To explore the influencing factors of amelogenesis imperfecta: the effect of melatonin on ALC cell line

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Research Article

Keywords: Melatonin, ALC cell line, enamel growth, Amelogenesis imperfecta, Wnt pathway

Posted Date: February 9th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1321628/v1>

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Abstract

Objective: Amelogenesis imperfecta is a common oral disease. In this study, ALC cells were cultured in vitro to study the factors affecting the growth and development of enamel to explore the influencing factors of amelogenesis imperfecta.

Methods: The effects of melatonin on the proliferation and differentiation of ALC cells were detected by CCK8, qRT-PCR, and alizarin red staining, etc. RNA-seq and qRT-PCR were used to identify the differentially expressed genes in ALC cells after melatonin treatment. Then, we focused on observing the important factors of the signaling pathway.

Results: Melatonin inhibited the proliferation of ALC cells in a dose-dependent manner and promoted the production of actin fibers. High concentration of melatonin significantly promoted the expression of enamel development related proteins and the formation of mineralized nodules. RNA-seq data showed that melatonin may induce transcriptional changes of Wnt signaling pathway in ALC cells.

Conclusion: This study suggests that melatonin plays a regulatory role in the proliferation, differentiation, and mineralization of the enamel, which may be one of the causes of enamel hypoplasia. Moreover, the main factors of the Wnt signaling pathway may play an important role in the process of melatonin in the growth and development of ALC lines.

Introduction

Amelogenesis imperfecta (AI), including enamel hypoplasia and hypomineralized enamel, is the most common dental dysplasia in the oral cavity. It will significantly increase the incidence of oral diseases such as caries, tooth fracture and tooth defect, and seriously affect the beauty, function and mental health of patients[1]. Among them, Enamel hypoplasia is due to the abnormality of ameloblasts, which can not produce a normal amount of enamel matrix. Clinically, it describes the decrease or even complete disappearance of enamel thickness[2]. Hypomineralized enamel is due to the failure of ameloblasts to completely calcify the matrix formed, and the clinical manifestation is that the enamel is white and opaque[3].

The etiology of enamel hypoplasia is complex. At present, the research on AI genetic factors has made a breakthrough. These discoveries have expanded proteins mutated in AI, from solely structural enamel matrix proteins, such as AMELX, ENAM and AMBN, to a series of other proteins, involved in diverse functions, such as cell adhesion[2, 4]. Environmental factors, such as severe nutritional disorders, endocrine disorders and periapical infection, will interfere with the normal development and mineralization of enamel [5]. For example, molar incisor hypoplasia (MIH), which affects only permanent incisors and first molars, may be caused by prenatal or early child exposure to endocrine disruptors [6]. The loss of circadian rhythm leads to the low degree of enamel mineralization in BALB/c neonatal mice[7].

Melatonin (MT) is mainly secreted by the pineal gland in the brain and is regulated by the suprachiasmatic nucleus (SCN). It is one of the neuroendogenous hormones affecting the biological cycle [8, 9]. It not only regulates the periodic rhythm, body temperature [10], and blood pressure [11], but also plays a role as an anti-cancer agent [12], and regulates endocrine system activities [13]. Melatonin produces a marked effect by activating the high-affinity melatonin receptor (MR) in target tissues. MT1 and MT2 are the two subtypes of MR in humans and mammals.

According to the research report, MR is expressed in human tooth germ and mouse tooth epithelial cell line HAT-7 [14], and when the suprachiasmatic nucleus with high expression of melatonin receptor in experimental animals is removed, the circadian growth and development of teeth immediately disappears [15]. Therefore, we put forward the hypothesis for the first time that the rhythm change of melatonin and its receptor may be one of the mechanisms of tooth dysplasia [16]. In order to test the hypothesis, we found that after changing the circadian rhythm of BALB/c pregnant mice, the protein levels of MT1 and MT2 in neonatal mouse tooth germs changed in varying degrees. We injected melatonin receptor antagonist into the peritoneum of BALB/c pregnant mice. It was found that the ameloblasts of mice at 7 and 10 days after birth were widely denatured, the degree of enamel mineralization was low, and the content of amelogenin was significantly reduced [7]. The previous experimental results show for the first time that melatonin and its receptor system are related to the periodic growth and development of enamel. However, we have not conducted in vitro experiments on melatonin treated ameloblasts, and have not explored the amelogenesis imperfecta etiology of from the cellular level.

