



An *in vitro* comparative evaluation of the osteogenic potential of antibiotics or neem-infused titanium platelet-rich fibrin

In vitro uporedna procena osteogenog potencijala antibiotika ili neem-a u infuziji titanijumom-pripremljenog fibrina obogaćenog trombocitima

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Abstract

Background/Aim. Titanium-prepared platelet-rich fibrin (T-PRF) has superior properties with respect to resorption time, thickness, growth factor holding capacity, and gradual release at the surgical site. The aim of this study was to evaluate the cell viability and osteogenic differentiation of an osteoblastic cell line MG-63 following exposure to eluates obtained from T-PRF clots injected with antibiotic gels [amoxicillin/clavulanic acid – amoxiclav gel (AMX), metronidazole – metronidazole gel (MET)], or herbal extracts (neem gel). **Methods.** In this *in vitro* study, blood was collected, and T-PRF clots were prepared. Antibiotic/herbal gels along with T-PRF clots were added to Dulbecco's Modified Eagle Medium – DMEM to obtain eluate. This eluate was later added to the cultured osteoblastic cells to check for cell viability using the [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] (MTT) assay on days 1, 3, and 7. Gene expression [alkaline phosphatase (ALP), osteopontin (OSP), bone morphogenetic protein-2 (BMP-2)] was checked on day 7 using quantitative polymerase chain reaction (qPCR), and alizarin red S (ARS) staining was used for late-stage osteogenic differentiation and matrix mineralization on day 21. Statistical analysis was performed using GraphPad Prism

software. One-way analysis of variance (ANOVA) was used for the MTT assay and for ARS staining, with Tukey's *post-hoc* test applied only for significant ANOVA results (on day 7 MTT and day 21 ARS data). The Kruskal-Wallis test was used for qPCR analysis. **Results.** Cell viability was significantly higher in the T-PRF + neem group on day 3 ($p = 0.0229$) and day 7 ($p = 0.0034$), while the T-PRF + AMX group showed significantly higher cell viability on day 7 ($p = 0.0142$) compared with T-PRF alone. The qPCR analysis showed significantly increased expression of OSP ($p = 0.0422$), ALP ($p = 0.0203$), and BMP-2 ($p = 0.0290$) in all tested groups (T-PRF + AMX/MET/neem) compared with the control group (T-PRF alone). Intergroup comparisons showed no significant differences, except between the T-PRF + neem group and the control group. ARS analysis at 21 days revealed the highest mineralization in T-PRF + neem, followed by T-PRF + MET and T-PRF + AMX. **Conclusion.** Both antibiotic- and herbal-extract-incorporated T-PRF clots demonstrated superior osteogenic potential compared to plain T-PRF alone. Therefore, T-PRF can serve as a sustained drug delivery system.

Keywords:

amoxicillin; clavulanic acid; drug delivery systems; plants; platelet-rich plasma.

Apstrakt

Uvod/Cilj. Titanijumom-pripremljen fibrin obogaćen trombocitima (*titanium-prepared platelet-rich fibrin* – T-PRF) ima superiorna svojstva u pogledu vremena resorpcije, debljine, kapaciteta zadržavanja faktora rasta i postepenog oslobađanja na mestu hirurške intervencije. Cilj rada bio je da se procene vijabilnost ćelija i osteogeno diferentovanje osteoblastne ćelijske linije MG-63 posle izlaganja eluatima

dobijenim iz ugrušaka T-PRF u koje su ubrizgani gelovi sa antibioticima [amoksicilom/klavulanskom kiselinom – amoksiklav gel (AMX), metronidazolom – metronidazol gel (MET)] ili biljnim ekstraktima (*neem* gel). **Metode.** U ovoj *in vitro* studiji, sakupljena je krv i pripremljeni su T-PRF ugrušci. Antibiotički/biljni gelovi, zajedno sa T-PRF ugrušcima, dodati su u *Dulbecco* modifikovani *Eagle* medijum – DMEM kako bi se dobio eluat. Ovaj eluat je kasnije dodat kultivisanim osteoblastnim ćelijama kako bi se procenila

vijabilnost ćelija 1, 3. i 7. dana primenom [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] (MTT) testa. Ekspresija gena [alkalna fosfataza (ALP), osteopontin (OSP), koštani morfogenetski protein-2 (*bone morphogenetic protein-2* – BMP-2)] proverena je 7. dana korišćenjem kvantitativne lančane reakcije polimeraze (*quantitative polymerase chain reaction* – qPCR), dok je bojenje alizarin crvenim S (*alizarin red S* – ARS) korišćeno za kasnu fazu osteogene diferencijacije i mineralizaciju matriksa 21. dana. Statistička analiza izvršena je korišćenjem GraphPad Prism softvera. Jednofaktorska analiza varijanse (*analysis of variance* – ANOVA) korišćena je za MTT test i za bojenje ARS, pri čemu je Tukey-ev *post-hoc* test primenjen samo za značajne rezultate ANOVA (podaci MTT testa 7. dana i bojenje ARS 21. dana). Kruskal-Wallis-ov test korišćen je za analizu qPCR. **Rezultati.** Značajno veća vijabilnost ćelija zabeležena je u grupi T-PRF + *neem* 3. dana ($p = 0,0229$) i 7. dana ($p = 0,0034$), dok je u grupi T-PRF + AMX značajno

veća vijabilnost pokazana 7. dana ($p = 0,0142$) u poređenju sa samim T-PRF. Analiza qPCR pokazala je značajno povećanu ekspresiju OSP ($p = 0,0422$), ALP ($p = 0,0203$) i BMP-2 ($p = 0,0290$) u svim testiranim grupama (T-PRF + AMX/MET/*neem*) u poređenju sa kontrolnom grupom (samo T-PRF). Međugrupna poređenja nisu pokazala značajne razlike, osim između grupe T-PRF + *neem* i kontrolne grupe. Analizom ARS 21. dana otkrivena je najveća mineralizacija u T-PRF + *neem*, zatim u T-PRF + MET i T-PRF + AMX. **Zaključak.** Ugrušci T-PRF kako sa dodatkom antibiotika tako i sa dodatkom biljnog ekstrakta pokazali su superiorniji osteogeni potencijal u poređenju sa samim T-PRF. Zbog toga, T-PRF se može primenjivati kao sistem za produženo oslobađanje lekova.

Ključne reči:

amoksicilin; klavulanska kiselina; lekovi, sistemi za isporuku; biljke; plazma bogata trombocitima.

Introduction

Platelet concentrates are simple, autologous therapies used to treat periodontitis (soft and hard tissue defects) and are applied in membrane¹, clot², or liquid³ forms depending on clinical need. Due to the presence of adhered cells and gradual release of various growth factors—such as platelet-derived growth factor, vascular endothelial growth factor, transforming growth factor (TGF)- β , epidermal growth factor, and insulin-like growth factor (IGF)—at the surgical site, better healing was observed with improved clinical and radiographic parameters⁴. Specifically, a decrease in probing pocket depth, a gain in clinical attachment level, and bone fill in intra-bony defects were noted^{5, 6}; additionally, a reduction in recession depth and width, alongside an increase in keratinized tissue width, occurred in gingival recession treatments^{7, 8}.

