Original Article

Comparative evaluation of the osteogenic capacity of second-generation platelet concentrates on dental pulp stem cells – An *ex vivo* study

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Abstract

Introduction: Clinical evidence of platelet-rich fibrin (PRF) benefits on bone repair is still emerging, prompting researchers to experiment with different PRF formulations as osteoconductive scaffolds.

Aims: This study compared the osteoconductive effects of injectable PRF (i-PRF) and leukocyte-rich PRF (L-PRF) on the differentiation of dental pulp stem cells (DPSCs) into osteoblasts.

Materials and Methods: Blood samples were collected from the volunteers to prepare L-PRF and i-PRF conditioned media (CM) by centrifugation. DPSCs were isolated from impacted third molars and cultured. Proliferation of DPSCs in response to L-PRF and i-PRF was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Osteoinductive potential was evaluated through alkaline phosphatase (ALP) activity, alizarin red S (ARS) staining, growth factor levels (vascular endothelial growth factor [VEGF], transforming growth factor [TGF-beta]), and cytokine expression (interleukin 6 [IL-6], IL-8) after 7 days.

Results: MTT assay results showed that both L-PRF and i-PRF increased DPSC proliferation relative to the control group. After 7 days in L-PRF and i-PRF CM, DPSCs exhibited increased ALP activity, higher red-colored calcium deposits with ARS staining, and elevated levels of VEGF and TGF-beta. In addition, higher concentrations of inflammatory cytokines IL-6 and IL-8 were observed in both L-PRF and i-PRF compared to the control.

Conclusions: Using both L-PRF and i-PRF as scaffolds can enhance the osteoinductive ability of stem cells, offering a potential strategy for regenerative therapies.

Keywords: Alizarin red S staining; alkaline phosphatase activity; dental pulp stem cells; injectable platelet-rich fibrin; leukocyte platelet-rich fibrin; osteogenic capacity; platelet by-products; platelet concentrates; platelet-rich fibrin; regenerativetreatments/therapies

INTRODUCTION

Tissue regeneration techniques recommend using concentrates from peripheral blood, such as platelet-rich fibrin (PRF) and platelet-rich plasma (PRP), to promote bone production and graft healing. Since clot formation is crucial in tissue repair, adding anticoagulants to PRP negatively affects wound healing. PRF, a second-generation autologous platelet concentrate, is produced without anticoagulants or other chemicals. Research shows that PRF has a stronger and longer-lasting impact on osteoblast differentiation and

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growth compared to PRP, due to its inherent osteoinductive and osteoconductive properties.[1-3]

At present, PRF stands out as the most frequently utilized platelet concentrate. Originating from the initial work of Choukroun *et al*.,[4] this method has undergone various protocol modifications over time. A multitude of PRF protocols have been documented in the literature, as outlined by Shah *et al*. These encompass variations such as L-PRF, advanced PRF (A-PRF), A-PRF+, and I-PRF.[4,5]

High levels of factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-β1), vascular endothelial growth factor (VEGF), interleukin 1 (IL-1) β, IL-4, and IL-6 have been detected in L-PRF. These factors promote tissue regeneration by supporting the proliferation and differentiation of fibroblasts, osteoblasts, endothelial cells, and chondrocytes.[6] Physiological polymerization sustains PRF bioactive levels for 28 days, allowing the gradual release of growth factors and cytokines.[7] Besides promoting cell migration and angiogenesis, L-PRF provides a scaffold that stabilizes implanted bone grafts.[8] VEGF, PDGF, and fibroblast growth factor mediate angiogenesis, enhanced by αvβ3 integrin activation, improving scaffolding function. The bio-functionality, cost, ease of preparation, and availability of L-PRF support its use in various regenerative applications. Leukocytes in L-PRF may also regulate inflammation and prevent infection.[9,10]

Introduced in 2014, injectable PRF (i-PRF) is a liquid blood derivative developed alongside the low-speed centrifugation concept (LSCC). It contains high concentrations of platelets, leukocytes, and growth factors. Recent research indicates that i-PRF forms a three-dimensional fibrin gel embedded with growth factors, type-I collagen, platelets, leukocytes, and osteocalcin. This gel has anti-biofilm and antibacterial properties, modulates inflammation, and promotes osteogenesis. Compared to PRF, i-PRF enhances bone regeneration, addresses gingival recession, and treats endodontic diseases more effectively.[11]

Substantial research has been done to confirm the effectiveness of I-PRF in bone regeneration processes.[1] In addition, research highlights the encouraging attributes of L-PRF that might help with both soft-tissue and hard-tissue regeneration; however, the inconsistent findings reported in the literature support[6] this indicates that an experimental strategy is required to confirm the osteogenic potentials of i-PRF in comparison to L-PRF. Hence, this research aims to evaluate the assessment of the osteoconductive effect of i-PRF and L-PRF on dental pulp stem cell (DPSC) development.

