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Original Article

Relaxin treatment stimulates the differentiation of mesenchymal stem cells into osteoblasts

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Abstract *Background/purpose:* Several studies have determined that relaxin stimulates differentiation and regulates the activity of mature osteoclasts, but little is known about its effect on the differentiation of mesenchymal cells towards the osteogenic lineage. Therefore, this study aimed to determine the effect of relaxin on the proliferation and differentiation of the osteoblastic lineage of mesenchymal cells derived from human dental pulp (hDPSC).
Materials and methods: In this in vitro study, hDPSC were characterized and treated with relaxin at different doses (10–80 ng/ml) and times (1–21 days). Morphology was assessed by microscopy, and proliferation was assessed using a resazurin assay. Osteoblastic differentiation was evaluated by Alizarin Red staining, alkaline phosphatase (ALP) labeling, and changes in the expression of the osteoblastic differentiation genes RUNX2 and BMP2.
Results: Relaxin treatment did not induce changes in the proliferation or viability of hDPSCs; however, larger cells and increased cytoplasmic prolongation were observed. Relaxin treatment (20 and 80 ng/ml) significantly increased calcified nodule formation on days 14 and 21. The cytochemical signals for ALP, RUNX2, and BMP2 gene expression were significantly ($P < 0.05$) increased by the relaxin treatment.
Conclusion: Relaxin treatment does not induce changes in hDPSC proliferation but induces morphological changes, increases ALP detection, calcified nodule formation, and increases

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expression of RUNX2 and BMP2, suggesting the induction of osteoblastic differentiation of hDPSC.
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Introduction

Alveolar bone tissue undergoes constant remodeling throughout life. This remodeling process is coordinated and balanced between the osteoblastic cells that form the bone tissue and osteoclasts that induce bone degradation.[1] Tooth movement requires alveolar bone remodeling and bone resorption and apposition phases. Studying the mechanisms that can accelerate tooth movement is emphasized to shorten treatment time. Conventional orthodontic treatments usually extend for an average of 1.5—3 years, depending on the degree of malocclusion, treatment mechanics, and host-related factors. Accelerating tooth movement and reducing treatment time would reduce the side effects associated with excessive treatment time in orthodontic treatment for conditions such as root resorption, caries, and periodontal disease.[2]

Bone metabolism is a regulated process that stimulates bone regeneration by inducing the formation of osteogenic cells, such as osteoblasts and osteocytes derived from mesenchymal stem cells from different sources in the human body.[3] Osteogenic stimulation accelerates bone production and matrix mineralization; these processes are modulated by various molecules such as vitamin D, prostaglandins, bisphosphonates, and hormones, including relaxin.[4]

Relaxin is an insulin-like hormone that reduces spontaneous contractions of the uterus, increases pubic area flexibility, and facilitates labor.[1,5] It is involved in peripheral blood monocyte differentiation, osteoclast survival, and activation.[6] Binding to specific receptors stimulates collagen turnover, angiogenesis, and anti-fibrosis.[7] Relaxin receptors are located in reproductive tissues and bone tissue cells such as osteoclasts, osteoblasts, and osteocytes.[6]

Relaxin predominantly binds to the relaxin/insulin-like peptide receptor (Rxfp)-1 or -2 of the peptide receptor family.[5] Rxfp1 is expressed in osteoclasts, whereas Rxfp1 and 2 are expressed in osteoblasts and peripheral blood mononuclear cells.[8] Relaxin induces osteoclastogenesis and activates mature osteoclasts through the upregulation of nuclear factor enhancer of activated B-cell kappa light chain kappa (NF-κB), NF-kappa-B receptor activator (Rank), tartrate-resistant acid phosphatase (TRAP), and cathepsins by binding to the Rxfp1 and influencing bone metabolism.[6]

In contrast, Rxfp2 mutations are associated with osteoporosis induction. The binding of insulin-like factor 3 to Rxfp2 induces an increase in osteoblast proliferation and the expression of genes related to bone formation, such as *bone morphogenic protein* (*BMP2*) and *runt-related transcription factor 2* (*RUNX2*).[1,9]

Despite the importance of relaxin in stimulating osteoclast formation, published results regarding its effect on accelerating tooth movement remain contradictory.[10,11] Little is known about the effects of relaxin on the differentiation of mesenchymal stem cells into osteoblasts in vitro or its involvement in bone formation and mineralization.

