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Melatonin regulates mitochondrial function and biogenesis during rat dental papilla cell differentiation

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of melatonin on mitochondria of dental papilla cells (DPCs) during the odontogenic differentiation process.

MATERIALS AND METHODS: Primary DPCs were obtained from the first molar dental papilla of neonatal rats and cultured in osteogenic (OS) or basal medium supplemented with melatonin at different concentrations (0, 1 pM, 0.1 nM, 10 nM, and 1 μM) for differentiation *in vitro*. Effects of melatonin on differentiation, mitochondrial respiratory function, and mitochondrial biogenesis of DPCs were analyzed.

RESULTS: Upon odontogenic induction, Alkaline phosphatase (ALP) activity, dentin sialophosphoprotein (DSPP), and dentin matrix protein (DMP1) expression were significantly enhanced, with a peaked expression at 10 nM of melatonin treatment. During DPCs differentiation, 10 nM melatonin could significantly induce the increase of intracellular Adenosine triphosphate (ATP), the decrease of the oxidized form of nicotinamide adenine dinucleotide (NAD+)/NADH ratio and reactive oxygen species (ROS). The mRNA and protein levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (TFAM) were significantly increased, and the peak level of expression was found in cells treated with 10 nM of melatonin. Furthermore, the mitochondria DNA (mtDNA) copy number was significantly decreased during DPCs differentiation.

CONCLUSIONS: These findings suggest that melatonin can promote the differentiation of rat DPCs and regulate mitochondrial energy metabolism, ROS scavenging, and mitochondrial biogenesis.

Key Words:
Dental papilla cell, Melatonin, Differentiation, Mitochondrial.

Introduction

The formation of dentin, known as dentinogenesis, is initiated by the differentiation of dental papilla cells (DPCs) into odontoblasts, which is controlled by epithelial-mesenchymal interactions[1-3]. Aside from dentin formation, DPCs are also able to differentiate into other types of cell, such as adipocytes[4,5], nerve cells[6], endothelial cells, and epithelial cells[7]. Due to their clonogenicity and ability for multi-lineage differentiation, DPCs could be ascribed to postnatal stem cells[8] and are used not only as a promising candidate for dental regeneration, but also as a source for cell-based therapy to treat liver disease[9] and cardiovascular disease[7].

Melatonin (N-acetyl-5-methoxytryptamine), an endogenous indolyl hormone, is primarily synthesized and secreted in the pineal gland, but is also produced in other organs, such as the retina, thymus, and spleen[10]. Melatonin plays a vital role in a variety of physiological processes, including circadian rhythms[11], seasonal reproduction[12], blood

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pressure regulation[13], immune function[14], metabolic regulation[15], and free radical scavenging[16]. Among the numerous functions of melatonin, regulating stem cell differentiation has been recently proposed[17-22], and the possible involvement of mitochondria in the effects of melatonin during differentiation has been established[19,21].

Mitochondria are crucial organelles in eukaryotes that are the sites for cellular respiration and energy production. In addition, mitochondria display differences in their abundance, morphology, and functions in response to environmental and cellular cues[23,24]. Several studies[24-27] have suggested that the regulation of mitochondria dynamics and function is essential for successful differentiation of stem cells. Mitochondrial biogenesis, the generation of new mitochondria, which involves the synthesis of the inner and outer mitochondrial membranes and mitochondrial encoded proteins, synthesis and imports of nuclear-encoded mitochondrial proteins, and replication of mitochondrial DNA (mtDNA)[28,29]. Mitochondrial biogenesis is a highly regulated cellular process controlled by the peroxisome proliferator activated receptor-gamma (PPARγ) coactivator-1 alpha (PGC-1α)[30]. PGC-1α plays a central role in the regulatory network governing the transcriptional control of mitochondrial biogenesis and respiratory function[30]. The co-activator controls multiple transcription factors, including nuclear respiratory factor 1 (NRF-1), which can regulate nuclear DNA-encoded proteins involved in mitochondrial respiration and oxidative phosphorylation[30,31]. Furthermore, NRF-1 can activate the expression of mitochondrial transcription factor A (TFAM), which translocate into the mitochondria to stimulate DNA replication and gene expression[32].

Previous works[33,34] have shown that melatonin promoted the odontogenic differentiation of DPCs. However, the underlying mechanism remains unclear. In this study, we investigated the effect of various concentrations of melatonin on mitochondrial function and biogenesis of DPCs during differentiation.

Materials and Methods

Cell Culture

The first molar dental papilla of neonatal Sprague-Dawley (SD) rats (derived from Sun Yat-sen University, Guangzhou, China) was extracted and cultured. The experimental protocol was approved by the Ethics Committee of Guanghua School and Hospital of Stomatology, Sun Yat-sen University, China and was in accordance with the Ethical Principles of Animal Experimentation. The dental papilla tissues were separated as previously described[5,33] and incubated in basal medium: α-Modified Eagle's Medium (α-MEM; Gibco, Life Technologies, MA, USA) with 20% fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The culture was maintained at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every three days.

In Vitro Differentiation

Cells were used at passage 2-3 for the following experiments. DPCs were induced to differentiate into odontoblasts in an osteo/odontogenic (OS) medium (basal medium, 0.1 mM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate; Sigma-Aldrich, St. Louis, MO, USA). The DPCs cells were cultured in OS or basal medium supplemented with melatonin (Sigma-Aldrich, St. Louis, MO, USA) at different concentrations (0, 1 pM, 0.1 nM, 10 nM, and 1 μM).

