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Acoustic Vibration Enhances Osteogenic Differentiation in Dental Mesenchymal Stem Cells

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ABSTRACT

Vibration-assisted orthodontic treatment accelerates tooth movement and reduces complications associated with prolonged interventions. While vibration has been shown to enhance osteogenic potential in bone marrow-derived mesenchymal stem cells (MSCs), its effects on dental tissue-derived MSCs remain unclear. This study investigated the impact of acoustic-frequency vibratory stimulation (AFVS) on gingival-tissue-derived MSCs (GT-MSCs) at 20 Hz and 60 Hz under both basal and osteogenic conditions. A custom vibratory platform was developed, and GT-MSCs were assessed for viability, proliferation, and osteogenic differentiation. Resazurin assay, Calcein-AM staining, and vimentin immunohistochemistry were used to evaluate cell viability, proliferation, and morphology, while Alizarin Red staining and calcium accumulation assays measured extracellular matrix mineralization at 7, 14, and 21 days. A Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) reaction was performed to quantify osteogenic markers (colagen type I [COL-I], osteopontin [OPN], and alkaline phosphatase [ALP]), and protein expression for COL-I and OPN was confirmed by immunohistochemistry. The results showed that AFVS at 20 Hz and 60 Hz enhanced osteogenic differentiation in GT-MSCs compare with other groups. Extracellular matrix mineralisation increased significantly, with 60 Hz resulting in the highest calcium deposition. Transcript levels of COL-I and OPN were markedly upregulated at 60 Hz, indicating a frequency-dependent response. Cell proliferation was also promoted, with optimal results observed at 60 Hz compare with other groups. These findings highlight the role of mechanical stimulation in enhancing the osteogenic potential of GT-MSCs, suggesting that AFVS is a promising tool for regenerative and orthodontic treatments. This study provides new insights into the frequency-specific effects of vibration, supporting the use of vibration therapy strategies in dental applications.

Keywords: *Acoustic-frequency vibratory stimulation; dental mesenchymal stem cells; gingival tissue mesenchymal stem cells; high-frequency vibration; osteogenic differentiation*

INTRODUCTION

Orthodontic treatment, commonly pursued for aesthetic and functional reasons, faces biological limitations, particularly concerning treatment duration (Felemban *et al.*, 2022). Bone and tissue remodeling are adaptive processes influenced by mechanical, chemical, and biological signals (Adamopoulos, 2018). Mesenchymal stem cells (MSCs) play a central role in this process, responding to mechanical forces by releasing bioactive trophic factors that stimulate resident cells to repair damaged tissues and to promote osteoblast proliferation, migration, and differentiation (Reiss *et al.*, 2020). However, prolonged orthodontic treatment can lead to complications such as pain, root resorption, and periodontal problems, highlighting the need for methods to accelerate tooth movement while minimising adverse effects (Zhang *et al.*, 2008; Talic, 2011; Liu *et al.* 2015; Bakdach & Hadad, 2020).

Among various methods explored, including surgical, pharmacological, and physical stimulation, vibrational devices such as AcceleDent® Aura and VPro5 have been introduced for its potential to accelerate orthodontic tooth movement and reduce pain during dental procedures (Judex & Pongkitwitoon, 2018; Telatar & Gungor, 2021). These devices aim to promote dental movement and bone remodeling through high-frequency vibrational stimulation (e.g., 30 Hz or 120 Hz), although their effectiveness is debatable (Telatar & Gungor 2021; Woodhouse *et al.* 2015). Previous reports suggest that local resonant vibration could accelerate orthodontic tooth movement; however, to determine the optimal parameters for such vibrational stimulation, it is necessary to identify the specific cells that respond to vibration.

Therefore, *in vitro* experiments are necessary for understanding the role that each cell type plays and while studies have examined vibrational effects on bone marrow-derived MSCs, little is known about how dental tissue-derived MSCs (DT-MSCs) particularly those from gingival tissue-derived mesenchymal stem cells (GT-MSCs) respond to such stimuli. We have described important properties of GT-MSCs related to their accessibility and immunosuppressive potential (Poblano-Pérez, Castro-Manrreza *et al.*, 2024; Poblano-Pérez, Monroy-García *et al.*, 2024). However, understanding their mechanoresponse is critical for optimising vibrational therapies in dentistry (Stewart *et al.*, 2020).

Current clinical guidelines for dental vibratory devices use fixed vibrational parameters (e.g., 30 Hz for 20 minutes or 120 Hz for 5 minutes) without considering frequency-dependent cellular responses. Preliminary evidence, primarily in long bones rather than craniofacial tissues, suggests that higher frequencies (e.g., > 60 Hz) may enhance osteogenic gene expression. However, the effects of lower frequencies (e.g., 20 Hz to 45 Hz) on DT-MSCs remain unexplored (Judex & Pongkitwitoon, 2018). This gap limits our ability to customise vibrational stimulation for the effective regeneration of maxillofacial tissue.

To address this, we developed an acoustic vibratory platform to systematically evaluate how different frequencies (20 Hz and 60 Hz) affect GT-MSCs differentiation and osteogenic potential. Two frequencies were selected: 20 Hz, which falls within the audible spectrum, and 60 Hz, which doubles the lower clinical frequency but remains below the 120 Hz threshold of the clinical device VPro5. We held acceleration and duration constant to isolate frequency-specific effects, avoiding the cell detachment

that we previously observed at higher frequencies *in vitro* (e.g., 120 Hz). The present study aims to determine whether vibration stimulation at these frequencies enhances osteogenic differentiation in GT-MSCs, providing insight into how DT-MSCs perceive mechanical signals.

By clarifying the relationship between vibration frequency and GT-MSCs behaviour, this work could tailor more effective clinical protocols, advancing orthodontics and regenerative dentistry.

MATERIAL AND METHODS

Acoustic Frequency Loading Platform

A mechanical vibration device was designed and constructed to evaluate the impact of mechanical vibrations on the response of GT-MSCs, based on previously reported methodologies (Broadbent *et al.*, 2010; Marędziak *et al.*, 2017; Beckingham *et al.*, 2019). The device consists of two main components: mechanical and electrical (Fig. 1).

