The Antimicrobial Peptide LL-37 as a Possible Adjunct for the Proliferation and Differentiation of Dental Pulp Stem Cells

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Abstract

Introduction: This study evaluated the biocompatibility of 5 and 10 μg/mL LL-37 *in vitro* and its effect on the differentiation of human dental pulp stem cells (DPSCs) into odontoblast-like cells. **Methods:** Cell viability, genotoxicity, nitric oxide production, cell cycle, dentine sialophosphoprotein (DSPP) production, and *DSPP* gene expression. **Results:** Concentrations of 5 and 10 μg/mL of LL-37 were not cytotoxic and generally increased cell viability, especially on the third day ($P < .05$). The tested concentrations did not induce genotoxicity ($P < .05$). LL-37 did not significantly alter nitrite production at either concentration. Cell cycle analysis revealed that 10 μg/mL of LL-37 arrested cells in G0/G1 ($P < .05$). The control group exhibited higher numbers of cells in other phases of the cell cycle ($P < .05$). The expression of the *DSPP* protein and gene was also higher in the 10 μg/mL of LL-37 group ($P < .05$). **Conclusions:** These results demonstrated that LL-37 was biocompatible at these concentrations and increased the number of viable cells, especially during the initial period. The 10 μg/mL concentration arrested the cell cycle and increased expression of the *DSPP* protein and gene, which indicates that this peptide contributes to odontoblastic differentiation. *(J Endod 2017;43:2048–2053)*

Key Words

Antimicrobial peptide, biocompatibility, dental pulp, differentiation, LL-37, stem cells

Significance

Molecules with antimicrobial and regenerative features have been investigated for regeneration of dental pulp complex. Our findings suggest that 10 μg/mL of LL-37 induced cell proliferation and may contribute to differentiation of dental pulp stem cells into odontoblast-like cells, being a possible adjunct for regeneration of immature permanent teeth after pulpal necrosis.

Progressive carious lesions may permanently damage the dental pulp via propagation of a sustained inflammatory response throughout the tissue, which leads to pulpal necrosis (1, 2). The development of immature permanent teeth is interrupted and makes these teeth more prone to fractures (3). Therefore, regeneration of these tissues would be extremely important for these teeth after decontamination of the root canal because it would provide continuity to tooth development.

Dental pulp, apical papilla, and inflamed periapical tissue are sources of stem cells (4). The induced bleeding in immature teeth with pulpal necrosis promotes a massive influx of undifferentiated mesenchymal stem cells into the root canal space, which may contribute to the regeneration of pulpal tissues (3, 5). This influx is induced in the revascularization technique, which despite promising findings (6), still presents limitations (7).

Regenerative endodontics using tissue engineering-based strategies was investigated. Different types of scaffolds, including electrospinning and self-assembly isolated or associated with biomolecules, were studied (7, 8). A variety of bioactive molecules, such as anticancer drugs, antibiotics, proteins, growth factors, and polysaccharides, were used to deliver drugs within a defined period (8, 9). A previous study proposed a regenerative protocol for immature permanent teeth that included root decontamination using irrigating antimicrobial solutions, followed by the insertion of a bioactive and biocompatible scaffold that contained antimicrobial substances. A bacteria-free environment is established, and scaffolds containing growth factors and/or undifferentiated mesenchymal cells are placed to induce the development of new pulp tissue and odontoblasts with the consequent formation of dentin and increased tooth wall thickness, which would restore tooth function in the oral cavity (7).

Cell lines obtained from the human dental pulp may be classified as DPSCs (human dental pulp stem cells) when obtained from permanent teeth and SHED (Stem cells

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from Human Exfoliated Deciduous) when obtained from primary teeth. These cells are promising for the regeneration of the dentin-pulp complex because these cells are accessible and offer small chances of immunological rejection and pathogen transmission (10). Some studies demonstrated that DPSCs differentiate into odontoblast-like cells when in contact with some substances (11, 12).

The human catalecidin gene (CAMP) encodes the LL-37 peptide (13), which is formed from the last 37 amino acid residues of the C-terminal human cationic antimicrobial peptide 18, which exhibits a linear structure without a disulfide bridge (14). LL-37 is also known as hCAP-18, FALL-39, or CAMP-human cationic antimicrobial peptide (13), and it is widely expressed by epithelial cells and various types of leukocytes, such as neutrophils (15–17).