Additionally, the previous discovery of the multilineage differentiation capacity of human dental pulp stem cells (hDPSCs) is an important factor for generating multipotent cells that are more readily available than bone marrow-derived mesenchymal stem cells (BMMSCs) with a higher proliferation rate because of their clonogenic, multipotent, and proliferative capacities in vitro and in vivo.[12,13] Therefore, this study aimed to determine the effect of relaxin on the induction of osteoblastic differentiation in hDPSCs in vitro.

Materials and methods

Isolation of mesenchymal cells from dental pulp

This in vitro experimental study was reviewed and approved by our Institutional Ethics Committee (CIE-159-2020). hDPSC were obtained from two independent cultures of the dental pulp from two donors, each with three replicates ($n = 6$). The donors were healthy individuals aged between 18 and 20 years who required premolar extraction for orthodontic treatment. The volunteers signed an informed consent form before surgery.

The protocol proposed by Grontos et al. modified by Baldión et al. was used to obtain mesenchymal cells from the pulp. The teeth were decontaminated using 5% sodium hypochlorite solution for 5 s and sectioned with a high-speed disk to obtain the total pulp. The intact pulp was placed in Dulbecco's modified Eagle medium, low glucose (DMEM) (Hyclone Thermo Fisher Scientific, Bremen, Germany) supplemented with antibiotics and fetal bovine serum (FBS) (Hyclone Thermo Fisher Scientific).

For the tissue dissociation process, dispase (4 mg/mL) (Invitrogen/Gibco Waltham, MA, USA) and collagenase (3 mg/mL) (Sigma-Aldrich, St Louis, MO, USA) were used for 16 h in a humidified atmosphere with 5% CO_2 at 37 °C. The cell suspension was then centrifuged. The cell pellet was resuspended in culture medium and seeded in 25 cm^2 flasks until 80% confluence was reached.[14,15]

Determination of changes in morphology, viability, and proliferation of human dental pulp stem cells treated with relaxin

The seeded mesenchymal cells were dissociated with 0.25% trypsin and seeded into 12-well microplates (5000 cells/

well). Initially, the cells were treated with 0 (control), 10, 20, 40, and 80 ng/mL relaxin (Recombinant Human Relaxin-2 (B-33/A-24) 3596-RN Biotechne, Minneapolis, MN, USA) for 120 h to determine the dose that induced the most evident changes. The dose range was selected based on previous literature reports.[16–18]

The proliferation of hDPSCs with or without relaxin treatment was assessed using a resazurin fluorometric assay. Resazurin is a nonfluorescent blue compound that is irreversibly reduced to resofurin (highly fluorescent pink). This transformation is associated with the activity of vital mitochondria. Resofurin diffuses into the medium, allowing the monitoring of cell proliferation and cell death. Cells cultured under different experimental conditions were incubated with 4.4 μg/well of resazurin solution at 37 °C for 4 h. Fluorescence was read using a Tecan Infinite M2000 Pro reader at 535–595 nm. Morphological changes induced by relaxin treatment were determined via light microscopy.[19]

Differentiation of human dental pulp stem cells toward osteoblastic lineage

Evaluation of in vitro mineralization

hDPSCs with or without relaxin treatment for 7, 14, and 21 days were fixed with paraformaldehyde (PFA) and stained with 2% Alizarin Red S following the protocol developed by Gregory et al. The dye was extracted by washing cells with PBS. Calcified nodule formation was analyzed using an inverted microscope. The absorbance of the extracted dye was quantified at 550 nm using an Infinite M200 multi-plate reader (Tecan).[20]

Alkaline phosphatase activity

A SigmaFast Red TR/naphthol AS-MX kit (Sigma) was used to detect ALP activity. The kit contains a chemical substrate that produces a reddish color in the presence of ALP inside the cells.

Expression of osteoblastic differentiation genes

After treatment with relaxin or differentiation medium (DM), RT-qPCR was performed to analyze changes in the expression of the osteoblastic differentiation marker genes *RUNX2* and *BMP2*.

