Trends in Biomaterials and Artificial Organs
sbaoi
journal homepage: www.biomaterials.org.in/tibao
ISSN 0971-1198

Original Research Article

Synergistic Effects of Theobromine Derived Copper Nanoparticles on Periodontal Ligament Cell Proliferation and Migration

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Received: *3 December 2025*
Accepted: *11 February 2026*
Published online: *24 February 2026*

Keywords: *theobromine, human, health, medicine, illness, regenerative dentistry, disease*

Regeneration of periodontal tissues relies on effective cell proliferation, migration, and matrix remodeling. Recent advances in nanomedicine have demonstrated that phytochemical-based nanoparticles may offer synergistic benefits in enhancing these cellular processes. This study explores the effects of Theobromine-Derived Copper Nanoparticles (Theobro-CuNPs) on human periodontal ligament fibroblasts (PDLFs), with a focus on proliferation, migration, and gene expression of key regenerative markers. Theobro-CuNPs were synthesized via a green chemistry method using theobromine as a reducing agent and copper sulfate as the metal precursor. Characterization of nanoparticles was conducted using SEM, UV-Vis spectroscopy and EDX analytical techniques. PDLFs were treated with Theobro-CuNPs using doses of 1, 5 and 10 µg/ml, for durations of either 24 or 48 hours. The MTT assay was utilized to evaluate cell proliferation, meanwhile migratory behavior assessment employed scratch wound assays. Gene expression levels of VEGF, COL1A1, FN1, and MMP2 were quantified using qRT-PCR. Characterization confirmed the successful formation of spherical, nanoscale Theobro-CuNPs with a distinct surface plasmon resonance was detected near 575 nanometers, confirming nanoparticles formation. MTT assay revealed a concentration-dependent improvement in cellular viability was observed to be peaking at 10 µg/mL. Scratch assay results showed significantly enhanced wound closure in the Theo-CuNP-treated group compared to controls. qRT-PCR demonstrated significant upregulation of VEGF (3.2-fold), COL1A1 (2.8-fold), FN1 (2.5-fold), and MMP2 (3.6-fold), indicating strong pro-regenerative and matrix-remodeling effects. Theobro-CuNPs significantly enhance periodontal ligament cell proliferation, migration, and the upregulation of critical genes involved in tissue regeneration. These results imply that Theobro-CuNPs hold potential as a bioactive therapeutic platform for periodontal tissue engineering and regenerative dentistry.

Introduction

Periodontal tissue regeneration remains a significant clinical challenge in dentistry due to the complex structural and functional nature of the periodontium, which comprises cementum, alveolar bone, gingiva, and the periodontal ligament (PDL). Damage to these tissues caused by trauma, periodontitis, or surgical intervention necessitates coordinated cellular responses, including proliferation, migration, and matrix remodeling, to restore functional integrity [1]. Among the various components of periodontal healing, the periodontal ligament plays a pivotal role as it serves as a dynamic interface between the tooth root and alveolar bone. Human periodontal ligament (PDL) fibroblasts are key mediators of periodontal regeneration due to their capacity to proliferate, migrate, and secrete extracellular matrix (ECM) components required for tissue reorganization and repair. The regenerative process is tightly regulated by several molecular pathways involving angiogenesis, matrix remodeling, and collagen synthesis. Following genes implicated in this pathway include vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP2), fibronectin 1 (FN1), and collagen type I alpha 1 (COL1A1), each playing essential roles. VEGF is a major angiogenic factor that promotes neovascularization and ensures sufficient oxygen and nutrient supply during wound healing. COL1A1 encodes type I collagen, the most abundant protein in periodontal ECM, providing structural stability and tensile strength. FN1 is a multifunctional glycoprotein that facilitates cell adhesion, migration, and ECM organization. MMP2, a member of the matrix metalloproteinase fam-

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https://doi.org/10.65795/ybvxq161

ily, is involved in ECM degradation, enabling cell migration and matrix turnover during regeneration. Collectively, these genes orchestrate the essential biological events underpinning periodontal ligament repair [2].

