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Effects of melatonin on the proliferation and differentiation of human dental pulp cells

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ABSTRACT

Objective: Effects of melatonin on the proliferation and differentiation of human dental pulp cells (hDPCs) remain unclear. The purpose of this study was to investigate the effect of melatonin on the proliferation and differentiation of the hDPCs.

Design: Primary hDPCs were obtained from the third molar of volunteer aged from 18 to 25. CCK8 assay evaluated the effect of melatonin upon cell proliferation at day 1, 2, 3, 4, 5. After 7 days' osteogenic induction with melatonin or vehicle, alkaline phosphatase (ALP) activity was measured with a commercial kit. Then levels of dentin sialophosphoprotein (DSPP) were determined by immunocytochemical staining and western blot analysis, followed by quantitative real-time reverse transcription-Polymerase chain reaction (qRT-PCR) to analyse mRNA levels of ALP and DSPP. Finally hDPCs exposed to osteogenic medium containing melatonin or vehicle for 14 days were stained with alizarin red to detect mineralization nodules formation.

Results: Melatonin significantly inhibited the proliferative ability of the hDPCs in a concentration- and time-dependent manner. The hDPCs cultured in osteogenic induction medium with melatonin presented an increase of ALP activity, expression of DSPP, mRNA levels of ALP and DSPP, and mineralization nodules formation.

Conclusions: These findings indicate that melatonin at physiological concentrations can inhibit proliferation and promote the differentiation of hDPCs, which might give some new insights into the mechanism of regulating DPCs to achieve dentine regeneration.

1. Introduction

Dental pulp tissue contains abundant dental pulp stem/progenitor cells of mesenchymal origin. The dental pulp cells (DPCs) have a potential for pluripotency (Gronthos et al., 2002; Morito et al., 2009; Werle, Chagastelles, Pranke, & Casagrandea, 2016) and can differentiate into dontoblast-like cells, which synthesize and secrete the collagenous and non-collagenous proteins such as osteopontin (OPN), alkaline phosphatase (ALP) and dentin sialophosphoprotein (DSPP) to form the reparative dentin after tooth injury (Cooper et al., 2010; Couble et al., 2000). The proteins have been used as important markers of odontoblastic/osteoblastic differentiation (Couble et al., 2000). Due to their regenerative potential, DPCs are used not only as a promising resource for dental regeneration (Lee et al., 2011) but also to study diseases such as ischemic vascular diseases (Yamaguchi et al., 2015; Yoo et al., 2013) and disorders of the nervous system (Foldes et al., 2016).

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone derived from amino acid tryptophan principally produced by the pineal gland (Reiter, 1991), which was initially studied in terms of its role in endocrine physiology regulating circadian (Vanecek, 1998). A large body of evidence indicates that melatonin is involved in a wide variety of physiological and pathological processes such as anti-inflammation (J.G. Li et al., 2015), antioxidant (Li, Cao, Wang, Dong, & Chen, 2015)

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and so on. Recent studies have demonstrated that melatonin regulates proliferation and differentiation of a variety of cell types (Chu et al., 2016; Codenotti et al., 2015; Luchetti et al., 2014). For example, melatonin was reported to maintain survival of mesenchymal stem cells and promote osteogenic differentiation in an inflammatory environment (X. Liu et al., 2013). Cho' study demonstrates that melatonin can promote the differentiation of human DPCs (hDPCs) into hepatocyte (Cho, Noh, Jue, Lee, & Kim, 2015). Moreover, our previous study has demonstrated that melatonin suppresses the proliferation and promotes odontogenic differentiation of rat dental papilla cells (J. Liu et al., 2013). However, effects of melatonin on hDPCs involved in proliferation and odontogenic differentiation still remain to be explored.

The aim of this study was thus to examine the in vitro response of hDPCs to melatonin. Proliferative and mineralizing activities of hDPCs induced by osteogenic medium were evaluated. The current study was undertaken to advance the understanding of the observed effects of melatonin and might give some new insights into the mechanism of regulating hDPCs.

2. Materials and methods

This experiment was approved by the Ethical Review Committee of Guanghua School of Stomatology, Hospital of Stomatology, Institute of Stomatological Research, Sun Yat-Sen University. And written informed consent was obtained from all subjects.