Total RNA was extracted following the manufacturer's instructions after 7, 14, and 21 days of treatment using a Quick-RNA MicroPrep kit (Zymo Research, Irvine, CA, USA). cDNA was obtained by reverse transcription using a Proto-Script II First Strand cDNA Synthesis Kit (New England Bio-Labs, Ipswich, MA, USA). The expression levels of *RUNX2* and *BMP2* were determined by RT-qPCR using the Luna Universal real-time RT-PCR master mix system (New England Biolabs) and the CFX96 Real-Time Thermal Cycler Detection System (Bio-Rad, Hercules, CA, USA). The primers used in this study are listed in Table 1. The amplification conditions were an initial 3 min at 95 °C; 50 cycles of 10 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C; and finally, 5 s at 65 °C and 5 s at 95 °C. PCR efficiencies were calculated using LinRegPCR (Academic Medical Center, Amsterdam, Netherlands), and relative expression was quantified based on the method described by Schefe.[21]

For all osteoblast differentiation induction experiments, the positive differentiation control comprised of hDPSCs that were cultured for 7, 14, and 21 days in a DM containing DMEM supplemented with 10% FBS, 0.1 μM dexamethasone (Sigma), 5 mM β-glycerophosphate (Santa Cruz, Dallas, TX, USA), and 50 μg/mL ascorbic acid (Sigma) with no addition of relaxin. The negative control was maintained in a culture medium lacking osteoblastic differentiation factors.[14]

Statistical analysis

Three independent experiments were performed in duplicate. The data are expressed as mean ± SD. Student's t-test and one-way ANOVA with post-hoc Tukey's test was used to analyze the differences between groups with a normal distribution. In addition, the Mann-Whitney U test was performed for groups with non-normal distributions. Statistical analysis was performed using SPSS software (version 21.0; SPSS, Chicago, IL, USA). A statistical value of $P < 0.05$ was considered a significant difference between experimental groups.

Results

Characterization of mesenchymal stem cells obtained from dental pulp

Immunophenotyping of the cells using flow cytometry revealed high expression of the cell surface markers CD105, CD90, and CD73 and low expression of the hematopoietic cell markers CD34/CD45 (Fig. 1A–E). hDPSCs adhered to a fibroblastoid morphology after 7 days of culture (Fig. 1F).

Effect of different treatment times and doses of relaxin on human dental pulp stem cells proliferation and morphology

Previous studies evaluating the effect of relaxin on the function of different cell types, such as dermal fibroblasts, cardiac fibroblasts, and the osteoblastic cell line UMR 106-0, used doses ranging from 10 to 100 ng/mL[16-18]; hence,

Table 1 Primers used in this study.

Gen	Forward primer	Reverse primer	Amplicon size (bp)
RUNX2	5′-CATCTAATGACACCACCAGGC-3′	5′-GCCTACAAAGGTGGGTTTGA-3′	168
BMP2	5′-CGAAACACAAACAGCGGAAAC-3′	5′-GCCACATCCAGTCGTTCCA-3′	97
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′GAAGATGGTGATGGATTTC-3′	226

Runt-related transcription factor 2 (*RUNX2*), bone morphogenic protein 2 (*BMP2*), and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