While conventional approaches such as guided tissue regeneration (GTR), bone grafting, and application of growth factors have shown moderate success in PDL repair, limitations such as rapid degradation, poor targeting, and high costs have stimulated interest in novel biomaterials. In recent years, the field of nanotechnology has shown tremendous promise in regenerative dentistry owing to their distinct characteristics such as increased cellular internalization, high surface-area-to-volume ratios, and capacity to influence cellular functions on a molecular scale. Metal-based nanoparticles, especially those derived from biocompatible metals like copper, have attracted attention for their dual functional properties - antimicrobial and pro-regenerative effects. Copper nanoparticles (CuNPs) exhibit strong pro-angiogenic and wound healing properties due to their capacity to stimulate VEGF expression, modulate redox signaling, and support cellular proliferation. However, the cytotoxicity and uncontrolled release of ions from metallic nanoparticles necessitate surface functionalization with bioactive molecules to ensure safe and targeted delivery [3]. In this context, theobromine, a naturally occurring methylxanthine found in cocoa beans, emerges as a promising candidate. Theobromine exhibits antioxidant, anti-inflammatory, and mineralizing properties that are particularly relevant to periodontal applications. Its ability to modulate cell signaling pathways, promote osteogenesis, and support fibroblast function makes it an ideal bio-reductant and capping agent for nanoparticle synthesis. Theobro-CuNPs represent a novel biomaterial with synergistic potential. The green synthesis approach using theobromine not only enhances the stability and biocompatibility of CuNPs but also imparts additional therapeutic benefits by preserving the functional groups of the bioactive capping agent [4]. Preliminary studies have suggested that such hybrid nanoparticles may accelerate wound healing, promote cell migration, and support angiogenesis, yet their specific role in modulating PDL cell functions has not been thoroughly investigated. Given the regenerative complexity of the periodontal ligament, it is essential to develop biomaterials that can simultaneously promote cell proliferation, directed migration, and matrix remodeling while minimizing inflammatory responses. The current study investigates the synergistic effects of Theobro-CuNPs on PDL fibroblast proliferation and migration, focusing on the expression of VEGF, COL1A1, FN1, and MMP2 as molecular markers of regeneration. The rationale for selecting these genes stems from their integral involvement in cell–ECM interaction, angiogenesis, and tissue remodeling—all key events in periodontal repair [5,6].

In vitro models using human PDL fibroblasts offer an ideal platform to evaluate the biological compatibility and functional outcomes of novel biomaterials. These cells respond sensitively to chemical and physical stimuli, making them suitable for assessing the effects of nanomaterials on wound healing dynamics. Cell proliferation can be examined using MTT method to determine mitogenic activity, whereas cell migration is commonly observed using scratch assays that measure directional motility, a prerequisite for tissue repair. Complementary molecular analyses using qRT-PCR allow the quantification of gene expression changes that underpin these phenotypic behaviors [7].

By integrating cellular and molecular assessments, this study aims to provide a comprehensive evaluation of the regenerative potential of Theobro-CuNPs in periodontal therapy. We hypothesize that Theobro-CuNPs will enhance PDL fibroblast proliferation and migration more effectively than copper or theobromine alone due to their combined biochemical and nanostructural features. Furthermore, we anticipate that Theobro-CuNPs will upregulate VEGF, COL1A1, and FN1, thereby promoting angiogenesis and matrix synthesis, while also enhancing MMP2 expression to facilitate matrix turnover and migration [8]. The broader implication of this research lies in its potential contribution to the development of multifunctional, naturally derived, and cost-effective nanomaterials for periodontal regeneration. Unlike synthetic growth factors and polymers, plant-based and bio-reduced nanoparticles offer a safer, eco-friendly alternative with reduced immunogenicity. Moreover, the dual ability of Theobro-CuNPs to support regeneration while potentially inhibiting bacterial colonization could make them particularly suitable for clinical applications in periodontally compromised sites. In conclusion, this study explores a novel biomimetic strategy by combining the regenerative potential of theobromine with the bioactivity of copper in nanoparticle form. Through in vitro evaluation of PDL fibroblast behavior and gene expression analysis, we aim to elucidate the synergistic effects of Theobro-CuNPs in promoting key regenerative processes. The outcomes of this study could pave the way for the future development of biologically active dental biomaterials aimed at improving periodontal repair and efficacy in therapeutic advances [9].

Materials and Methods

Chemicals and reagents

Sigma-Aldrich from which Theobromine (>98% purity) was procured, and used as a natural reducing and capping agent for nanoparticle synthesis. Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), used as the metal precursor, was also obtained from Sigma-Aldrich. Analytical-grade ethanol, methanol, and deionized water were used for nanoparticle synthesis and washing steps. Gibco-sourced components such as DMEM, FBS, antibiotics (penicillin-streptomycin), and trypsin-EDTA were used for cell culture maintenance. Thermo Fisher Scientific provided TRIzol reagent, RevertAid First Strand cDNA synthesis kit, and SYBR Green qPCR Master Mix for RNA extraction and real-time PCR. Custom primers for VEGF, COL1A1, FN1, MMP2, and GAPDH were synthesized by Eurofins Genomics (India). Additional reagents including MTT powder, DMSO, phosphate-buffered saline (PBS), and culture-grade plasticware were obtained from HiMedia Laboratories. All reagents used were of molecular biology or cell culture grade and handled under sterile conditions as per standard protocols.

Synthesis of Theobro-CuNPs

Theobro-CuNPs were synthesized through a green chemistry method using theobromine as both a reducing and stabilizing agent. In this process, 100 mL of 1 mM copper sulfate ($CuSO_4$) solution was mixed with 10 mL of a 1% theobromine extract prepared in distilled water. The solution was maintained at 70°C under constant stirring for 2 hours, resulting in a noticeable change in colour–indicative of copper nanoparticle formation. At 12,000 rpm for 20 minutes the evolved colloidal suspension was centrifuged to separate the nanoparticles, followed by triple washing with deionized water to eliminate unbound residues and impurities. The final nanoparticle pellet was dried and stored at 4°C in airtight vials for subsequent characterization and biological assays [10].

