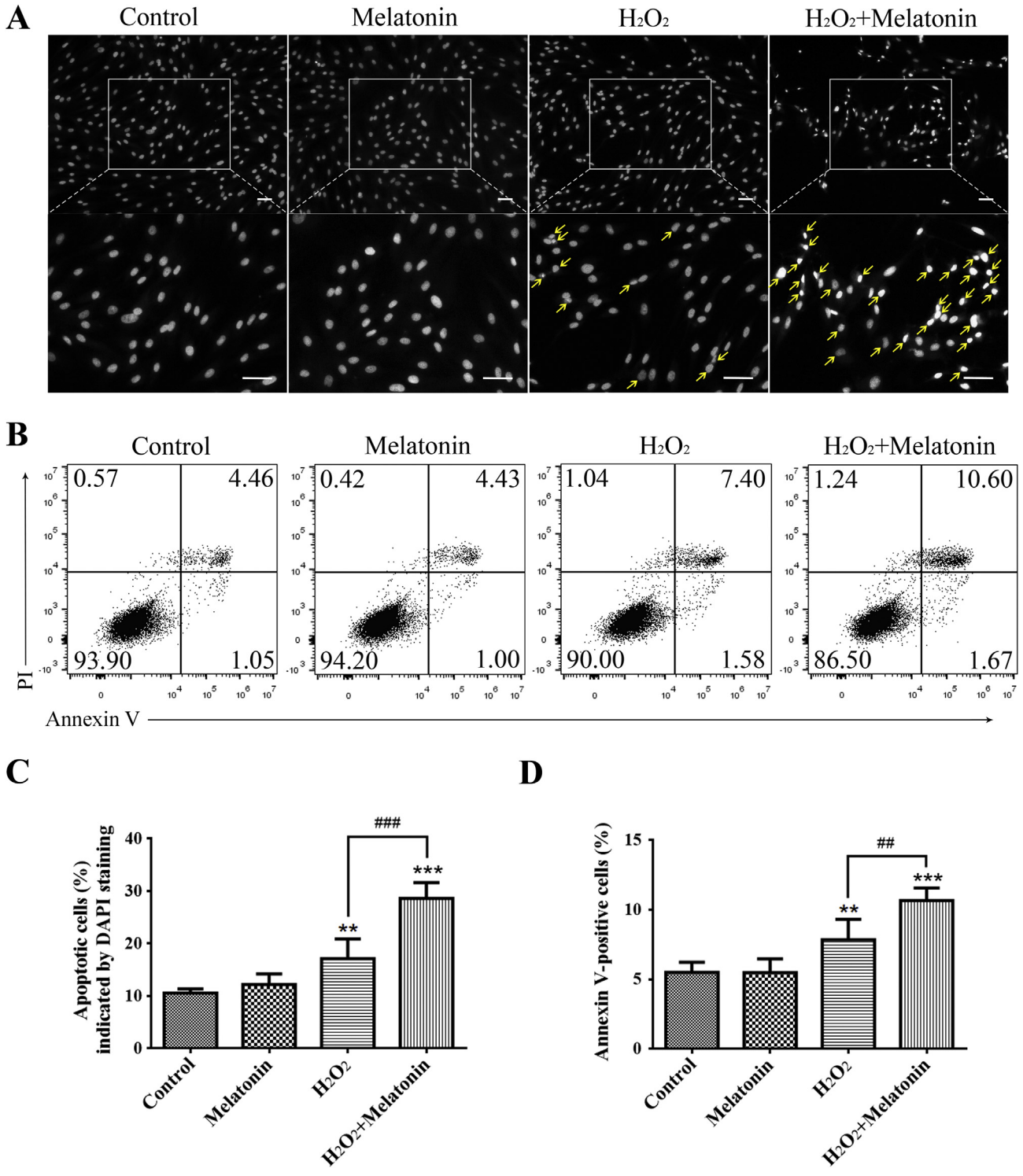

Figure 3 Effect of melatonin on H_2O_2-induced apoptosis in human dental pulp cells (hDPCs). (A) Morphological changes in nuclei of hDPCs were evaluated by DAPI staining. Apoptotic cells are indicated by the arrows; scale bars = 50 μm. (B) Representative dot plots of different groups based on Annexin V/PI staining, as measured by flow cytometry. The fluorescence intensity for Annexin V/FITC is plotted on the x-axis, with PI plotted on the y-axis. The rate of apoptosis is indicated by DAPI staining (C) and by percentages of Annexin V-positive cells (D). Data are presented as the mean ± SD ($n = 4$), $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, vs. H_2O_2 alone.