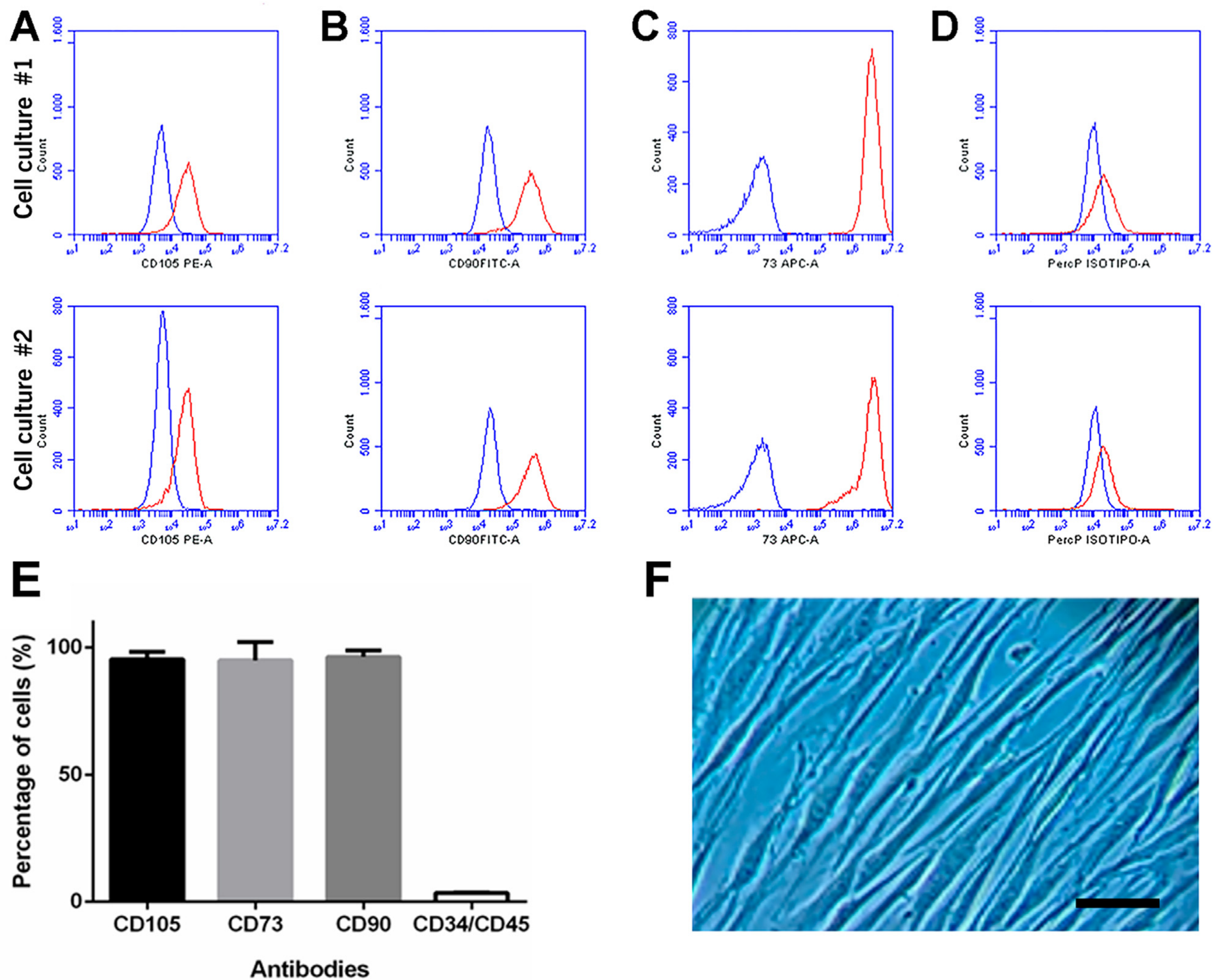

Figure 1 **Characterization of hDPSCs.** Flow cytometry histograms of two primary cultures of hDPSCs with surface markers positive for (A) CD105, (B) CD90, (C) CD73, and (D) negative for CD34 and CD45. (E) Percentage of cells positive for each antibody. 95% of cells were positive for CD73, CD90, CD105 and less than 2% positive for CD34 and CD45. (F) Photograph of hDPSC after 48 h of culture. Values are presented as averages and standard deviations (SD), from two independent experiments. Bar: 200 μm.

four different concentrations (10, 20, 40, and 80 ng/mL) were selected to determine the most efficient dose to induce changes in cell proliferation, morphology, and differentiation. Compared to the control group, treatment with each of the four doses of relaxin for 120 h did not induce significant changes in proliferation or cell viability in any of the cell types (Fig. 2A).

Effect of relaxin on human dental pulp stem differentiation

Evaluation of in vitro mineralization

To evaluate relaxin treatment-induced cell differentiation, Alizarin Red staining was performed to detect calcified nodule formation, which was evident in hDPSCs as early as 7 days of culture in the presence of osteogenic DM; however, hDPSCs treated with relaxin at the same time showed the opposite effect. At 14 days, calcified nodule formation was observed in hDPSCs treated with the two selected doses of RLN, which was more evident at 21 days in all experimental groups than in the control group (Fig. 3). After 14 days of treatment with 80 ng/mL relaxin and DM, the absorbance of Alizarin Red extracted from the DPSCs was significantly higher than that of the untreated control group after 21 days. DPSCs treated with DM showed absorbance values similar to those obtained with 80 ng/mL relaxin (Fig. 3).

Detection of alkaline phosphatase activity

Untreated control cells and those treated with relaxin (20 and 80 ng/mL) and DM were evaluated by histochemical analysis for ALP activity after 7, 14, and 21 days of treatment. As shown in Fig. 4, positive ALP labeling was evident at 14 days and was much more apparent at 21 days post-treatment with relaxin and DM in hDPSCs (Fig. 4).