The ameloblast-lineage cell line (ALC) was originally obtained from the organ of mandibular molar tooth germ of newborn (C57BL/6J) mice by Akira Nakata et al. [17]. ALC cells are a spontaneously immortalized ameloblast cell line and are thought to be in the early stages of maturation [17, 18]. They consist of enamel epithelioid structure formation, have ameloblast characteristics, and express amelogenin-specific genes, such as amelogenin X-linked (AMELX), enamelin (ENAM), and odontogenic ameloblast-associated protein (ODAM). AMELX and ENAM are two proteins that play important roles in the development of enamel in mammals [19]. ODAM is relatively small in amount but still plays a regulatory role [20]. All of them participate in the nucleation process of hydroxyapatite and promote enamel mineralization of enamel [21, 22]. An increase in the secretion, causes the enamel layer to gradually thicken [23].

Wnt signaling pathways were reported to play a key role in the differentiation and mineralization of early enamel cells [24]. The Wnt signaling pathway can be divided into the β -catenin-dependent canonical pathway and β -catenin-independent pathway. In the canonical pathway, ligands, such as WNT protein WNT-1 and WNT-10a, 10b, FZD protein, and low-density lipoprotein receptor-related protein (LRP), constitute complexes that inhibit the degradation of β -catenin in cells. As the concentration of β -catenin increases, LEF in the nucleus is activated, which then transmits information through downstream target genes Cyclin D1, C-myc, and C-Jun [25, 26], to promote the growth and development of the tooth enamel [27].

In this study, the effects of melatonin on the proliferation, differentiation, and mineralization in ALC was studied in vitro. The effects of Luzindole, a melatonin receptor antagonist, on ALC cells were also studied. The mechanism of action of melatonin on ALC was preliminarily explored to provide new ideas for the study of the tooth development.

Materials And Methods

Cell culture and treatment

ALC, an ameloblast line derived from the mandibular molar tooth germ of C57BL/6J mice, was obtained from the Ninth People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine (Shanghai, China). The appearance of epithelial cells indicates heterogeneous cell changes in ameloblasts, including differentiation, secretion, transition, and maturation [18]. ALC cells were cultured in a standard Dulbecco's modified Eagle's medium (DMEM, high glucose, no phosphates, Invitrogen, USA) containing 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin complex (Invitrogen) at 37°C in a humidified atmosphere under 5% CO₂ in the air. The medium was changed twice a week. When the cells reached 70% confluence, they were digested, counted, and transferred to 10 cm culture dishes.

Cell Proliferation Assay

ALC cells were seeded in 96 well plates at a density of 1×10^4 cells/well, and cultured in a standard medium (DMEM as described earlier) at various concentrations of melatonin medium (standard medium containing 10% FBS and 10^{-10} , 10^{-8} , 10^{-6} , 10^{-4} , and 10^{-3} M of melatonin) [Sigma-Aldrich, USA] for 24, 48, 72, and 96 h. Among them, the solution was changed every 48 h with the same concentration. At each scheduled time, 10 µl CCK-8 solution (Yeasen, Shanghai, China) was added into each well under dark conditions, and then incubated at 37°C for 2 h. The absorbance at 450 nm was determined using a microplate reader (Tecan, Austria).

Laser scanning confocal microscopy

The morphology of the cells was observed using a laser scanning confocal microscope (LSCM). ALC cells were seeded into 24 well plates (1×10^4 cells/well) and cultured for 48 or 96 hours in a standard medium containing melatonin concentrations of 0, 10^{-10} , 10^{-8} , 10^{-6} , 10^{-4} , and 10^{-3} M respectively. At the planned time, the samples were fixed in 4% paraformaldehyde, the membrane was broken with 0.5% Triton X-100, and then stained with rhodamine-labeled phalloidin (1 µg/ml; Sigma Aldrich) and 4,6-diamino-2-phenylindole (DAPI, 1 µg/ml), and finally sealed with Antifade Mounting Medium. LSCM (FV1200; Olympus Company, Japan) was used to observe the samples. The number and morphology of ALC cells were analyzed at a scale of 50 µM.

