Original Article

Enriched advanced platelet-rich fibrin plus gold nanoparticles against *Enterococcus faecalis* for its potential use in revascularization for necrotic immature permanent teeth

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Abstract

Background: Regenerative endodontic procedures allow reinforcement of root canal wall and continuation of root development, opening new therapeutic possibilities. The root canal system of infected teeth is colonized by a variety of microorganisms, which hinder the regenerative process, leading to treatment failure if not adequately addressed, thereby requiring careful attention to microbial control.

Aim and Objective: The aim of the study was to assess the antimicrobial activity of advanced platelet-rich fibrin (A-PRF) and gold nanoparticles (AuNps) against *Enterococcus faecalis*.

Materials and Methods: Intravenous blood (5–6 ml) was drawn from four healthy individuals, and A-PRF was prepared through centrifugation at 1500 revolutions per minute (rpm) for 14 min. A-PRF was doped with 3 μl of AuNps and centrifuged at 1000 rpm for 1 min. Antimicrobial activity was assessed using disk diffusion; inhibition zones were measured. For minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), A-PRF + AuNps were added to the microbial broth at varying concentrations to determine growth inhibition and microbial death.

Results: Disk diffusion assays revealed significant antibacterial effects against *E. faecalis*. Norfloxacin displayed the highest mean zone of inhibition (20.33 ± 1.53 mm), followed by the Test group (A-PRF + AuNPs) (19.33 ± 0.58 mm). Multiple comparisons indicated significant differences ($P < 0.001$). MIC of A-PRF + AuNPs against *E. faecalis* was 0.031 mg/ml, with MBC at 0.015 mg/ml.

Conclusion: The addition of AuNPs to A-PRF offers the potential for sustained growth factor release while maintaining the sterility of the canal, leading to successful revitalization and regeneration. The combined use of A-PRF + AuNps shows promise for enhancing revascularization in necrotic immature permanent teeth.

Keywords: Advanced platelet-rich fibrin; antimicrobial activity; gold nanoparticles; regenerative endodontics

INTRODUCTION

Regenerative endodontic therapy focuses on restoring and regenerating the tissues within damaged or necrotic dental pulp and dentin. This involves encouraging the growth of dentin, pulp tissue, and root structures to restore

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the normal functions of the pulp. Following effective disinfection, it is possible to stimulate the development of pulp-like tissue using various dental stem cells, aided by appropriate growth factors and a scaffold medium.[1,2] Regenerative techniques such as revascularization and revitalization enable reinforcement of the root canal wall and the continued development of the root, introducing novel therapeutic opportunities.

Pulp revascularization refers to the capability of the remaining pulp tissue, stem cells located at the apex of the tooth, and stem cells in the periodontal area to undergo differentiation and create a living tissue that is rich in blood vessels and connective elements.[3]

Microbial control is essential to achieve a successful outcome and continued root development. *Enterococcus faecalis* is the most frequently isolated bacterial species in root canals of endodontically failed teeth.[4] *E. faecalis* possesses various survival mechanisms in diverse conditions, including its capacity to withstand various disinfection methods, form biofilms, thrive in regions inaccessible to root canal cleaning procedures, and exhibit synergistic reactions of different strains.[5,6]

Platelet-rich fibrin (PRF) fibrin matrix comprises platelets and leukocytes, along with an array of growth factors and cytokines such as transforming growth factor-beta1, platelet-derived growth factor, vascular endothelial growth factor, interleukin (IL)-1 β, IL-4, and IL-6.[7]

The fibrin generated in the latter phases of the coagulation process, coupled with cytokines discharged by platelets, renders PRF an exceptionally biocompatible matrix, particularly in areas of injury where the fibrin structure serves as a storage unit for tissue growth factors. These growth factors play a direct role in stimulating the expansion and specialization of osteoblasts, endothelial cells, chondrocytes, and different types of fibroblasts.[8,9]

Advanced PRF (A-PRF) represents an enhanced version of PRF, characterized by higher levels of PRF cells and concentrations of growth factors exceeding normal physiological levels. These elements play a crucial role in facilitating tissue recovery and the mending process.[10,11]