MATERIALS AND METHODS

The current study included four male and four female systemically healthy individuals between the age group of 20 and 30 years who met certain inclusion and exclusion criteria. Ethical approval was obtained from the institution (CSP/19/SEP/80/304), and each patient submitted a written consent form to take part in the research.

Based on the pilot study, the sample size determination was done. From the eight patients, two samples of blood were taken (one sample for L-PRF and one sample for i-PRF). For this study, a total of 16 samples were used.

Inclusion criteria

The individuals between the age group of 20 and 30 years who were free from systemic health issues, not on any medications, and did not engage in smoking were included in the study.

Exclusion criteria

Individuals who, drink, are on anticoagulant medication, are using bisphosphonates, are pregnant or nursing, or have taken antibiotics within the last 3 months were excluded from the study.

Methodology

Source and cell culture of dental pulp stem cells

DPSCs were sourced from patients reporting to the department of maxillofacial surgery for impacted third molar removal. The study made the use of two donors. Under the Ethical Committee's guidelines, both donors signed written informed consent. The obtained normal teeth were immediately transported to the cell culture laboratory in Hank's balanced salt solution to isolate DPSCs. In sterile circumstances, mesenchymal stem cells (MSCs) were extracted using a previously described method by Naz *et al*.[12] [Supplementary File].

Blood sampling and preparation of platelet-rich fibrin

Venous blood was drawn by venipuncture in the antecubital vein of the forearm, 8 mL for each sample. In the next step, blood samples were transferred into glass-coated vacuum tubes. A tabletop (Eppendorf) centrifuge was used to centrifuge the blood sample right away. It was set at 3200 rpm for 12 min to prepare the L-PRF[13] and 700 rpm for 7 min to prepare the i-PRF[14] [Supplementary File] tubes were allocated under a sterile laminar flow hood and PRF mass was taken out using a sterile cotton plier and kept at −20°C until use.

Preparing platelet-rich fibrin-derived conditioned medium

To evaluate the paracrine activity PRF on osteogenic differentiation of human DPSCs, we prepared a PRF-derived conditioned medium. PRF masses were incubated in 8 mL Dulbecco modified essential medium (DMEM)/LG for 72 h. The medium was then collected, centrifuged for 10 min at 1200 rpm to exclude debris, and sterilized by using 0.2 μm-microfilters.

Assessments

Experimental groups

After 24 h, cells were allocated into three different groups as follows:

- Group 1: Control cells that received DMEM/F-12
- Group 2: Cells were given L-PRF-derived conditioned media (L-PRF CM)
- Group 3: Cells were given i-PRF derived CM (i-PRF CM).

MTT assay

The proliferation of DPSCs was investigated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. DPSCs were initially plated at a density of 2.5×10^4 cells per well in 96 well plates. After 24 h, cells were divided into three groups: the control group received DMEM/F-12; Group 2 received L-PRF CM; and Group 3 received i-PRF CM. The cells were maintained for 7 days and the percentage of proliferation was then studied.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) production was measured using an ALP assay kit following the manufacturer's instructions. DPSCs were cultured in 24 well plates with 500 µL of DMEM/LG containing 10% fetal bovine serum at 37°C and 5% CO_2. After 24 h, the media were replaced with CM from L-PRF and i-PRF and maintained for 7 days, with media changes every 3–4 days. After the incubation period, supernatants were collected and centrifuged for 10 min at 2500 rpm. ALP content was calculated using the p-nitrophenyl phosphate method.

Alizarin red S staining

To detect extracellular calcium deposition, DPSCs were stained with alizarin red S (ARS) solution after treatment with L-PRF and i-PRF-CM. After 7 days of incubation, DPSCs were washed with peripheral blood smear (PBS) twice, fixed with 100% methanol for 10 min, and stained with 0.1% ARS solution for 30 min. The cells were then washed with PBS twice (10 min each) and examined under a light microscope. For semi-quantitative evaluation, the stained cells were dried, washed with 5% HCl for 20 min, and the contents transferred to a 96-well plate. Absorbance was measured at 405 nm and standardized to the cell count.

