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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