2.1. Cell cultures and identification

HDPCs were collected from healthy, non-carious human third molars extracted from patients aged 18 to 25 at the Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University. After collection, teeth were washed with 70% ethanol and then rinsed with sterile phosphate-buffered saline (PBS, pH 7.4).The pulp tissues were retrieved from pulp chamber aseptically and minced into small clumps and digested with solution of 3 mg/ml collagenase type I for 20 min at 37 °C. Following digestion, the pulpal tissue explants were cultured in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and antibiotics (10U/L penicillin and 100 mg/L streptomycin) in an atmosphere containing95% O_2 and5% CO_2 at 37 °C with medium change every 3 days. Upon 80% confluences, cells were digested using 0.25% trypsin with 0.25 mmol/L EDTA and serially passaged for further experiments. Distinct digestion method was applied to purify cells. Passages 3 and 5 were used for all experiments. Immunocytochemical stainings for cytokeratin (an intermediate filaments present in all epithelial cells , 1 : 100, BOSTER, China) and vimentin (an intermediate filament protein expressed in mesenchymal cells , 1 : 100, BOSTER, China) were performed to identify the origin of hDPCs. HEK293T cells stained for cytokeratin served as positive control and the two primary antibodies replaced by PBS served as negative control. Otherwise, several mesenchymal markers (CD44, CD73, CD90, CD105) were performed in hDPCs by flow cytometry (Beckman Coulter, USA). All other reagents were purchased from Gibco unless otherwise stated.

2.2. Cell proliferation assay

Cell proliferation was analyzed by Cell Counting Kit-8 (CCK8, Dojindo, Japan). The hDPCs were seeded in 96-well plates at a density of 2×10^3 cells/well and allowed to adhere for 24 h prior to supplementation with varying concentrations of melatonin (Sigma, St Louis, MO, USA) (0, 10^{-12}, 10^{-10}, 10^{-8}M). After 1, 2, 3, 4, 5 days, the media were removed and cells were treated with 10% CCK-8 in new media for 2 h at 37 °C. Absorbance at 450 nm was measured using a spectrophotometer (Tecan, Grodig, Austria). Each experiment was performed in four parallel wells, and the mean optical density (OD) values were measured. All samples were run in quadruplicate with data for 3 independent donors collected together.

2.3. Quantitative assay of alkaline phosphatase (ALP) activity

To determine melatonin induced differentiation of hDPCs, ALP activity was assessed. The hDPCswere seeded into 96-well plates at a density of 3×10^3 cells/well and exposed to (OS-M-), (OS-M +), (OS + M–), (OS + M +) for 7 days. Two of the treatment groups consisted of basal growth medium (OS-) containing either 0.05% ethanol vehicle (OS-M-) or 10^{-8}M melatonin (OS-M +). The remaining two groups were exposed to osteogenic medium (OS +) in the absence (OS + M-) or presence (OS + M +) of melatonin. ALP activity was measured using a commercial ALP kit (Abcam, UK) according to the manufacturer's instructions. The cells were lysed, extracted and incubated with 0.5 mg/mL *p*-nitrophenyl phosphate solution. The production of *p*-nitrophenol in the presence of ALP was measured by monitoring light absorbance at 405 nm using a spectrophotometer. Each experiment was performed in four parallel wells, and OD values were measured. All samples were run in quadruplicate with data for 3 independent donors collected together.

2.4. Immunocytochemistry and western blot analysis for dentin sialophosphoprotein (DSPP)

Cells were seeded in 48-well culture plates at a cell density of 1×10^4 cells/well and after 24 h incubation they were exposed to the following conditions for 7 days: (OS-M-), (OS-M +), (OS + M–), (OS + M +). Subsequently cells were fixed with ice-cold 4% paraformaldehyde for 20 min and permeablized with 0.3% Triton X-100 for 10 min. Nonspecific binding was blocked with 10% goat serum for 20 min, and then primary antibody DSPP (1:50, mouse monoclonal antibody, Santa Cruz, USA) was added at 4 °C overnight. After removal of primary antibody solution, cells were washed with PBS three times and were incubated with peroxidase-conjugated goat anti-mouse polyvalent antibody for 1 h at room temperature. The immunoreactive product was visualized by incubation with 3,3-diaminobenzidin (DAB). The images were viewed with an inverted phase contrast microscope Axiovert 40 (Zeiss, Jena, Germany) and analyzed with Image J (NIH, USA), by which the relative optical density was obtained.