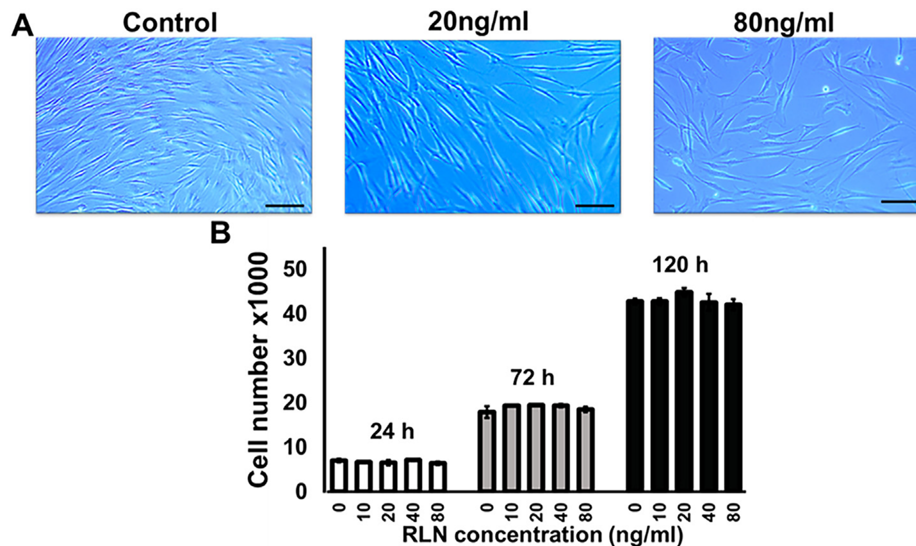

Figure 2 Changes in morphology and cell number induced by treatment with different doses of relaxin for 120 h. (A) Morphological changes were evident from 72 h onwards where more elongated cells with larger cell prolongations were observed when compared to the group of cells not treated with relaxin (Control). (B) No significant reduction in the number of hDPSC was observed in any of the doses (10, 20, 40, and 80 ng/ml) or times analyzed (24–120 h) in relation to the Control group (0 ng/ml). Data are expressed as averages$\pm$ SD. Bar: 200 μm.

Evaluation of changes in the expression of osteoblastic differentiation genes

Changes in the expression of two cell differentiation marker genes, *RUNX2* and *BMP2*, were determined. A significant increase in expression was observed under all experimental conditions. However, cells treated with 80 ng/mL relaxin showed a 45-fold increase in *RUNX2* expression and a 60-fold increase in *BMP2* expression after 14 days. This increase was more pronounced than when 20 ng/mL of relaxin was applied. After 21 d, the expression of both genes was reduced in cells treated with relaxin (Fig. 5).

Discussion

The present study evaluated the effects of relaxin on hDPSC proliferation, viability, and differentiation. Relaxin did not induce significant changes in the proliferation or viability of these cells but induced morphological changes. Additionally, we found an osteoblastic phenotype in treated cells with mineralization activity, ALP expression, and increased transcription of specific genes, such as *RUNX2* and *BMP2*.

Regarding morphology, Henneman et al. reported that RLN treatment of periodontal ligament-derived fibroblasts did not affect DNA quantity or cell number or induce cytotoxic effects in these cells at doses up to 500 ng/mL.[25] However, changes in cell proliferation were not observed in rat cardiac fibroblasts or human dermal fibroblasts treated with relaxin.[16,26]

Relaxin plays a physiological role in the growth and differentiation of the reproductive tract during pregnancy. It may be essential for bone physiology, disease, and metastasis.[1,7,22,23] relaxin has been suggested to participate in bone remodeling by stimulating osteoblastic and osteoclastic activities and driving bone mineralization and resorption processes. The balance between these two processes is finely tuned by the modulation of anti-osteoclastogenic factors released by osteoblasts, which are coordinated by relaxins.[24]

Here, we used light microscopy to determine whether relaxin induced morphological changes in hDPSC. The cells presented a spindle-shaped and fibroblastoid morphology with longer extensions and a larger cytoplasm. However, the induction of morphological changes seemed to depend on the cell type studied, as relaxin treatment did not induce morphological changes in periodontal ligament cells.[25]

Relaxin influences bone metabolism by inducing osteoclastogenesis and activating mature osteoclasts through activation of NF-κB, Rank, TRAP, and cathepsins through Rxfp1[6,27]; however, its effect on the osteogenesis process is not yet fully understood.