Characterization of Theobro-CuNPs

The synthesized Theobro-CuNPs were characterized using a suite of spectroscopic and microscopic methods. ZEISS EVO18 SEM was used to perform Scanning Electron Microscopy (SEM) to observe the surface morphology and size distribution of the nanoparticles. The images revealed that the Theobro-CuNPs were predominantly spherical with slight agglomeration, and the estimated size of the average particle was found to be in the range of 40–70 nm.Energy Dispersive X-ray Analysis (EDX) attached to the SEM system was used to ascertain the nanoparticles and their elemental composition. The EDX spectra confirmed the presence of copper as the major element, with a characteristic peak at ~8 keV, along with traces of oxygen and carbon, indicating capping by theobromine and minimal impurities. Shimadzu UV-2600 spectrophotometer was used to conduct a UV–Visible Spectroscopy. The Theobro-CuNPs exhibited a characteristic surface plasmon resonance (SPR) peak at approximately 570–580 nm, which is indicative of copper nanoparticle formation and confirms their optical stability.These characterization techniques validated the successful synthesis of Theobro-CuNPs with expected nanoscale features, elemental purity, and characteristic absorbance properties [11].

Cell culture and treatment protocol

Human periodontal ligament fibroblasts (PDLFs) were obtained from a certified commercial cell line repository (e.g., ATCC) and maintained in

Table 1: Primers used

Gene	Forward Primer (5' ? 3')	Reverse Primer (5' ? 3')	Function
VEGF	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA	Angiogenesis and vascular development
COL1A1	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC	Type I collagen; ECM structural protein
FN1	TGCAGCTGATCCTGAGGTTT	CTTGACCTGAGTTGGTGTGG	Fibronectin; cell adhesion and ECM
MMP2	TTCAGGATGCAGTTTGCCTG	GTGTGTTGCAGCTGATGTCC	Matrix remodeling and cell migration
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC	Housekeeping gene (internal control)

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 1% L-glutamine. The cells were cultured under standard conditions in a humidified incubator at 37°C with 5% CO, . For experimental procedures, PDLFs were seeded in 6-well culture plates and allowed to reach approximately 70–80% confluency. The cells were then treated with Theobro-CuNPs at concentrations of 1 μg/mL, 5 μg/mL, and 10 μg/mL for 24 or 48 hours, depending on the assay requirements. Untreated cells were maintained as negative controls, while additional groups treated with copper sulfate alone or theobromine alone were included as comparative controls to evaluate the synergistic effects of the nanoparticle [12].

Cell proliferation assay (MTT)

MTT assay was employed to determine metabolic activity and proliferation levels of PDLFs after exposure to treatment with Theobro-CuNPs. After the designated treatment phase, to each well containing the cells, 20 μL of MTT solution (5 mg/mL in PBS) was added and incubated at 37°C for 4 hours. During this period, viable cells reduced the MTT to insoluble purple formazan crystals. At the end of the incubation, the medium was carefully discharged, and to dissolve the resulting formazan crystals, 100 μL of dimethyl sulfoxide (DMSO) was added to each well. To allow full stabilization, the plate was incubated on a gentle shaker for 10 minutes and absorbance was measured at 570 nm using a microplate reader. The absorbance readings were adjusted relative to untreated control values to ensure comparative analysis [13].

Evaluation of cell migration by scratch assay

Human periodontal ligament fibroblasts were seeded in 6-well plates and grown until a consistent monolayer was established. A straight scratch was introduced across the center of each well using a sterile pipette tip to simulate a wound. To eliminate floating cells and debris, the culture wells were gently washed using phosphate-buffered saline (PBS). Subsequently, fresh growth medium containing Theobro-CuNPs at concentrations of 1, 5, and 10 μg/mL was added to the wells. Images of the scratch area were captured at 0 hours (immediately after scratching) and again at 12 and 24 hours to monitor cell migration into the wound area. The reduction in the scratch width over time was used as an indicator of the migratory capacity of the treated cells compared to untreated controls [14].

Gene expression analysis by qRT-PCR

RNA was extracted from human periodontal ligament fibroblasts (PDLFs) utilising TRIzol reagent (Invitrogen USA), following the instructions provided by the manufacturer. The concentration and purity of the RNA were assessed spectrophotometrically, and equal quantities of RNA were reverse transcribed into cDNA using a commercially available RevertAid First Strand cDNA Synthesis Kit. SYBR Green-based qRT-PCR was conducted to measure relative mRNA expression: VEGF, COL1A1, FN1, and MMP2 (table 1). GAPDH served as the housekeeping gene for normalization of expression levels. The amplification reactions were performed in triplicates, and relative quantification followed the 2^-ΔΔCt method. Changes in gene expression were analyzed to determine the effect of Theobro-CuNPs on angiogenesis, extracellular matrix production, fibronectin synthesis, and matrix remodeling. The results were compared with both untreated control and mono-component (copper or theobromine) treated groups to assess the synergistic effect of Theobro-CuNPs[15].