The specific modifications in the design and construction of the mechanical vibration device are as follows: frequencies of 20 Hz and 60 Hz, with an amplitude of 0.3 mm, were calibrated using a three-axis accelerometer (AX3; Axivity, Newcastle upon Tyne, United Kingdom) and an MD03032 oscilloscope (Tektronix, Beaverton, OR, USA). Displacement was calculated using the root mean square error value and the known calibrations of both the oscilloscope and accelerometer. The following equation was used to determine the optimal power amplifier volume for achieving the desired amplitude:

$$D = \frac{GA}{2\pi^2 F}$$

where D = displacement (m), G = gravity (constant at 9.81 m/s^2), A = acceleration (g), and F = frequency (Hz). A displacement less than 30% from the non-vibrated platform was considered optimal for our experiments, in which the plate maintained a central position over the speaker.

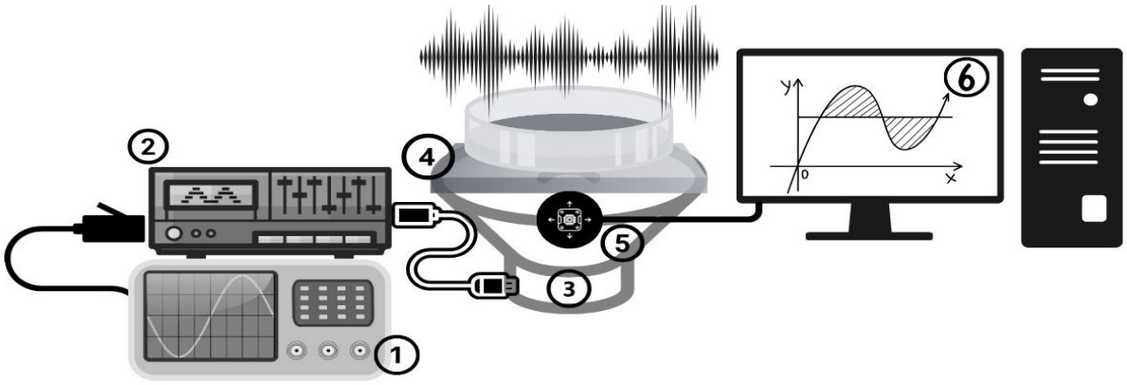

Fig. 1 Vibratory platform. The vibratory platform consists of a mechanical unit and an electrical unit. The setup includes a function generator (1) connected to an audio amplifier (2), sinusoidal signals generated by the system are sent to a 300-watt speaker (3), which transmits vibrations to the culture plates via a polymethyl methacrylate platform (4). Vibrations at frequencies of 20 Hz and 60 Hz, with an amplitude of 0.3 mm, were calibrated using a three-axis accelerometer (5) that measured vibrations in three dimensions (X, Y, Z) at a sampling rate of 3,200 Hz. The accelerometer was connected to an oscilloscope (6), which recorded its output. Displacement was calculated using the root mean square error value and known calibrations for both the oscilloscope and accelerometer.

Cell Culture of GT-MSCs

Human GT-MSCs were obtained from an existing biorepository (de la Rosa-Ruiz *et al.*, 2019). The collection, characterisation, and preservation of this biorepository were previously approved by the Ethics Committee of the Faculty of Dentistry, UNAM, in accordance with the Declaration of Helsinki and NOM-012-SSA3-2012 regulations (Ethics Committee Approval number: CIE/1110/2017). These cells were previously characterised according to the criteria established by the International Society for Cell Therapy. Morphologically, they exhibit a fibroblast-like appearance and possess the capacity to differentiate into adipogenic, osteogenic, and chondrogenic lineages as we have previously published (de la Rosa-Ruiz *et al.*, 2019).

GT-MSCs were cultured in a growth factor-free medium, specifically Dulbecco's Modified Eagle Medium with low glucose (DMEM-lw; Gibco, Grand Island, NY, USA), supplemented with 1% antibiotics (penicillin-streptomycin; Gibco), 2 mM glutamine (Glutamax; Gibco, Invitrogen), and 10% fetal bovine serum (Gibco). After reaching a sub-confluent state of 60%, the cells were used at passage 3 or 4 and seeded at a density of 1.5×10^4 cells/cm^2 in either 12- or 96-well culture plates. The cells were then incubated at 37°C for 24 hours before vibration stimuli were applied.

Vibrational Exposure

Culture plates were subjected to sinusoidal vibrations at 20 Hz or 60 Hz frequencies for 20 minutes daily over a consecutive 21-day period, under either basal or osteogenic medium. The control condition, designated as 0 Hz, consisted of culture plates placed on the platform without activation for 20 minutes. This control included cells cultured in basal medium (negative control) and cells cultured in osteogenic medium (positive control). Each osteogenic and basal experimental condition was performed in triplicate, with three or four replicates per trial.

Cell Viability Assessment

Cell viability was assessed using a resazurin assay following exposure to mechanical vibrations at 0 Hz, 20 Hz and 60 Hz over 1, 3, 5, and 7 days. Cells were seeded in 96-well plates, and at each designated time point, the culture medium was replaced with a mixture of medium and resazurin solution at a 10:1 ratio (44 μM in phosphate-buffered saline [PBS]). After a 4-hour incubation, the conversion of resazurin to resorufin was measured using an enzyme-linked immunosorbent assay plate reader at an optical density (OD) of 570 nm. For each experiment, 16 wells located in the centre of the plate were used, with 4 wells selected for OD readings at each time point. The experiment was performed in triplicate, with three or four replicates per trial.

Staining with fluorescein diacetate (Calcein-AM, CMFDA, Cat. C7025; Gibco, Invitrogen) was used to visualise viable cells, as well as the attachment and distribution patterns of GT-MSCs after 7 days of vibratory stimulation. Cells were cultured for 7 days and stimulated with 0 Hz, 20Hz, or 60 Hz. Following stimulation, culture wells were rinsed with PBS, and cells were stained by incubating them with CMFDA (5 mg/mL) at a 1:1,000 final dilution in phenol-red-free basal culture medium for 1 hour at 37°C and 5% CO_2. After incubation, the cells were washed with PBS and visualised using a fluorescent microscope (AmScope, Irvine, CA, USA) with an excitation wavelength of 485 nm. This qualitative analysis was performed to assess cell distribution, adhesion coverage, and morphology via optical microscopy. Four wells were stained for each experimental or control group, and the procedure was performed in triplicate.

Osteogenic Differentiation

GT-MSCs were seeded into 12-well culture plates (Corning Inc., Corning, NY, USA) at a density of 1.5×10^4 cells/cm^2. After 24 hours of incubation, osteogenic induction was initiated using StemPro osteogenic medium (Gibco). The cells were incubated for 21 days, with the medium changed twice a week. Each osteogenic and basal experimental condition was seeded in 12-well plates, with 6 wells used per condition. These were considered as replicates, and the experiment was performed in triplicate.