Cells were seeded in 6-well plates at a cell density of 1×10^5 cells/well and treated the same as above for 7 days. Then Cells were lysed in RIPA buffer containing protease inhibitor and the protein concentration was measured using the BCA protein assay (Beyotime, China). Forty micrograms of total protein was separated by 10% SDS–PAGE electrophoresis, then transferred to PVDF membrane, followed by blocking in 5% skim milk in TBST buffer at room temperature for 1 h. Membranes were incubated with anti-DSPP antibody (1:500) and anti-GAPDH antibody (1:5000, Proteintech, USA) overnight at 4 °C and then with secondary antibodies to mouse (1:5000, LI-COR, USA) at room temperature for 1 h. Finally the bands were detected on an Odyssey infrared imaging system (Odyssey LI-COR Biosciences, Lincoln, NE). GAPDH was measured as an internal control. The relative optical density which stands for the relative protein expression levels in images was analyzed with Image J (NIH, USA).

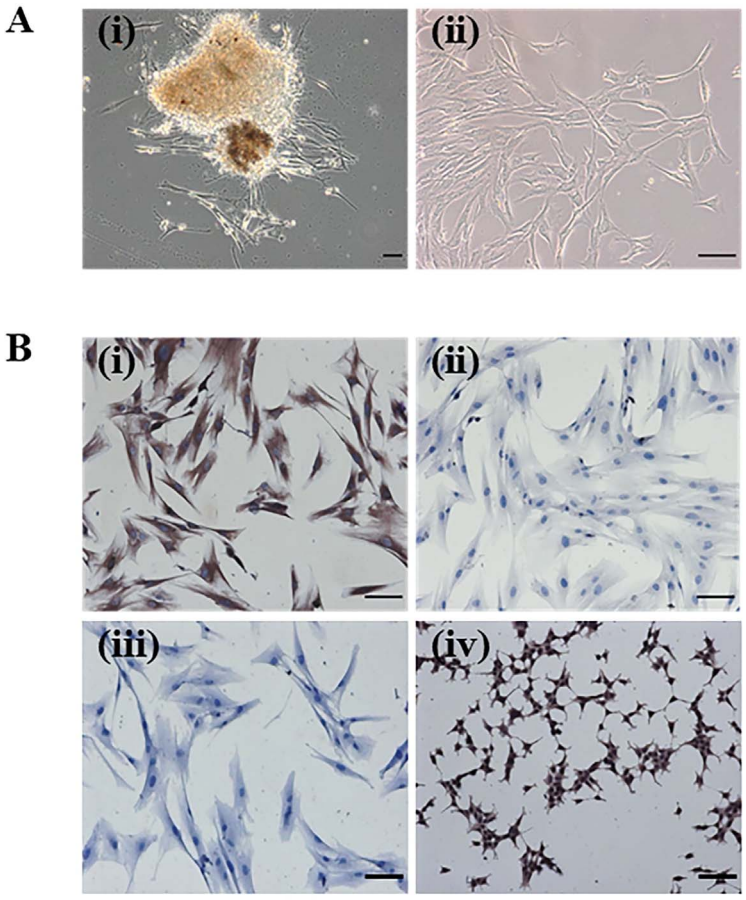
2.5. Quantitative real-time reverse transcription-Polymerase chain reaction (qRT-PCR)

HDPCs of passage 3 were seeded and cultured in osteogenic medium with melatonin (10^{-8}M) or vehicle for 7 days, namely the same treatments and groups as in Section 2.4. Subsequently total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. 1 μg of RNA and transcriptor first strand cDNA synthesis kit (Roche) was used for cDNA synthesis.Then cDNA was amplified in a final volume of 20 μL containing 6 μL of DNase/RNase free water, 10 μLof SYBR Green I Master (Roche) and 0.5 μM of specific

Table 1
Primer sequences involved for RT-PCR.