Gold nanoparticles (AuNPs) are one of the highly promising and extensively investigated tools in the field of nanomedicine.[12] Their applications span across various areas, including being employed as therapeutic agents, drug delivery systems, tools for photothermal therapy, diagnostic agents, and imaging agents. Their minute size aligns with biological components and their simple preparation, substantial surface area, and ease of functionalization make them particularly valuable.[13]

The distinct biological and optical traits of AuNPs, combined with their straightforward synthesis methods, have led to their utilization in both disease diagnosis and treatment.[14,15] They exhibit the ability to stimulate osteogenic differentiation and bone mineralization. Incorporating AuNPs onto the surface of dental implants has demonstrated a significant enhancement in osteoblastic differentiation and the generation of bone tissue around the implant. The influence of AuNPs' size and shape on differentiating human periodontal ligament stem cells into osteoblasts has been previously established.[16,17]

The utilization of A-PRF membranes in pulp revascularization has exhibited encouraging results. This approach has demonstrated the potential to promote the growth of additional thickness of root dentin, a critical factor in strengthening immature teeth.[18]

Therefore, this study was done to assess that addition of AuNps to A-PRF to form A-PRF + AuNps could prove to be beneficial to maintain sterility of the root canal along with sustained release of growth factors essential for revascularization.

MATERIALS AND METHODS

Sample collection and preparation

Four young and healthy patients of the same blood group O + ve, were selected. Patients with a history of systemic disorders, drug use, and other complications were excluded. Informed written consent was obtained as per the ethical committee protocol. 5–6 ml of intravenous blood was drawn from the cubital fossa of the right hand and transferred into sterile vacutainers without anticoagulant.

Preparation of advanced platelet-rich fibrin

Sterile vacutainer tubes were centrifuged at 1500 revolutions per minute (rpm) for 14 min [Figure 1a]. Blood settles into a structured fibrin clot in the middle of the tube, just between the red corpuscles at the bottom

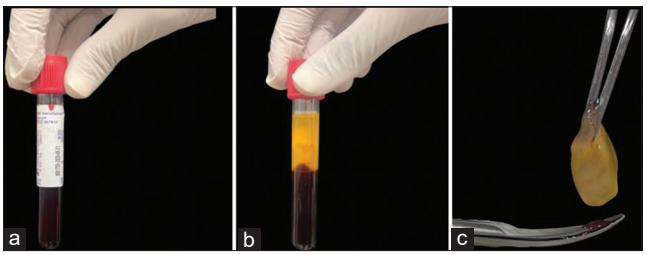

Figure 1: Preparation of advanced platelet-rich fibrin (A-PRF). (a) Sterile vacutainer tubes centrifuged at 1500 revolutions per minute (rpm) for 14 min. (b) Blood settles into layers: lower red fraction (red blood cells), upper straw-colored cellular plasma, middle fraction: Fibrin clot. (c) A-PRF: Middle fraction was collected, 2 mm below lower dividing line. RBCs: Red blood cells, A-PRF: Advanced Platelet Rich Fibrin

and acellular plasma (platelet-poor plasma) at the top [Figure 1b]. After the removal of platelet-poor plasma, a middle fraction of A-PRF was separated from the red corpuscles base, preserving a small red blood cell layer using sterile tweezers and scissors [Figure 1c].

Formulation of advanced platelet-rich fibrin + gold nanoparticles

The obtained A-PRF was then placed in a fresh sterile vacutainer and 3 µl of AuNps (777137 Sigma-Aldrich) were added using a sterile micropipette. The vacutainer containing A-PRF-AuNps was subjected to centrifugation at 1000 rpm for 1 min.

Evaluation of advanced platelet-rich fibrin + gold nanoparticles s for antimicrobial activity using disk diffusion method

E. faecalis cultures were inoculated in a nutrient agar plate and sub-cultured at 37ºC for 24 h, bacteria was evenly spread on agar plates, and the sample-loaded discs with 100ul/ml were placed on the plates followed by incubation at 37°C for 24 h. Inhibition zones were examined around the disc and measured with a ruler in mm.