Alizarin Red S Staining

To detect calcium deposition, Alizarin Red S (ARS) staining was performed after exposing GT-MSCs to mechanical vibrations at frequencies of 0 Hz, 20 Hz, and 60 Hz for 7, 14, and 21 days under either osteogenic or basal conditions. Calcium nodules indicate that GT-MSCs have differentiated into an osteoblast-like phenotype. Brightly stained red areas represent nucleation sites of crystals with high calcium content; a positive ARS result suggests the initiation of extracellular matrix mineralisation. At the end of each time point (days 7, 14, and 21), the culture medium was removed, and the cells were washed twice with PBS and fixed in 4% paraformaldehyde overnight at 4°C.

The cells were rinsed twice with PBS and once with a 9% NaCl solution, followed by a 15-minute incubation in 100% cold methanol. After methanol incubation, a 2% ARS working solution (pH 4.3; Sigma-Aldrich) was prepared and added to each well and incubated for 45 minutes at room temperature in the dark to allow staining, after which representative photographs were taken using an inverted optical microscope equipped with a camera.

The experiments were conducted under both osteogenic and basal medium conditions. Each experimental condition was seeded in 12-well plates, with 6 wells used per condition. These were considered as replicates, and the experiment was performed in triplicate.

Calcium Accumulation Assay

To quantify the amount of ARS red dye bound to calcium deposits in each well, the dye was extracted using acetic acid. Following ARS staining, 500 µL of 10% acetic acid was added to each well and incubated for 30 minutes, then heated at 85°C for 10 minutes. After cooling on ice for 5 minutes, the samples were centrifuged at 13,000 rpm for 15 minutes. Subsequently, 125 µL of ammonium hydroxide was added to each tube, and the solution was homogenised. The concentration of ARS was then measured at a wavelength of 405 nm.

Additionally, to verify cell morphology following osteogenic induction and vibratory stimulation at frequencies of 20 Hz and 60 Hz, immunohistochemical staining was performed using a vimentin antibody (1:100 dilution, SC-6260; Santa Cruz Biotechnology, Dallas, TX, USA). The procedure followed the manufacturer's instructions using the BioSB kit (BSB-0001; BioSB Inc., Santa Barbara, CA, USA). Each experimental condition, both osteogenic and basal, was seeded in 12-well plates, with 6 wells allocated per condition. These were considered replicates, and the experiment was performed in osteogenic and basal experimental conditions in triplicate.

Total RNA Extraction and Reverse-Transcription Quantitative Polymerase Chain Reaction

Total RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) were performed based on previously described methodologies (Fajardo-Orduña *et al.*, 2017). Briefly, total RNA was extracted from GT-MSCs

cells subjected to mechanical vibrations at frequencies of 0 Hz, 20 Hz, and 60 Hz after 7, 14, and 21 days of culture, using either osteogenic or basal medium. Four wells were used for each experimental condition, and the extraction was carried out using the Isolate II RNA Mini Kit (Bioline; Meridian Biosciences, Cincinnati, OH, USA), following the manufacturer's instructions.

Next, 1 µg of RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The reaction conditions included an annealing step at 25°C for 5 minutes, followed by an extension at 42°C for 1 hour, all conducted in a 20-µl reaction volume. RNA and DNA quantification were performed using the BioTek Epoch Spectrophotometer (Agilent, Santa Clara, CA, USA).

For mRNA quantification, 10 ng of complementary DNA was amplified using the 2X Forget-Me-Not™ Universal Probe qPCR Master Mix (Biotium, Fremont, CA, USA) and specific primers were employed for the following targets: alkaline phosphatase ([ALP] Forward: agaaccccaaaggcttcttc; Reverse: cttggcttttccttcatggt), osteopontin ([OPN] Forward: gagggcttggttgtcagc; Reverse: caattctcatggtagtgagttttcc), and colagen type I ([COL-I] Forward: ctggagaggctggtactgct; Reverse: agcaccaagaagaccctgag). The amplification protocol included an initial pre-incubation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds and annealing at 60°C for 1 minute. All reactions were performed using the LightCycler 480 System (Roche Diagnostics, Basel, Switzerland).

PCR specificity was confirmed through gene expression analysis using crossing points, defined as the point where fluorescence significantly exceeds background levels. Crossing point values were determined using the second derivative maximum method. Expression calculations were performed using the ΔΔCt method with crossing point values calculated via the Fit Point method in the LightCycler 480 SW 1.5 software. Hypoxanthine phosphoribosyl transferase ([HPRT]. Forward: tgaccttgatttattttgcatacc; Reverse: cgagcaagacgttcagtcct) was used as the housekeeping gene. RT-qPCR tests were conducted in triplicate for each negative or positive control and mechanically stimulated groups.

Additionally, immunohistochemical staining for COL-I and OPN was performed to correlate with functional osteogenic differentiation. Each experimental group, including those for osteogenic differentiation and basal conditions, was seeded in 12-well plates, with 6 wells per condition. These were considered as replicates, and the experiment was performed in triplicate.

Statistical Analysis

Data collected from each experiment were expressed as means with standard deviations, derived from three independent experiments. The Kruskal–Wallis test or one-way ANOVA on ranks was applied to all datasets. Dunn's post hoc test was used to compare pairs of sample groups for viability assays. Tukey's multiple comparison test was used for all other assessments to determine the statistical significance of differences between pairs of means. Additionally, multiple pairwise comparisons with Bonferroni correction were performed when analysing three or four experimental groups against the control group. All statistical analyses were conducted using Prism Software version 10.3.1 (GraphPad Software, San Diego, CA, USA), with a p-value of ≤ 0.05 considered statistically significant.

RESULTS

Characterisation of Vibrational Stimulation

The vibrational stimulation system produced vertical mechanical oscillations in the form of harmonic sine waves at two distinct frequencies: 20 Hz (peak amplitude: 7.4 µm) and 60 Hz (peak amplitude: 6 µm), as registered in Table 1. The sine waves exhibited consistent periodicity through the characterisation methodology proposed in the present study, confirming stable and controlled delivery of mechanical stimuli.

Vibration Enhances GT-MSCs Viability and Proliferation

An initial assessment of the effects of these vibrations on the viability of GT-MSCs was conducted over 1, 3, 5, or 7 days of exposure to vibrations at 20 Hz or 60 Hz. This was compared to the cell viability under static conditions (control, 0 Hz) using the resazurin assay (Fig. 2).