The utilization of medications as part of surgical and non-surgical periodontal therapy has been employed for many years. Medications such as amoxicillin, metronidazole, doxycycline, clindamycin, metformin, atorvastatin, and rosuvastatin have been administered systemically (as oral tablets) or locally (*via* local drug delivery systems) during non-surgical therapies, reporting positive clinical outcomes^{9, 10}. These medications affect osteogenesis differently and interact with osteoblastic cells in distinct ways. For instance, the antibiotic metronidazole activates the bone morphogenetic protein (BMP)-2 and runt-related transcription factor 2 (Runx2) pathways, aiding in bone cell differentiation and activity. Additionally, it reduces inflammation, which can change the environment and promote new bone formation with bone cells¹¹.

Casellas et al.¹² showed that amoxicillin/clavulanate had a positive effect on active ulcerative colitis, reducing inflammatory mediator production and, in turn, intraluminal interleukin (IL)-8 and other inflammatory mediators. Furthermore, Balcazar-Ochoa et al.¹³ claimed that clavulanate has both anti-inflammatory and pain-relieving qualities. It was never easy to maintain the prolonged release

of these medications at the surgical site. Because of this, scientists have employed several delivery methods, such as collagen membranes, microspheres, gelatin sponges, carbopolymers, etc.¹⁴. However, due to concerns regarding synthetic production, increased antigenicity, and potential allergic responses, there has been a continuous search for superior, autogenous materials to develop sustained drug delivery systems (SDDS). Leukocyte- and platelet-rich fibrin – PRF (L-PRF), a second-generation platelet concentrate, has been shown to enhance soft and hard tissue healing while reducing postoperative pain. Its three-dimensional fibrin structure serves as a reservoir for growth factor, enabling their sustained and gradual release. It can be prepared easily without adding anticoagulants and is purely autogenous¹⁵. Based on these promising findings, Polak et al.¹⁶ incorporated vancomycin, penicillin, and other antibiotics into L-PRF and evaluated its antimicrobial activity against *Staphylococcus* (*S.*) *aureus* and *Fusobacterium nucleatum*. They found that antibiotic-loaded L-PRF exhibited significantly greater antimicrobial activity than L-PRF alone. In their study on third molar surgery, Bennardo et al.¹⁷ evaluated antibiotics combined with L-PRF; their findings indicated lower postoperative pain and infection rates compared to L-PRF alone.

Despite the promising clinical outcomes associated with L-PRF, its relatively short resorption time, the potential for silica contamination, and the risk of glass test tube fracture prompted the development and investigation of improved biomaterials. As a result, scientists were drawn to titanium (Ti), which has seen widespread use in medicine and dentistry. Tunali et al.¹⁸ introduced Ti-prepared PRF (T-PRF) in 2013. This inert substance passivates into a layer of Ti dioxide, which triggers platelet activation and the creation of a three-dimensional fibrin clot. T-PRF is a second-generation platelet concentrate made by centrifuging blood in medical-grade Ti tubes. Compared to L-PRF, T-PRF eliminates silica contamination and exhibits a longer resorption time of up to 21 days. The tubes are sterile, reusable, and unbreakable.

Previous histological investigations^{19–22} comparing T-PRF and L-PRF found that T-PRF had more fibrin, a denser fibrin network, and superior cellular entrapment compared to L-PRF. Studies have shown that T-PRF persists at the surgical site for approximately 21 days, indicating a longer resorption time than earlier PRF preparations¹⁹. Due to the hard and soft tissue characteristics of T-PRF when used as a biomaterial, Ercan et al.²³ chose to employ T-PRF as their SDDS. They used doxycycline hyclate liquid injected into T-PRF clots and tested medication release and antimicrobial activity. They concluded that T-PRF retained the medication better, showed a sustained release profile based on drug kinetics, and exhibited superior antimicrobial activity as evidenced by its impact on *S. aureus* and *Pseudomonas (P.) aeruginosa* bacteria. The successful impregnation of drugs into T-PRF was verified by scanning electron microscopy (SEM).

In a related experiment, Indurkar and Purohit²⁴ administered a 3 mg/mL doxycycline solution into the T-PRF. The antimicrobial activity of the T-PRF membrane was also evaluated using *P. aeruginosa* and *S. aureus*. They concluded that T-PRF alone has antimicrobial properties and that the addition of antibiotics improved them. They also stated that T-PRF's activity may be used to treat peri-implantitis and periodontitis. Herbal items such as aloe vera, neem, curcumin, and mangosteen were also used as mouthwashes, gels, etc., and the results were positive. Clinical variables such as the plaque index, gingival index, enhanced clinical attachment level, and reduced probing pocket depth were assessed²⁵. Neem, curcumin, and aloe vera gel were used in a number of trials^{26–29} and produced positive outcomes. Therefore, in chronic periodontitis, topical applications of curcumin or aloe vera may be regarded as adjuncts to scaling and root planing.

It is always preferable to experiment on cell lines before starting a clinical trial of a new treatment protocol. As a result, test groups may interact with cultured cells in an environment that allows researchers to determine whether the experiment had any negative effects on the cells³⁰. Additionally, researchers can determine whether the cells are actively dividing when they are treated. Sumida et al.³¹ conducted an *in vitro* experiment in 2019 on rat cells that showed PRF boosted osteoprotegerin levels in MC3T3-E1 cell cultures, indicating that PRF played a significant role in the initial stages of osteogenesis by regulating osteoblastic differentiation. Esmaeilnejad et al.³² investigated the effects of L-PRF and advanced PRF (A-PRF) on the osteoblastic cell line MG-63. Their findings demonstrated that A-PRF was more effective at promoting osteoblastic proliferation and mineralization, whereas L-PRF predominantly stimulated cellular proliferation. According to the authors, no research has been done on the osteogenic potential of T-PRF clots containing neem gel or antibiotics (amoxicillin/clavulanate or metronidazole) in osteoblast cells.

The aim of this *in vitro* study was to compare the osteogenic differentiation of osteoblastic cell lines exposed to neem-embedded T-PRF and antibiotic-embedded T-PRF.

Methods

Study design, sample collection, and ethical clearance

This study was an *in vitro* experiment approved by the Department of Periodontics, Saveetha Institute of Technical and Medical Sciences (SIMATS), and Saveetha Dental College and Hospital, Chennai, India. The study was conducted in accordance with the protocol authorized by the institutional and/or licensing Committees of Saveetha Dental College and Hospital and was properly reviewed by Saveetha University, Chennai, India (No. SRB/SDC/PhD/PERIO-2251/24/245, from August 8, 2024). Blood sample collection adhered to proper protocols and regulations. The study followed appropriate ethical norms and was conducted in accordance with the Declaration of Helsinki. Confidentiality was maintained to safeguard the volunteers' privacy. Verbal and written informed consent was obtained from the subjects and/or legal guardians before conducting the study. The pros and cons of the study protocol were explained, and after acceptance, the blood-drawing procedure was performed.

Inclusion and exclusion criteria

A healthy, 31-year-old male with no blood-related problems, willing to donate blood and take part in the study, was recruited for the study. As this was an initial exploratory study, only one individual's blood was considered. Subjects who were not interested, unwilling to donate blood, or on any sort of medication were excluded.

Study groups

Groups consisted of T-PRF + AMX, T-PRF + MET, T-PRF + neem, and T-PRF clot without any additional substances, which was used as the control.

Procedure and total sample size of study

Triplication was done for each group, so a total of 12 experiments (i.e., 36 tests) were performed for the [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] (MTT) assay at three different time intervals (on day 1, 3, and 7) for viability of osteoblast cells (MG-63). For quantitative polymerase chain reaction (qPCR), 12 experiments were done on day 7 [gene expression for osteopontin (OSP), alkaline phosphatase (ALP), and BMP-2]. Triplication was also performed for alizarin red S (ARS) staining. For mineralization assessment, 12 experiments were done on day 21. The study adhered to the Checklist for Reporting *In-vitro* Studies – CRIS guidelines to the extent possible.