Results

Characterization of Theobro-CuNPs

The characterization of the synthesized Theobro-CuNPs confirmed successful nanoparticle formation with desirable physicochemical properties. Scanning Electron Microscopy (SEM) analysis showed that the nanoparticles had a mostly spherical shape with moderate dispersion, with particle sizes ranging between 40 and 70 nm (figure 1). A surface plasmon resonance peak was detected via UV-Vis spectroscopy at around 570–580 nm, confirming nanoparticle characteristics (figure 2). Scanning Electron Microscopy (SEM) analysis showed that the nanoparticles had a mostly spherical shape with moderate dispersion, with particle sizes ranging between 40 and 70 nm. Energy Dispersive X-ray (EDX) analysis verified the elemental makeup of the synthesized particles, a prominent copper peak near 8 keV, along with minor signals for oxygen and carbon, which were attributed to the capping effect of theobromine and residual plant-based components (figure 3). These findings collectively validated the successful green synthesis of Theobro-CuNPs with defined morphology, elemental purity, and surface characteristics conducive to biological applications.

Representative Scanning Electron Microscopy (SEM) image of Theobro-CuNPs displaying a uniform, spherical morphology with slight aggregation. The nanoparticle diameter was estimated to be within the 40–70 nm range, indicating successful nanoscale synthesis ideal for cellular interaction and tissue regeneration studies.

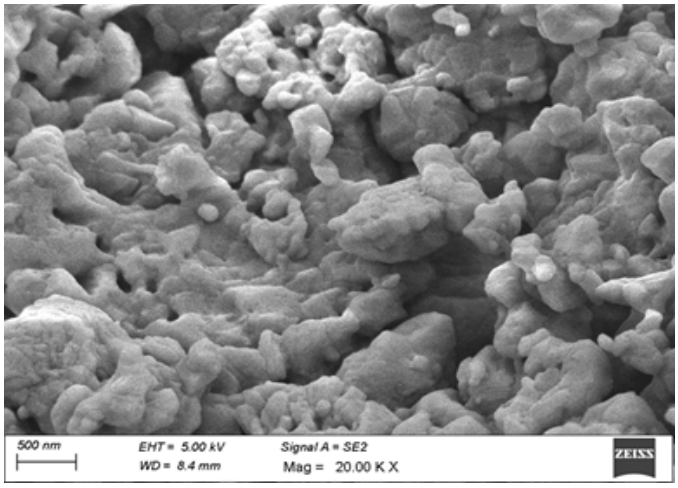

Figure1: SEM Image of Theobro-CuNPs

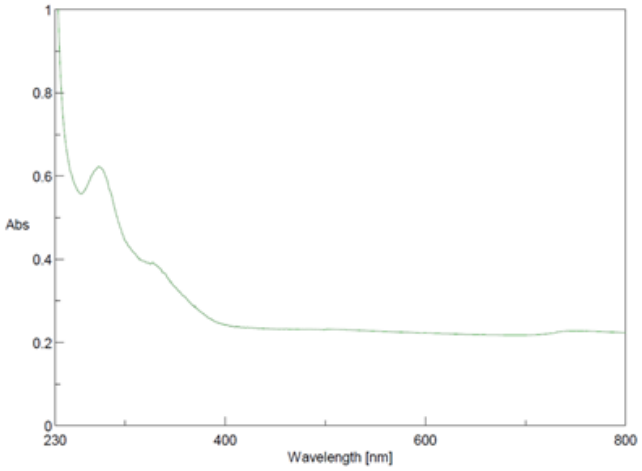

Figure 2: UV–Visible Spectroscopy of Theobro-CuNPs

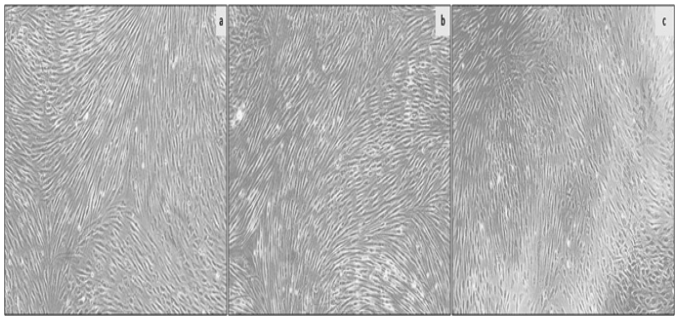

Figure 4: Morphological Evaluation of PDLFs Post-Treatment with Theobro-CuNPs a) Untreated control cells displaying normal fibroblastic morphology; b) Cells treated with 1 µg/mL Theobro-CuNPs showing mild enhancement in cell density and spreading; c) Cells treated with 10 µg/mL Theobro-CuNPs exhibiting prominent proliferation, dense confluency, and extended cytoplasmic processes, indicative of enhanced biocompatibility and cellular activity

A surface plasmon resonance peak was detected via UV-Vis spectroscopy of Theobro-CuNPs at around ~575 nm, confirming nanoparticle characteristics.