Table 1 Readings on the frequency and displacement of the platform

Input frequency	Mean	Maximum displacement	Minimum displacement	RMS	Acceleration	
					Value	Mean
20 Hz	19.95 Hz	20.05 Hz	19.83 Hz	7.4 µm	232 mV	237 m
60 Hz	60.04 Hz	60.41 Hz	58.88 Hz	6.0 µm	202 mV	196 m

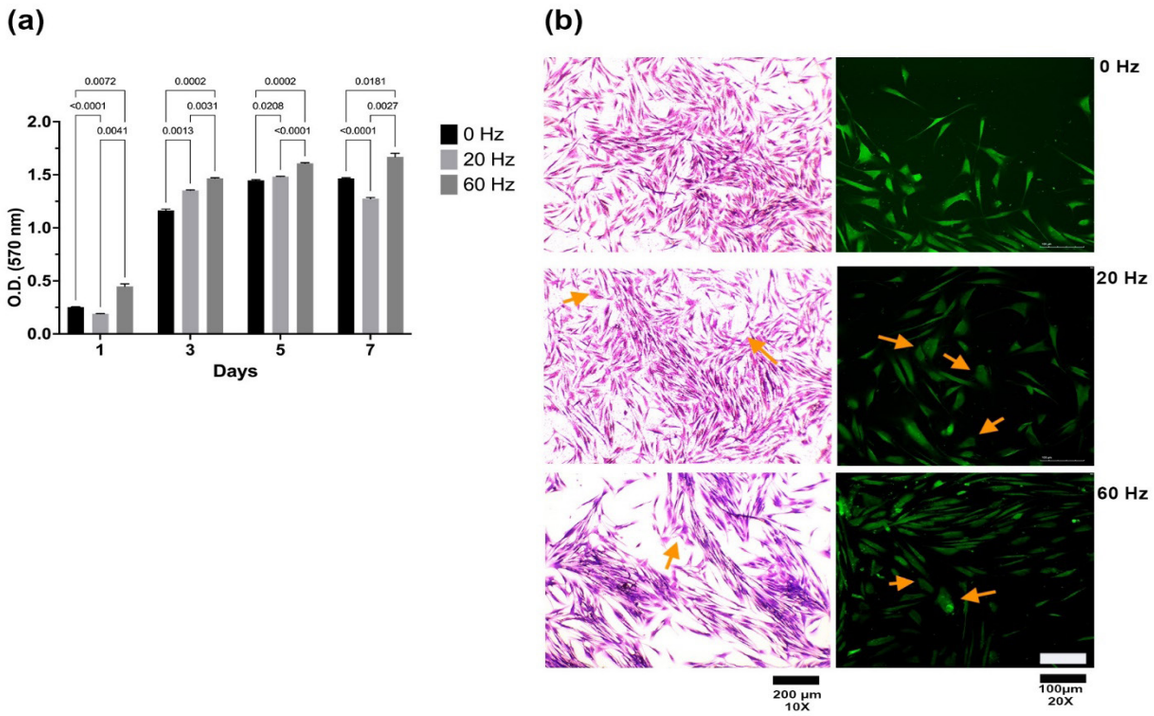

Fig. 2 Effects of vibratory stimulation on gingival tissue-derived mesenchymal stem cells (GT-MSCs). (a) Graphical representation of the resazurin assay at 1, 3, 5, and 7 days of culture under vibratory stimulation. Resazurin reduction reflects cellular metabolic activity and is proportional to the number of viable cells in culture. In cells stimulated at 20 Hz, resazurin reduction increased at days 3 and 5, followed by a decrease in viability at day 7 compared with the control. Cells stimulated at 60 Hz

showed increased viability at all time points (1, 3, 5, and 7 days), with the strongest effect observed on day 7 compared with the control. Statistical analysis was performed using one-way ANOVA on ranks, followed by Dunn's post hoc test to compare sample groups. Statistically significant results ($p \leq 0.05$) are indicated in brackets (number of families: 4; number of comparisons per family: 4; F(6, 16) = 157.0.9). Results are presented as the mean ± standard error of the mean from three independent experiments with four replicates per group (n = 12 per group). (b) GT-MSCs stained with crystal violet after 7 days of stimulation retained a fibroblastoid appearance similar to non-vibrated cells. However, vibrated cells also exhibited round and polyhedral shapes (orange arrows, left panel), which were more evident at higher magnification (orange arrows, right panel). Calcein-AM staining showed bright green fluorescence in the cell membranes, indicating cell viability following 7 days of vibratory stimulation. Left panel: 10× magnification, scale bar = 200 µm; right panel: 20× magnification, scale bar = 100 µm.

The results showed that vibratory stimulation enhanced resazurin reduction at 3 days of culture: at 20 Hz, the mean OD was 1.354 ± 0.0036, and at 60 Hz, the mean OD was 1.467 ± 0.0052. These findings indicate a positive effect on cell viability compared with the control group at 0 Hz, which had a mean OD of 1.165 ± 0.0108. This was followed by a further increase in resazurin reduction after 5 days of culture at 20 Hz (mean OD: 1.482 ± 0.0032) and at 60 Hz (mean OD: 1.608 ± 0.0055) compared with the control group (mean OD: 1.447 ± 0.0072). The 60 Hz group exhibited the highest cell growth by day 7, showing a 13.7% increase (mean OD:1.6683 ± 0.0345) over the control group at 0 Hz (mean OD: 1.4663 ± 0.0064), and a 22.02% increase over the 20 Hz group (mean OD: 1.275 ± 0.0105) by day 7 (Fig. 2a).

Calcein-AM staining showed that cells were positive for the stain, indicating that the GT-MSCs were viable and proliferating. Crystal violet staining confirmed that cells maintained a spindle and fibroblast-like morphology after 7 days in culture, with some cells adopting a round, polyhedral, or cuboidal shape, along with a spindle or fusiform, prominent nucleus (Fig. 2b).

In the 60 Hz osteogenic induction group, spheroid cell aggregates formed by day 7 but disassembled following vibrational stimulation. Nevertheless, GT-MSCs continued to populate the plate throughout the experiment, suggesting that detachment did not significantly impair proliferation.

The results suggest that stimulation at 20 Hz and 60 Hz enhances the viability and proliferation of GT-MSCs compared with non-vibrated conditions, with 60 Hz showing the highest cell growth by day 7, as confirmed by resazurin reduction, Calcein AM staining, and maintained cell morphology.