Genes	GeneBank No.	Sequences (5'-3')	Product size (bp)
ALP	XM_017000903.1	F: TAAGGACATCGCCTACCAGCTC R: TCTTCCAGGTGTCAACGAGGT	170
DSPP	NM_014208.3	F: AGTTCCTCAAAGCAAACCACTG R: CCTCCTACTTCTGCCCACTTAG	219
GAPDH	NM_001289746.1	F: GAGTCAACGGATTTGGTCGT R: TTGATTTTGGAGGGATCTCG	238

primers, which were detailed in Table 1.Thermocycling conditions consisted of 95 °C for 5m, 35 cycles at 95 °C for 10 s, at 60 °C for 20 s, and at 72 °C for 20s, followed by 1 cycle at 95 °C for 5s, at 65 °C for 1 m, and at 97 °C, and then a final 10 s cooling at 40 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was applied as a housekeeping gene as a reference. The final data for gene expression of ALP and DSPP were analyzed by using the ΔΔCt method.



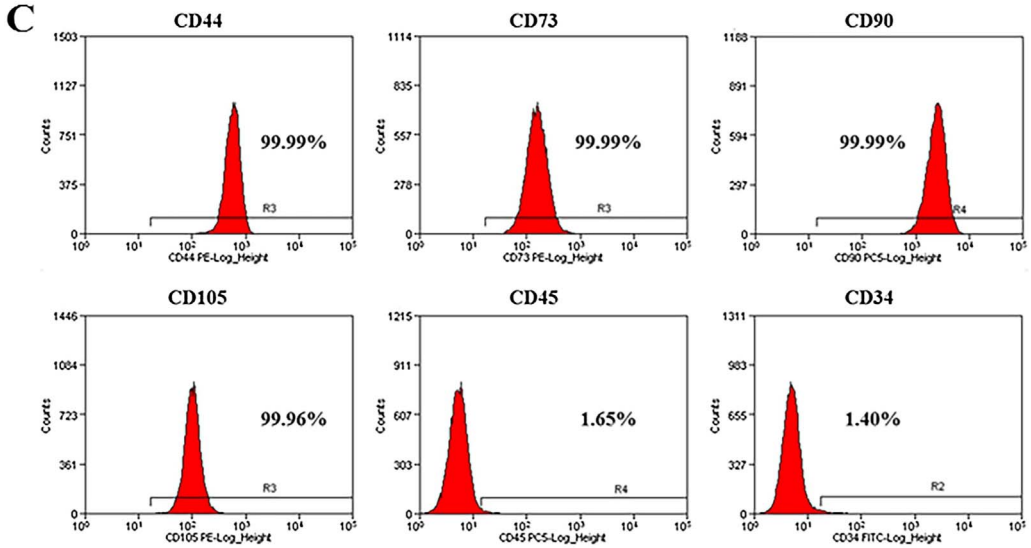


Fig. 1. Human dental pulp cells (hDPCs)' isolation, culture and identification. (A) HDPCs' primary culture: cells were migrated from pulp tissue block after about 7 days' culture, whose morphology was typical of fibroblast-like cells (i) and hDPCs grew to near confluent state after 14 days' incubation (ii). (B) Identification of hDPCs: all cells showed positive immunostaining for vimentin (i) and were negative for cytokeratin (ii). PBS replaced the primary antibodies served as negative control (iii) and HEK293T cells stained for cytokeratin served as positive control (iv). (C) Cell surface markers of hDPCs: cells were positive for CD44, CD73, CD90 and CD105 while negative for CD34 and CD45. Scale bars = 100 μm.

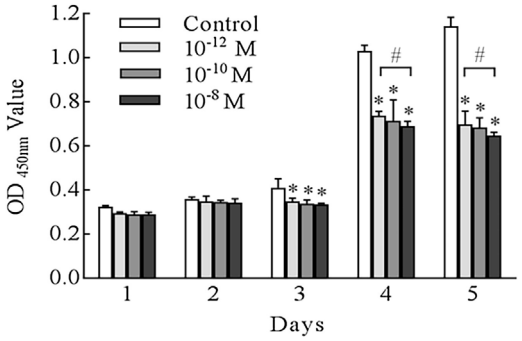


Fig. 2. Effect of melatonin on hDPCs' proliferation. Cells were treated with various concentrations of melatonin (0, 10^{-12}, 10^{-10}, 10^{-8}M) for 1, 2, 3, 4, 5 days respectively. Subsequently the cells viability was measured by CCK8 method. Each value is presented as mean ± SD for triplicate cultures. (*$p < 0.05$, compared to control, #$p < 0.05$, compared to 10^{-8}M).