Growth factor profile

Supernatant culture medium in 1 mL was collected and the amount of released growth factor TGF-beta, VEGF was quantified using the enzyme-linked immunosorbent assay (ELISA) assay at 1, 3, and 7 days.

Cytokine expression

The concentrations of ILs, IL-6, and IL-8 were determined using the ELISA assay after 7 days.

Statistical analysis

The data were analyzed using the IBM SPSS version 25. (IBM, Armonk, NY, USA). ANOVA followed by *post hoc* test was used to compare the values of all variables among the three groups. $P \leq 0.05$ was considered statistically significant and statistical significance was designated as ^{ns}nonsignificant, $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ represent significant data.

RESULTS

MTT assay for proliferation

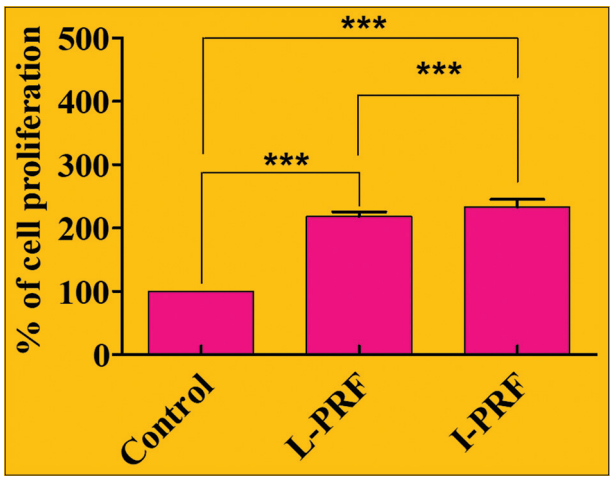
The mean of DPSC proliferation in percentage was found as 100, 218, and 248 for control, L-PRF, and i-PRF, respectively [Graph 1]. We found a statistically significant ($P < 0.001$) difference in the proliferation of DPSC in the both L-PRF and i-PRF group compared to the control. Microscopic changes depicted the same findings [Figure 1].

Alkaline phosphatase activity

Based on the results, the mean concentration of ALP in the supernatant media from the control, L-PRF, and i-PRF groups was 70 U/L, 152 U/L, and 164 U/L, respectively [Graph 2]. A statistically significant difference ($P < 0.001$) in the released ALP content of the L-PRF and i-PRF group compared to the control.

Alizarin red staining

Based on microscopic bright field imaging, no calcium and/or very little deposition was observed in the control group [Figure 2]. The relative mean intensity of ARS was found to be 16, 32, and 36 for the control, L-PRF, and I-PRF groups, respectively [Graph 3]. A statistically significant



Graph 1: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis of dental pulp stem cells treated with control, leukocyte-rich platelet-rich fibrin (PRF) conditioned media (CM) and injectable-PRF CM groups over 7 days. ***$P < 0.001$. L-PRF: Leukocyte-rich platelet-rich fibrin, I-PRF: Injectable platelet-rich fibrin