Assessment of advanced platelet-rich fibrin + gold nanoparticles for minimum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of antimicrobial agent, which will inhibit microbial growth. Constantly increasing the % concentration of test agent A-PRF + AuNps to microbial-rich broth in a series of tubes. Turbidity of tubes indicates the amount of microbe growth. Least turbid or clear, tubes correlating with the absence of microbes.

Minimum bactericidal concentration (MBC) is the lowest level of antimicrobial agent that results in microbial death. Ten milliliters were taken from the well obtained from the MIC experiment spread on Mueller–Hinton Agar plates. The number of colonies was counted after 18–24 h of incubation at 37°C. The concentration of sample that produces <10 colonies was considered as MBC value.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) for Windows Version 22.0 Released 2013. Armonk, NY, USA: IBM Corp. was used to perform statistical analyses. Descriptive statistical analysis was done, and mean and standard deviation were calculated for each zone of inhibition (ZOI) value. One-way analysis of variance was performed, followed by Tukey's *post hoc* test, which was used to compare the mean ZOI for *E. faecalis* between three groups. The level of significance was set at $P < 0.05$.

RESULTS

Disk diffusion

As shown in Table 1, the antibacterial activities of the negative control, norfloxacin-10 µg (control), and A-PRF + AuNp (test) group were tested against clinical pathogenic bacteria *E. faecalis*. The mean ZOI for *E. faecalis* by the control group was 0.00 ± 0.00 mm, for the norfloxacin group, it was 20.33 ± 1.53 mm and for the Test group was 19.33 ± 0.58 mm. This mean difference in the ZOI between the three groups was statistically significant at $P < 0.001$. Multiple comparisons of the mean difference between groups revealed that the norfloxacin group showed significantly higher mean ZOI as compared to the control group and the mean difference was statistically significant at $P < 0.001$. This was then followed next by the test group showing significantly higher mean ZOI as compared to the control group and the mean difference was statistically significant at $P < 0.001$. However, the mean difference in ZOI between norfloxacin and the test group did not show any significant difference ($P = 0.47$). This infers that the mean ZOI for *E. faecalis* was least in the control group and highest in the norfloxacin group, which was comparable with the test group [Figure 2].

Minimum inhibitory concentration and minimum bactericidal concentration

Table 2 shows the observed growth rate and absorbance readings measured at 600 nm in treated and untreated bacterial conditions after 24 h of treatment with a given test sample with different concentrations. MIC activity of A-PRF + AuNp against the *E. faecalis* is 0.031 mg/ml. MBC growth of *E. faecalis* with A-PRF + AuNp is 0.015 mg/ml.

DISCUSSION

In this study, the antimicrobial activity of A-PRF and AuNps against *E. faecalis* was assessed. Platelet concentrates are advantageous in regeneration, attributed to longer, and rich exposure to growth factors. A-PRF is a specialized form of platelet concentrate that is used in regenerative medicine and various dental and medical procedures. A-PRF is an evolution of the basic PRF concept and involves modifications in the preparation process to enhance its regenerative potential. A-PRF released the maximum

Table 1: Standard deviation of zone of inhibition in mm from disc diffusion test

	Zone of inhibition±SD (mm)
Sample	*Enterococcus faecalis*
NC	0
Norfloxacin-10 µg	20.33±1.53
A-PRF+AuNps-100 µL/mL	19.33±0.58

ZOI values of NC, Norfloxacin-10 µg (control), and A-PRF+AuNp (test) group tested against *E. faecalis*. ZOI: Zone of Inhibition, NC: Negative Control, SD: Standard deviation, A-PRF+AuNps: Advanced Platelet Rich Fibrin + Gold Nanoparticles, *E. Faecalis*: *Enterococcus faecalis*