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

qRT-PCR was used to detect the expression of melatonin receptors, such as the melatonin 1a receptor (MT1, Mel1aR) and melatonin 1b receptor (MT2, Mel1bR), along with genes related to enamel formation (AMELX, ENAM, and ODAM). The cells were incubated in a 12-well plate at the concentration of 1×10^5 cells/well for 24 hours, the media were removed and cells were treated with a standard medium containing various concentrations (0, 10^{-4} , and 10^{-3} M) of melatonin, and cultured for 1, 2, 3, 4, and 5 days. Following the manufacturer's instructions, total RNA was extracted and isolated with Invitrogen TRIZOL reagents (Thermo Fisher Scientific, Germany). cDNA synthesis was translated from 1 µg total RNA using Hifair® II 1st Strand cDNA Synthesis SuperMix (Yeasten). Then, cDNA was amplified in a 20 µL reaction mixture using Hieff UNICON® qPCR SYBR Green Master Mix (low Rox, Yeasen). The final volume of 20 µL contained 10 µL of SYBR Green Master Mix (Low Rox), 7.2 µL of DNase/RNase-free water, and 0.2 µM of specific primers. Their sequences are detailed in Table 1. Thermocycling conditions consisted of 95°C for 5 min, 40 cycles of 95°C for 10s and 60°C for 20s each, followed by 1 cycle at 95°C for 15s, 65°C for 1 min, 95°C for 15s, and then stored at 4°C. The β-ACTIN gene was used as a housekeeping gene to compare the quantity and quality of cDNA. Finally, the relative expression of the gene to be tested was determined by the $\Delta\Delta C_t$ method.

Table 1
Primer sequences used for qRT-PCR.

Genes	GeneBank No.	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	Products size (bp)
MT1	NM_026642	GAGTCTGTAGTTGTCGCAGTTG GGCCTTTTTTGAAGGAAGAGGA	246
MT2	NM_205822	GTTATGGCCCTGTTTGTGAGA CAATAGTTCCGGCCTCAAATCAA	125
AMELX	NM_001081978	GATGGCTGCACCACCAAATC CTGAAGGGTGTGACTCGGG	65
ENAM	NM_017468	TGCAGAAATCCGACTTCTCCT CATCTGGAATGGCATGGCA	114
ODAM	NM_027128	ATCAATTTGGATTTCGCACCACC AGTTGGATCTATCCCAGAAGTGA	242
WNT4	NM_009523	AGACGTGCGAGAAACTCAAAG GGAAGTGGTATTGGCACTCCT	126
WNT6	NM_009526	GCAAGACTGGGGGTTCGAG CCTGACAACCACACTGTAGGAG	202
β -catenin	NM_001081655	GTGTACCCATATCCCAGCCC TCCTGCCCCACATCTCTCAG	239
LEF	NM_172143	ATGGTTAAAGAATACACGGACGC CTGGCAGCCTGGATTTTTGT	163
Cyclin D1	NM_026562	ATGGAAGGACCCTTGAGGC CTTCACGGCTTGCTCGTTCT	123
c-Jun	AJ315350	GTCCTCCATAAATGCCTGTTCC GATGCAACCCACTGACCAGAT	60
β -ACTIN	NM_027493	ATGACCCAAGCCGAGAAGG CGGCCAAGTCTTAGAGTTGTTG	185

Quantitative assay of alkaline phosphatase (ALP) activity

The differentiation and mineralization of ALC cells were detected by ALP activity. ALC cells were seeded into 6-well plates at a density of 1×10^5 cells/well, and then cultured in different concentrations of

melatonin medium (0, 100, 200, 500, and 1000 μ M) for 7 days. ALP activity of samples cultured for 7 days was measured with the Alkaline Phosphatase Assay Kit (Beyotime Biotechnology) according to the manufacturer's instructions. After cell lysis and extraction, the cells were incubated with 10 μ l p-nitrophenol solution (10mM) for 30 min. In the presence of alkaline phosphatase, the absorbance at 450 nm was monitored by a microplate reader.