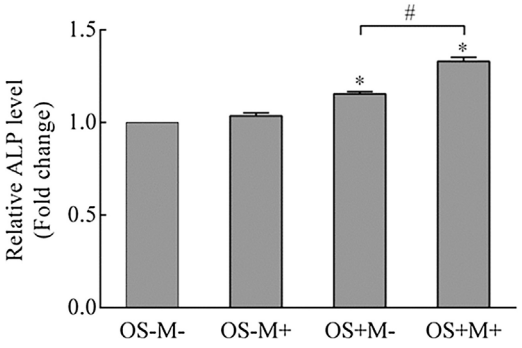


Fig. 3. Alkaline phosphatase (ALP) activity in hDPCs under melatonin treatments was determined by colorimetric assay on day 7. Each value is presented as mean ± SD for triplicate experiments. (*$p < 0.05$, compared to OS-M-, #$p < 0.05$, compared to OS + M-).

2.6. Alizarin red S staining and calcium concentration determination

To assess the effect of melatonin on hDPCs' mineralization in vitro, cells were inoculated into a 6-well plate and cultured in conditional media with the same grouping as above. After 14 days, cells were rinsed with PBS and fixed in 4% paraformaldehyde for 20 min, then stained with 1% Alizarin Red S solution (Cyagen, China) for 5 min at room temperature. After being washed with PBSto remove unbound stains, cells were air-dried before being photographed. For quantitative evaluation, stain was extracted with 100 nM cetylpyridinum chloride (Sigma-Aldrich, USA) for 10 min at room temperature, and the absorbance of the supernatant was measured at 562 nm. The data were collected from three same conditioned wells independently.

2.7. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical difference was analyzed by one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered to be significant. Statistical analysis was performed using SPSS 19.0 (IBM Corp. USA).

3. Results

The small pieces of pulp tissue were cultured, from which spindle-like shaped hDPCs with extensive cellular processes grew out by day 7 (Fig. 1A (i)). Cells were subcultured and further purified by distinct digestion in vitro, then used for functional experiments once they grew to complete confluence (Fig. 1A (ii)). HDPCs involved in this study showed a positive vimentin and a negative cytokeratin staining by the method of immunocytochemistry (Fig. 1B (i), (ii)). Negative control which replaced the primary antibodie with PBS and Positive control

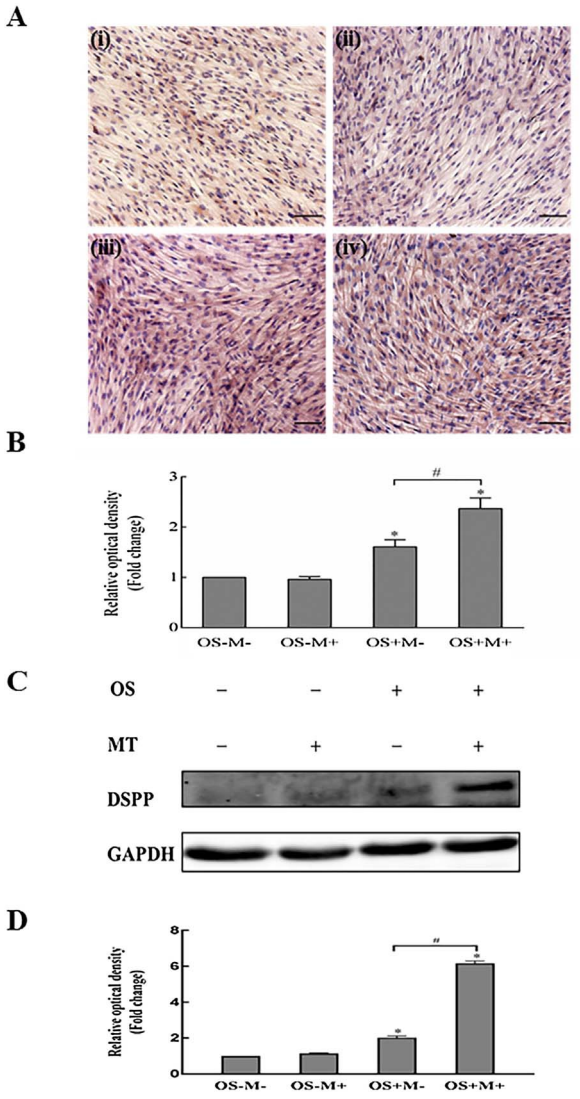


Fig. 4. Effect of melatonin on protein levels of dentin sialophosphoprotein (DSPP) in hDPCs.
(A) Results of immunocytochemistry for DSPP in the (OS-M-) (i), (OS-M +) (ii), (OS + M–) (iii), (OS + M +) (iv) group. (B).The relative optical density was examined by Image J. (C) Results of western blot analysis for DSPP in five groups. (D) The relative optical density was examined by Image J (ii). Each value is presented as mean ± SD. (*$p < 0.05$, compared to OS-M-, #$p < 0.05$, compared to OS + M-). Scale bars = 100 μm.