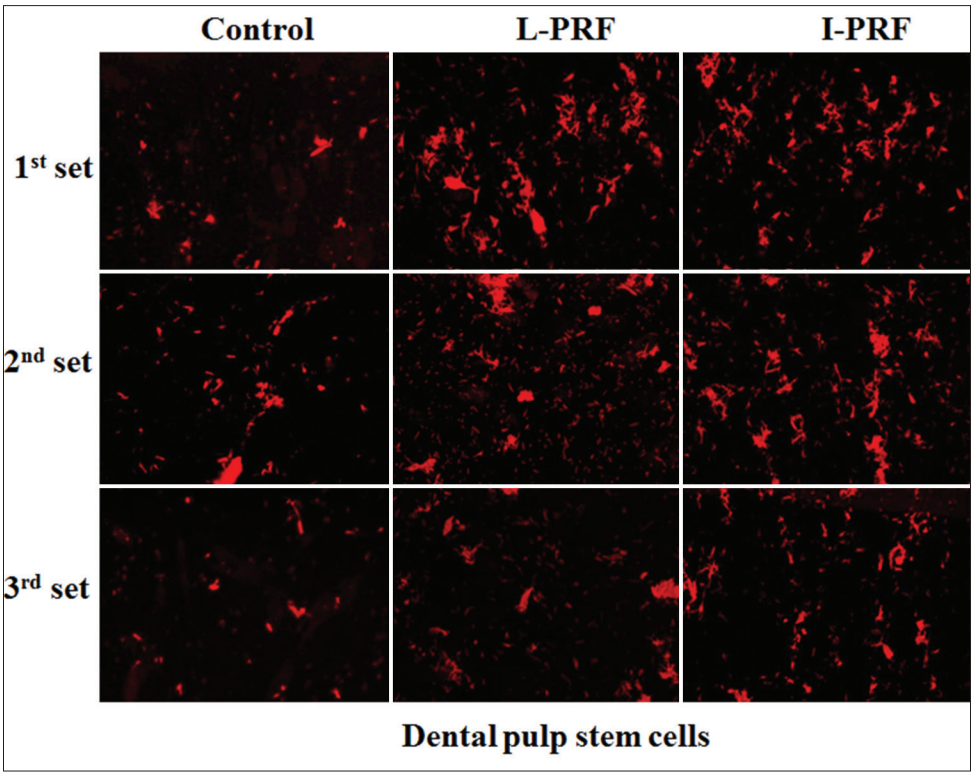


Figure 1: Microscopic examination of morphological changes in dental pulp stem cells after 7 days of incubation with control, leukocyte-rich platelet-rich fibrin (PRF) conditioned media (CM) and injectable-PRF CM. L-PRF: Leukocyte-rich platelet-rich fibrin, I-PRF: Injectable platelet-rich fibrin

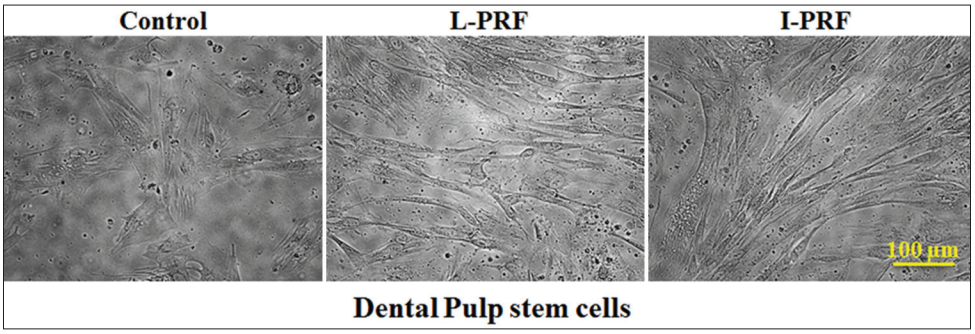


Figure 2: Microscopic analysis of calcium deposition by alizarin red S staining

difference ($P < 0.001$) in calcium deposit in the L-PRF and i-PRF groups compared to the control.

Growth factor release profile

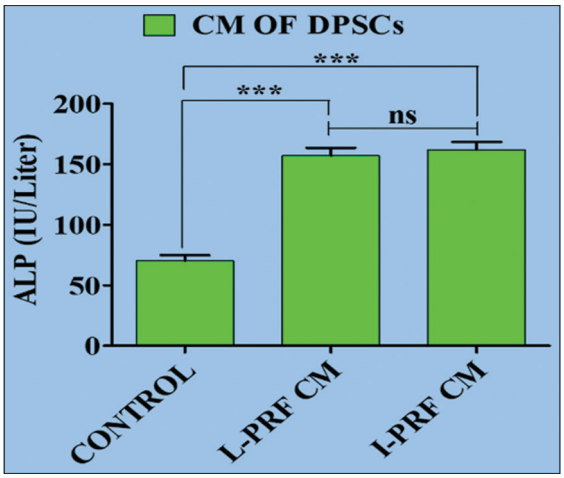
VEGF and TGF-beta growth factor release were analyzed using the ELISA. On day 7, it was seen that higher release of VEGF and TGF-beta in both L-PRF and i-PRF groups as compared to the control [Graph 4]. It was also found that on the 7th day, significantly higher ($P < 0.001$) levels of VEGF and TGF-beta were released from both L-PRF and i-PRF when compared to the control.