LL-37 inhibits lipopolysaccharide activity (15) and increases cytokine production of dendritic cells (15). This peptide also exhibits antimicrobial activity (18, 19), induces the migration of human cells from the pulp to sites where LL-37 is located (20), and stimulates the migration of undifferentiated rat mesenchymal cells, which is important for bone repair (21). This peptide induces angiogenesis (21–23) via an increase in vascular endothelial growth factor (VEGF) (21, 23) and the consequent formation of capillary structures (22). Some authors have suggested that LL-37 may contribute to the regeneration of the dentin-pulp complex based on these properties (20, 23).

These findings suggest that LL-37 is important to the regeneration of the dentin-pulp complex because it could (1) promote undifferentiated mesenchymal stem cell migration of the apical papilla in immature permanent teeth, (2) provide a suitable environment for angiogenesis, and (3) inhibit bacterial proliferation due to its antimicrobial activity. Current studies are searching for molecules with antimicrobial and regenerative features. Therefore, we investigated whether the antimicrobial peptide LL-37 also induces the differentiation of DPSCs into odontoblast-like cells.

The present study evaluated the biocompatibility of the antimicrobial peptide LL-37 at 5 and 10 μg/mL and its possible effect on DPSC differentiation.

Materials and Methods

Cell Culture and LL-37 Peptide

Human DPSCs (Lonza, Walkersville, MD) were cultured in Dulbecco's modified Eagle medium (DMEM; Cultilab, Curitiba, Brazil) supplemented with 10% fetal bovine serum (FBS; Cultilab), penicillin (100 U/mL) (Cultilab), and streptomycin (100 μg/mL) (Cultilab). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Culture medium was changed every 2 days, and the development of the cells was evaluated using inverted-phase microscopy (Axiovert 40C; Carl Zeiss Microimaging GmbH, Jena, Germany). DPSCs from the second to the sixth passage were used in the following experiments. The LL-37 peptide (Sigma-Aldrich, St Louis, MO) was diluted in DMEM to 10 and 5 μg/mL. DMEM supplemented with 10% FBS, penicillin, and streptomycin was used as a control in all experiments and designated "pure DMEM."

Cell Viability Assay

DPSCs were seeded into 24-well plates at a density of 2×10^4 cells per well to evaluate cell viability. Cells were incubated with 5 and 10 μg/mL of LL-37, pure DMEM, or 10% DMSO (dimethyl sulfoxide; Sigma-Aldrich) diluted in DMEM (positive control). Cell viability was assessed after 3 or 7 days via the addition of MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium; Sigma-Aldrich) for 60 minutes. MTT was removed, and DMSO was added to the wells for 10 minutes. The resulting optical density of the solution was measured in a spectrophotometer (Biotek-EL808IU; BioTek Instruments, Winooski, VT) at 570 nm. Absorbance data were normalized to the control group (= 100%).

Cell Cycle

Cells were seeded into 6-well plates at a density of 1×10^5 cells per well, maintained for 24 hours, and incubated with 5 and 10 μg/mL of LL-37 and pure DMEM. Cells were washed in Dulbecco's phosphate-buffered saline (Sigma-Aldrich) on the third day, placed on ice, fixed in 70% ethanol, and maintained at −20°C overnight. Cells were resuspended in propidium iodide (Sigma-Aldrich) and incubated in the absence of light for 30 minutes. The cell cycle was analyzed using specific software on the cytometer (Tali Image-based Cytometer, Invitrogen, Carlsbad, CA). All the steps were performed according to the manufacturer's recommendation.

Genotoxicity Test

DPSCs were seeded into 24-well plates at a density of 2×10^4 cells per well to assess genotoxicity. Cells were incubated with 5 and 10 μg/mL of LL-37, pure DMEM, or ethyl methane sulfonate (EMS; Sigma-Aldrich) at 5 mM diluted in DMEM, which was used as a positive control. Cells were fixed in 4% formaldehyde on the seventh day, and a Fluoroshield solution with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was added to the wells. Cells were photographed using a digital camera (Sony F828 digital, Cyber-Shot, 8.0 megapixels; Sony Corporation, Tokyo, Japan) coupled to an inverted light microscope. At least 10 photos per well were obtained, and the number of micronuclei was determined in 2000 cells per well using ImageJ software (National Institutes of Health, Bethesda, MD). Micronuclei were identified as DNA structures contained in the cytoplasm, totally separated from the main nucleus, with an area smaller than one-third of the main nucleus area. Mitotic and apoptotic cells were not considered in the count.