which used HEK293T cells stained for cytokeratin were shown in Fig. 1B (iii) and (iv) respectively. Besides hDPCs involved were positively expressed mesenchymal markers, such as CD44, CD73, CD90 and CD105, while negatively expressed hematopoietic cell markers, such as CD34 and CD45 (Fig. 1C).

The CCK-8 results showed that hDPCs incubated with melatonin (10^{-12}, 10^{-10}, 10^{-8}M) had a significantly lower proliferation rate than that of the control group at days 3, 4 and 5 of culture in a concentration- and time-dependent manner (*$p < 0.05$) (Fig. 2).

The differentiation potential of hDPCs with melatonin was measured by performing ALP activity, protein level of odontoblast markers (DSPP) assay, qRT-PCR for gene expression of ALP and DSPP, and mineralized nodule formation. As shown in Fig. 3, the ALP activity increased significantly (*$p < 0.05$) in osteogenic medium supplemented with vehicle (OS + M-) or melatonin (OS + M +) when compared with cells exposed to growth medium containing vehicle (OS-M-) or melatonin (OS-M +). Melatonin added to basal medium (OS-M +) did not significantly increase ALP activity in the vehicle group (OS-M-). But

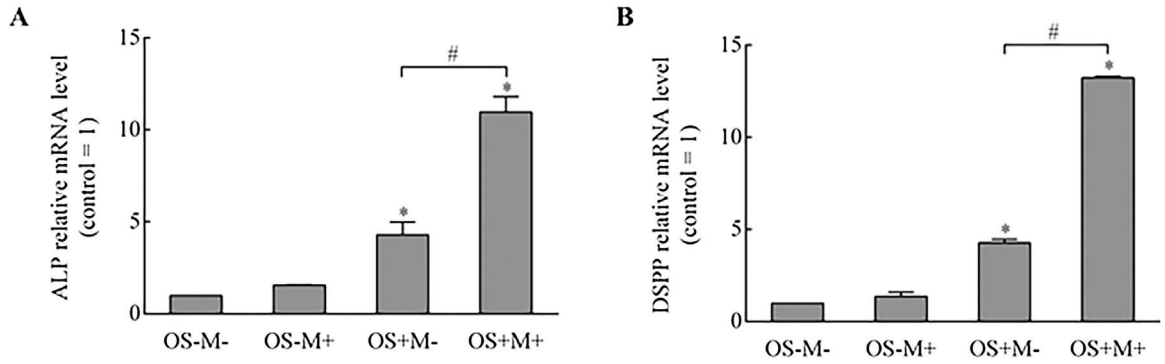
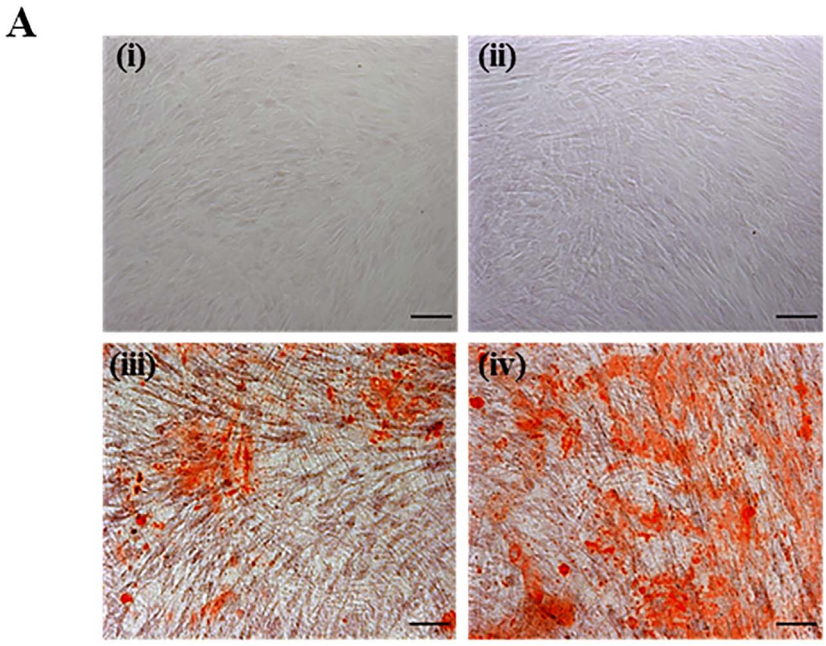