Cytokine expression

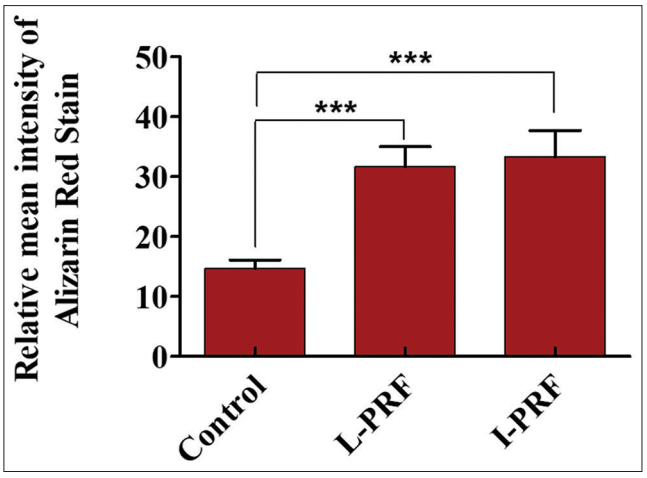
The concentrations of inflammatory cytokines in control, L-PRF, and I-PRF preparations are shown in Graph 4. IL-6 and IL-8 in all three preparations were under detectable levels, it showed higher levels in L-PRF and I-PRF as compared to the control, whereas it showed a similar level in both L-PRF and I-PRF. There were no significant differences between L-PRF and i-PRF, but a statistically significant difference ($P < 0.001$) was observed in IL concentration in the L-PRF and i-PRF group compared to the control.

DISCUSSION

PRF is widely used in pulp tissue engineering and various surgical fields. Although its effectiveness in pulpal and periapical regeneration is under-researched, PRF has shown success in sinus lifts, extraction socket healing, and managing periapical abscesses. It promotes root



Graph 2: Alkaline phosphatase activity of dental pulp stem cells treated with conditioned media from control, injectable platelet-rich fibrin (PRF) and leukocyte-rich-PRF groups after 7 days. ***$P < 0.001$, ns means not significant. L-PRF: Leukocyte-rich platelet-rich fibrin, I-PRF: Injectable platelet-rich fibrin, CM: Conditioned media, ALP: Alkaline phosphatase



Graph 3: Measuring the existence of calcium deposition by alizarin red S staining. ***$P < 0.001$. L-PRF: Leukocyte-rich platelet-rich fibrin, I-PRF: Injectable platelet-rich fibrin

lengthening, periapical lesion regression, dentinal wall thickening, apical closure, and has antimicrobial properties. Modifications have enhanced PRF's regenerative success.[15,16]

L-PRF is a modern platelet concentrate, easy, and inexpensive to prepare for clinical use. *In vitro*, L-PRF membranes release significant amounts of PDGF-AB, TGF-β, VEGF, cytokines, and fibronectin over 7 days. Compared to PRP, L-PRF releases over 15 times more VEGF and more than twice the TGF-β1. Leukocytes, essential for tissue regeneration, are more abundant in A-PRF, which shows higher growth factor release than standard L-PRF, highlighting its potential in regenerative applications.[10,13]

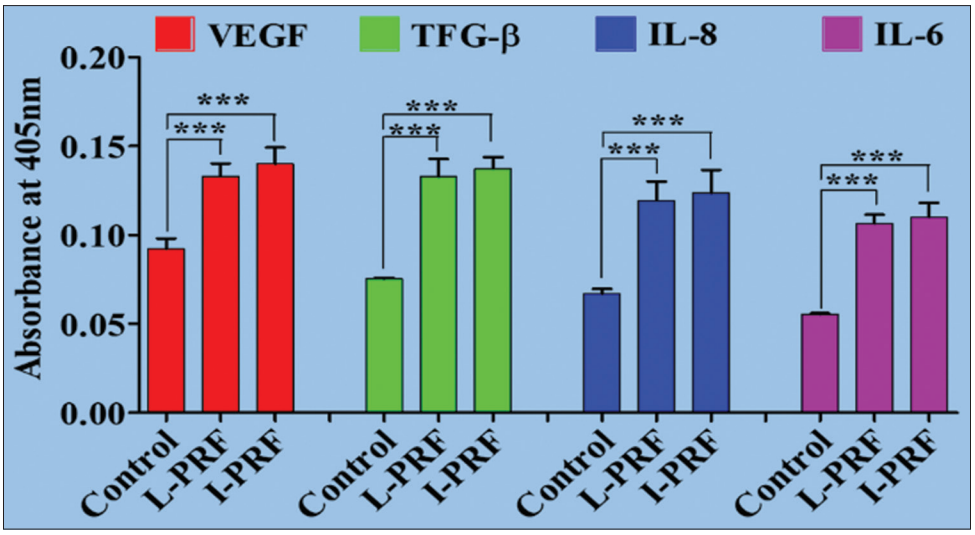
To obtain PRF with a stronger pro-regenerative efficacy, the LSCC was created. The i-PRF was created in 2014. The shortcomings of the solid PRF matrix are addressed by i-PRF since it is a flexible matrix.[11] The creation of an i-PRF formulation, or "i-PRF," has been developed to provide clinicians with a simple-to-use liquid platelet concentration that can be used alone or conveniently combined with a variety of biomaterials.[14]