Energy Dispersive X-ray (EDX) spectrum of Theobro-CuNPs showing strong copper (Cu) peaks around 8 keV, along with minor peaks corresponding to oxygen and carbon. This confirms the elemental composition of the nanoparticles and suggests the presence of organic components from theobromine acting as a capping agent.

Morphological assessment of PDLFs following Theobro-CuNPs treatment

The treatment of human periodontal ligament fibroblasts (PDLFs) with Theobro-CuNPs resulted in notable improvements in both cell morphology and viability, as observed under phase-contrast microscopy (figure 4). At the lowest concentration of 1 µg/mL, cells maintained healthy spindle-shaped morphology with a slight increase in confluency compared to the untreated control. At 5 µg/mL, a marked enhancement in cell density and spreading was observed, indicating stimulated proliferation. The most pronounced effect was seen at 10 µg/mL, where PDLFs exhibited robust growth with dense cellular architecture and active cytoplasmic extensions, reflecting enhanced cellular activity and biocompatibility. In contrast, cells treated with copper sulfate or theobromine alone showed only moderate growth stimulation, suggesting that the combined nanoparticle formulation elicited a superior proliferative response. No signs of cytotoxicity or morphological abnormalities were observed at any tested concentration, supporting the safety and regenerative potential of Theobro-CuNPs in periodontal cell models.

Dose-dependent proliferative response of PDL to Theobro-CuNPs (MTT Assay)

Cell proliferation of PDLFs treated with Theobro-CuNPs was quantified using the MTT assay using a dose range of 1 to 100 µg/mL. Following 24-hour treatment, cell viability improved in a concentration-dependant manner. At 1 µg/mL, cells exhibited a slight but consistent increase in metabolic activity (~110%) compared to untreated control (figure 5). This proliferative effect was significantly enhanced at 5 µg/mL and peaked at 10 µg/mL, with cell viability reaching approximately 140–150%, indicating optimal stimulation of cell growth. At the upper end of the tested concentrations (50 and 100 µg/mL), a reduction in viability was observed, suggesting a threshold beyond which cytotoxic effects may begin to occur. These results indicate that Theobro-CuNPs promote cell proliferation at lower doses, while excessively high

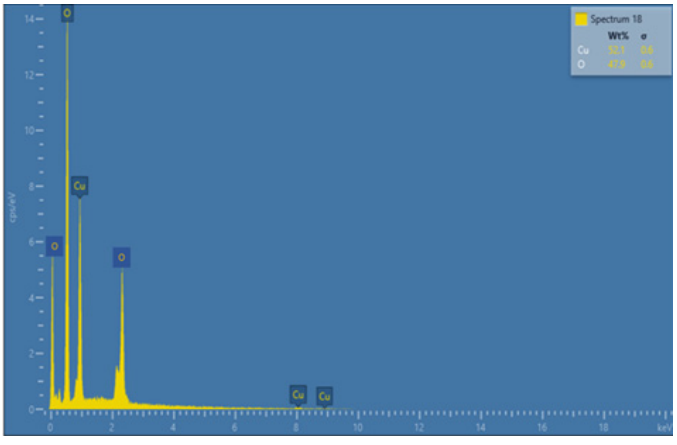

Figure 3. EDX Spectral Analysis

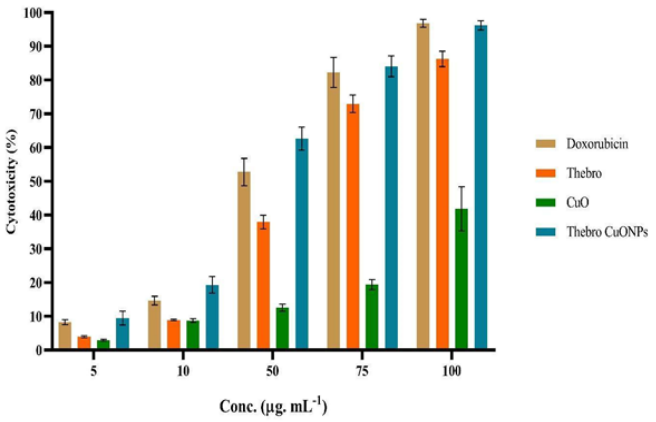

Figure 5: MTT Assay Showing Cell Viability of PDLFs Treated with Theobro-CuNPs

concentrations may impair cell viability, underscoring the importance of dosage optimization for regenerative applications.

Bar graph representing the percentage viability of human periodontal ligament fibroblasts (PDLFs) after 24-hour treatment with Theobro-CuNPs at concentrations of doses 1 to 100 μg/mL. Maximum proliferation was observed at 10 μg/mL, while upper end tested cncentrations (50 and 100 μg/mL) showed reduced viability. Values are expressed as mean ± SD (n = 3); $p < 0.05$, $\mathbf{p} < 0.01$ vs. control group.