Fig. 5. Effect of melatonin on mRNA levels of ALP and DSPP in hDPCs.
The hDPCs of passage 3 were exposed in conditions as follows: (OS-M-), (OS-M +), (OS + M–) and (OS + M +) for 7 days. (A, B) The mRNA levels of ALP and DSPP were evaluated by qRT-PCR analysis. Each value is presented as mean ± SD. ($^{*}p < 0.05$, compared to OS-M-, $^{\#}p < 0.05$, compared to OS + M-).



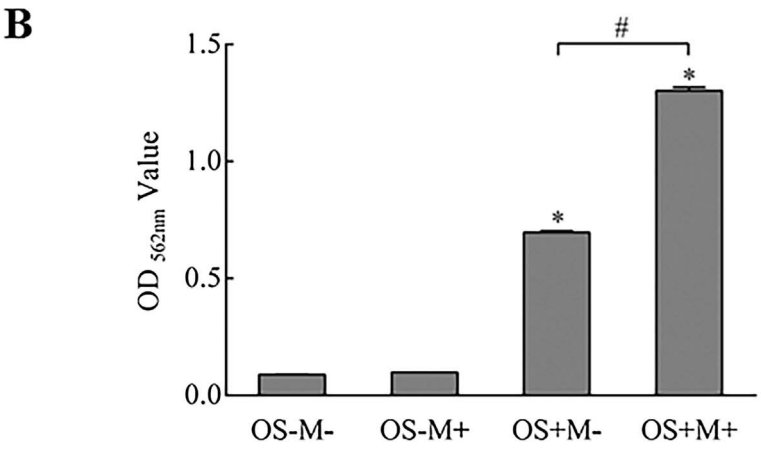


Fig. 6. Effect of melatonin on the mineralized nodules formation in hDPCs.
Cells were incubated with the condition medium (OS-M- (i), OS-M + (ii), OS + M– (iii), OS + M + (iv)) for 14 days. (B) Quantification of Alizarin red S of hDPCs cultures. Each value is presented as mean ± SD. ($^{*}p < 0.05$, compared to OS-M-, $^{\#}p < 0.05$, compared to OS + M-). Scale bars = 100 μm.

when cells were incubated with melatonin in combination with the osteogenic medium (OS + M +), higher ALP activity was detected compared to that of cells exposed to osteogenic medium with vehicle (OS + M-) ($^{\#}p < 0.05$). The results of both immunocytochemistry and western blot showed that melatonin significantly increased DSPP protein expression of hDPCs in osteogenic medium compared with the cells in basal medium (Fig. 4A, B, C, D). qRT-PCR analysis revealed that growth medium containing melatonin (OS-M +) did not increase mRNA levels of ALP and DSPP, while osteogenic medium with vehicle (OS + M-) could increase mRNA levels of ALP and DSPP, but the relative value is smaller than the melatonin in combination with the osteogenic medium group (OS + M +) ($^{\#}p < 0.05$, Fig. 5A, B). At day 14 the mineralized nodules formation was increased significantly in the group containing osteogenic medium with vehicle than in the group

containing osteogenic medium with melatonin ($^{\#}p < 0.05$, Fig. 6C, D), which was not significantly affected in the group containing basal medium with vehicle or melatonin (Fig. 6A, B).