Quantification of Nitric Oxide

DPSCs were seeded into 24-well plates at a density of 2×10^4 cells per well and maintained for 24 hours before incubation with 5 and 10 μg/mL of LL-37 and pure DMEM for 7 days. Nitric oxide production was quantified indirectly as the concentration of nitrite detected using Griess reagent (Sigma-Aldrich). Supernatants (100 μL) were added to 96-well plates with the same volume of Griess reagent. Nitrite concentrations were calculated from a standard curve, which was created based on standard samples of nitrite.

Indirect Enzyme-Linked Immunosorbent Assay

Cells were seeded into 6-well plates at a density of 1×10^5 cells per well, maintained for 24 hours, and incubated with 5 and 10 μg/mL of LL-37 and pure DMEM. Supernatants were collected after 3 days for the detection and quantification of dentine sialophosphoprotein (DSPP) protein (Fine test; Lab Research, Miami, FL) using an indirect enzyme-linked immunosorbent assay. Presensitized microtiter plates were used according to the manufacturer's recommendation. The resulting optical density of the solution was measured at 450 nm using a spectrophotometer. A standard curve was created based on standard samples of DSPP, and protein levels were determined in μg/mL.

Quantitative Real-Time Polymerase Chain Reaction

Cells were seeded in 6-well culture plates at a density of 1×10^5 cells per well and exposed to medium containing 5 and 10 μg/mL of LL-37 and pure DMEM for 3 days. Total RNA was isolated

using TRIzol reagent (Ambion, Inc, Carlsbad, CA) as recommended by the manufacturer. The extracted total RNA (1 μg) was treated with DNase I (Turbo DNase Treatment and Removal Reagents; Ambion Inc) and transcribed into complementary DNA (cDNA) using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen), according to the protocols recommended by the manufacturer. Transcribed cDNAs were amplified for relative quantification of *DSSP* gene expression using *GAPDH* as the reference gene. The present study examined 3 reference genes, *ACTB*, *GAPDH*, and *TUBA6* (24), in all experimental groups. The obtained results were analyzed at http://www.leonxie.com/referencegene.phpe, and the selected reference gene was *GAPDH* (data not shown). Table 1 details the primer sequences. Quantitative real-time PCR (qPCR) was performed using the Platinum SYBR Green qPCR SuperMix-UDG Kit (Applied Biosystems, Framingham, MA) in the StepOnePlus apparatus (Applied Biosystems, Framingham, MA). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from the qPCR experiment (25).

Statistical Analysis

Each experiment was repeated at least 3 times. Kruskal-Wallis followed by Mann-Whitney *U* test and analysis of variance followed by Tukey test were used to compare groups. Statistical significance was set at $P < .05$. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc, San Diego, CA).

Results

LL-37 Is Not Cytotoxic and Increases Cell Viability

The absorbance values at 10 μg/mL were significantly different from those of the control group at 3 (P = .0006) and 7 days ($P < .0001$), with a higher percentage in the treated group. Absorbance values at 5 μg/mL were significantly different from the control group at 3 days (P = .012), but values were also higher than 100% on the seventh day (P = .95). The positive control DMSO was cytotoxic at 3 and 7 days ($P < .0001$) and exhibited absorbance values below 40% at both times. Figure 1*A* shows the differences between groups and the same concentrations at different times.

LL-37 Arrests the Cell Cycle

Cytometry was performed to complement the cytotoxicity assay and observe whether LL-37 arrested the cell cycle. The number of cells in G0/G1 and S phases in the group treated with 10 μg/mL of LL-37 was significantly different from that in the control group (P = .0064), with a greater number of cells in G0/G1 and fewer cells in S phases compared with the control group. A greater number of cells were in G2/M phases in the control group compared with the groups treated with 5 and 10 μg/mL of LL-37 (P = .032 and P = .0021, respectively) (Fig. 1*B*).