Antibiotic gels and neem gel preparations

Amoxiclav gel (AMX) (TBD003-1vl Biomall, India), metronidazole (Cat No. 210340050, Thermo Fisher Scientific), and neem gel were commercially prepared from Periobiologics Lab, Hyderabad, India, in insulin syringes using

weight/weight percentage. AMX was prepared by mixing amoxicillin, clavulanate, carboxymethyl cellulose (Cat No. 332640010, Thermo Fisher Scientific), magnesium stearate (Cat No. PA13 66000 Pharmaffiliates), polycarbophil powder, and glycerine (Cat No. 2143-03, Thermo Fisher Scientific) to form an 18% concentration. Metronidazole gel (MET) was prepared using Poloxamer 123 (Cat No. FP106449, Biosynth Inc.), propylene glycol, methylisothiazolinone (CAS No. 2682-20-4, Santa Cruz, India), and distilled water in the required quantity to prepare a 30% concentration. To prepare 100 g of neem extract, 20 g of neem leaves (CAS No. 84696-25-3, Bureau of Indian Standards) were boiled in 1 L of water; further, 20 g Carbopol 934P (Himedia), calcium chloride 0.02%, and sodium chloride 0.9% were added to form a 12% concentration neem gel.

Preparation of titanium-prepared platelet-rich fibrin clots and their incorporation with antibiotic gels or neem gel to form eluates

Twenty mL of blood was collected through the antecubital vein from a healthy volunteer and centrifuged at 3,500 rotations *per min* for 15 min (Remi 8C, New Delhi, India) (modified Tunali et al.¹⁸ protocol) to obtain T-PRF in sterile medical-grade Ti tubes (Grade 2, Supra Alloys Company, Camarillo, USA). This T-PRF preparation protocol was adapted from the previously published articles of Bhattacharya et al.^{21, 22}. Clots were carefully retrieved using sterile tweezers from Ti tubes and placed on Petri dishes, which were later transferred to Eppendorf tubes for lyophilization. Lyophilized T-PRF clots were mixed with antibiotic gel or neem gel in Dulbecco's Modified Eagle Medium (DMEM), incubated to obtain a proper eluate, and exposed

to the wells of osteoblast cells on days 1, 3, and 7 to perform the MTT assay and qPCR on day 7.

Osteoblast culture

DMEM supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin was used to culture MG-63 (osteosarcoma cell line) osteoblast cells obtained from the National Center for Cell Science, Pune, India. They were grown in a humidified atmosphere at 37 °C with 5% CO₂.

MTT assay

The MTT assay was used to assess cell viability and proliferation on days 1, 3, and 7. After seeding 10,000 osteoblast cells *per well* in 96-well plates, the corresponding T-PRF clot eluate containing AMX, MET, and neem was incubated separately, and the results were compared to the T-PRF clot eluate alone. Cells were cultured for 4 hrs after adding MTT solution (5 mg/mL) at all time points, then incubated again for 4 hrs at 37 °C to calculate percentage viability. Dimethyl sulfoxide was used to dissolve the formazan crystals, and absorbance at 570 nm was measured using a microplate reader [Synergy™ hybrid Multi-Mode Reader (BioTek, Winooski, VT, US)] (Figures 1 and 2).

The percent cell viability was calculated using the resulting equation³³:

$$\text{Cell viability (\%)} = \frac{\text{OD}(\text{test sample}) - \text{OD}(\text{blank})}{\text{OD}(\text{PC}) - \text{OD}(\text{blank})} \times 100$$

where OD stands for optical density, and PC is the positive control.

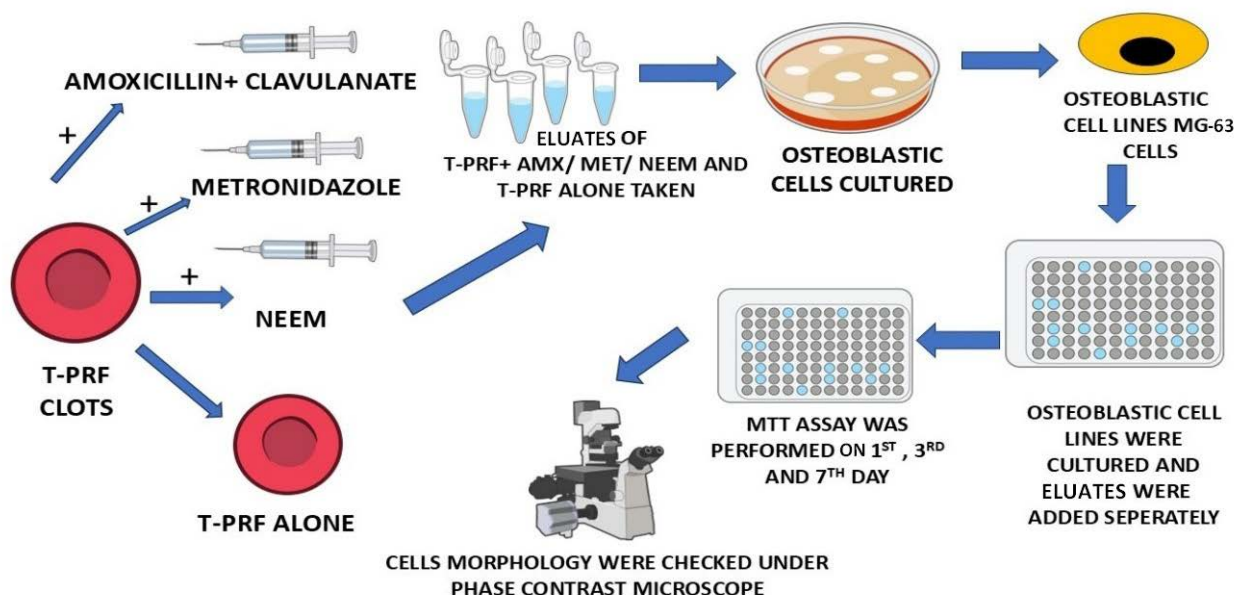


Fig. 1 – Schematic representation of the MTT assay procedure of the study and the method of incorporating T-PRF and AMX/MET/neem eluates.

MTT – [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide];