Enhanced migratory response of PDL induced by Theobro-CuNPs (scratch assay)

The scratch wound healing assay demonstrated a clear enhancement in the migratory capacity of human periodontal ligament fibroblasts (PDLFs) following treatment with Theobro-CuNPs. In the untreated control group, the average wound closure after 24 hours was approximately 18.82%, indicating baseline cell migration (figure 6). Cells treated with theobromine alone showed improved migration, with an average wound closure of 40.97%, while copper nanoparticles (CuNPs) alone resulted in a further increase to 46.29%. Notably, the Theo-CuNP-treated group exhibited the most significant enhancement in migration, achieving an average wound closure of 61.67%, suggesting a strong synergistic effect. These results highlight the superior capacity of Theobro-CuNPs to promote fibroblast migration, a critical factor in periodontal tissue regeneration.

Upregulation of regenerative genes in PDLFs by Theobro-CuNPs (qRT-PCR analysis)

The gene expression analysis revealed a marked upregulation of key regenerative markers in PDLFs treated with Theobro-CuNPs. At the optimal concentration of 10/ μg/mL, VEGF levels rose by nearly 3.2 times relative to untreated samples($p < 0.01$), indicating strong pro-angiogenic activity. COL1A1, which encodes type I collagen essential for extracellular matrix formation, was upregulated by 2.8-fold ($p < 0.05$), suggesting enhanced collagen deposition. Similarly, FN1 was upregulated by 2.5-fold increase ($p < 0.05$), reflecting improved cellular adhesion and matrix organization. The most notable change was observed in MMP2, with a 3.6-fold upregulation ($p < 0.01$), indicating active matrix remodeling and support for cell migration. These results suggest that Theobro-CuNPs effectively stimulate molecular pathways involved in angiogenesis, matrix synthesis, and remodeling—critical for periodontal tissue repair and regeneration (figure 7).

Bar graph showing the relative mRNA expression levels of VEGF, COL1A1, FN1, and MMP2 in human periodontal ligament fibroblasts (PDLFs) following treatment with Theobro-CuNPs at 10 μg/mL. All genes were significantly upregulated juxtaposed to the untreated control, with VEGF and MMP2 showing the highest fold increases. Data are expressed as mean ± SD (n = 3); $p < 0.05$, $p < 0.01$ vs. control.

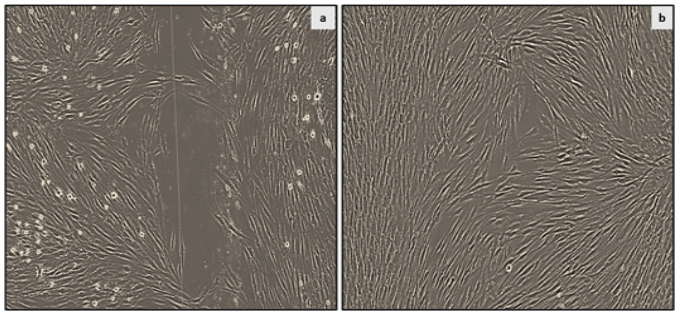

Figure 6: Scratch Wound Healing Assay of PDL Treated with Theobro-CuNPs. a) Control group showing moderate wound closure with limited cell migration after 24 hours; b) Theo-CuNP-treated group displaying significant wound closure, indicating enhanced fibroblast migration and regenerative potential

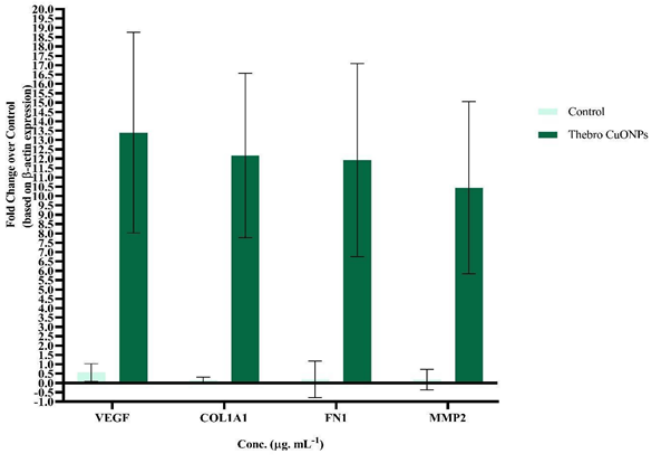

Figure 7: Gene Expression Analysis of PDL Treated with Theobro-CuNPs

Discussion

The present study explored the biocompatibility and regenerative efficacy of Theobro-CuNPs on human periodontal ligament fibroblasts (PDLFs), with emphasis on their ability to promote cell proliferation, migration, and gene expression linked to periodontal tissue repair. The use of green synthesis strategies involving natural plant-based compounds like theobromine has attracted significant attention in recent times, owing to their environmentally sustainable synthesis, affordability, and low toxicity toward cells[16]. In this context, theobromine, a methylxanthine alkaloid extracted from cacao, offers antioxidant, anti-inflammatory, and tissue-stimulating properties that complement the known pro-angiogenic and antimicrobial effects of copper ions.The synthesis of Theobro-CuNPs via the reduction of copper sulfate using a theobromine extract yielded stable, spherical nanoparticles in the 40–70 nm range as confirmed by SEM. A distinct SPR signal was observed in the UV–Vis spectrum showing a characteristic surface plasmon resonance peak at ~575 nm, consistent with the expected optical behavior of copper nanoparticles. The EDX spectrum confirmed the presence of elemental copper and minor amounts of oxygen and carbon, the latter likely originating from the theobromine capping matrix. These results validated the formation of biofunctional copper nanoparticles through a clean and efficient green synthesis approach suitable for biomedical applications [17].