Immunocytochemical Staining of Cytokeratin and Vimentin

DPCs were fixed with cool 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100 for 10 min, and then treated with 0.3% hydrogen peroxide (H_2O_2) for 30 min to inhibit endogenous peroxidase activity. The cells were then blocked with 5% goat serum for 30 min at room temperature. The cells were incubated with a mouse anti-cytokeratin antibody (1:100, Boster, China) or a mouse anti-vimentin antibody (1:100, Boster) for 2 h at 37°C, followed by incubation with rabbit anti-mouse immunoglobulin (IgG H+ L, 1:100, Boster) for 1 h at 37°C. Primary antibodies replaced by phosphate-buffered saline (PBS) served as negative control and HEK293T cells stained for cytokeratin served as a positive control. The cells were then stained with 3,3′-diaminobenzidine (DAB) for 3 minutes and hematoxylin for 1 min. Images were captured with an inverted microscope (Axio Observer Z1; Carl Zeiss AG, Oberkochen, Germany).

Alkaline Phosphatase (ALP) Activity Assay

DPCs were cultured in OS or basal medium supplemented with various concentrations of

melatonin (0, 1 pM, 0.1 nM, 10 nM, and 1 μM) for three days. Then, the ALP activity, a marker of odontoblast differentiation, was measured using a commercial ALP kit (Jiancheng, Nanjing, China), following the manufacturer's instructions[5,33]. The generation of p-nitrophenol in the presence of ALP was determined by measuring the optical density (OD) at a wavelength of 520 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan, Grodig, Austria). The OD was normalized to protein concentration.

Measurement of ATP Content

DPCs were cultured in OS or basal medium supplemented with various concentrations of melatonin (0, 1 pM, 0.1 nM, 10 nM, and 1 μM) for seven days. Then, the intracellular ATP content of the DPCs was determined by using an ATP Colorimetric/Fluorometric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions[5]. The OD of the fluorescent signal (FLU, λex = 535/ λem=5 87 nm) was measured using the Promega GloMax Multiplus Plate Reader (Promega Corporation, Madison, WI, USA). The fluorescent intensity was normalized to the total cell number.

NAD+/NADH Assay

The NAD^+ is reduced to NADH by accepting electrons or hydrogen atoms from oxidized compounds in dehydrogenase reactions. DPCs were cultured in OS or basal medium supplemented with various concentrations of melatonin (0, 1 pM, 0.1 nM, 10 nM, and 1 μM) for seven days. The intracellular NAD^+/NADH ratio was then measured in the DPCs using a NAD^+/NADH quantification kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions[5]. NAD_{total} (NADH and NAD^+) and NADH were detected following the manufacturer's instructions. The absorbance was measured at 450 nm wavelength in an ELISA plate reader (Tecan, Grödig, Austria). The NAD^+/NADH ratio was calculated as: [NAD_{total}- NADH]/NADH.

Measurement of Intracellular ROS

DPCs were cultured in OS or basal medium supplemented with various concentrations of melatonin (0, 1 pM, 0.1 nM, 10 nM, and 1 μM) for seven days. The DPCs were then prepared as single-cell suspensions (1×10^4 cells) using trypsin/EDTA, resuspended in α-MEM containing 2% fetal bovine serum (FBS) and 5 μM CellROX® Green Reagent (Invitrogen, Carlsbad, CA, USA), and incubated at 37°C for 60 min. The cell suspensions were then washed three times with phosphate-buffered saline (PBS). Then, the fluorescent intensity of each group was measured by flow cytometry (Beckman Coulter, Brea, CA, USA). The level of ROS in each group was indicated as the mean of fluorescent intensity[5].

Measurement of MtDNA Copy Number

Quantitative Real Time PCR (qPCR) was used to determine the mtDNA copy number. Total DNA was extracted from treated cells using a DNA extract kit (Omega Bio-Tek, Norcross, GA, USA). Two different segments of mtDNA were magnified: NADH dehydrogenase subunit 1 (*Nd1*), encoded by the heavy chain of mtDNA, and NADH dehydrogenase subunit 6 (*Nd6*), encoded by the light chain of mtDNA. The nuclear gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplicon was used as an internal standard. The gene-specific primers in this study are listed in Table I.

Quantitative Real Time PCR Analysis

Total mRNA was extracted using TRIzol (Life Technologies), and purity and concentration of extracted RNA were measured, followed by reverse transcription to synthesize cDNA. First-strand cDNA was synthesized using the PrimeScript™ RT Master Mix (TaKaRa, Otsu, Shiga, Japan). PCR system was prepared using SYBR Green I Mastermix (Roche Applied Science, Penzberg, Bayern, Germany). The gene-specific primers used in this study are listed in Table I. The qPCR reaction conditions were as follows: 1 cycle at 95°C for 5 min, followed by 45 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. Ct values were processed using the $2^{-\Delta\Delta CT}$ method. The relative expression level of each gene was normalized to internal control β-actin.