Vibration Promotes Osteogenic Differentiation and Mineralisation

Extracellular matrix mineralisation (ASR staining)

GT-MSCs were induced to undergo osteogenesis and were subjected to acoustic frequency vibratory stimulation (AFVS) at frequencies of 0 Hz, 20 Hz, or 60 Hz, with a static culture condition at 0 Hz serving as the control. Mineralisation of the extracellular matrix (ECM) was assessed using ASR, a marker of osteogenic differentiation, on days 7, 14, and 21 (Fig. 3). Visual examination revealed that the mineralised matrix appeared more robust in the vibration-stimulated groups (Fig. 3a). To quantify calcium deposition in the mineralised matrix, a calcium accumulation assay was performed by extracting the ARS dye and measuring the absorbance on the days when calcium nodules were visible, specifically on days 14 and 21 (Fig. 3b).

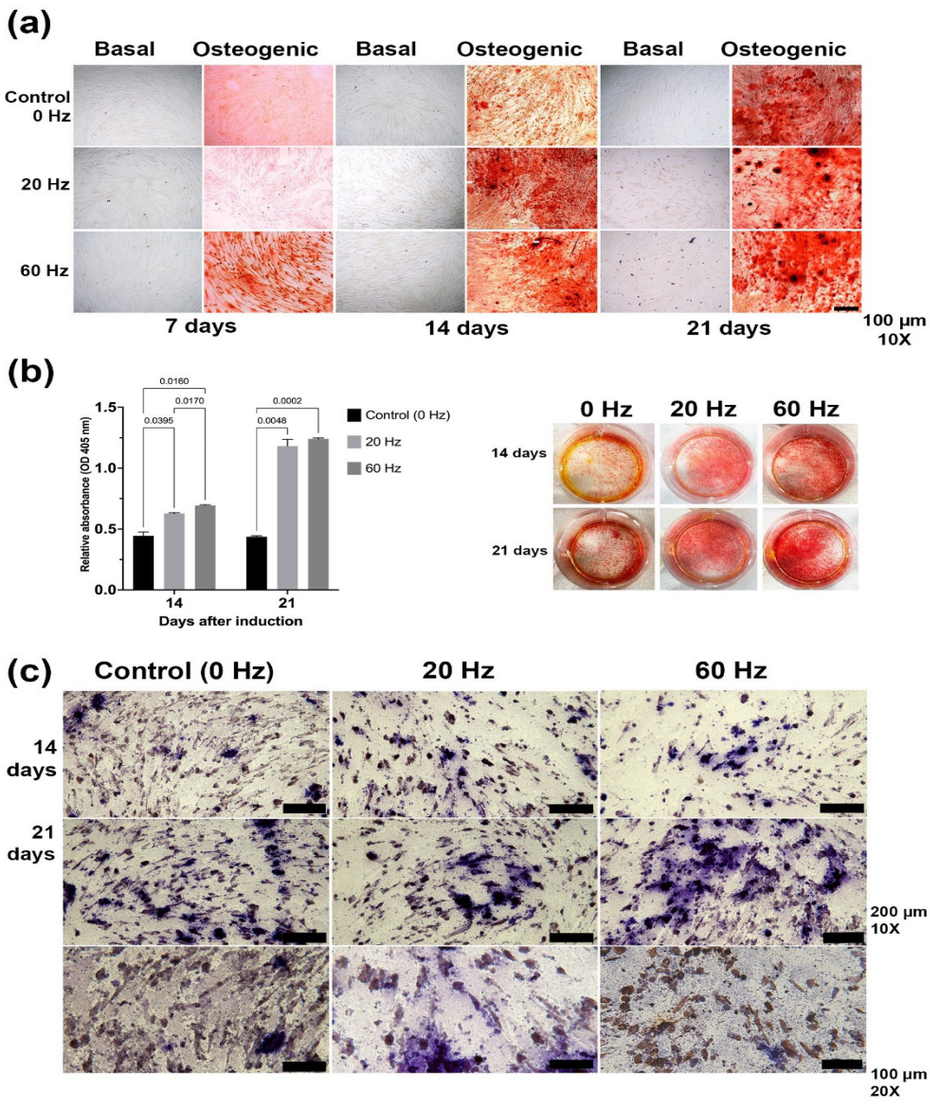

Fig. 3 Effects of vibratory stimulation on mineralised matrix formation in gingival tissue-derived mesenchymal stem cells (GT-MSCs). (a) Alizarin Red staining of GT-MSCs cultured under vibratory stimulation at 20 Hz and 60 Hz in both basal and osteogenic media for 7, 14, and 21 days. In basal medium, GT-MSCs did not form a mineralised matrix at any frequency. Non-vibrated and 60 Hz-stimulated cells maintained a fibroblastoid morphology. Notably, cells stimulated at 20 Hz displayed altered morphology by day 21 but remained negative for mineral deposition. By contrast, under osteogenic conditions, positive staining for mineralised nodules was observed at days 7, 14, and 21, with larger and more prominent nodules in the 20 Hz and 60 Hz groups compared with non-vibrated controls. (b) Calcium accumulation assay results showed increased calcium deposition in cells stimulated at 20 Hz (p = 0.0395) and 60 Hz (p = 0.0160) after 14 days, with even greater significance after 21 days (20 Hz: p = 0.0048; 60 Hz: p = 0.0002). (c) Immunohistochemical staining for vimentin revealed that vibrated cells predominantly exhibited a cuboidal morphology, whereas non-vibrated cells displayed more varied shapes. By day 21, non-vibrated cells retained a fibroblastic appearance, while vibrated cells maintained a cuboidal morphology with larger nuclei around areas of mineralised matrix. Magnification: 10× and 20×; scale bars: 200 µm and 100 µm, respectively.

The absorbance of ARS dye extraction revealed increased calcium deposition at 20 Hz (mean OD: 0.6276 ± 0.0046) and 60 Hz (mean OD: 0.6936 ± 0.0049) on day 14, compared with the control group (mean OD: 0.4443 ± 0.0313). By day 21, calcium accumulation further increased in the vibrated groups: 20 Hz (mean OD: 1.182 ± 0.0044) and 60 Hz (mean OD: 1.241 ± 0.0058), compared with the non-vibrated cells in osteogenic medium (mean OD: 0.4663 ± 0.0081). Interestingly, the calcium assay showed no significant increase in calcium content from day 14 to day 21 in the non-vibrated groups (Fig. 3b). The findings indicate that vibratory stimulation at 20 Hz and 60 Hz significantly enhanced calcium deposition and mineralisation in GT-MSCs undergoing osteogenesis, compared with the non-vibrated control, with noticeable improvements in the mineralised extracellular matrix observed by days 14 and 21, as confirmed by ARS staining and calcium deposition.