The cell viability results using the MTT assay revealed a dose-dependent proliferative effect of Theobro-CuNPs on PDLFs. At concentrations of 1–10 μg/mL, cell viability significantly increased, with the highest proliferation observed at 10 μg/mL (~150% relative to control). This finding indicates that the combined effects of copper and theobromine create a favorable microenvironment that stimulates cellular metabolism and mitotic activity. Theobromine alone promoted moderate proliferation, consistent with its known role in enhancing cellular energy metabolism and modulating inflammatory responses. Copper nanoparticles, although beneficial in low doses, can exhibit cytotoxicity at higher concentrations due to the generation of reactive oxygen species (ROS). However, when conjugated with a phytochemical such as theobromine, the resulting nanoparticle system appears to maintain cellular homeostasis more effectively. The findings reinforce the notion that nanoparticle design based on synergistic biofunctionalization improves cellular tolerance and activity. A scratch assay was employed to evaluate cell migration, a crucial process involved in tissue repair and periodontal regeneration. The untreated control cells showed minimal migration (~18.8% closure), while treatment with theobromine and CuNPs alone resulted in moderate wound closure (~41% and ~46%, respectively). Theo-CuNP-treated cells, however, demonstrated significant migration (~61.7% closure), outperforming the individual treatments. These observations suggest that Theobro-CuNPs enhance

cytoskeletal reorganization and intercellular communication required for directed motility. The superior migration observed may be attributed to the synergistic modulation of signaling cascades by copper ions (which can activate angiogenic and growth factor pathways) and theobromine (which can stabilize redox conditions and reduce pro-inflammatory mediators) [18].

At the molecular level, qRT-PCR analysis provided further insights into the mechanisms underlying the observed bioactivity. The gene expression of VEGF, a key mediator of angiogenesis, was markedly upregulated (3.2-fold), supporting the hypothesis that Theobro-CuNPs can stimulate neovascularization—an essential process for supplying nutrients and oxygen to regenerating tissues. Enhanced VEGF expression is also critical for fibroblast migration, extracellular matrix (ECM) remodeling, and the mobilization of precursor cells toward the damaged tissue area. Upregulation of COL1A1 (2.8-fold) indicated increased synthesis of type I collagen, the principal component of the periodontal ECM. This is vital for restoring tissue architecture and function following injury. Likewise, FN1, which encodes fibronectin, was upregulated by 2.5-fold. Fibronectin is essential for facilitating cell adhesion, guiding migration and organizing the extracellular matrix, especially during early stages of tissue repair. The increased expression of MMP2 (3.6-fold), a matrix metalloproteinase responsible for degrading ECM components, implies dynamic remodeling and restructuring of the extracellular space, allowing for cell movement and re-epithelialization. The coordinated upregulation of these genes reflects an active and favorable regenerative environment promoted by Theobro-CuNPs. These findings align with prior studies demonstrating that metal-based nanoparticles can influence cell proliferation and gene expression, but this study uniquely highlights the amplified effect achieved through theobromine capping. The enhancement of biological functions by such a hybrid nanoparticle system may stem from the controlled release of copper ions, antioxidant stabilization by theobromine, and the provision of bioactive cues that mimic physiological conditions [19].

Importantly, at the most effective concentrations (1–10 μg/mL), the nanoparticles exhibited no signs of cytotoxicity, indicating that the nanoparticles are safe for use in regenerative applications. However, at higher concentrations (e”50 μg/mL), a slight decline in cell viability was noted, underscoring the importance of dose optimization and nanoparticle surface modification in minimizing adverse effects. The findings of this study support the emerging paradigm that multifunctional nanoparticles, especially those derived from phytochemicals, can synergize therapeutic effects and serve as potential alternatives to traditional regenerative therapies or scaffolds. Moreover, Theobro-CuNPs demonstrated beneficial effects on early-stage indicators such as cell proliferation and motility, as well as on later-stage gene expression associated with tissue regeneration. Additional in vivo experiments and histological assessments are needed to validate tissue integration, support angiogenic potential and ensure long-term compatibility [20].

Conclusion

This study demonstrates that Theobro-CuNPs, synthesized through a green approach, significantly promote the proliferation and migration of human periodontal ligament fibroblasts. The nanoparticles also upregulate genes critical to angiogenesis (VEGF), matrix synthesis (COL1A1 and FN1), and ECM remodeling (MMP2) were notably upregulated. The combined bioactivity of copper and theobromine enhances cellular behavior beyond that of either component alone, highlighting the synergistic effect. These findings support the application of Theobro-CuNPs as a promising biomaterial for periodontal tissue engineering and other regenerative therapies.