T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

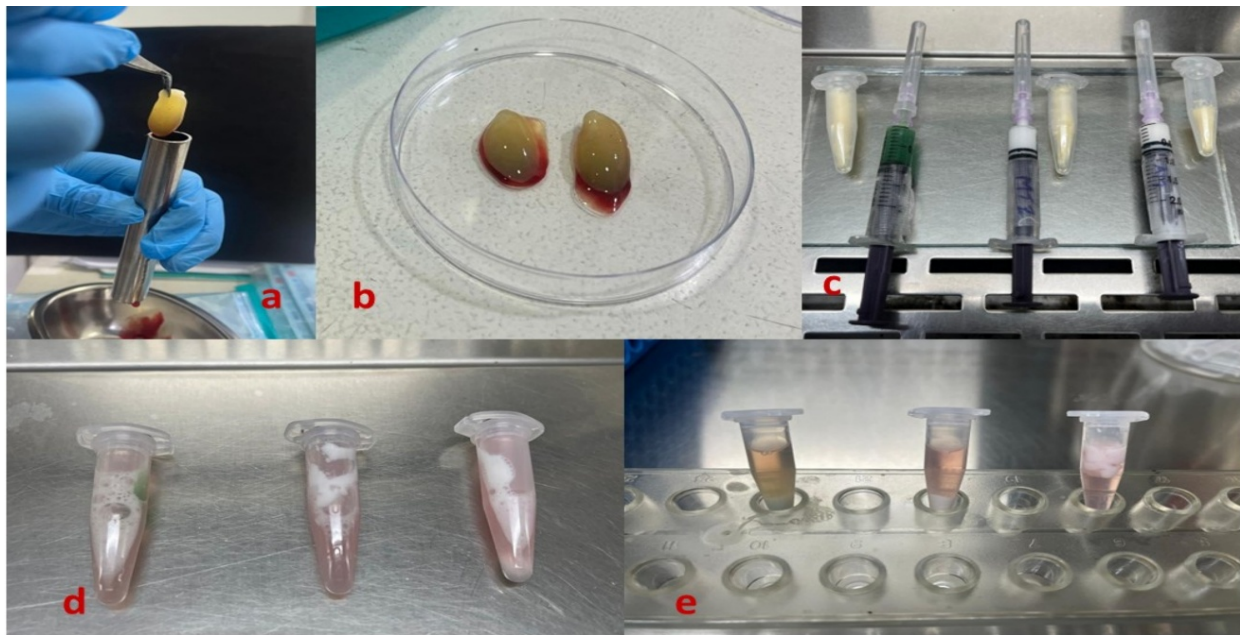


Fig. 2 – a) T-PRF retrieval from tubes; b) T-PRF placed on culture plates; c) T-PRF sample eluate in Eppendorf tubes; d) the immediate (AMX/MET) and neem eluates mixed with T-PRF eluate after vortexing; e) the final eluates of both test and control groups in an Eppendorf tube, which was ready to use on MG-63 osteoblastic cells.
T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

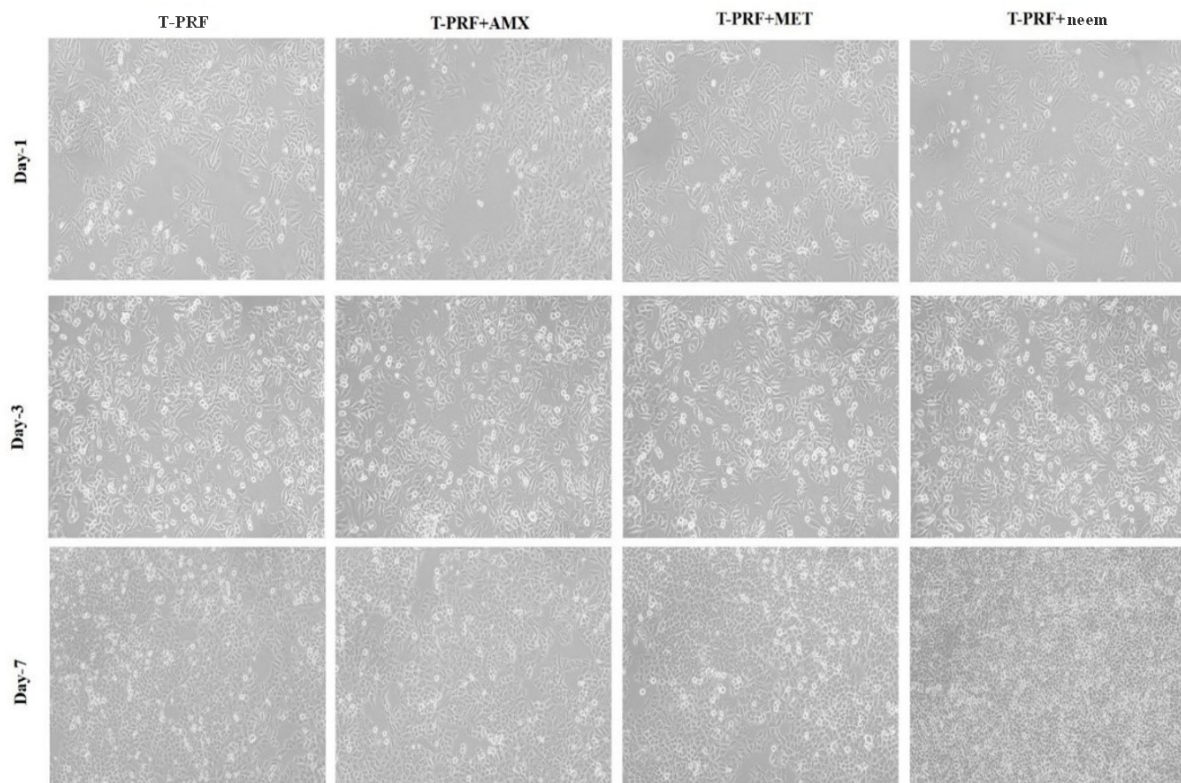


Fig. 3 – The biocompatibility of T-PRF gels incorporated with AMX, MET, and neem gels, along with the control group on osteoblast cells, was evaluated by morphological assessment using phase contrast (×20 magnification).
T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

Further biocompatibility of T-PRF eluate incorporated with AMX/MET/neem, along with T-PRF eluate alone, on os-

teoblasts was morphologically assessed and visualized under a phase contrast microscope at ×20 magnification (Figure 3).

Ribonucleic acid isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for osteogenic markers

Total ribonucleic acid (RNA) was extracted using TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA). A NanoDrop™ ND 100 (Thermo Fisher Scientific, Waltham, MA, USA) was used for ultraviolet spectrometry at 260 and 280 nm to measure RNA quantity and purity. In addition, 500 ng of RNA was converted into complementary deoxyribonucleic acid – DNA (cDNA) using the PrimeScript™ RT Reagent Kit (Perfect Real Time, Takara, Shiga, Japan). Using Bio-Rad Connect real-time PCR equipment (Bio-Rad, Hercules, CA, USA) and KAPA SYBR™ Fast Master Mix (2×) Universal (Kapa Biosystems, Sigma, Darmstadt, Germany), qPCR analysis was conducted. Three indicators associated with osteo-

genesis were selected for analysis: BMP-2, OSP, and ALP, and primer design was employed using primer blast software (Table 1). β-actin was used as a housekeeping gene (Figure 4). Three independent qPCR experiments were carried out for three osteogenic markers: OSP, ALP, and BMP-2. To maximize PCR amplification properties, primer sets were employed. OSP and BMP-2 were subjected to a real-time PCR reaction at 95 °C for 3 min, followed by 40 amplification cycles at 95 °C for 3 sec and 58 °C for 30 sec. After the reaction was run at 95 °C for 3 min, 40 amplification cycles were carried out for ALP at 95 °C for 3 seconds and 60 °C for 30 sec. The information was assessed using Bio-Rad CFX Manager software version 3.0. Relative expression levels of OSP, ALP, and BMP-2 were quantified using the double delta cycle threshold ($2^{-\Delta\Delta Ct}$) method, which was the most convenient way of assessment ^{34, 35}.

Table 1
The forward and reverse primers used for checking the PCR in the present study

Gene	Forward 5'-3'	Reverse 5'-3'
β-actin (housekeeping)	TCGTGTTGGATTCTGGGGAC	ACGAAGGAATAGCCACGCTC
OSP	CCTGGCTGAATTCTGAGGGAC	TATAGGATCTGGGTGCAGGCT
ALP	TGGGCATTGTGACTACCACTCGG	CCTCTGGTGGCATCTCGTTATCC
BMP-2	TGCTAGTAACTTTGGCCATGATG	GCGTTCCGCTGTTTGTGTT

PCR – polymerase chain reaction; OSP – osteopontin; ALP – alkaline phosphatase; BMP-2 – bone morphogenetic protein-2.