Western Blotting Analysis

An equal amount of protein from each sample was loaded and separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with Tris-Buffered Saline containing 0.05% Tween-20 (TBS-T) and 5% (w/v) milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with antibodies against dentin sialophosphoprotein

Table I. Quantitative real time reverse transcription polymerase chain reaction primers.

Genes	Primers	Sequences (5'-3')
Alp	Forward	GACAAGAAGCCCTTCACAGC
	Reverse	ACTGGGCCTGGTAGTTGTTG
Dspp	Forward	ACGCCACTAACGACGATTC
	Reverse	CCTCCTACGGCTATCGACTC
Dmp1	Forward	ACCAAAATACTGAATCTGAAAGCTC
	Reverse	TGCTGTCCGTGTGGTCACTA
Nd1	Forward	GCAGGACCATTCGCCCTATT
	Reverse	AAAACGGGGGTAGGATGCTC
Nd6	Forward	CTCCTCAGTAGCCATAGCAGT
	Reverse	GTTGTCTAGCGTTGGCGTTG
Gapdh	Forward	CGGCCAAATCTGAGGCAAGA
	Reverse	TTTTGTGATGCGTGTGTAGCG
Pgc-1α	Forward	ACCCACAGGATCAGAACAAACC
	Reverse	GACAAATGCTCTTTGCTTTATTGC
Nrf-1	Forward	GACCATCAGCAAAGCCGTGA
	Reverse	ACGTAAGCTCTGCCTGGTTG
Tfam	Forward	ATCATGACGAGTTCTGCCGT
	Reverse	ACTTCACAAACCCGCACGAA
β-actin	Forward	CAGCACTGTGTTGGCATA
	Reverse	CGGTCAGGTCATCACTATC

(DSPP; 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), dentin matrix protein (DMP1; 1:500, Novus Biologicals, Littleton, CO, USA), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α; 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), nuclear respiratory factor 1 (NRF-1; 1:1000, Abcam, Cambridge, MA, USA), mitochondrial transcription factor A (TFAM; 1;100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1:1000, Beyotime). Next, the membranes were washed with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibody (Beyotime) at room temperature for 1 h. The results were analyzed using an ImageQuant Las 4000 mini imaging system and software, according to the manufacturer's instructions. Band intensities were quantified by using ImageJ 1.36b (NIH, Bethesda, MD, USA).

Statistical Analysis

All experimental data are presented as the mean ± standard deviation (SD). A one-way (ANOVA) was used for the comparison among groups and Fisher's least significant difference (LSD) was used for Post-Hoc analysis. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 19.0 (SPSS, Chicago, IL, USA).

Results

Isolation and Identification of DPCs

To establish primary rat DPCs, we extracted and cultured the first molar dental papilla from SD rats. After 48 h of culture, DPCs were seen around the small pieces of the dental papilla in a 25-cm^2 flask and exhibited a homogenous, large, polygonal fibroblastic morphology (Figure 1A). These DPCs were positive for the expression of the mesenchymal cell marker, vimentin (Figure 1B), and negative for the expression of the epithelial cell marker, cytokeratin (Figure 1C). This indicates that the cells we cultured were of mesenchymal origin. Negative and positive controls are shown in Figure 1D and E, respectively. Thus, our results demonstrate that we can successfully isolate and culture primary rat DPCs, which are of mesenchymal origin.

Melatonin Enhanced the Odontogenic Differentiation of DPCs

To determine the effect of melatonin on the odontogenic differentiation of DPCs, ALP activity, and *Alp* mRNA expression were assessed at day three of odontogenic induction. We observed that ALP activity was significantly enhanced in DPCs exposed to OS medium with 0, 10 nM, and 1 μM of melatonin. Particularly, the maximal increase in the ALP activity was observed in the 10 nM melatonin-treated group (Figure 2A). The *Alp* mRNA