TABLE 1. Primer Sequence Used for Real-Time Polymerase Chain Reaction in This Study

Gene	Sequence 5′–3′	Reference
DSSP	(F*) GTCCTAGTGGGAATGGAGCA (R†) CCTGGCCCTTGCTGTTATTA	This study
GAPDH	(F*) CTCTGCTCCTCCTGTTCGAC (R†) TTGACTCCGACCTTCACCTT	Rentoft et al (24)
ACTB	(F*) CCAACCGCGAGAAGATGAC (R†) CAGAGGCGTACAGGGATAGC	Rentoft et al (24)
TUBA	(F*) CCGGGCAGTGTTTGTAGACT (R†) TTGCCTGTGATGAGTTGCTC	Rentoft et al (24)

*Indicates a forward primer.

†Indicates a reverse primer.

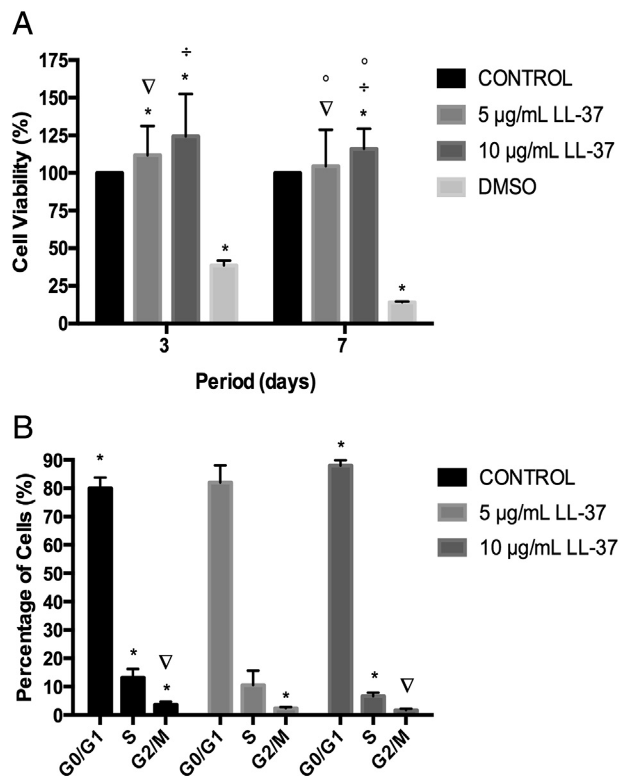

Figure 1. Data from MTT test and cell cycle after exposure of cells to 5 and 10 μg/mL of LL-37. (*A*) LL-37 was not cytotoxic and increased cell viability in DPSCs. *Statistical significance compared with control group (= 100%). °Statistical significance between LL-37 concentrations at the same time period. ∇ and ÷ indicate statistical significance between the same concentrations of LL-37 at different time periods. (*B*) LL-37 at 10 μg/mL arrested the cells in G0/GI. * and ∇ indicate the statistical significance of the same phases of cell cycle between groups.

LL-37 Does Not Induce Genotoxicity

The number of micronuclei (Fig. 2) in the positive control (EMS group) was higher than that in the control group (P = .028). The number of micronuclei in cells exposed to 5 μg/mL and 10 μg/mL of LL-37 was not significantly different than the control group (P = .48 and .51, respectively), and no differences were observed in micronuclei formation between the 2 concentrations (P = .31) (Fig. 3*A*).

Exposure to LL-37 Does Not Alter Nitric Oxide Production

Nitrite production by cells treated with 5 and 10 μg/mL of LL-37 was not significantly different from the control group (P = .83 and P = .93, respectively). There was no significant difference in nitrite production from cells exposed to 5 μg/mL of LL-37 compared with 10 μg/mL of LL-37 (P = .54) (Fig. 3*B*).

LL-37 Enhances DSPP Protein Production and *DSPP* Gene Expression

DSPP production from cells treated with 10 μg/mL of LL-37 was statistically greater than that of the control group (P = .02). The quantity of DSPP in cells treated with 5 μg/mL of LL-37 was not different from that of the control group, but a greater production of DSPP was observed in the treated group (Fig. 4*A*).