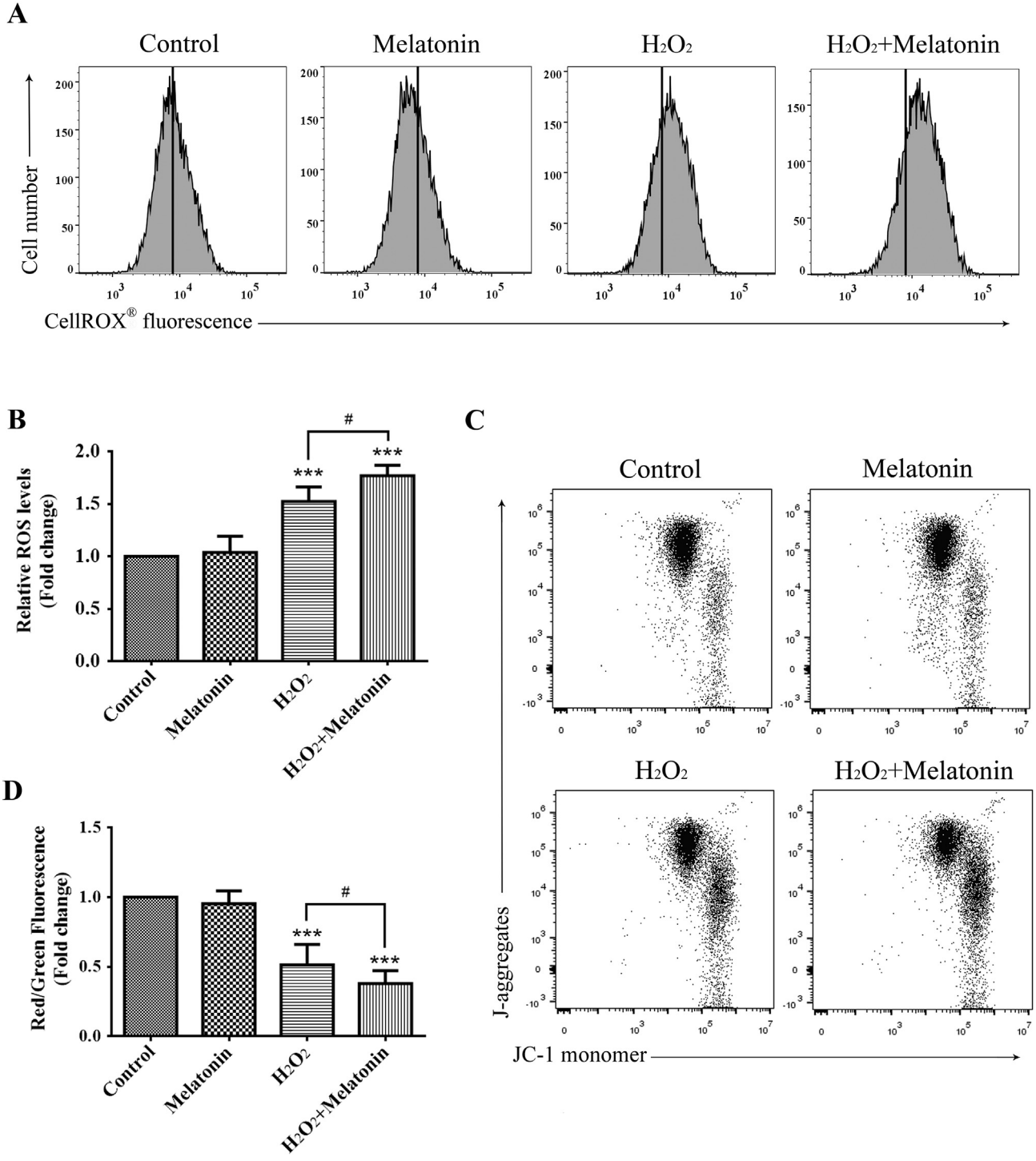

Figure 4 Effect of melatonin on H_2O_2-induced reactive oxygen species (ROS) production and mitochondrial membrane potential loss in human dental pulp cells (hDPCs). (A) Representative histogram of CellROX® Green fluorescence intensities. The mean fluorescence values for the vehicle control cells are indicated by vertical lines in each graph. (B) Quantitative evaluation of ROS in hDPCs. Data are presented as the mean ± SD ($n = 4$). ***$P < 0.001$, vs. control; $^{\#}P < 0.05$, vs. H_2O_2 alone. (C) Representative dot plots of different groups based on JC-1 staining. Fluorescence intensity for JC-1 monomers (green) is plotted on the x-axis, with J-aggregates (red) plotted on the y-axis. (D) Mitochondrial membrane potential ($\Delta\psi m$), presented as red/green fluorescence, is shown. Data are presented as the mean ± SD ($n = 6$). ***$P < 0.001$, vs. control; $^{\#}P < 0.05$, vs. H_2O_2 alone.

Discussion

Our study investigated the effect of melatonin on apoptosis of hDPCs, and H_2O_2 was used for this. Loss of cell viability is one characteristic of apoptotic cells and is a useful trait to monitor apoptosis.[22] Based on cell viability assays, H_2O_2 at 500 μmol/L, which reduced viability of hDPCs by approximately 40%, was selected for other experiments. Our previous study showed that physiological concentrations of melatonin (10^{-12}, 10^{-10}, 10^{-8} mol/L) could inhibit proliferation of hDPCs.[18] In the present study, the administration of physiological concentrations of melatonin for 24 h reduced hDPCs viability by 16–20%, and synergistically enhanced the H_2O_2-induced reduction in viability of hDPCs in a concentration-dependent manner.