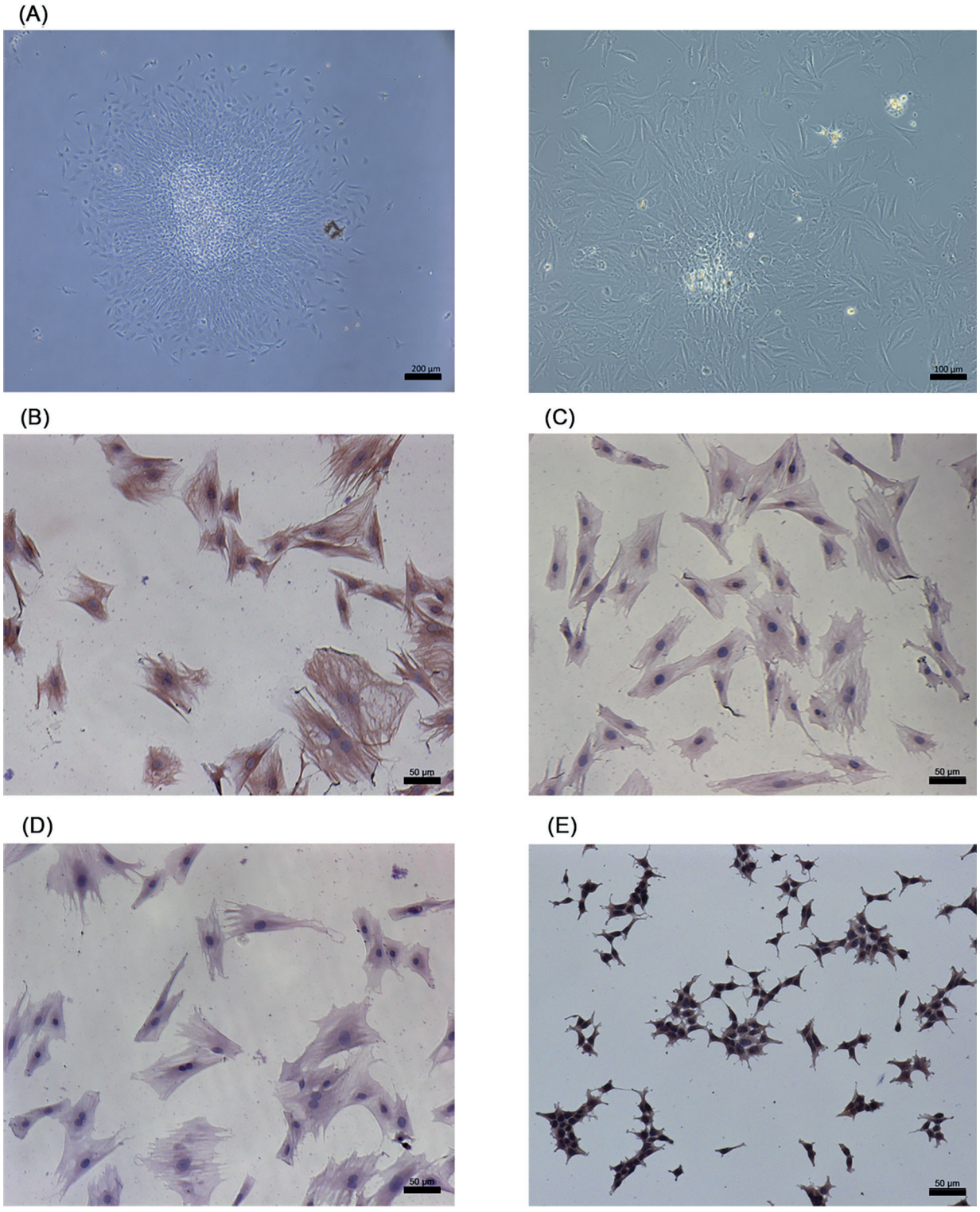

Figure 1. The isolation, culture, and identification of rat dental papilla cells (rat DPCs). **A,** Rat DPCs migrated from dental papilla tissue pieces after two days in culture. **B,** Immunocytochemical staining showed that DPCs were positive for expression of vimentin (mesenchymal cell marker). **C,** Immunocytochemical staining showed that DPCs were negative for expression of cytokeratin (epithelial cell marker). **D,** Primary antibodies replaced by PBS served as negative control. E, HEK293T cells stained for cytokeratin served as positive control.