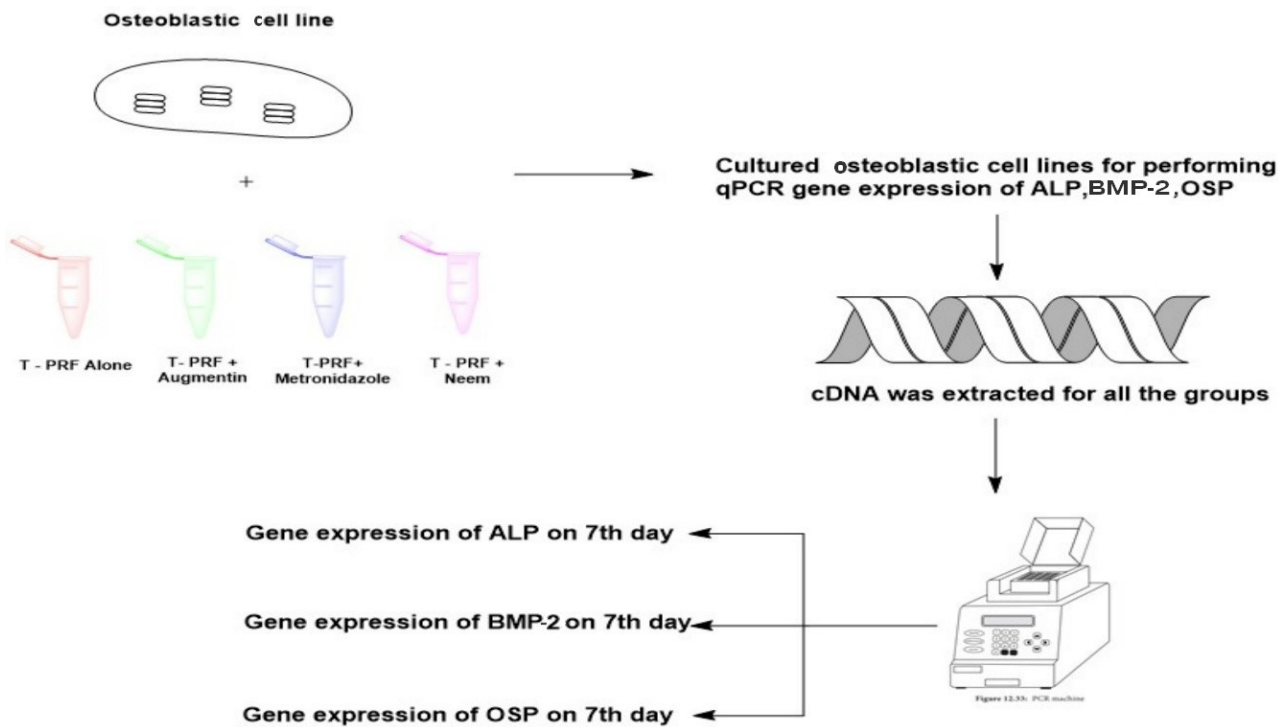


Fig. 4 – Schematic representation of the qPCR protocol of the study.
T-PRF – titanium-prepared platelet-rich fibrin; qPCR – quantitative polymerase chain reaction; ALP – alkaline phosphatase; BMP-2 – bone morphogenetic protein; OSP – osteopontin; cDNA – complementary deoxyribonucleic acid.

Alizarin red S staining assay

To evaluate late-stage osteogenic differentiation and matrix mineralization, ARS staining was performed on day 21. Human osteoblasts were cultured in 24-well plates and exposed to four experimental conditions: T-PRF alone, amoxiclav-incorporated (AMX) T-PRF, metronidazole-incorporated (MET) T-PRF, and neem gel-incorporated T-PRF clots. At the end of the incubation period, cells were washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde for 15 min at room temperature, and subsequently stained with 2% ARS (pH 4.2) for 20 min. Excess dye was removed by washing with distilled water. For quantification, bound dye was solubilized using 10% cetylpyridinium chloride, and the absorbance was measured at 405 nm using a microplate reader. Mineralization levels were compared across groups to assess calcium deposition and osteogenic potential.

Statistical analysis

GraphPad Prism software was used for statistical analysis, with all collected data imported directly into the program. One-way analysis of variance (ANOVA) test was performed to assess the time changes for MTT assay (on day 1, 3, and 7) with *post-hoc* Fisher's least significant difference – LSD test on day 7. Data from qPCR analysis (7th

day) and ARS (21st day) were initially checked for normalization using the Shapiro-Wilk test. Because the data were non-normally distributed, the Kruskal-Wallis test was employed for qPCR analysis. Dunn's *post-hoc* multiple comparison test with Bonferroni adjustment was also performed for qPCR. One-way ANOVA test with *post-hoc* honestly significant difference – HSD test was performed for ARS analysis. The value of $p < 0.05$ was considered statistically significant.

Results

MTT assay (days 1, 3, and 7)

On days 1 and 3, overall ANOVA results were non-significant ($p > 0.05$); therefore, *post-hoc* comparisons were not performed. Compared with T-PRF alone, T-PRF + AMX/MET showed no statistically significant differences on days 1 and 3. In contrast, the T-PRF + neem group showed a statistically significant difference on day 3 ($p = 0.0229$) compared with T-PRF alone. On day 7, overall ANOVA results showed a significant difference among groups ($F = 9.42$, $p = 0.001$). *Post-hoc* and ANOVA analyses revealed significantly higher viability for T-PRF + neem ($p = 0.0034$) and T-PRF + AMX ($p = 0.0142$) when compared with T-PRF alone. Comparisons between test groups (neem vs. AMX or MET) were non-significant ($p > 0.05$) (Tables 2 and 3 and Figure 5).

Table 2

Mean percentages of MTT assay of T-PRF injected with antibiotics, neem gel, and control on days 1, 3, and 7

Day	T-PRF control, %	T-PRF + AMX, %	T-PRF + MET, %	T-PRF + neem, %	F	p-value
1	100 ± 0	91.64 ± 4.82	97.86 ± 3.40	97.10 ± 2.92	2.334	0.150
<i>p</i> -value	0	0.070	0.079	0.1517		
3	102.36 ± 0	103.96 ± 5.39	106.58 ± 1.40	111.51 ± 3.41	2.827	0.107
<i>p</i> -value	0	0.325	0.286	0.0229*		
7	108.66 ± 0	120.29 ± 4.94	122.12 ± 1.93	125.55 ± 2.50	9.42	0.001*
<i>p</i> -value	0	0.142*	0.286	0.0034*		

MTT – [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide]; T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

Values are given as mean ± standard error.

Note: *Statistically significant ($p < 0.05$).

Table 3

Least significant difference (LSD) *post-hoc* comparisons among the groups on day 7

Pairwise comparison	p-value
T-PRF + AMX vs. T-PRF alone	0.0142*
T-PRF + MET vs. T-PRF alone	0.286
T-PRF + neem vs. T-PRF alone	0.0034*
T-PRF + AMX vs. T-PRF + MET	0.603
T-PRF + AMX vs. T-PRF + neem	0.136
T-PRF + MET vs. T-PRF + neem	0.821

T-PRF – titanium-prepared platelet-rich fibrin;

AMX – amoxiclav gel; MET – metronidazole gel.

Note: *Statistically significant difference ($p < 0.05$) based on Fisher's LSD *post-hoc* test.