References

1. Wen, S., Zheng, X., Yin, W., et al.: Dental stem cell dynamics in periodontal ligament regeneration: from mechanism to application. Stem Cell Research & Therapy, 15, 389 (2024).
2. Park, H., Patil, TV., Dutta, SD., et al., Extracellular MatrixBioinspired Anisotropic Topographical Cues of Electrospun Nanofibers: A Strategy of Wound Healing through Macrophage Polarization. Advanced Healthcare Materials,13, 2304114 (2024).
3. Parihar, AS., Nandanan, N., Patadiya, HH., et al., Efficacy of 3D-Printed Scaffolds in Guided Tissue Regeneration for Combined Endo-Perio Lesions: A Clinical and Radiographic Study. Journal of Pharmacy and Bioallied Sciences,17, S568-S570 (2025).
4. Fitoz, A., Yazan, Z., Önal, M., Simultaneous Trace Electrochemical Determination of Xanthine Theophylline and Theobromine with a Novel Sensor Based on a Composite Including Metal Oxide Nanoparticle Multiwalled Carbon Nanotube and NanoNamontmorillonite Clay. Electroanalysis, 33, 2226-2234,(2021).
5. Biswas, S., Gangadaran, P., Dhara, C., et al., Extracellular Vesicles in Osteogenesis: A Comprehensive Review of Mechanisms and Therapeutic Potential for Bone Regeneration. Current Issues in Molecular Biology, 47, 675, (2025).
6. Senthil, R., Bevacizumab-conjugated curcumin nanoparticles promote cytotoxicity and apoptosis in human malignant oral keratinocytes in vitro. Journal of Oral and Maxillofacial Surgery, (2025).
7. Dandagi, P., Martin, TM., Babu, Y.,In silico and glioblastoma cell line evaluation of thioflavin-derived zinc nanoparticles targeting beclin protein. Cureus,16,(2024).
8. Sureendar, B., Chellappa, LR., In Vitro Evaluation of Antifungal Activity of a Dental Varnish Formulated with Ginger and Rosemary Mediated Titanium Oxide Nanoparticles. Journal of Pioneering Medical Sciences, 14, 278-282 (2025).
9. Umakanth, K., Martin, TM., An In Vitro and In Silico Study of Luteolin-Loaded Zinc Oxide Nanoparticles: Enhancing Bioactivity and Efficacy for Advanced Therapeutic Applications Against Cariogenic Microorganisms. Cureus,16, (2024).
10. Alvarez-Yanamango, E., Obregon, D., Ibañez, A., A Comprehensive Review of the Development of Green Extraction Methods and Encapsulation of Theobromine from Cocoa Bean Shells for Nutraceutical Applications. Food Engineering Reviews, 1-22 (2025).
11. Sebastian, S., Martin, TM., Kumar, MSK., Thymoquinone-Loaded Zinc Nanoparticles Mitigate Inflammation and Inhibit Glioblastoma Progression: A Novel Therapeutic Approach, (2025).
12. Jayaraman, S., Veeraraghavan, V.P., Deoxyelephantopin induces cell death in oral cancer cells via the downregulation of AKT1-mTOR-mediated mechanisms. Journal of Oral and Maxillofacial Pathology, 29, 193-205 (2025).
13. Srimathi, B., Roy, A., Elumalai, P., Chemotherapeutic Effect of Rutin on TGF-â/SMAD2 Signalling Molecules Gene Expression in Oral Cancer Cells. Oral & Maxillofacial Pathology Journal, 15 (2024).
14. Mouritzen, M.V., Jenssen, H, Optimized scratch assay for in vitro testing of cell migration with an automated optical camera. Journal of visualized experiments, JoVE, 57691, (2018).
15. Sagar, S., Ramani, P., Jeyaraj, G., Jayaraman, S., Moses, S. Decoding the Biochemical Pathways of Orthodontic Tooth Movement: A Focus on Salivary IL-17A and 1, 25-Dihydroxycholecalciferol, (2025).
16. Nanci, A., Bosshardt, D.D., Structure of periodontal tissues in health and disease. PERIODONTOLOGY, 40,11 (2006).
17. Payra, M., Mohanraj, K.G., Martin,T.M.,Modulation of Inflammation in McCoy Cells by Zinc Nanoparticles Conjugated With â-Chitosan, Cureus, 16,(2024).
18. Zhang, M., Zhang, H., Jia, L., et al, Health benefits and mechanisms of theobromine. Journal of Functional Foods, 115, 106126 (2024).
19. Martin, T.M., Seaweeds and Their Secondary Metabolites: A Promising Drug Candidate With Novel Mechanisms Against Cancers and Tumor Angiogenesis, Cureus, 16 (2024).
20. Sacchi, V., Mittermayr, R., Hartinger, J., et al, Long-lasting fibrin matrices ensure stable and functional angiogenesis by highly tunable, sustained delivery of recombinant VEGF164. Proceedings of the National Academy of Sciences, 111, 6952-6957 (2014).