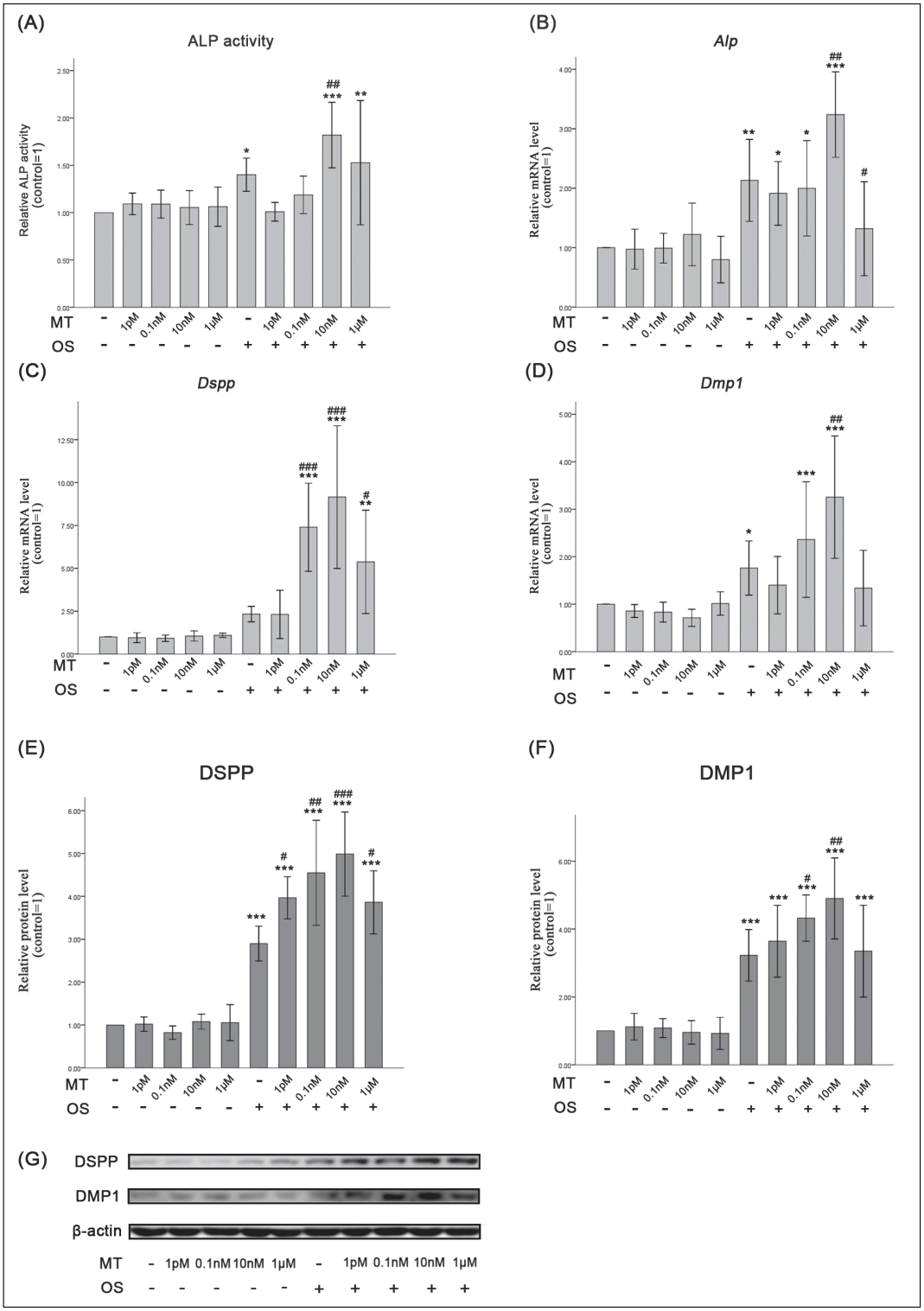

Figure 2. The effect of melatonin on the odontogenic differentiation of rat DPCs. **A,** ALP activity of rat DPCs was assessed. **B,** MRNA level of *Alp* was measured by qPCR. **C,** MRNA level of *Dspp* was measured by qPCR. **D,** MRNA level of *Dmp1* was measured by qPCR. **E,** Relative protein expression of DSPP was analyzed by Western blotting. **F,** Relative protein expression of DMP1 was analyzed by Western blotting. **G,** Representative immunoblots of DSPP and DMP1 via Western blotting were shown, and β-actin was used as the internal control. All the data are shown as the mean ± SD (n = 5). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ *vs*. basal medium without melatonin group (OS-, MT-); # $p < 0.05$; ## $p < 0.01$, and ### $p < 0.001$ *vs.* odontogenic medium without melatonin group (OS+, MT-).

levels were significantly increased in DPCs exposed to OS medium with 0, 0.1 nM, 10 nM, and 1 μM of melatonin, with a peaked expression level at 10 nM (Figure 2B). Next, we detected the expression of DSPP and DMP1, which are considered closely associated with odontogenic differentiation, by qPCR and Western blot at day 14 of odontogenic induction. QPCR analysis showed that *Dspp* mRNA levels dose-dependently increased in cells incubated in OS medium with melatonin at a range from 0 to 10 nM (Figure 2C). Cultures treated with OS medium demonstrated higher *Dmp1* mRNA levels compared with the basal medium group, also with a peaked expression level at 10 nM melatonin (Figure 2D). Western blot analysis revealed that changes in DSPP and DMP1 protein levels were consistent with the results of mRNA expression (Figure 2E, F, G). From our results, we noticed that melatonin alone did not influence the differentiation of DPCs; thus, we did not include any "melatonin only" group in the subsequent experiments.

Melatonin Enhanced Mitochondrial Respiratory Function of DPCs During Odontogenic Induction

To access the effect of melatonin on the respiratory function of DPCs during odontogenic induction, we firstly analyzed the intracellular ATP content, after seven days of osteogenic induction. The ATP content of cells incubated with OS medium was significantly increased compared to untreated cells, which indicated the upregulation of aerobic mitochondrial metabolism. Specifically, we observed the maximal increase in ATP content with melatonin treatment at a concentration of 10 nM (Figure 3A). NADH and its oxidized form NAD^+ are known as classic molecules involving in mitochondrial respiratory function and oxidative metabolism, which are the major electron donors for the electron transport chain. So, the cellular NAD^+/NADH ratio is a key indicator reflecting the overall redox state of the cell. Our results demonstrated a significant reduc-