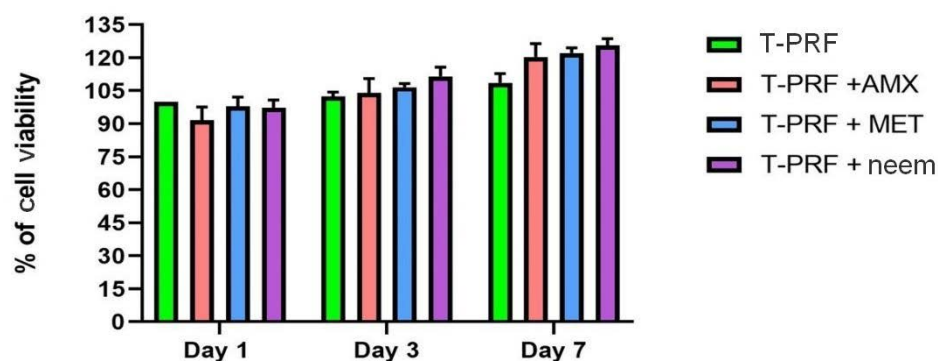


Fig. 5 – Biocompatibility of T-PRF clots incorporated with AMX, MET, and neem gels, along with the control group (T-PRF alone), on osteoblast cells was evaluated on days 1, 3, and 7 by MTT assay. T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel; MTT – [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide].

Table 4

Mean gene expression levels of OSP, ALP, and BMP-2 measured by qPCR in the T-PRF treatment groups on day 7

Groups	OSP	ALP	BMP-2
T-PRF alone	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
T-PRF + AMX	3.43 ± 0.80	3.03 ± 0.11	2.53 ± 0.75
T-PRF + MET	4.83 ± 1.09	3.49 ± 0.29	3.20 ± 0.85
T-PRF + neem	5.33 ± 0.39	13.31 ± 2.03	6.61 ± 1.59
<i>H</i> -test	8.191	9.804	9.024
<i>p</i> -value	0.0422*	0.0203*	0.0290*

OSP – osteopontin; ALP – alkaline phosphatase; BMP-2 – bone morphogenetic protein-2; qPCR – quantitative polymerase chain reaction; T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

Values are given as mean ± standard error.

Note: *Statistically significant difference ($p < 0.05$). *H* – primary test statistic for the Kruskal-Wallis test.

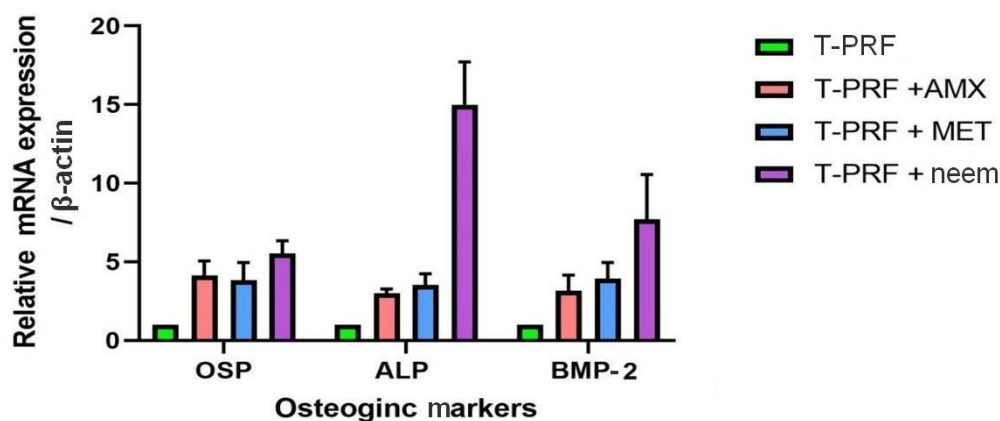


Fig. 6 – Expression of selected osteoblastic markers based on relative mRNA levels (qPCR) in osteoblast cells continuously treated with T-PRF clots incorporated with AMX, MET, and neem gels, along with the control group, for 7 days.

Note: Average values ± standard deviation are plotted, indicating significantly higher levels in the T-PRF + neem group when compared with other groups.

mRNA – messenger ribonucleic acid; qPCR – quantitative polymerase chain reaction; T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

Quantitative polymerase chain reaction (day 7)

All test groups (T-PRF + AMX/MET/neem) showed a significant increase in OSP expression ($p = 0.0422$)

compared to the control group (T-PRF alone). A similar trend of significantly increased ALP ($p = 0.0203$) and BMP-2 ($p = 0.0290$) levels across all test groups was noted compared with the control group (Table 4 and Figure 6).

However, when Dunn's *post-hoc* test was performed for group comparison, all differences were non-significant, except for the comparison between the T-PRF + neem group and T-PRF alone (Table 5).

Alizarin red S staining (day 21)

On day 21, ARS analysis revealed statistically significant differences among all groups ($p < 0.001$). T-PRF + neem demonstrated the highest mineralization and calcium deposition, followed by T-PRF + MET and T-PRF + AMX, while T-PRF alone showed the lowest values (Table 6).

Post-hoc tests confirmed significant differences for each test group compared to T-PRF alone ($p < 0.05$). When comparisons were performed among the test groups, a

significant difference ($p = 0.0133$) was reported for T-PRF + neem compared with T-PRF + AMX, while the remaining comparisons were non-significant ($p > 0.05$) (Table 7). The results showed increased calcium deposition in both T-PRF + AMX and T-PRF + MET compared to T-PRF alone. The neem-incorporated T-PRF group demonstrated relatively higher mineralization than the T-PRF + AMX/MET groups, suggesting enhanced late-stage osteogenic activity. This mineralization evidence provides functional confirmation of osteogenic differentiation when observed under a phase-contrast microscope (Figure 7). Thus, the T-PRF + neem group exhibited greater mineralization and a stronger osteoinductive effect than T-PRF alone. The second highest representation was recorded for T-PRF + MET, followed by T-PRF + AMX, with T-PRF alone showing the lowest values (Figure 8).

Table 5

Dunn's *post-hoc* multiple comparison test (Bonferroni-adjusted) for gene expression of qPCR analysis

Comparisons	OSP	ALP	BMP-2
T-PRF vs. T-PRF + AMX	1.0000	1.0000	1.0000
T-PRF vs. T-PRF + MET	0.2407	0.4085	0.4085
T-PRF vs. T-PRF + neem	0.0372*	0.0125*	0.0182*
T-PRF + AMX vs. T-PRF + MET	1.0000	1.0000	1.0000
T-PRF + AMX vs. T-PRF + neem	1.0000	0.4085	0.6624
T-PRF + MET vs. T-PRF + neem	1.0000	1.0000	1.0000

qPCR – quantitative polymerase chain reaction; OSP – osteopontin; ALP – alkaline phosphatase; BMP-2 – bone morphogenetic protein; T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

Note: *Statistically significant ($p < 0.05$).

Table 6

Mean values of ARS staining among the groups on day 21 (one-way ANOVA)

Values	T-PRF	T-PRF + AMX	T-PRF + MET	T-PRF + neem	<i>F</i>	<i>p</i> -value
Mean ± SE	0.33 ± 0	0.82 ± 0.02	1.01 ± 0.06	1.50 ± 0.04	266.154	< 0.0001*
<i>p</i> -value	0	0.0018*	0.0023*	0.0006*		

ARS – alizarin red S; ANOVA – analysis of variance; T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel; SE – standard error.

Note: *Statistically significant ($p < 0.05$).