Alizarin red S staining

Alizarin red S staining was used to examine the effects of melatonin and its receptor inhibitor, luzindole, on mineralized matrix formation in ALC cells. The cells were inoculated in 6-well plates at a density of 1×10^5 cells/well for 24 h. Half of the samples were cultured in different concentrations of melatonin medium (the same grouping conditions as the fifth paragraph), and the other half of the samples were exposed to (L-M-), (L+M-), (L-M+), or (L+M+) for 28 days. Among them, the control group utilized the standard medium (L-M-), and the three treatment groups involved the standard medium containing luzindole (L+M-), 10^{-3} M melatonin (L-M+), or both (L+M+). After 28 days, the cells were fixed with 4% paraformaldehyde for 30 min and stained with alizarin red solution for 10 min at room temperature. After the staining was cleaned with distilled water, the cells were air-dried. Images were taken using an inverted phase-contrast microscope (Leica Microsystems, Germany).

RNA Sequencing and qRT-PCR

Shanghai Majorbio Bio-pharm Technology was responsible for RNA isolation, cDNA library construction, sequencing, and transcriptome data analysis. ALC cells were seeded into 60 mm culture dishes at a density of 2×10^5 cells/well, cultured in standard medium and melatonin medium supplemented with 10^{-3} M for 3 days, and RNA was extracted with TRIzol. ND-2000 (NanoDrop Technologies) and 2100 Bioanalyzer (Agilent) methods were used to determine the quality of RNA samples, to ensure the use of qualified samples ($OD_{260/280} = 1.8-2.2$, $OD_{260/230} \geq 2.0$, $Rin \geq 6.5$, $28S:18S \geq 1.0$, $>2 \mu$ g) for transcriptome sequencing library. The RNA library prepared by TruSeqTM RNA sample preparation Kit from Illumina (San Diego, CA) was sequenced with an Illumina HiSeqxten/NovaSeq 6000 sequencer. The reading length of the sequence was PE 150. The clean reads were then compared with the reference genome using TopHat software (<http://tophat.cbcb.umd.edu/>, version 2.0.0) [28]. The R statistical package software EdgeR was used for differential expression analysis.

RT-PCR was performed using the same cDNA as that used for RNA sequencing. The primers used are listed in Table 1.

Statistical analysis

All experiments were repeated three times, and each experiment was performed in four parallel wells. All data were analyzed using SPSS ver.19.0 (IBM, Armonk, NY, USA) and expressed as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and Dunn's multiple comparison test was used to analyze statistical differences. When $p < 0.05$, the difference was considered statistically significant.

Data availability

The sequences in this study have been deposited in the NCBI-SRA under BioProject PRJNA 774042, and the accession number is SRP16555451 for mouse.

Results

Effect of Melatonin on the Proliferation of ALC cells

In order to evaluate the effect of melatonin on the proliferation of ALC cells, six concentration gradients of 0, 10^{-10} , 10^{-8} , 10^{-6} , 10^{-4} and 10^{-3} M were set up, and the absorbance value at 450 nm was detected by the CCK-8 method. Melatonin inhibited the proliferation of ALC cells in a time-dependent (Fig. 1A) and dose-dependent manner (Fig. 1B).

Effect of Melatonin on the Morphology of ALC cells

The morphology of ALC cells was investigated via rhodamine-labeled phalloidin / DAPI staining and confocal image analysis. After 48 h of melatonin treatment, the changes in cell number and myofilament protein in the experimental group were not obvious compared with the control group. However, ALC cell morphology differed remarkably between melatonin medium and standard medium after 96 h. At the 96 h time point, cells in melatonin showed plenty of pseudopods and more actin filaments compared to those in the standard medium. With the increase in melatonin concentration, cell numbers were significantly decreased. (Fig. 2)

Effect of Melatonin on the Differentiation of ALC Cells

RT-PCR was used to analyze the expression of amelogenin genes in different concentrations of melatonin (Fig. 3). In the experimental group, 10^{-4} M melatonin concentration had no significant effect on the amelogenin gene and melatonin receptors after 5 days. However, the expression of MT1 and MT2 in 10^{-3} M melatonin showed an obvious and same change trend, reaching the peak for both on the third day, and was 70 times and 20 times higher than that of the control group (standard medium), respectively. AMELX, ENAM, and ODAM expression levels increased significantly in cells cultured in the medium containing 10^{-3} M melatonin ($P < 0.05$). The expression of AMELX and melatonin receptors showed the same trend, reaching a peak on the third day, and the expression level of AMELX was 50 times higher than that of the control group. The expression levels of ENAM and ODAM were 5 times and 1–2 times higher than those in cells in the standard medium, but the changing trend was different from that of the melatonin receptor. As a result, melatonin has been shown to increase the mRNA expression of these amelogenin genes.