4. Discussion

The present data demonstrate that melatonin significantly inhibited the proliferative ability of the hDPCs in a concentration- and time-dependent manner. There are some studies showing that melatonin modulates the proliferative ability of different kinds of cells. For example, melatonin has been shown to inhibit the proliferation of human breast cancer cells (Dai, Inscho, Yuan, & Hill, 2002; Mao et al., 2010) and umbilical vein endothelial cells (Alvarez-Garcia, Gonzalez, Alonso-Gonzalez, Martinez-Campa, & Cos, 2013) in vitro. The present results agree with previous findings (Liu et al., 2013) which showed that melatonin suppresses the proliferation of rat dental papilla cells. In contrast, some studies have suggested that melatonin induces proliferation in Schwann cell (Chang et al., 2014) and normal human bone cells (Nakade, Koyama, Ariji, Yajima, & Kaku, 1999; Satomura et al., 2007). In the present study, melatonin's proliferation-inhibting effect on hDPCs is at physiological concentrations (10^{-12}-10^{-8}M). Pharmacological concentrations of melatonin (10^{-6}–10^{-4}M) failed to inhibit human osteoblastic cell line hFOB 1.19 proliferation (Xiong, Zhu, Ge, Liu & Yuan, 2015). These evidences indicate that melatonin's inhibiting effect at physiological concentrations in vitro may be specific only for certain types of cells. The exact mechanism remains to be determined.

Teeth involved are collected from the third molar of the healthy young adult, where dental pulp is obtained. Generally hDPCs is derived from mesenchymal tissue, which was confirmed by immunocytochemistry and flow cytometry in this study. And it is composed of various cells including immunocompetent cells, undifferentiated mesenchymal cells and stem cells (B.N. Lee et al., 2016), which can be induced to differentiate into odontoblast-like cells and form dentine-like mineral structures in vitro (Lee, Kang, Mahapatra, & Kim, 2016). To estimate the effects of melatonin on the odontogenic differentiation of hDPCs, we determined ALP activity, protein levels of DSPP, mRNA level of ALP and DSPP as markers of odontogenic differentiation. The results showed that melatonin-treated hDPCs exhibited much higher activity and expression of odontogenic differentiation, which indicated that melatonin plays a promoting role in the mineralization of reparative dentine. There are some studies showing that melatonin at pharmacological doses enhances human osteoblastic differentiation in vitro and promotes mouse cortical bone formation in vivo (Satomura et al., 2007). In addition, experiments show that melatonin promotes the differentiation of precursor cells to neuronal cells (Sotthibundhu, Phansuwan-Pujito, & Govitrapong, 2010). Conversely, some investigators report that melatonin at high concentrations can inhibit the differentiation of chondrocytes (Zhong et al., 2013). Indeed it has been recently shown that physiological concentrations of melatonin blocked the differentiation of T cells in human and mouse (Farez et al., 2015). As mentioned above, the obtained results from animal and cellular studies are inconsistent. We did not determine the effect of pharmacological concentrations of melatonin on the differentiation of hDPCs, which remains to be verified

It is well known that some important effects of melatonin are mediated through activation of specific receptors including two membrane-associated receptors (MT1 and MT2 receptor) and the other two nuclear receptors (Becker-Andre et al., 1994; Dubocovich & Markowska, 2005; Mailliet et al., 2005). In the present study, we did not detect melatonin receptors in hDPCs, although immunohistochemical analysis revealed that MT1 receptor was expressed in secretory ameloblasts, the cells of the stratum intermedium and stellate reticulum, external dental epithelial cells, odontoblasts, and dental sac cells (Kumasaka et al., 2010). Thus, the effects of melatonin on proliferation and differentiation of hDPCs might be mediated by a non-receptor-dependent pathway. As an indoleamine, melatonin can play a role in cell growth regulation through some other signaling pathways, which is confirmed in SK-MEL-1 cells (Cabrera, Negrin, Estevez, Loro, Reiter & Quintana, 2010) and colon cancer cells (Wang et al., 2013). Much is known about the role of melatonin in regulating cell proliferation and differentiation, but the mechanisms underlying melatonin's actions on hDPCs should be elucidated further in our study of the future.

In summary, this study demonstrates that melatonin at physiological concentrations can inhibit proliferation and promote the odontogenic differentiation of hDPCs. The effects of melatonin on hDPCs might give some new insights into the mechanism of regulating hDPCs to achieve dentine regeneration and promote specific cell-oriented differentiation to restore injured tissue. Therefore, given these effects on hDPCs, the consequence of melatonin-treated hDPCs needs to be considered, and whether melatonin has therapeutic potential warrants further investigation.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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