DAPI is a blue fluorescent nuclear stain that can be used to visualize nuclear changes such as nuclear fragmented bodies and condensed or deformed nuclei during apoptosis or necrosis.[22] Moreover, in apoptotic cells, membrane phosphatidyl serine (PS) from the inner leaflet of the plasma membrane is translocated to the outer component. Annexin V-FITC can specifically interact with PS, serving as a sensitive probe of cells undergoing apoptosis.[23] In this study, we found that melatonin enhances H_2O_2-induced morphological changes in nuclei of hDPCs based on DAPI staining and elevated the proportion of Annexin V-positive cells in the presence of H_2O_2. These results were consistent with those of cell viability assays. Thus, we suggest that melatonin can enhance H_2O_2-induced apoptosis in hDPCs.

There are three main mechanisms that regulate apoptosis, namely the death receptor pathway, mitochondrial pathway, and endoplasmic reticulum pathway.[24] Excessive H_2O_2 causes apoptosis via the mitochondrial death pathway, which commences with a transient increase in mitochondrial membrane hyperpolarization, causing a collapse in the $\Delta\Psi m$, mitochondrial translocation of the pro-apoptotic proteins Bax and Bad, and release of cytochrome c from mitochondria to the cytoplasm.[24,25] Melatonin is a lipophilic molecule that can penetrate cellular and mitochondrial membranes, and was found to accumulate in mitochondria, indicating that this organelle might be a major target for melatonin. Further, it might interact with mitochondrial transition pores to elevate ROS production,[26] and was found to induce mitochondrial permeability transition pores in human myeloid HL-60 cells.[27] In our study, melatonin upregulated H_2O_2-induced intracellular ROS production and enhanced associated $\Delta\Psi m$ loss in hDPCs. We thus hypothesized that melatonin might influence H_2O_2-induced apoptosis of hDPCs through the mitochondrial pathway. Further studies will focus on the expression of mitochondrial pathway proteins such as Bcl-2, Bax, and cytochrome c.

The effect of melatonin on apoptosis is different in various cell types. For most tumor cells, it enhances apoptosis. Paradoxically, its effect is opposite in most normal cells. The neuroprotective effects of melatonin have been mostly studied, as it can reduce apoptosis in various neural cells.[14] In addition, such effects were also observed in epithelial cells (e.g. retinal pigment epithelium cells,[28] lens epithelial cells,[29] cardiac microvascular endothelial cells)[30] and stem cells (e.g. bone marrow stem cells,[20] adipose-derived stem cells,[21] haemopoietic cells).[31] Interestingly, it was reported that melatonin enhances H_2O_2-induced apoptosis in human promyelocytic leukemia HL-60 cells, but increases the viability of healthy leucocytes exposed to H_2O_2.[32] This dual effect might be explained by the different activity of respiratory complexes of diverse cell types.[12] In a recent study, by measuring relevant mitochondrial respiration parameters in mesenchymal stem cells (MSCs) from different human tissues, MSCs from dental pulp were found to have higher mitochondrial respiratory abilities than MSCs from bone marrow and adipose.[33] Thus, we speculated that mitochondrial respiratory abilities of hDPCs might be more similar to those of tumor cells, which could explain the enhanced H_2O_2-induced apoptosis of hDPCs with melatonin in our study.

Additionally, the effect of melatonin on apoptosis might be treatment-specific, as different concentrations could have disparate effects. At micro/millimolar levels (pharmacological concentrations, used in most studies), which are relatively higher than the physiological concentrations applied in this study, melatonin could directly scavenge hydroxyl and peroxyl radicals. However, in the picomolar range, melatonin has higher affinity for membrane receptors, the stimulation of which could decrease intracellular cAMP levels, thus activating different signal transduction pathways. In the nanomolar range, melatonin can activate the nuclear receptors RZR/RORα and RZRβ to regulate gene transcription.[34] It was found that addition of the RZR/RORα ligand CGP52608 could increase cell death in an ovarian carcinoma cell line.[35] Consequently, under physiological conditions, the effect of melatonin on apoptosis might be different.

H_2O_2 is commonly used in dental treatments such as tooth bleaching and root canal irrigating. It can penetrate enamel and dentine and reach the dental pulp chamber where hDPCs exist; moreover, the concentration of H_2O_2 that reaches the pulp chamber is higher in restorative teeth.[36] Melatonin levels in the human body change with age and are low in infants, increasing significantly to peak at 1–3 years of age, and subsequently declining during puberty and in old age.[37,38] As we found that physiological concentrations of melatonin enhance H_2O_2-induced toxicity of hDPCs in a concentration-dependent manner, the overuse of H_2O_2 in dental procedures might be more harmful to the dental pulp in children and adolescents with higher melatonin levels than in adults. Consequently, H_2O_2 should be used with caution in pediatric dental patients.

In conclusion, our study showed that melatonin at physiological concentrations enhances the H_2O_2-induced loss of viability in hDPCs and promotes H_2O_2-induced apoptosis, with elevated intracellular ROS levels, and $\Delta\Psi m$ loss. Further studies are needed to examine whether melatonin enhances H_2O_2-induced apoptosis of hDPCs through the mitochondrial pathway.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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