Effect of Melatonin on the mineralization of ALC cells

ALP activity

Alkaline phosphatase (ALP) activity was used to evaluate the mineralization of ALC cells in different concentrations of melatonin medium (0, 100, 200, 500, and 1000 μ M). The ALP activity of ALC cells in the 1×10^{-3} M and 5×10^{-4} M melatonin mediums was significantly higher than that in the standard medium (P

< 0.05). With the increase in melatonin concentration, the ALP activity of ALC cells gradually increased, indicating an enhancement of mineralization potential (Fig. 4A).

Alizarin red S staining

Alizarin red staining was used to evaluate mineralized nodule formation in ALC cells exposed to different concentrations of melatonin medium and melatonin receptor antagonist. Only a few calcified nodules were stained in the control group (standard medium). There were more calcified nodules in the 1×10^{-4} M, 2×10^{-4} M, and 5×10^{-4} M groups. After repeated experiments, dense and obvious calcified nodules were produced in the 1×10^{-3} M melatonin medium. Compared with the control group and the low-concentration melatonin group, the red staining became darker and the number of calcified nodules increased significantly in this medium. With the increase in melatonin concentration, calcified nodules of ALC cells were dense and obvious (Fig. 4B).

In the other half of the samples, ALC cells in standard medium (L-M-) produced a small amount of calcification, and those in medium supplemented with Luzindole (L+M-) had no obvious calcified nodules, with the alizarin red staining yielding negative results. The calcified nodules of MLT in the absence of Luzindole (L-M+) were significantly increased and dense. In the presence of Luzindole (L+M+), calcified nodules decreased and alizarin red staining was weakly positive, which suggested that the mineralization of ALC cells depends on melatonin receptor MT1 and MT2. The results showed that melatonin promoted the mineralization of ALC cells, and Luzindole inhibited the mineralization of ALC cells (Fig. 4C).

Signaling pathway of melatonin in ALC cells

Differential expression analysis by RNA-seq

In order to investigate this phenotype, we performed high-throughput sequencing of ALC cells treated with or without melatonin for 3 days to understand how melatonin alters gene expression. After comparing the two groups according to the requirements of “multiple of difference in the project ≥ 2 , significance level $P < 0.001$ ”, 679 up-regulated genes and 675 down-regulated genes were identified (Fig. 5A). This result indicated the great alterations in gene expression during differentiation after melatonin treated ALCs. To comprehensively depict gene functional information and link genome information, KEGG enrichment was explored to classify genes according to their pathways or functions (Fig. 5B). Among the pathways with the most abundant genes and P adjusted < 0.05 , the Wnt signaling pathway, which is closely related to tooth growth and development, attracted our attention. According to the results of RNA-seq, melatonin induced the expression of Wnt signaling pathway factors in ALC cells at least twice as much as that in the control group (Fig. 5C). These genes were Wnt genes (Wnt1, Wnt4, Wnt5b, Wnt6, Wnt7b, Wnt10a, and Wnt11), Frizzled family genes (Fzd1, Fzd3, Fzd4, and Fzd5), secreted frizzled-related genes (Sfrp2 and Sfrp4), and the cAMP response element-binding protein (CBP)-encoding gene, among others. Among them, the expression of Sfrp2, Sfrp4, Fzd1, Fzd3, Fzd4, and Fzd5 genes in the melatonin group increased significantly, while that of Wnt4, Wnt5b, Wnt6, Wnt7b, and Wnt10a genes decreased significantly.