Table 7

The *post-hoc* pairwise comparisons among all the groups for ARS staining on day 21

Group-wise comparison	<i>p</i> -value
T-PRF + AMX vs. T-PRF + MET	0.7484
T-PRF + AMX vs. T-PRF + neem	0.0133*
T-PRF + MET vs. T-PRF + neem	0.0645

ARS – alizarin red S; T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

Note: *Statistically significant ($p < 0.05$).

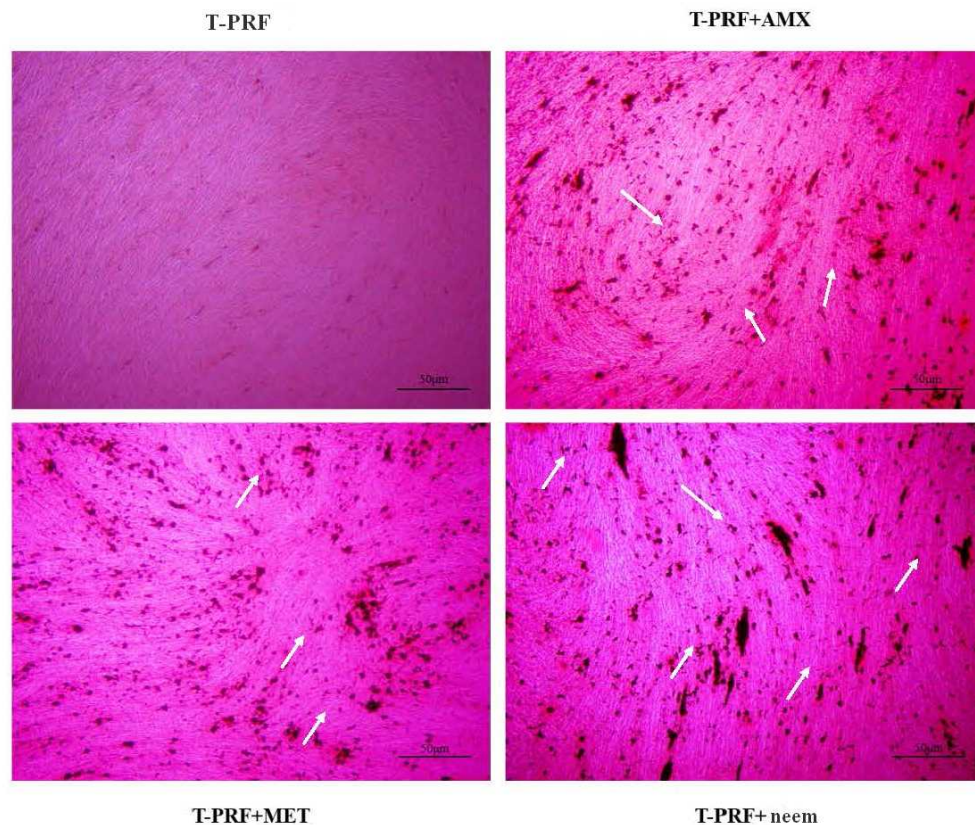


Fig. 7 – Alizarin red S staining of osteoblast cultures treated with T-PRF alone (control), antibiotic-incorporated (AMX/MET) T-PRF, and neem-incorporated T-PRF clots after 21 days of incubation at 50 μm scale bar at ×20 magnification. The staining and white arrows indicate calcium-rich mineral deposition, a hallmark of osteogenic differentiation and suggesting an enhanced osteogenic potential.

T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel.

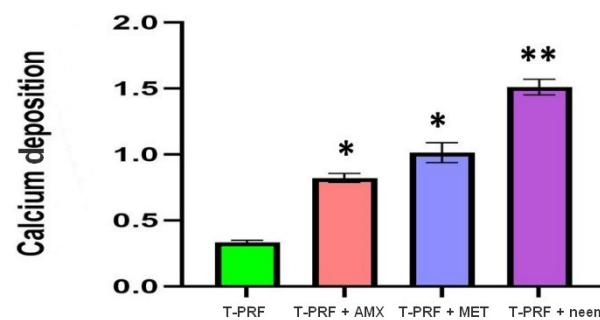


Fig. 8 – Quantitative analysis of mineral deposition by measuring ARS stain eluted from osteoblast cultures at day 21. Absorbance was recorded at 405 nm. Both the antibiotic + T-PRF and neem + T-PRF groups showed significantly higher calcium deposition compared to the control, with the neem group exhibiting the highest mineralization.

Data represent mean ± standard error.

ARS – alizarin red S; T-PRF – titanium-prepared platelet-rich fibrin; AMX – amoxiclav gel; MET – metronidazole gel; n – number.

Note: ** Indicates statistical significance for the T-PRF + neem group compared with all groups, and * also indicates statistical significance of T-PRF + AMX/MET when compared with T-PRF alone (control).

Discussion

To assess cell viability in osteoblastic cells, the current study looked at a novel therapy regimen that included herbal extract and antibiotics in T-PRF for gene expression and

differentiation potential. Cell viability and differentiation were determined using MTT; qPCR was used to assess gene expression, and verification of mineralization was done by ARS staining. The study utilized MG-63 cells—human osteosarcoma cells. These osteosarcoma cell lines, in

contrast to normal osteoblast cells, have a different route that includes fewer or faulty interactions with immune cells. This immunological response may also be influenced by the microenvironment of carcinoma cell lines and the absence of significant expression of major histocompatibility complex class I (MHC I). This may partially explain the absence of detectable abnormalities in the tests and the lack of observed immunological reactions³⁶. This research offers a method for identifying T-PRF as SDDS. The specific antibiotics used in the current study were selected because they represent established standard treatments for periodontitis³⁷.

Additionally, the rationale behind utilizing neem extract in the current study was its antibacterial, antioxidant, and analgesic effects. The current study used MG-63 osteosarcoma cells because of their replication as osteoblastic cells and their assistance with the study protocols. According to a histological analysis by Gummaluri et al.³⁸, a thin fibrin network formed when neem gel was injected into T-PRF clots. These characteristics were not statistically significant for T-PRF alone, and the cells had a 26–75% score with a scattered or layered arrangement and a wider distribution. This suggested that herbal extracts could be indirectly integrated into T-PRF without significantly changing the fibrin structure.

Gummaluri et al.³⁹ further conducted a histological study evaluating the incorporation of antibiotic gels, such as AMX and MET, and neem herbal extract gel into T-PRF. The research examined the results of adding AMX/MET or neem to T-PRF using light microscopy and SEM. It was determined that adding drugs or herbal extract into clots did not change the fibrin structure. The haziness captured in the SEM images suggested that these antibiotics or herbal extracts had successfully penetrated and adhered to the T-PRF fibrin surface, creating a system for continuous drug release. Since platelet concentrates are known to retain growth factors and release them over a period of time, Gummaluri et al.⁴⁰ conducted another study in which they incorporated neem and *Triphala (T.) indica* gels into T-PRF clots. The release of IGF-1, vitamin C, and azadirachtin was evaluated at 6 hrs, 72 hrs, 7 days, and 14 days using individual assay methods. The findings indicated that both herbal groups demonstrated consistent release of vitamin C and azadirachtin throughout the duration of the experiment, with no significant differences between them. Even IGF-1 production was not suppressed. This demonstrates that T-PRF may be employed as an SDDS. The majority of published studies were limited to light microscopy and SEM. Previous research findings were connected with the literature on the interactions or combination forms of antibiotics and T-PRF^{38–40}. Due to the scarcity of information on T-PRF clots on osteoblast cells treated with AMX, MET, or neem, the present study was performed.