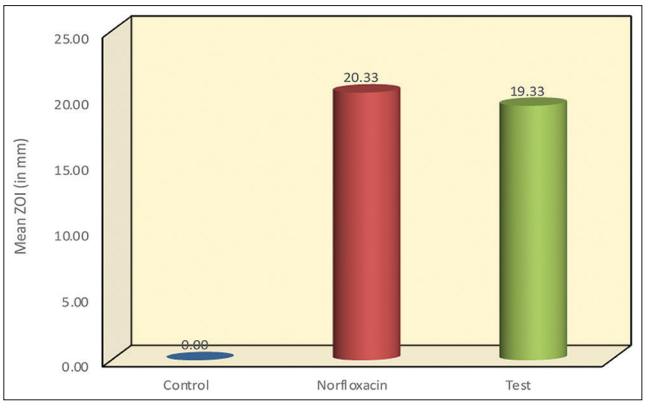

Figure 2: Mean zone of Inhibition (ZOI). Mean difference of ZOI between the Positive Control and Test group did not show any significant difference (P = 0.47). This infers that the mean ZOI for *Enterococcus faecalis* was least in Control group and highest in the Norfloxacin group which was comparable with the Test group. Image Credits: Dr. Sophia Saud. ZOI: Zone of inhibition

Table 2: Minimum inhibitory concentration and minimum bactericidal concentration using 9 Dilutions: (1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015, 0.007, and 0.003 mg/mL)

Sample concentration (mg/mL)	Growth rate (24hr)	OD at 600 nm
1	–	0.001
0.5	–	0.001
0.25	–	0.001
0.125	–	0.001
0.062	–	0.001
0.031	+	0.561
0.015	+	0.487
0.007	++	0.569
0.003	+++	0.740
BC	++++	0.928
NC (blank)	–	0.001
Positive control	–	0

Growth rate and optical density measured at 600 nm after 24 h of treatment with 9 dilutions of given test sample against E. faecalis. NC: Negative control, BC: Blank control, OD: Optical Density, Positive (+): Indicating growth, Negative (–): Indicating absence of growth

amount of growth factors over a longer duration when compared to either PRF or PRP due to the unique fibrin matrix structure allows for a sustained and controlled release of growth factors, potentially enhancing its regenerative effects; this extended release provides cells with a longer exposure to the growth factors, which can support various cellular processes involved in tissue repair and regeneration.[10]

AuNps have shown antibacterial activity against various oral pathogens, including bacteria commonly associated with dental infections.[19] They can disrupt bacterial cell membranes, induce oxidative stress, and inhibit bacterial growth. This antimicrobial property makes AuNps potentially useful in the treatment of dental infections. AuNps can serve as an alternative antibacterial agent due to growing bacterial resistance and side effects of antibiotics.[20]

The antibacterial tests performed indicated that the combination of A-PRF + AuNPs has demonstrated effective antibacterial properties against *E. faecalis*. The mean ZOI was comparable to that of the Norfloxacin group, suggesting that the A-PRF + AuNPs combination indeed possesses significant antibacterial potency. This finding highlights the potential of combining regenerative biomaterials like A-PRF with nanoparticles to create novel therapeutic approaches.

In immature teeth, as the mechanical removal of microorganisms is not recommended due to the fragility of the thin root walls, requiring decontamination restricted to the use of irrigant solutions and intracanal medication and the use of this novel agent as an intracanal medicament could potentially address the challenge of decontamination. Its antibacterial properties could help control microbial growth without the need for mechanical intervention. As it possessed the capability for sustained release of growth factors, coupled with its antibacterial effects, it could contribute to enhanced tissue regeneration within the immature tooth. The gradual and controlled release of growth factors supports the development of dental pulp tissue. The sterilizing properties of the test agent are particularly important in maintaining a sterile environment within the root canal, reducing the risk of further infection and promoting a conducive environment for regeneration. Successful revitalization and regeneration can be achieved by providing a controlled environment that encourages both sterilization and regenerative processes.

The limitation of the study is that the experimental sample of A-PRF + AuNPs was tested against *E. faecalis*, which was the sole target microorganism used; the effect may be different in other strains of bacteria since endodontic infections are polymicrobial. Future research could focus on evaluating its proliferative and cytotoxic effects on the surrounding tissue, the stability of AuNps in A-PRF over time and assessing their effectiveness in clinical settings.

CONCLUSION

The observed results showed the potential antibacterial potency of A-PRF + AuNPs against *E. faecalis*, similar to the positive control. The addition of AuNps to A-PRF can potentially aid in the sustained release of growth factors while maintaining the sterility of the canal, resulting in successful revitalization and regeneration. AuNps to A-PRF can be beneficial for its use in revascularization for necrotic immature permanent teeth.

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Conflicts of interest

There are no conflicts of interest.

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