Morphological changes (Vimentin immunohistochemistry)

Cell morphology of the cells was evaluated using vimentin immunohistochemistry on GT-MSCs subjected to vibrations at 20 Hz and 60 Hz and compared with non-vibrated cells (0 Hz) cultured in osteogenic medium at days 14 and 21 (Fig. 3c). The results showed that vimentin expression in GT-MSCs revealed a predominantly large fibroblastoid morphology in GT-MSCs vibrated for 14 days, with some cells displaying a spindle shape and having a more compact cytoplasm. However, by day 21, the cell population had shifted predominantly to cuboidal and spindle-shaped morphologies.

In the vibrated groups at 20 Hz spindle-shaped 60 Hz, there was a notable presence of smaller cells compared with the non-vibrated group (0 Hz) culture in basal medium, which predominantly exhibited a cuboidal morphology. Furthermore, at 21 days, cells stimulated with 60 Hz were mainly round and cuboidal, although a few spindle-shaped cells were still observed (Fig. 3c). The round morphology was not associated with apoptotic characteristics. The results indicate that acoustic vibrational stimulation at 20 Hz and 60 Hz caused morphological changes in GT-MSCs, with cells shifting from a predominant fibroblastoid and spindle shape at 14 days to a more cuboidal and round morphology by day 21, without morphological signs of apoptosis.

Gene Expression Under Vibration

Basal medium conditions

To evaluate the effect of AFVS on osteogenic differentiation, we analysed the expression levels of osteogenic genes under both osteogenic and basal conditions at 7, 14, and 21 days (Fig. 4, right panel). In the basal medium, the expression levels of COL-I, ALP, and OPN were either absent or minimally detectable in both vibrated and non-vibrated groups at 7, 14, and 21 days, in contrast to GT-MSCs cultured in an osteogenic medium (Fig. 4a, 4b, 4c). However, GT-MSCs exposed to 20 Hz and 60 Hz in basal medium exhibited increased OPN expression after 21 days compared to the non-vibrated control (0 Hz), although these levels remained lower than those observed in the osteogenic medium control group (Fig. 4c, right panel). The results suggest that in basal medium conditions, GT-MSCs subjected to 20 Hz and 60 Hz vibrations exhibited increased OPN expression after 21 days, compared to the non-vibrated control; however, overall osteogenic gene expression was minimal compared with GT-MSCs undergoing osteogenesis.