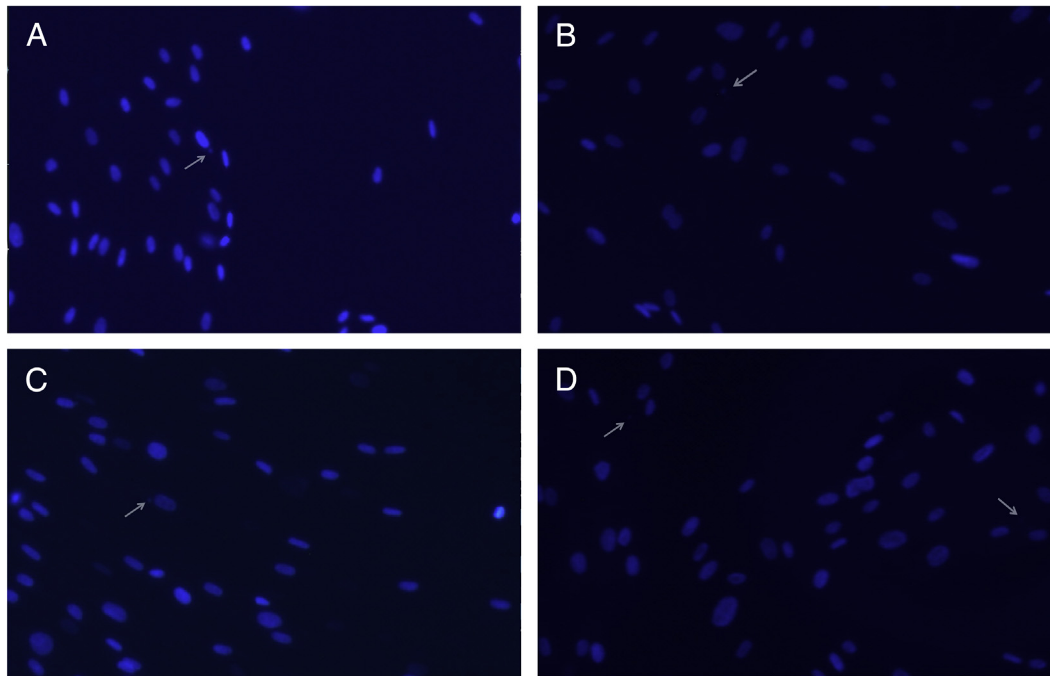

Figure 2. Nuclei and micronuclei (*arrows*) in the genotoxicity assay after exposure of cells to 5 μg/mL of LL-37 (*A*); 10 μg/mL of LL-37 (*B*); pure DMEM (*C*), and EMS (*D*). Fluoroshield with DAPI immunofluorescence staining, original magnification ×200.

We assessed the production of the odontogenic marker *DSPP* using qPCR to elucidate the mechanisms of LL-37 stimulation of DPSCs. Treatment with 10 μg/mL LL-37 induced a significant increase in *DSPP* gene expression (2.9-fold) compared with the control group ($P < .0001$). There was no statistically significant difference between the control group and the 5 μg/mL of LL-37 group ($P = .2634$) (Fig. 4*B*).

Discussion

Current research efforts include searching for scaffolds that release molecules with antimicrobial and regenerative functions to support the regeneration of the dentin-pulp complex of immature permanent teeth after pulp necrosis (7). Numerous bioactive molecules were investigated for drug delivery (9). The present study investigated the biocompatibility of the antimicrobial peptide LL-37 and its ability to induce the differentiation of DPSCs into odontoblast-like cells. Previous studies suggested that LL-37 may be an effective adjunct for the regeneration of the dentin-pulp complex (21, 23).

A previous study demonstrated that LL-37 increased the expression of VEGF in pulp cells in a dose-dependent manner, and better results were obtained at 10 μg/mL, which was the highest concentration evaluated (23). Another study demonstrated a significant proliferation of rat calvaria mesenchymal stem cells (MSCs) after contact with 10 μg/mL LL-37 for 24 hours (26). Shaykhiev et al (27). observed that LL-37 stimulated the proliferation of a cell line derived from bronchial mucoepidermoid carcinoma (NCI-H292) cells in a dose-dependent manner, but LL-37 was cytotoxic to these cells at 20 μg/mL. Therefore, the present study examined 5 and 10 μg/mL of LL-37.