Wnt signaling pathway tested by RT-PCR

The regulation of factors of the Wnt signaling pathway was also confirmed by qRT-PCR analysis (Fig. 5D), including the WNT genes, conduction factor β -catenin, the transcription factor LEF, and the downstream factors c-Jun N-terminal kinase (c-Jun) and Cyclin D1. Compared with the control group, the expression levels of WNT4 and WNT6 in 10^{-3} M melatonin were significantly decreased, which was consistent with the results of RNA-seq. Although the expression of β -catenin and LEF did not change significantly in RNA-seq, we also detected the expression of β -catenin and LEF because they are key factors in the Wnt canonical pathway. However, the expression of β -catenin and LEF increased significantly in the experimental group, which was 27 times and 12 times higher, respectively. The downstream factor cyclin D1 had no significant difference in expression level, while the expression level of the c-Jun gene was approximately 2 times of that of the control group. The change in expression of these factors confirmed that the important factors of the Wnt/ β -catenin signaling pathway may play a role in the effect of melatonin on the growth and differentiation of ALC cells.

Discussion

The aim of this study was to evaluate the effect of melatonin on ameloblasts and to explore the signaling pathway of melatonin. The proliferation, differentiation, and mineralization of ameloblast-lineage cells, induced by melatonin, were studied using various techniques. The most widely used ameloblast-like cells are the ameloblast-like cells (LS-8) and the ameloblast-lineage cell line (ALC). By comparing the expression profiles of LS-8 cells and ALC cells, Sarkar et al. found that LS-8 cells expressed more genes representing secretory-stage activities (amelx, AMBN, ENAM, and MMP20), while ALC expressed more genes representing the maturation-stage activities (ODAM and KLK4). In addition, ALC cells in the basic medium could form calcified nodules, while LS-8 cells did not [29]. To better understand the differentiation and mineralization of ameloblasts, ALC was selected.

At present, research on melatonin in oral hard tissue mainly focuses on promoting the growth and development of dentin and alveolar bone, as well as the induction and differentiation of dental pulp stem cells [30–32]. However, the effect of melatonin on ameloblasts remains unclear. In this study, we confirmed that a melatonin concentration of 10^{-3} M inhibited the proliferation of ALC cells in a time- and concentration-dependent manner. Melatonin has been shown to effectively modulate the proliferation of various types of cells [33]. For example, 1 mM melatonin inhibits the proliferation of breast cancer MCF-7 [34] and umbilical vein endothelial cells [35] in vitro. Melatonin (10^{-6} – 10^{-4} M) has no obvious effect on the proliferation of human osteoblast cell line hFOB1.19 [36], but melatonin (10^{-12} – 10^{-10} M) has an obvious inhibitory effect on the proliferation of human dental pulp cells or HDPCs [37]. This evidence suggests that melatonin may have different sensitivity and specificity in inhibiting cell proliferation in vitro. In order to observe the effect of melatonin on ALC cells more intuitively, the morphology of ALC cells will be detected by laser scanning confocal microscopy. We showed that ALC cells were more evenly distributed and produced more pseudopodia in the melatonin medium than in the standard medium. To

some extent, with the increase in melatonin concentration, myofilament proteins were more densely arranged and interwoven, but the number of cells had decreased at the same time, which was similar to the results of the cell proliferation experiment. The changes in cell number and myofilament protein levels were not obvious at 48 h, compared with 96 h. Therefore, we speculate that melatonin promotes actin fiber production and increases cytoskeleton construction in a time- and concentration-dependent manner.

Exogenous melatonin can restore and stabilize the circadian rhythm of mice with the pineal gland removed [38]. In our previous work, the loss of circadian rhythm mainly inhibited enamel formation and AMELX expression in mice through the melatonin receptor [7]. The role of ALC cells in the formation of mineralized tissues is similar to that of mouse ameloblasts. Therefore, we hypothesized that melatonin may promote the amelogenic behavior of ALC cells. According to the experimental results, melatonin receptors MT1 and MT2 are present and stably expressed in ALC cell lines, suggesting that ameloblasts may be one of the targets of melatonin. We found that the expression of amelogenes in ALC cells was significantly enhanced by 10^{-3} M melatonin. In the present study, we analyzed three important enamel matrix proteins, amelogenin X-linked (AMELX), enamelin (ENAM), and odontogenic ameloblast-associated protein (ODAM). AMELX is secreted by ameloblasts, accounting for approximately 95% of enamel matrix proteins. It is the most important matrix for enamel formation, maturation, and mineralization. It mainly regulates the crystallographic structure and thickness of the enamel [39]. ENAM is an important component of non-amelogenin, which can promote crystal nucleation, and the crystal size increases with an increase in its concentration [40, 41]. ODA is also produced by ameloblasts, at low levels. Although it is not clearly established, ODA is considered one of the regulators of enamel mineralization and crystal elongation [20]. The expression curve of AMELX mRNA was consistent with that of MT1 and MT2 mRNA, which peaked on the third day and then began to decline. This suggests that melatonin may promote the expression of AMELX through its receptors MT1 and MT2, thus stimulating the differentiation of enamel cells, which is consistent with previous experimental results in vivo. However, the expression curve of ENAM and ODA was relatively stable within 5 days. There was a significant difference compared with the control group, and it did not change with the melatonin receptor expression. This suggests that melatonin also promotes the expression of ENAM and ODA, but it may be through a receptor-independent pathway.