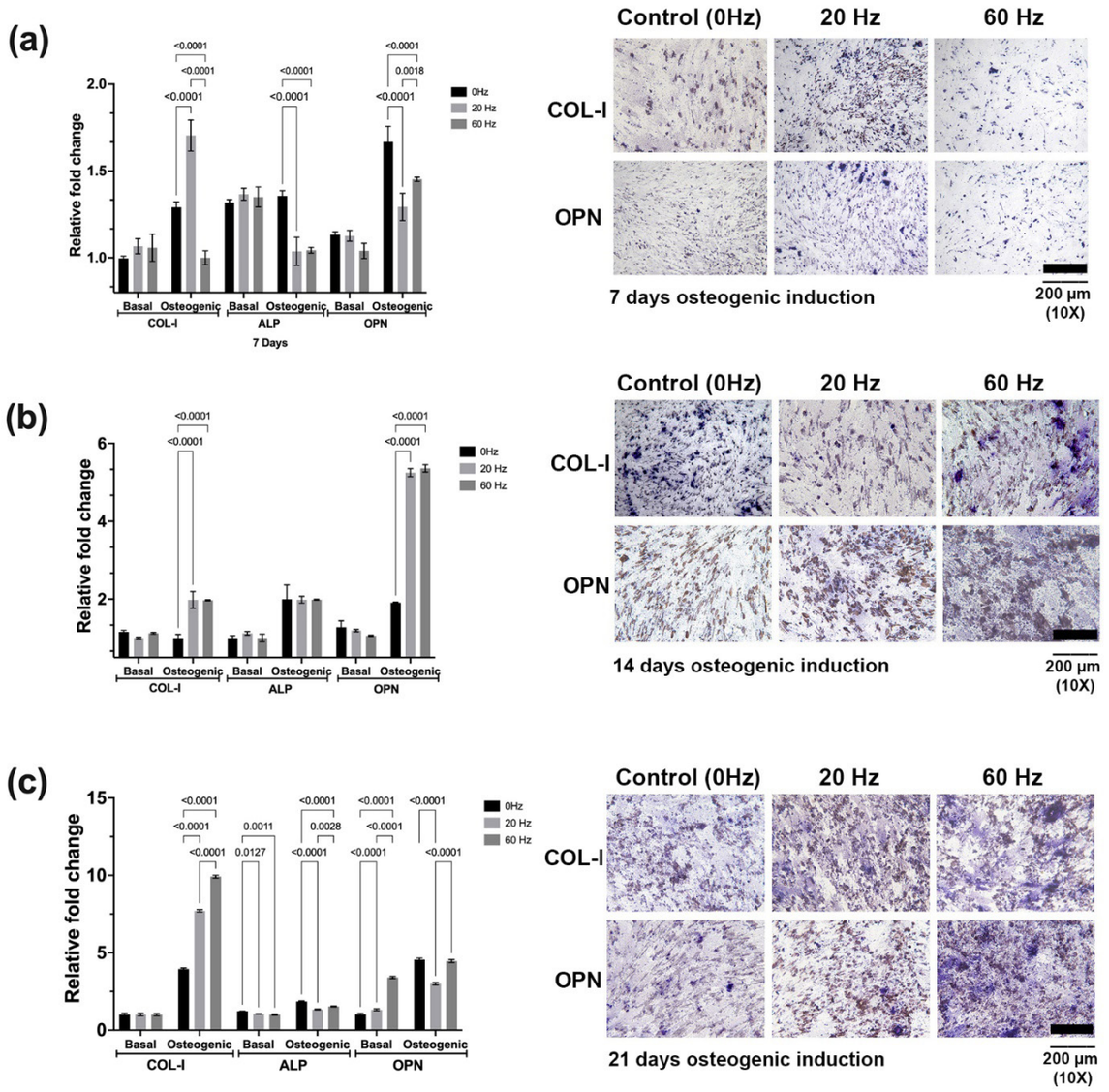

Fig. 4 Effects of vibratory stimulation on gene and protein expression of gingival tissue mesenchymal stem cells (GT-MSCs). (a), (b), and (c), left panels: Quantitative RT-PCR analysis of mRNA expression for collagen type I (COL-I), alkaline phosphatase (ALP), and osteopontin (OPN) after 7, 14, and 21 days of culture under basal or osteogenic conditions. Comparisons were made between vibrated cells at 20 Hz and 60 Hz and non-vibrated cells (0 Hz). Relative gene expression was normalised to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT-1). (a), (b), and (c), right panels: immunohistochemical analysis of COL-I and OPN protein expression in GT-MSCs cultured under osteogenic conditions, either under static conditions (0 Hz) or following vibratory stimulation at 20 Hz or 60 Hz. Results from one-way ANOVA are presented as the mean and standard deviation from three independent experiments (n = 9 per group). Brackets indicate statistically significant differences, with $p > 0.05$ considered significant.

Osteogenic medium

By contrast, groups cultured in osteogenic medium exhibited a more pronounced response to AFVS. At 20 Hz, COL-I expression increased significantly by 32% on day 7 (1.7041 ± 0.0895) compared with the static control (1.2909 ± 0.0309), and by 98.1% on day 14 (1.9818 ± 0.2148) compared with the control (1.0000 ± 0.0937) (Fig. 4a, 2b).

On day 14, vibration stimuli at both 20 Hz and 60 Hz increased COL-I by 98.18% (1.9818 ± 0.2148) and 91.125 (1.9712 ± 0.0156) respectably, compared with the control (1.0000 ± 0.0937; p = <0.0001) (Fig. 4b). By day 21 COL-I expression showed a 1.9-fold increased (7.7099 ± 0.0728) in the 20 Hz vibrated group, and a 2.5-fold (9.9122 ± 0.09283) increased in the 60 Hz group compared with the control group (3.9466 ± 0.0639; p = < 0.0001) (Fig. 4b, right panel).

On day 14, vibrational stimulation at both 20 Hz and 60 Hz resulted in a 2.08-fold increase (5.2500 ± 0.1045) and a 2.75-fold increase (5.36 ± 0.0958) in COL-I and OPN levels, respectively, compared to the control (1.909 ± 0.0153). By day 21, OPN mRNA expression of OPN was similar at 0 Hz (4.5500 ± 0.0995) and 60 Hz (4.4628 ± 0.0925), while a 34.15% decrease was observed at 20 Hz (2.9966 ± 0.07925) compared with the control group. Additionally, both frequencies inhibited ALP expression at day 7 (Fig. 4a). By day 14, ALP expression was similar across all groups: 0 Hz (1.9980 ± 0.03654), 20 Hz (1.9864 ± 0.0886), and 60 Hz (1.9853 ± 0.0124) under osteogenic induction (Fig. 4b). Finally, by day 21, ALP expression was reduced by 27.8% at 20 Hz (1.3358 ± 0.06324) and 17.2% at 60 Hz (1.5336 ± 0.0264) compared with the 0 Hz control group (1.8521 ± 0.0375) (Fig. 4c). Differentiation experiments suggest that under osteogenic conditions, AFVS significantly enhanced COL-I expression over time, especially at 60 Hz, while also increasing OPN by day 14 but reducing ALP expression by day 21.

Immunohistochemical Confirmation of Osteogenic Markers

Immunohistochemistry for COL-I and OPN was performed on the osteogenic induction groups, confirming functional osteogenic differentiation at 14 and 21 days (Fig. 4, right panels). COL-I protein was observed in the cytoplasm and within the mineralised matrix surrounding positive cells (Fig. 4, left panel). Similarly, OPN was detected in the cytoplasm and around the mineralised matrix, with occasional nuclear staining observed in the 20 Hz group. Notably, a more intense OPN expression was seen in the vibrated cells at both 20 Hz and 60 Hz on day 14 (Fig. 4b, right panel).

After 21 days, COL-I and OPN protein expression increased in the vibrated groups compared to non-vibrated cells (0 Hz) (Fig. 4c, right panel). Immunohistochemistry was not performed on the basal medium groups because no matrix formation was observed in either the stimulated or non-vibrated cells. Immunohistochemistry confirmed that vibratory stimulation at 20 Hz and 60 Hz enhanced the expression of osteogenic markers COL-I and OPN in GT-MSCs under osteogenic induction, with increased protein presence in the cytoplasm and mineralised matrix after 14 and 21 days, compared with the non-vibrated control (0 Hz), while no matrix formation was observed in basal medium groups.

DISCUSSION

Vibratory stimulation offers a flexible approach for studying the biology of DT-MSCs under dynamic conditions (Ambattu & Yeo, 2023). Sound frequency ranges from infrasound (below 20 Hz) to hypersound (above 1 GHz), allowing distinct

wave propagation through various sound generation devices (Pei *et al*., 2011; Ambattu & Yeo, 2023). Currently, vibration therapy is used as a part of integrative medicine in orthodontic treatment through devices such as AcceleDent and VPro5, although their clinical outcomes remain debatable (Judex & Pongkitwitoon, 2018). On the other hand, most *in vitro* studies on vibratory stimuli within the infrasound and audible frequency ranges had focused primarily on cell proliferation, migration, and, in some cases, apoptosis (Ambattu & Yeo, 2023).

This study offers valuable insights into the effects of AFVS on GT-MSCs, demonstrating that vibration at 60 Hz significantly enhances both proliferation and osteogenic differentiation, as evidenced by increased COL-I and OPN expression, and greater calcium deposition compared to lower frequencies (Chen *et al*., 2015; Baskan *et al*., 2017; Benjakul *et al*., 2019). The findings of the present study align with previous work in BM-MSCs, where higher frequencies tend to favour osteogenesis over adipogenesis, while lower frequencies, specifically those below 30 Hz, may have the opposite effect, enhancing adipogenesis via the p38 MAPK signaling pathway (Lau *et al*., 2010; Tirkkonen *et al*., 2011; Uzer *et al*., 2013; McClarren & Olabisi, 2018; Mehta *et al*., 2018; Steppe *et al*., 2020). Similarly, DT-MSCs, such as periodontal ligament-derived MSCs, have demonstrated significant chondrogenic and neurogenic potential following vibratory stimulation, particularly when encapsulated in alginate hydrogels with sustained transforming growth factor-β release (Robey, 2017; Omidvar *et al*., 2019; Bakdach & Hadad, 2020).