MTT assay is a commonly used method to assess cell proliferation and viability. It is a relatively simple, low-cost, and widely used method to measure cell proliferation. A statistically significant difference was found in our present study in the proliferation of DPSC in the L-PRF CM and i-PRF CM group compared to the control which signifies that both L-PRF and i-PRF caused higher stimulation of DPSCs proliferation as compared to the control group.

The ALP activity test in this study examined odontoblastic differentiation and mineralization effects. ALP activity, a biochemical indicator of osteoblasts and new bone production, releases phosphate ions that combine with calcium to form hydroxyapatite.[17] The study measured ALP release from DPSCs after 7 days of incubation in control, L-PRF, and i-PRF groups. Both L-PRF and i-PRF groups showed significantly higher ALP release compared to the control, indicating their potential to enhance osteogenic differentiation. ALP is an early marker of osteo/odontogenic differentiation, with substantial expression observed 1-week postinduction.[18] Another study noted that i-PRF increased fibroblast migration and production of PDGF, TGF-β, and collagen1, markers of osteoblastic development in DPSCs.[14]

Under *in vitro* conditions, newly developed osteoblasts generated structured extracellular matrix (ECM) with calcium-rich deposits, as revealed by positive alizarin red staining, a common method to evaluate ECM mineralization – a key step in bone formation.[19] This study observed that DPSCs exposed to all three experimental groups produced and deposited red ECM around the cells. Microscopic imaging showed that the L-PRF and i-PRF groups had significantly higher deposition than the control. These results indicate that both L-PRF and i-PRF enhance the osteoblastic activity of DPSCs by promoting calcific deposits, aligning with the findings by Graziano *et al*.[20] and Kermani *et al*.,[21] who assessed DPSC differentiation potential through positive alizarin red staining of ECM with calcium-rich deposits.

ELISA is a sensitive and specific method to measure the growth factor release profiles. This study analyzed the release of TGF-beta and VEGF. TGF-beta plays a crucial role in bone remodeling by inducing MSC differentiation into osteoblasts, inhibiting osteoclast differentiation, regulating ECM protein deposition, and promoting



Graph 4: Enzyme-linked immunosorbent assay quantification of vascular endothelial growth factor, transforming growth factor-beta, interleukin 6 (IL-6) and IL-8 on 7th day. ***P < 0.001. L-PRF: Leukocyte-rich platelet-rich fibrin, I-PRF: Injectable platelet-rich fibrin, VEGF: Vascular endothelial growth factor, TFG-β: Transforming growth factor-beta, IL-6: Interleukin 6

new bone formation.[22] VEGF also induces osteoblast differentiation from MSCs, enhances osteoblast survival, and interacts with other growth factors to promote differentiation.[23,24] The study found a statistically significant difference in TGF-beta and VEGF release between the L-PRF, i-PRF, and control groups, indicating that both L-PRF and i-PRF promote higher release of these growth factors. Similar results were observed by Ravi and Santhanakrishnan, who compared the release profile of PDGF-AA from A-PRF, T-PRF, and L-PRF.[25]