In addition to regulating the biomolecules of the enamel organic matrix, melatonin can further manipulate the amelogenin behaviors of ameloblasts. High concentrations of melatonin, especially 10^{-3} M, improved ameloblast functions, as confirmed by microscopic observations of ALP synthesis and mineralized nodule formation. According to the observed ALP activity levels, melatonin can promote ALP activity in ALC cells in a concentration-dependent manner. According to the enamel formation patterns observed at different concentrations of melatonin, the number of calcified nodules and the positive staining degree of Alizarin Red increased with increasing melatonin concentration. As ALC cell lines have the ability to form mineralization, we speculate that melatonin mainly promotes the formation of calcified nodules in ameloblasts rather than by induction. At the same time, luzindole, a melatonin receptor inhibitor,

significantly inhibited the calcification of ALC, which suggested that melatonin could regulate the calcification of ameloblasts through MT1 and MT2.

According to the above conclusions, it can be concluded that melatonin inhibits proliferation and promotes the differentiation of ALC cells. Therefore, it is particularly important to further explore the specific molecular mechanisms of melatonin in the growth and development of tooth enamel cells [23]. Studies have shown that Wnt/ β -catenin signaling pathways are critical for bone and tooth formation and development, and are involved in various stages of tooth morphogenesis, such as epithelial-mesenchymal interaction, tooth-specific cell proliferation, migration, and differentiation [42]. Signaling factors in various Wnt pathways are expressed in the inner enamel epithelium and ameloblast cells [43]. Activation of Wnt/ β -catenin signaling may stimulate osteoblast differentiation and osteogenesis [44]. The inactivation of β -catenin during the development of odontoblast cells can lead to a deficiency in the roots of the molar [45, 46].

In this study, RNA-seq data showed that the genes associated with the Wnt pathway changed significantly, indicating that the activity of Wnt signaling pathway was significantly changed during melatonin treatment of ALCS. Interestingly, the expression of the FZD receptor was significantly increased, and the WNT ligand was generally decreased. This finding was consistent with the results of qRT-PCR, in which Wnt4 and Wnt6 were 7- and 11-fold downregulated, respectively. Studies have shown that members of the Wnt5a family, including Wnt4, Wnt5a, and Wnt11, not only effectively activate the canonical pathway, but also inhibit it (Maye, Zheng et al. 2004). Therefore, we speculated that melatonin activates the canonical pathway by inhibiting the expression of Wnt5a family factors. We then used qRT-PCR to detect the significant signaling molecules involved in the Wnt/ β -catenin signaling pathway. Among them, the levels of β -catenin and LEF-1 were significantly upregulated to activate downstream factors. The expression of c-Jun decreased, and that of Cyclin D1 did not change significantly. These findings indicated that melatonin may pass through Wnt/ β -Catenin pathway to play a role in ALCs. However, there are many signaling pathways of melatonin in enamel development, which need to be further explored and verified. At the same time, cell lines are unable to simulate the environment in vivo, so future studies will focus on the validation of these hypotheses using vivo and animal models for pre-clinical trials.

Conclusion

Melatonin can inhibit the proliferation of ALC cells and promote differentiation and mineralization. The up-regulation of proteins related to ameloblast growth and development was affected by the important factors of the Wnt/ β -catenin signaling pathway. The findings of this study provide an experimental basis for the study of tissue engineering of tooth germ and the factors influencing enamel development abnormality.