Osteogenesis is a complex process involving calcium mineral deposition on a secreted collagen matrix. Signaling molecules, such as Runx2, Bmp2, and ALP, regulate osteogenesis during osteoblast differentiation.[8] ALP activity increases intracellular phosphates, triggering phosphorylation of the Erk1 cascade, which favors cell proliferation and differentiation.[28] Relaxin receptors trigger cAMP signaling, increasing nitrous oxide production and Erk1 phosphorylation.[29] Duarte et al. reported that relaxin induces osteogenic differentiation and increases mineralization through the activation of ALP, Runx2, and Bmp2 by activating the ERK signaling pathway, primarily via Rxfp2.[8]

When analyzing the induction of hDPSC differentiation, we observed increased ALP labeling and calcified nodule formation after 14 and 21 days of treatment with 20 and 80 ng/mL relaxin, respectively. These findings are similar to

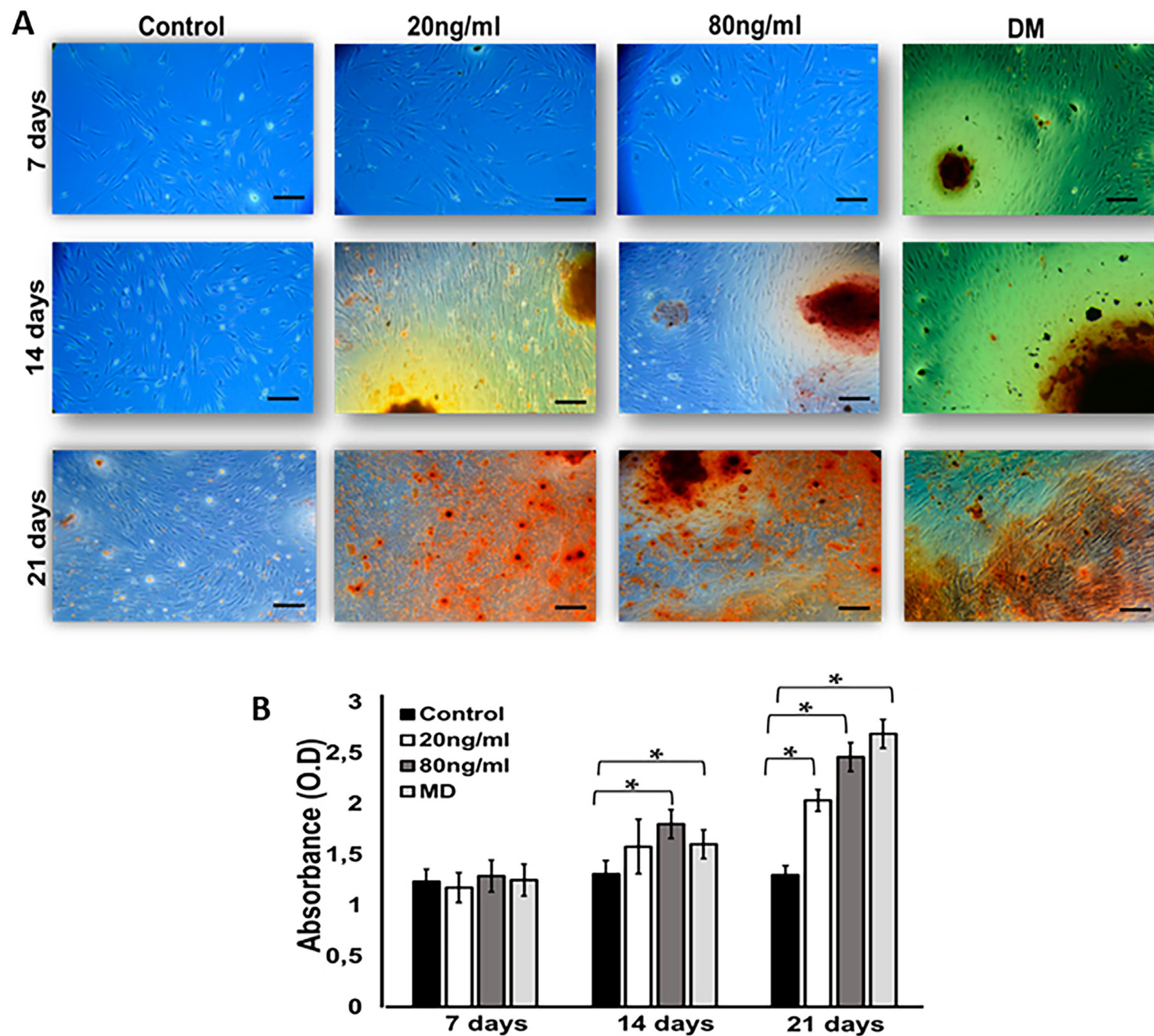

Figure 3 **Extracellular matrix mineralization induced by relaxin treatment.** (A) Mineralization was determined by Alizarin Red staining of hDPSC treated with 0 ng/ml (Control), 20 ng/ml, and 80 ng/ml relaxin and osteogenic differentiation medium (DM). Strong matrix staining was observed in the microphotographs, indicating the apparent formation of calcified nodules after 14 days of treatment. Bar: 200 μm. (B) Alizarin red staining was performed and quantified from hDPSC under different doses of relaxin (20 ng/ml and 80 ng/ml) and DM at 7, 14, and 21 days. $^{*}P < 0.05$. Data are expressed as averages± SD.