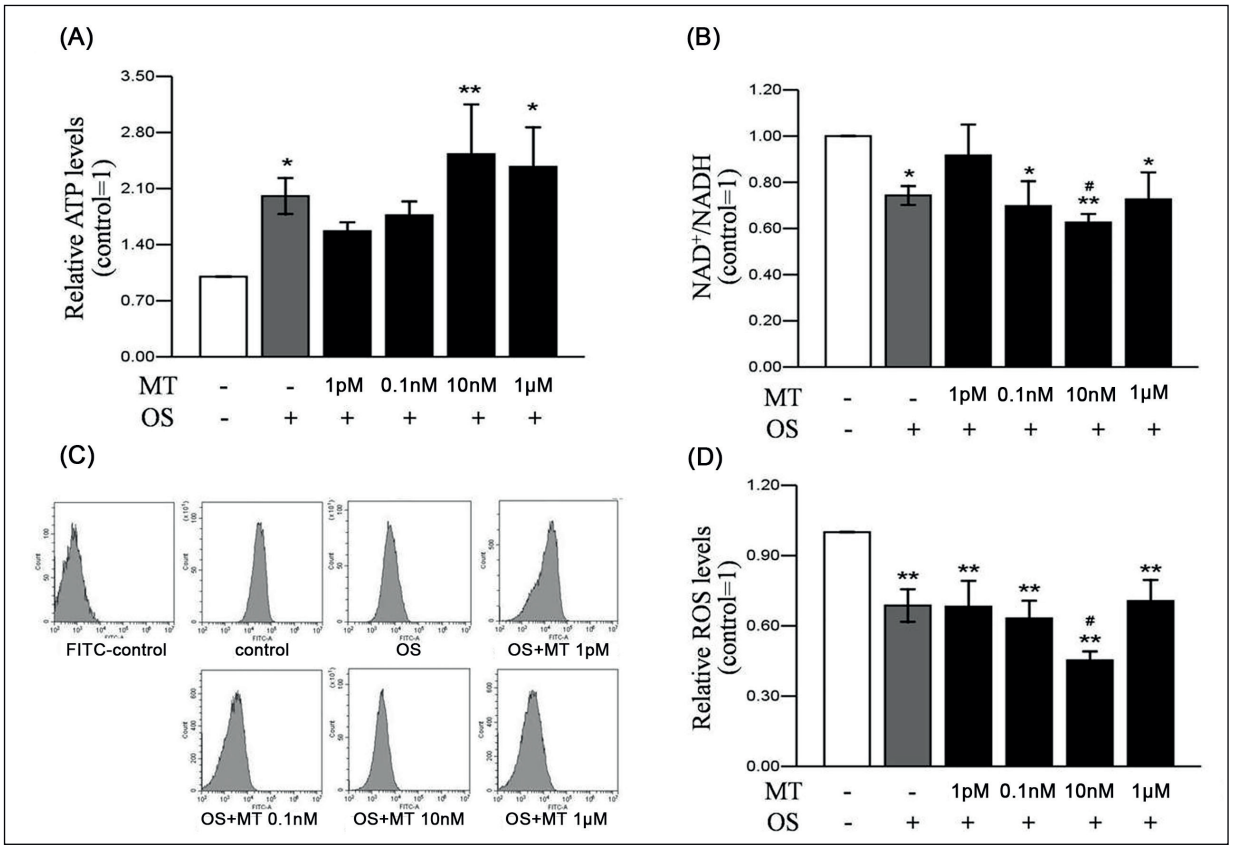

Figure 3. The effect of melatonin on the mitochondrial respiratory function of DPCs during odontogenic induction. **A,** Intracellular ATP content in each group was detected. **B,** The NAD^+/NADH ratio in each group was detected. **C,** ROS production in rat DPCs was measured by flow cytometry. **D,** Quantitative evaluation of ROS in rat DPCs. Data are shown as the mean ± SD (n = 5). *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs.* basal medium without melatonin group (OS-, MT-); # $p < 0.05$ *vs.* odontogenic medium without melatonin group (OS+, MT-).

tion of the NAD^+/NADH ratio in DPCs cultured in OS medium compared within basal medium, with the minimum level at 10 nM concentration of melatonin (Figure 3B). We further investigated the ROS, which is mainly produced from oxidative phosphorylation in mitochondria during ATP synthesis. Of note, a significant reduction of the level of ROS production in DPCs cultured in OS medium compared within basal medium, with the minimum level at 10 nM concentration of melatonin (Figure 3C, D).

The Effect of Melatonin on the Mitochondrial Biogenesis of DPCs During Odontogenic Induction

To determine if the melatonin has an impact on mitochondrial biogenesis of DPCs during odontogenic induction, mtDNA content, a reflection of mitochondrial biogenesis within in a cell, was examined after seven days of osteogenic induction. The mtDNA content in the OS medium groups decreased significantly compared with that in the basal medium group (Figure 4A). We then examined the expression levels of three crucial genes associated with mitochondrial biogenesis, including *Pgc-1α*, *Nrf-1* and *Tfam*. Culture treated with OS medium demonstrated increased gene expression compared with basal medium group, except for the OS medium with 1 μM melatonin group. Particularly, the maximal increase was observed in OS medium with 10 nM melatonin group (Figure 4B). Western blot analysis results showed a significant increase in the protein levels of PGC-1α, NRF-1, and TFAM in OS medium with 10 nM melatonin group compared with that OS medium group and basal medium group (Figure 4C, D).

Discussion

Teeth develop through a series of morphologically distinct stages, which are commonly composed of the bud, cap, bell and finally maturation stages[34-36]. During the cap stage, the neural crest-derived ectomesenchyme cells become partly surrounded by the epithelium and form the dental papilla. During the bell stage, the DPCs which interact with the dental epithelium differentiate into odontoblasts[34]. But the mechanisms for the initiation of odontogenic differentiation is still not elucidated fully. Thus, DPCs are appropriate sources for studying the underlying mechanisms.