The current study findings show a mixed pattern compared with Manzano-Moreno et al.⁴¹, in terms of the results of AMX-incorporated T-PRF. Manzano-Moreno et al.⁴¹ concluded in their study that plain amoxicillin had a beneficial impact on osteoblastic differentiation at concentrations up to 400 µg/mL, which resulted in higher

expression of TGF-β receptor type 1, type I collagen, and osteocalcin. They also found that 150 g/mL clindamycin is far more effective at sterilizing the intraoral autogenous grafts. Their research also showed that clindamycin was a superior antibiotic. These two studies differed mostly in the type of materials utilized and the distinct application of T-PRF, which potentially demonstrated increased viability and greater gene expression of BMP-2, ALP, and OSP. Given the favorable findings regarding cell viability and gene expression in the present study, it is possible that the anti-inflammatory effects of amoxicillin/clavulanate contributed to the observed effects in the T-PRF + AMX group, potentially explaining the favorable gene expression and osteogenic effects observed in this group^{12, 13}. The current results regarding the T-PRF + AMX group can be compared to Bilginaylar et al.⁴², who stated that antibiotic-loaded PRF had better outcomes—including accelerated tissue healing and reduced postoperative pain and trismus—compared to PRF alone. They also concluded that antibiotic-incorporated PRF reduced inflammation and bacterial load. The difference from the previously quoted study was the type of platelet concentrates used; the method of injecting antibiotics or herbal extract was different, but the addition of antibiotics and herbal extract had good cell viability and gene expressions.

The current study findings of MET integrated T-PRF for qPCR and MTT assay were similar to those of Duggal et al.⁴³, where they came to the conclusion that at 7 days, MET showed positivity for ALP and BMP activity, along with increased osteoblastic differentiation. Osteogenic differentiation and the development of mineralized nodules were additional outcomes seen three weeks after therapy. When MET was employed, the messenger ribonucleic acid – mRNA levels of osteogenic genes were elevated in bone mesenchymal stem cells. The nitroimidazole ring and the diminished anti-inflammatory impact may be responsible for the beneficial osteogenic action of the current study. The MET effect may also involve activation of the Wnt pathway, which is thought to be responsible for the expression of osteogenic indicators such as BMP-2 or Runx2, essential transcription factors for hard tissue formation, development, differentiation, and regeneration.

Current research indicates that both neem-incorporated (T-PRF + neem) and antibiotic-incorporated (T-PRF + AMX/MET) clots significantly enhance osteoblast viability and osteogenic differentiation compared to the control. Notably, the T-PRF + neem group demonstrated substantially superior outcomes numerically. The bioactive substances in neem, such as azadirachtin, nimbidin, nimbolide, and nimbin, may be responsible for the neem-incorporated T-PRF's better performance, which have been demonstrated to regulate cell responses, improve antibacterial and anti-inflammatory qualities, and aid bone healing. Neem components also reduce inflammatory mediators like tumour necrosis factor (TNF)-α, IL-1, and IL-6 that would decrease osteogenesis. It also shows down-regulation of receptor activator of nuclear factor kappa beta ligand – RANKL and helps in up-regulation of osteoprotegerin⁴⁴.

The results of the current study were further supported by qPCR analysis, which revealed higher gene expression of key osteogenic markers, such as OSP, ALP, and BMP-2, in all test groups compared with T-PRF alone, with the highest expression observed in the T-PRF + neem group. The T-PRF + neem group showed significantly higher expression of all three markers than the T-PRF-alone group. Neem-integrated T-PRF is indicative of a more positive osteogenic environment, as it supports bone formation and osteoblast differentiation. Neem gel produced better osteogenic potential than the antibiotic-incorporated T-PRF gels. Though the presence of antibiotics may have contributed to the positive osteogenic potential, the discrepancy among group results may be attributed to prolonged periods of cellular stress or restricted proliferation^{45, 46}.

In the present study, the antibiotics and the herbal extract were added post-centrifugation. Cell viability and gene expression were then evaluated in osteoblastic cell lines, demonstrating promising outcomes. These findings can be indirectly compared to a recent systematic review by Niemczyk et al.⁴⁶, which examined 13 *in vitro* experiments. In those studies, antibiotics were incorporated into the PRF using various techniques, including direct addition prior to blood centrifugation⁴⁷. It was determined that systemic administration of antibiotics was the most effective approach. The addition of antibiotics prior to centrifugation enhanced the antimicrobial activity of PRF and resulted in larger zones of inhibition. The antimicrobial properties of PRF were superior to those of many alternative materials, including collagen sponges. As a result, they also claimed that antibiotic-loaded PRF is a powerful tool for targeted antimicrobial delivery and may be a viable choice in clinical practice. Thus, the current study demonstrates that the T-PRF + neem group provides a treatment strategy for dentistry to utilize herbal extracts in future non-surgical and surgical periodontal treatments. Neem components have many positive properties, such as anti-inflammatory, antibacterial, antioxidant, and antiviral, which are very important for good wound healing (both soft and hard tissues)⁴⁸. Assessing the ALP, BMP-2, and OSP gene expressions with late-stage ARS mineralization assays was a key strength of the current research to determine the importance of this novel treatment protocol and also to use T-PRF as SDDS.

Current study results of herbal extract neem shared a similar pattern with that of a recent study performed by Gummaluri et al.⁴⁹. *Mangosteen rind* and *T. indica* gel extracts were injected into T-PRF clots and checked for MTT assay, qPCR gene expressions, and ARS staining, where they concluded both that the treatment groups showed similar cell viability and that T-PRF + *T. indica* showed better gene expression. They also concluded that

these herbal extracts added to T-PRF and subjected to future human trials might be better for hard tissue healing. Therefore, this suggests that T-PRF can be used as SDDS.

Limitation of the study

A limitation of the present study was the small sample size. The MTT assay, gene expressions, and ARS analyses performed in the current study had restricted gene and time points, which may affect the study outcomes. Checking gene expressions both pre- and post-treatment at multiple time points can help in the proper establishment of a comprehensive picture of the biological process (i.e., multi-time-point gene expression would offer deeper insight into the kinetics of osteogenic differentiation). Another limitation of the study was the use of MG-63 osteosarcoma cells instead of primary human osteoblasts. Further, a larger sample size with more replicates would improve statistical validity. Experiments with human osteoblast cells and *in vivo* trials might have enhanced the outcomes of the study. This study was an initial exploratory study, and *in vitro* model applicability is confined to testing the initial lab findings. Translating *in vitro* results to clinical settings through animal and clinical trials will provide better-established scientific outcomes to roll out this treatment strategy of T-PRF as SDDS into routine dental practice.

Conclusion

The present study concluded that both antibiotic (amoxicillin/clavulanate or metronidazole) and neem-incorporated titanium-platelet rich fibrin clots demonstrated osteogenic potential compared with titanium-platelet rich fibrin clots alone. Given the growing concern over antibiotic resistance, neem extract offers a promising alternative for bone regeneration without compromising antimicrobial properties. The enhanced osteoblast proliferation and elevated expression of osteogenic markers with neem extract suggest its potential as a biocompatible, osteogenic agent for regenerative therapies. Further *in vitro* studies using primary cells and additional blood donors, as well as subsequent validation in animal models and randomized clinical trials for *in vivo* use, are recommended to confirm these findings.

Conflicts of interest

The authors declare no conflict of interest.

Funding

There was no external source of funding.

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Received on December 3, 2025

Revised on July 2, 2026

Revised on July 22, 2026

Accepted on August 19, 2026

Online First October 2026