Declarations

Funding

We are grateful to those who volunteered to participate in this study. This work was supported by grants from STCSM (18ZR1422700;20ZR1431300).

Author Contributions

Jing Pan: Data curation; Formal analysis; Methodology; Resources; Software; Validation; Visualization; Writing-original draft; Qianhui Ren: Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing-original draft; Zhao Yang: Data curation; Formal analysis; Visualization; Ying Guo: Investigation; Methodology; Software; Kubin Kwon: Validation; Visualization; Investigation; Checheng Shen: Investigation; Validation; Data curation; Yueying Wang: Conceptualization; Formal analysis; Methodology; Methodology; Supervision; Writing-review & editing; Fang Ji: Conceptualization; Formal analysis; Methodology; Visualization; Resources; Supervision; Project administration; Writing-review & editing.

Declaration of competing interest

All the authors declare no potential conflict of interest.

Data availability

Data will be made available on request.

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Figures

Figure 1

Effect of melatonin on ALC cell lines' proliferation. Cells were treated with various concentrations of melatonin (0, 10^{-10} , 10^{-8} , 10^{-6} , 10^{-4} and 10^{-3} M) for 24, 48, 72 and 96 hours respectively. Subsequently the cells viability was measured via CCK8 assay. Each value was presented as mean $\pm$ SD for triplicate cultures. (A) Growth curve of ALC cells in different concentrations of MT at different time periods. (B) The proliferation rate of ALC cells in different concentrations of MT at different time periods. (* $p < 0.05$, ** $P < 0.001$, compared to control)

Figure 2

Effect of melatonin on ALC cell lines' morphology. Cells were treated with various concentrations of melatonin (0, 10^{-10} , 10^{-8} , 10^{-6} , 10^{-4} and 10^{-3} M) for 48 and 96 hours respectively. Subsequently the cells were observed by laser scanning confocal microscope (LSCM). Magnification = 400×; scale bar = 50μm. Cells were treated with various concentrations of melatonin for 48h (A) and for 96h (B), respectively.

Figure 3

Effect of melatonin on Expression of amelogenic genes in ALC cell lines. Cells were treated with various concentrations of melatonin (0, 10^{-4} and 10^{-3} M) for 3days. Subsequently expression of amelogenic genes and melatonin receptors was measured in qRT-PCR.

Figure 4

Effect of melatonin on ALC cell lines' mineralization. (A) Alkaline phosphatase (ALP) activity in ALC cell lines under melatonin treatments(0,100, 200, 500 and 1000μM)was determined on day 7. Each value was presented as mean ± SD for triplicate experiments. (*p<0.05,**P<0.001,comparedto control). (B) Effect of melatonin on the mineralized nodules formation in ALC cell lines.Cells were treated with various concentrations of melatonin (0,100, 200, 500 and1000μM) for 28 days.(C) Effect of melatonin and Luzindole on the formation of mineralized nodule of ALC cell lines. Cells were incubated with the condition medium ((L-M-), (L+M-), (L-M+), (L+M+)) for 28 days.

Figure 5

Melatonin caused transcriptional alteration of genes related to tooth development in ALC cell line. M: melatonin, C: control. (A) Volcano plots with 'log2fold change' on x-axis and '-log10 p-value' on Y-axis revealed significant changes in [melatonin (n = 2) relative to Control (n = 2)] genes in ALC cell lines. Each dot in the volcano plots represents a detected gene. In the melatonin treated group, the genes with significantly increased [fold change>2, P < 0.001] were shown in red, while those in melatonin treated group with significantly decreased [fold change<2, P < 0.001] were shown in blue. (B) KEGG enrichment analysis of differentially expressed proteins in ALC cell lines (Only selectively extracted from the top 30

KEGG signaling pathways). The vertical axis indicates the enrichment of KEGG signaling pathways. (C) The heat map of Wnt signaling pathway related gene expression in the control group and melatonin treated ALC cell lines for 3 days by RNA-seq method. (D) The main factors of Wnt canonical pathway in ALC cell lines treated with melatonin for 3 days was analyzed by qRT-PCR.