those obtained by Duarte et al. who treated MC3T3-E1 mouse calvarial osteoblasts with 20 ng/mL relaxin. They found a significant increase in Runx2 expression at 48 h and BMP2 expression at 72 and 120 h in cells treated simultaneously with relaxin and DM. Additionally, they observed a significant increase in ALP activity at 24 h and 120 h in relaxin-treated cultures maintained in DM.[8]

Previously, it was reported that relaxin treatment increased the BMP-2-induced in vitro differentiation and mineralization of mouse bone marrow stem cells (BMSCs). Relaxin increased BMP2-induced Runx2 expression and activity via Smad and p38 phosphorylation. In vivo studies have shown that RLN administration increases BMP2-induced bone formation.[30] However, the molecular mechanisms mediating relaxin activity in the BMP2 signaling pathway are not clearly understood and must be investigated in depth.

In this study, calcified nodule formation was measured as the amount of Alizarin Red obtained from cells and quantified using the absorbance of hDPSCs treated with 80 ng/mL relaxin, which was very similar to that observed in DM-treated cells. This suggests that relaxin induces a mineralizing phenotype in hDPSCs without the need for simultaneous treatment with an osteogenesis-inducing medium, as previously reported.[8]

When ALP activity was determined in DPSCs, positive cytochemical labeling for ALP was observed starting at 14 days of treatment and became more pronounced after 21 days of treatment at the evaluated relaxin concentrations. Although ALP is an early marker of osteoblast differentiation,[31] we demonstrated positive labeling as early as 14 and 21 days after relaxin treatment in DPSCs. These results are similar to that of Wrobel et al. where ALP activity increased from day 14 to day 21 in human bone-derived cells (HBDCs)