Recent studies have demonstrated that melatonin can modulate the differentiation of a variety of cell types, such as the pluripotent mesenchymal stem cells[17,37-39], neural stem cells[21,22], preadipocytes[19,20], spermatogenic cells[40,41], dental pulp cells[42]. In this study, we found that the expression of odontogenic-specific markers, ALP, DSPP, and DMP1 was not influenced by melatonin alone. However, osteogenic medium supplemented with melatonin caused changes in the expression of odontogenic-specific biomarkers. These results support the earlier observations in dental papilla cells[33]. Compared with the previous researches, we present more convincing evidence through the relative quantitative studies on the transcription and translation levels of the key factors related to the odontogenic differentiation, which is necessary to evaluate the effects of different concentrations of melatonin on the differentiation. Results indicate that melatonin may influence the differentiation process in differentiated odontoblasts rather than undifferentiated DPCs. Furthermore, we found that melatonin at physiological concentrations (1 pM to 10 nM) promoted differentiation in a concentration-dependent manner. In contrast, Mendivil-Perez et al[21] demonstrated that pharmacological concentrations of melatonin (25-100 μM), but not physiological concentrations (10 nM), were able to stimulate neural differentiation of neural stem cells. Zhou et al[43] showed that pharmacological concentrations of melatonin suppressed osteoclast differentiation in a concentration-dependent manner. Thus, the effect of melatonin on cellular differentiation differs depending on the cell type and concentration of melatonin treatment. Our results demonstrate that physiological concentrations of melatonin are able to regulate the differentiation of DPCs.

Although the underlying mechanisms of how melatonin influences differentiation are still poorly understood, the mitochondrial function most likely plays a role. As is well known, ATP is a kind of indispensable energy for vital activities, and mitochondria are the important sites for ATP production; therefore, ATP level is usually used as one of the indicators to evaluate mitochondrial function. NADH is one of the main electron donors in the mitochondrial respiratory chain, and its oxidation state is NAD+, NAD+/NADH represents the redox state in mitochondria, which is one of the best parameters to characterize mitochondrial function in vivo. In this study, we measured the ATP level and NAD+/NADH ratio in DPCs supplemented with melatonin to evaluate melatonin's influence