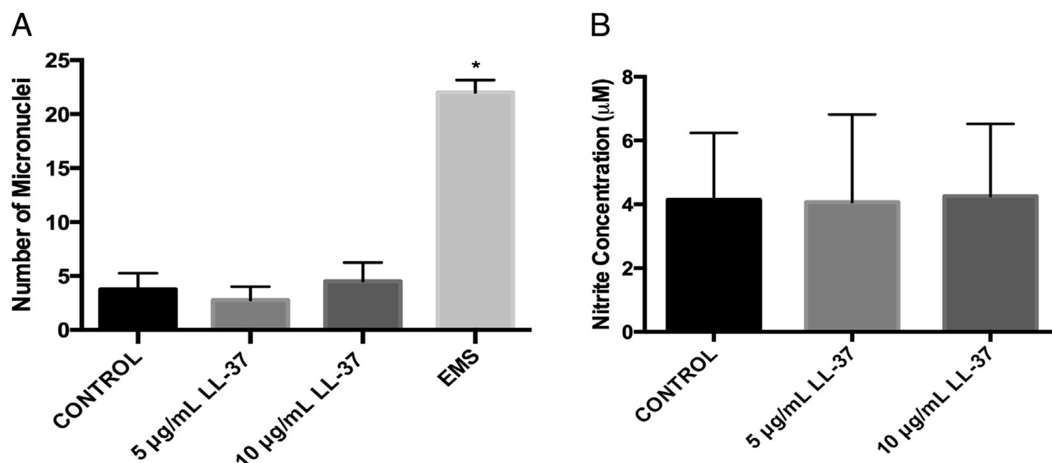

Figure 3. Number of micronuclei and nitrite concentrations (μM) after exposure of cells to 5 and 10 μg/mL of LL-37 for 7 days. (*A*) This peptide did not induce genotoxicity in DPSCs. *Statistical significance compared with the control group. (*B*) The production of nitric oxide by DPSCs was not altered after exposure to this peptide.

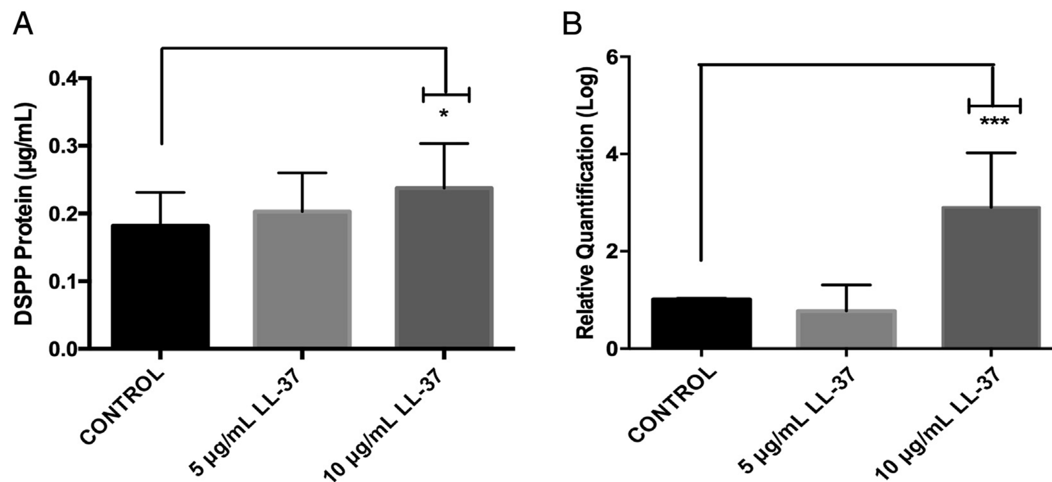

Figure 4. Data from direct enzyme-linked immunosorbent assay and qPCR after exposure of cells to 5 and 10 μg/mL of LL-37 for 3 days. (*A*) LL-37 at 10 μg/mL enhances the production of DSPP by DPSCs, and (*B*) LL-37 at 10 μg/mL enhances *DSPP* gene expression by DPSCs. *Statistical significance between groups ($P < .005$). ***$P < .001$.

Our results demonstrated that 5 and 10 μg/mL of LL-37 was not cytotoxic or genotoxic to DPSCs. A previous study evaluated the cytotoxicity of LL-37 after 24 hours of contact with endothelial cells and concluded that LL-37 was not cytotoxic at 5 μg/mL to 10 μg/mL. LL-37 at 50 and 500 ng/mL slightly increased cell viability (22).