Much of the existing research has been conducted using differentiation media, as in the current study, and some researchers suggest that the observed changes in protein profiles may primarily be attributed to chemical rather than acoustic stimuli (McClarren & Olabisi, 2018; García-López *et al*., 2020; Ambattu & Yeo, 2023). However, the present results indicate that vibration alone, even without an osteogenic medium, can modestly upregulate osteogenic markers, such as OPN, indicating that AFVS has direct effects on GT-MSCs. They also confirm that vibratory stimulation is non-toxic, suggesting that vibration therapy is safe for clinical applications.

The mechanical stimulation parameters used in this study were carefully characterised using acoustic engineering approaches, confirming that sinusoidal waves were delivered perpendicular to the culture plate and were based on the challenges pointed out in earlier research (Broadbent *et al*., 2010; Marędziak *et al*., 2017; Beckingham *et al*., 2019). Although the harmonics generated were slightly higher than the intended output frequency, these likely did not affect the displacement of the culture wells and allowed for a direct evaluation of the vibrational effects *in vitro*.

Vibration protocols in current studies vary significantly, with frequencies ranging from 30 Hz to 800 Hz applied to different stem cell types, including human and rat BM-MSCs. Vibration durations have included continuous stimulation over 7 days or sessions of 30 minutes to 1 hour daily for up to 14 days (Lau *et al*., 2010; Nikukar *et al*., 2013; Chen *et al*., 2015). Recent research has also explored higher frequencies (up to 1.5 MHz) for human alveolar and mandibular fracture-derived MSCs, though such frequencies are not typical in clinical applications (Zhang *et al*., 2012; Ota *et al*., 2016; Bakdach & Hadad 2020; Ambattu & Yeo, 2023). A protocol with every-other-day stimulation was adopted to maintain culture integrity over 21 days, thereby avoiding the risks of monolayer detachment associated with daily vibration. This intermittent method showed positive effects, suggesting clinical protocols could benefit from less frequent use.

Current research has demonstrated the regenerative and immunomodulatory potential of GT-MSCs for dental and regenerative medicine applications (Poblano-

Pérez, Castro-Manrreza *et al.*, 2024; Poblano-Pérez, Monroy-García *et al.*, 2024). However, to the best of our knowledge, no studies have directly examined the effects of vibratory therapy on GT-MSCs, as opposed to its interaction with vibrational orthodontic devices.

The implications of this research are significant for both regenerative medicine and orthodontic practices. In this regard, GT-MSCs can be easily obtained from discarded periodontal tissues and provide a valuable yet underutilised model for exploring the effects of vibrational stimulation and enhancing our understanding of maxillofacial tissue biology (Poblano-Pérez, Castro-Manrreza *et al.*, 2024). Notably, the observed improvement in osteogenic differentiation at higher frequencies indicates that vibratory devices, such as those used in orthodontics, could be optimized to enhance therapeutic outcomes (Judex & Pongkitwitoon, 2018).

Additionally, we observed that higher frequencies (60 Hz) produced acceleration peaks translated into broad horizontal sinusoidal waves, whereas lower frequencies generated acceleration peaks with more pronounced vertical stretch waves, resulting in greater displacement of the plates. These observations suggest that the displacement produced by clinical devices should be considered before incorporating vibration therapy into orthodontic treatment because it may also influence patient discomfort.

A significant limitation of both previously reported findings and the present study is the lack of a clear explanation for the fundamental mechanisms by which acoustic stimulation drives the differentiation process. Current literature acknowledges that, in the context of bone remodeling in maxillofacial tissues, vibration can upregulate RANK in osteoclasts, RUNX2 in osteoblasts, and fibroblast growth factor 2 in periodontal ligament cells, key markers associated with cellular differentiation and activity (Lau *et al.*, 2010; Judex & Pongkitwitoon, 2018). Also, interconnected network of $Ca2^{+}$-dependent signaling, integrin/focal adhesion kinase activation, and Wnt/ß-catenin regulation orchestrates the mechanosensitive differentiation of stem cells into bone-forming osteoblasts (Steppe *et al.*, 2021; Pisheh *et al.*, 2022; Ambattu & Yeo, 2023). Hence, understanding the mechanotransducive pathways, will be necessary for refining vibration therapies.

Despite the promising findings of the present study, several limitations must also be acknowledged. First, the present study examined only two vibration frequencies (20 Hz and 60 Hz) without assessing intermediate frequencies or amplitudes, which may have provided a deeper understanding of the optimal parameters. Second, our in vitro study should be fully validated with 3D cell culture models to better mimic *in vivo* conditions. Third, the heterogeneity of vibratory parameters in previous research makes it difficult to make proper conclusions, even if we had included different MSC sources, which limited the broader applicability and relevance of the findings. Lastly, while *in vitro* results are encouraging, their clinical relevance remains to be verified by high-quality clinical trials in the future.

Future research should expand the range of frequencies and amplitudes tested, incorporate various dental-derived MSC sources, and utilise advanced 3D culture systems to mimic clinical scenarios better. Continued investigation into the signaling pathways activated by mechanical stimulation will further elucidate the mechanisms underlying MSC differentiation and support the translation of these findings into clinical practice. Ultimately, integrating vibratory therapy into regenerative and orthodontic protocols holds promise for accelerating tissue turnover and improving patient outcomes in maxillofacial medicine.

CONCLUSION

In the present study, differentiation markers were carefully selected to investigate osteogenic differentiation, and the results suggest that GT-MSCs responded to vibrational stimuli, with the effects depending on the frequency applied. Higher frequencies, such as 60 Hz, had a more pronounced effect on the osteogenic differentiation of GT-MSCs than did lower frequencies (e.g., 30 Hz). Although vibration alone did not induce osteogenic differentiation, higher-frequency stimulation (60 Hz) led to an increase in osteogenic gene expression after 21 days in culture. The results enable us to conclude that the gene expression data collected in this study accurately reflect the response of GT-MSCs to vibration. To the best of our knowledge, this is the first study to analyse the effects of AFVS on GT-MSCs at 20 and 60 Hz, contributing to a better understanding of the biology of different DT-MSCs. Moreover, GT-MSCs may serve as a valuable model for maxillofacial tissue research and hold potential for improving current therapies or developing new therapeutic strategies for the maxillofacial region.

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