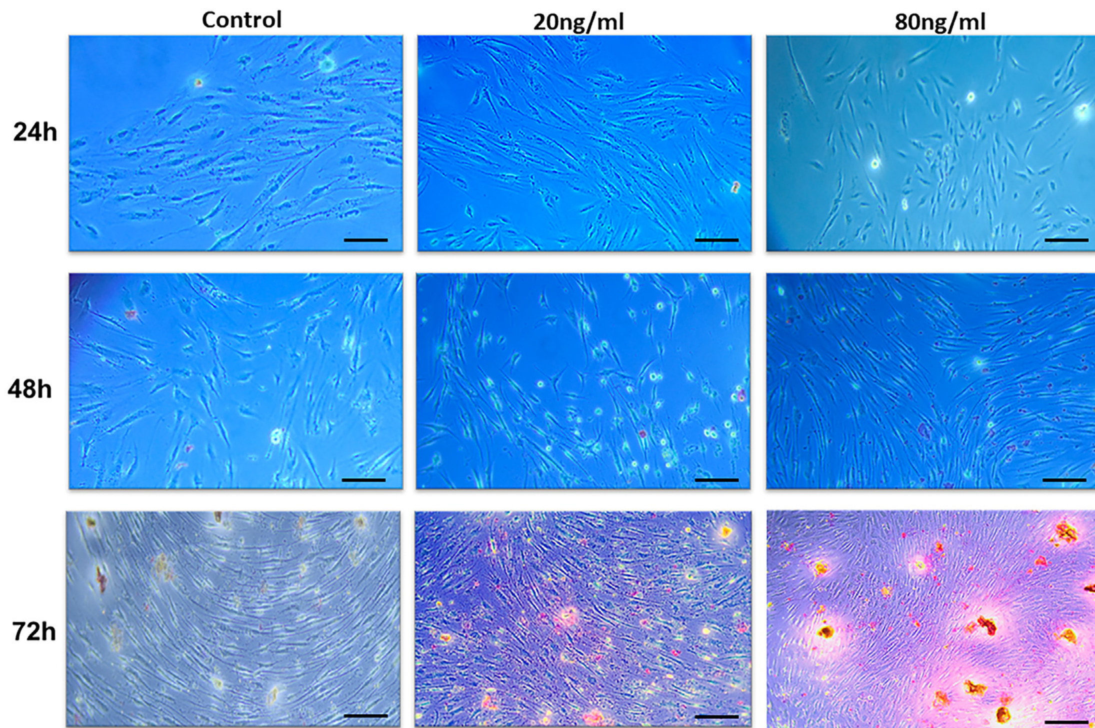

Figure 4 Detection of ALP activity in hDPSC. Cells without treatment (Control) and treated with relaxin 20 ng/ml and 80 ng/ml, were subjected to ALP detection by immunohistochemistry at 7, 14, and 21 days of treatment. Bar: 200 μm.

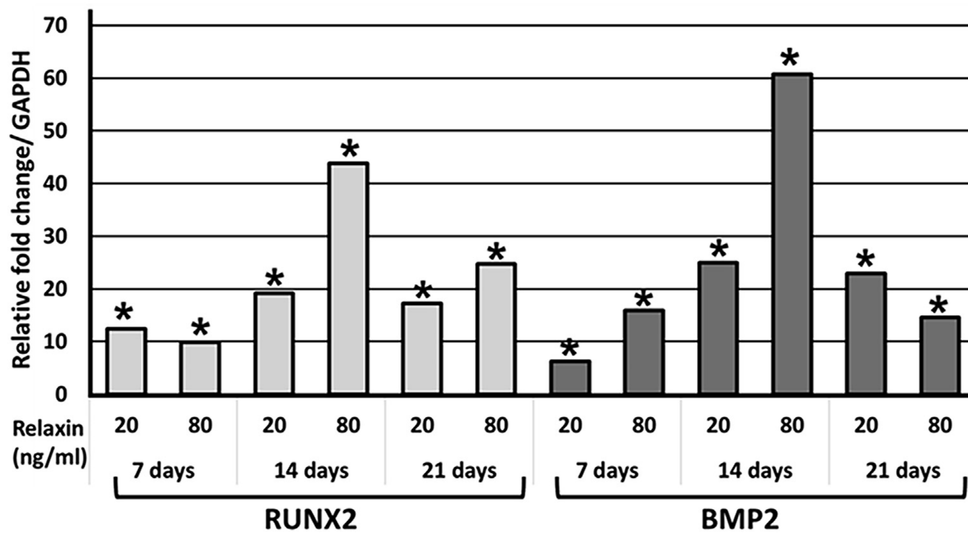

Figure 5 Quantification of the relative expression of osteoblastic differentiation genes. Quantification of the RUNX2 gene and BMP2 was performed in cells treated with relaxin 20 ng/ml or 80 ng/ml at 7, 14, and 21 days of treatment. Data were normalized to the GAPDH gene. $^{*}P < 0.05$.

induced osteogenic differentiation.[31] The ability of relaxin to induce ALP activity in DPSCs is an important finding as it has been previously established that the magnitude of ALP induction during osteogenic differentiation in vitro predicts osteogenic differentiation capacity in vivo.[30]

Collectively, our results suggest that relaxin treatment induces differentiation toward osteoblastic lineage in hDPSCs independent of the relaxin dose used. However, further in vitro and in vivo studies are required to understand the cellular and molecular processes that modulate relaxin-mediated osteogenesis. This could allow for the future use of relaxin as a therapeutic option in the field of bone regeneration and as a molecule that induces bone remodeling.

Declaration of competing interest

The authors declare that they have no competing interests.

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