LL-37 increased cell viability in the present study, which suggests that it stimulated DPSC proliferation, especially at 10 μg/mL, as previously observed in MSCs after 24 hours (26). This increase was more evident after 3 days, which suggests that LL-37 favors proliferation during the early stages of contact.

A greater number of cells were observed in the G0/G1 phase, which was demonstrated in previous studies of the cell cycle in DPSCs (11, 12). DPSC proliferation provides an appropriate number of cells for cell differentiation (28). A previous study demonstrated that ATP (adenosine 50-triphosphate) stimulated cell proliferation at low concentrations but inhibited proliferation at high concentrations by arresting the cells in the G0/G1 stage of the cell cycle, which supports odontoblastic differentiation and mineralization (12). Our study demonstrated that LL-37 at 10 μg/mL also affected cell cycle progression by arresting the cells in G0/G1, and fewer cells were observed in the S and G2/M phases. This result may be related to differentiation, as previously suggested (12), because more viable cells were present at 10 μg/mL LL-37.

No previous study evaluated the genotoxic potential of LL-37, but one study reviewed the role of this peptide in carcinogenesis. These authors observed that the overexpression of LL-37 promoted the development and progression of ovarian, lung, and breast cancers. However, LL-37 overexpression suppressed tumorigenesis in colon and gastric cancers (29). Neither concentration of this peptide exhibited a genotoxic potential in DPSCs in our study.

Many cells, principally neutrophils, produce LL-37 under inflammatory conditions (16, 17, 19), and nitric oxide is also produced under these conditions (30). This study investigated the potential relationship between these components. Nitric oxide is produced in large quantities in response to infection and inflammatory stimuli in various tissues, including the dental pulp. Mechanical stimulation during tooth preparation also may induce the production of this oxide. In 2016, Yasuhara et al (30) suggested that nitric oxide played a role in odontoblastic differentiation and the subsequent formation of reparative dentin while suppressing the growth and accelerating apoptosis in a dose-dependent manner. The present study could not establish this type of relationship because neither concentration of LL-37 interfered with DPSC production of nitric oxide.

The cellular morphology, the secreted matrix, and the cellular gene expression pattern define the primary odontoblast phenotype, which leads to the synthesis and secretion of the dentin matrix proteins. A variety of molecules contribute to the signaling cascade that results in odontoblast-like differentiation (31).

DSPP is a noncollagenous matrix of odontoblasts encoded by the *DSPP* gene and proteolytically cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) (32). DPP and DSP are involved in dentin mineralization. DPP plays an important role in early mineralization, and DSP is related to dentin maturation (33).

Some studies demonstrated increased expression of *DSPP* and *DMP-1* genes (34, 35) and DSP protein (34) after specific treatment associated with the use of mineralizing-inducing agents. Soares et al (35) observed that the biomembrane composed of chitosan/matrix of collagen incorporated with calcium aluminate microparticles increased the expression of these genes in DPSCs after 28 days. However, previous studies also demonstrated that DPSCs increased the expression of *DSPP* and *DMP-1* genes depending on the stimulus without the use of mineralizing-inducing agents (11, 12), and this methodology was successfully adopted in the present work. Wang et al (12) observed that the expression of DSPP and DMP-1 genes increased after treatment with ATP for 24 and 48 hours. Umemura et al (11) observed an increased expression of these genes and their respective proteins after 24 hours of hyaluronic acid treatment without mineralizing-inducing agents. We demonstrated that 10 μg/mL of LL-37 increased the expression of the *DSSP* gene and protein in a dose-dependent manner, which indicates that this peptide plays a role in the differentiation of stem cells into odontoblasts. These findings suggest that these important dentinogenesis markers and proteins are expressed during early periods under favorable conditions, even in the absence of mineralizing-inducing medium.

Taken together, our findings indicate that 10 μg/mL LL-37 increased the number of viable cells, likely via the induction of cell proliferation. The cell cycle was arrested in the group treated with 10 μg/mL of LL-37, which is likely related to the early differentiation in the group that previously proliferated more than others. LL-37 is biocompatible, increases the number of viable cells, and contributes to odontoblastic differentiation in a dose-dependent manner.

Further studies are needed to elucidate whether LL-37 enables full odontoblastic differentiation, with consequent dentin deposition. This peptide is a promising new alternative for dental pulp proliferation and differentiation.

Acknowledgments

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