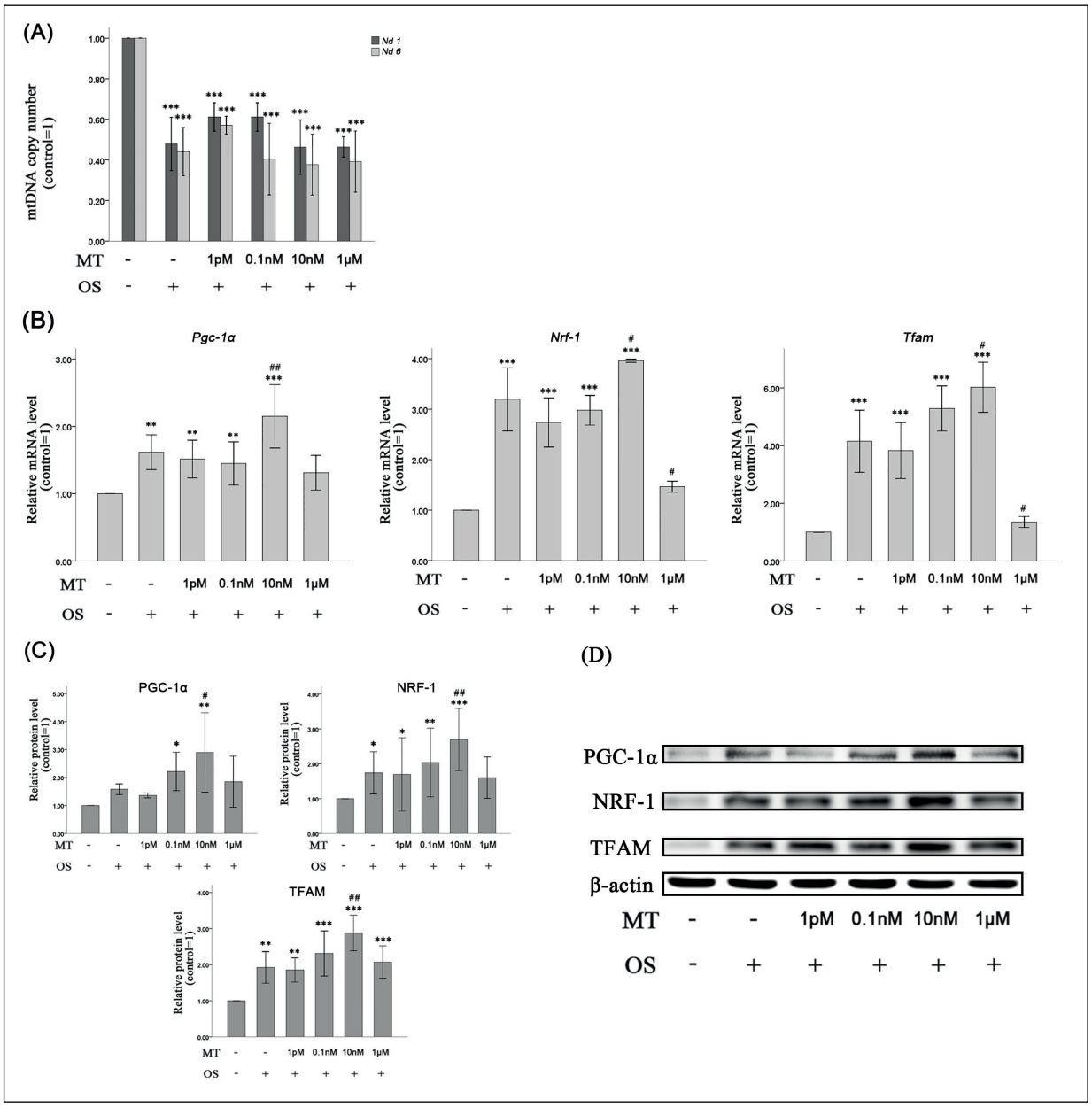

Figure 4. Effect of melatonin on mitochondrial biogenesis within DPCs during odontogenic induction. **A,** MtDNA level was measured using qPCR. **B,** MRNA levels of *Pgc-1α*, *Nrf-1*, and *Tfam* were analyzed by qPCR. **C,** Relative protein expression of PGC-1α, NRF-1, and TFAM was analyzed by Western blotting. **D,** Representative immunoblots of PGC-1α, NRF-1, and TFAM via Western blotting were shown, and β-actin was used as the internal control. Data are shown as the mean ± SD (n = 3) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs.* basal medium without melatonin group (OS-, MT-); # $p < 0.05$; ## $p < 0.01$ *vs.* odontogenic medium without melatonin group (OS+, MT-).

on aerobic respiration. Our results showed that an increase in the ATP level and a decrease in the NAD+/NADH ratio occurred during the differentiation of rat DPCs, and physiological doses (10 nM) of melatonin were able to intensify these changes. These findings demonstrated a dramatic difference in the metabolic phenotype of rat DPCs and their differentiated progenies, and moreover, physiological doses (10 nM) of melatonin supplementation positively modulated the mitochondrial respiratory function in differentiated cells. These results corroborated the findings that melatonin promoted the synthesis of respiratory chain complexes I and IV during the differentiation of dental

papilla cells[33]. Oxidative phosphorylation generates the majority of ATP within cells, but is also a major cellular source of ROS. Previously, scholars[21,44-46] have shown that there is an elevation in the ROS level during the differentiation of various cell types. Of note, in our study, a reduction of ROS level during the differentiation of rat DPCs was observed compared with the undifferentiated DPCs, especially under the condition with physiological doses (10 nM) of melatonin. In agreement, Chen et al[27,47] reported that a decrease of ROS level could be observed in human mesenchymal stem cells (hMSCs) upon osteogenic induction, which could be attributed to an increase in antioxidants, such as NADH, catalase, and manganese superoxide dismutase. The possibility that the further decline in ROS level due to melatonin (10 nM) is that melatonin is a potent free radical scavenger and a mitochondrial-targeted antioxidant[48,49]. These data suggest that melatonin may play a dual role in enhancing mitochondrial respiratory function and ROS scavenging during differentiation.

Numerous studies[25,27,50-52] have indicated that mitochondrial biogenesis is the key regulatory event in the differentiation of different cell types, including human embryonic stem cells, human mesenchymal stem cells. Notably, we found that upregulation of PGC-1α, NRF-1, and TFAM during odontogenic differentiation, and physiological concentration (10 nM) of melatonin further increased the expression. Furthermore, it's worth noting that the increase of PGC-1α, NRF-1, and TFAM during differentiation was inhibited by pharmacological concentration (1 μM) of melatonin, which suggested that melatonin may regulate mitochondrial biogenesis during odontogenic differentiation depending on the concentrations. These results corroborated the findings of the previously published work[19] regarding the preadipocytes, which demonstrated that melatonin promotes mitochondrial biogenesis during adipogenesis. The exact mechanisms by which melatonin targets mitochondrial biogenesis are not currently available. Previous studies[53-58] suggested that melatonin may initiate mitochondrial biogenesis through the AMP-activated protein kinase (AMPK)-dependent upregulation of PGC-1α expression or by the deacetylation of PGC-1α via the NAD+-dependent deacetylase Sirtuin 1.

Mitochondrial biogenesis involves numerous processes, which includes replication of mtDNA and maintenance of mitochondrial mass[32]. In our work, it was surprising that expression of the mitochondrial genes *Nd1* and *Nd6*, which reflected the replication of mtDNA, decreased at day 7 of odontogenic differentiation. In agreement, Chen et al[59] also showed that the mtDNA copy number revealed a dynamic change with an initial decline upon osteogenic induction (on day 7) in hMSCs. Although increased TFAM expression is typically associated with an increase in mtDNA transcription and replication[60], several groups reported that increased TFAM molecules had inhibitory effects on the transcription and replication of mtDNA because of extensive packaging of the mtDNA[61,62]. The discrepancy between TFAM expression and mtDNA was also observed during spontaneous differentiation of human embryonic stem cells[25]. We noticed that no decline in mtDNA occurred due to melatonin during differentiation. These findings suggest that mtDNA copy number is regulated by not only the three crucial genes associated with mitochondrial biogenesis, but also another regulator factors. Further effort should be made to investigate whether melatonin has an effect on another regulator factors associated with mtDNA copy number.

Conclusions

We demonstrated that physiological doses of melatonin contributed to the differentiation of rat DPCs, and promoted the mitochondrial energy metabolism, ROS scavenging, and mitochondrial biogenesis. In our report, we confirmed the optimal concentration of melatonin for enhancing the DPCs odontogenic differentiation and mitochondrial function and biogenesis and may facilitate the application of DPCs in tissue engineering. However, further investigation is required in order to identify the mechanism regarding the melatonin-mediated effects on mitochondrial dynamics during differentiation. Additionally, these findings provide new insight into the modulation of DPCs differentiation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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