Osteoblastogenesis is tightly regulated by cytokine networks in both healthy and pathological conditions. This study examined IL-6 and IL-8 levels. IL-6 can promote osteoblastogenesis by increasing RUNX2 and ALP expression and enhancing matrix mineralization in certain cells.[26] Conversely, IL-6 can suppress osteoblastogenesis and matrix mineralization in preosteoblastic cells by down-regulating markers like RUNX2, OSX, and OCN.[27] IL-8 is known for its high bone marrow stem cell recruitment efficiency and significant role in tissue regeneration, making it potentially valuable for gradual bone healing. Both *in vitro* and *in vivo* experiments that analyzed Sox9 and COL2 confirmed the results that IL-8 enhances bone regeneration via CXCR2-mediated PI3k/Akt signaling pathway.[28] The current study found statistically significant differences in IL-6 and IL-8 levels between L-PRF, i-PRF, and control groups, indicating that both L-PRF and i-PRF promote higher releases of these cytokines. This suggests that L-PRF and i-PRF equally enhance osteogenic activity in DPSCs. Further research on the roles and synergistic effects of IL-6 and IL-8 with osteogenic growth factors in bone regeneration is warranted.

CONCLUSIONS

To conclude, the results of this investigation show that both i-PRF liquid form and L-PRF membrane form showed the potential for proliferation and osteoblastic differentiation in capable of tissue regeneration with the ability to activate DPSCs, also both having equal ability to release various growth factors and cytokines hence can be considered as a potential scaffold in regenerative endodontics for pulpal and periapical regeneration.

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Conflicts of interest
There are no conflicts of interest.

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SUPPLEMENTARY FILE

Dental pulp stem cells isolation and culture

Extracted permanent teeth were collected. After disinfecting with 3% sodium hypochlorite solution for 2 min, tooth was rinsed with ×1 phosphate buffer saline (PBS) and dried using cotton gauze. A cut around the cementoenamel junction was made using a sterilized dental diamond fissure burs (MANI, Inc., USA) along with high speed hand piece (NSK, USA) under copious water supply to decoronate the tooth to expose the pulp chamber. Sectioned teeth were placed into the transport media containing basic medium Dulbecco modified essential medium F12 (DMEM-F12) supplemented with 20% fetal bovine serum (FBS) and penicillin 500 U/mL, streptomycin 500 μg/mL, amphotericin B 1.25 μg/mL (Sigma Aldrich, Merck, USA). Samples then placed on ice were transferred to research laboratories for subsequent processing and culture. Using aseptic condition, 100 mm petri plate (sterilin) was set up for processing of each tooth in a biohazard laminar flow hood. Extracted tooth was decanted in a petri plate. Tooth was hold with the help of a sterile forceps and gently extirpated out DP tissue using endodontic H-file #30 (MANI, Inc., USA). DP tissue was placed in ×1 PBS containing 1% antibiotic antimycotic solution (Sigma Aldrich) in a petri plate for 10–20 min and was washed twice with ×1 PBS (Sigma Aldrich, Merck, USA) each for 10 min. Then were transferred into a new petri plate containing DMEM-F12 with 20% FBS. Minced into 1–2 mm^3 pieces using surgical blade #20 (Feather, WAPI, USA) as demonstrated in. DP minced fragments were plated in a T-25 flask (Thermo Scientific, USA) containing DMEM-F12 supplemented with 20% FBS, penicillin 100 U/mL, streptomycin 100 μg/mL, amphotericin B 0.25 μg/mL, 1 mm sodium pyruvate, and 2 mm L-glutamine (Sigma Aldrich). Explants were cultured at 37°C in a humidified incubator with 5% CO_2. Cultures were observed daily under inverted microscope (Olympus Corp, USA) for any contamination and cell growth through migration from explant. Micrographs were captured using DSL3 standalone microscope camera controller (Nikon, Japan) at different magnifications. Cells were considered present when a fibroblastic morphology cell.[12]

Preparation protocol for leukocyte-platelet-rich fibrin

The L-PRF was prepared through a single centrifugation of blood according to the protocol of Dohan Ehrenfest *et al*. for a period of 12 min at 2700 rpm. Blood was taken in 9 mL tubes, immediately centrifuged. After centrifugation, each L-PRF clot was separated from the portion of red blood cells (red thrombus), obtaining a fibrin clot with a red small portion in order to include the "buffy" coat richer in large leukocytes.[13]

Preparation protocol for injectable platelet rich fibrin

For i-PRF preparation, two tubes of 10 mL of whole blood without anticoagulant were centrifuged at 700 rpm for 3 min (×60 g) at room temperature by a Eppendorf Centrifuge. The upper liquid layer was collected as i-PRF.[14]

Supplementary Table 1: Intergroup comparison of values using ANOVA

Variable	Group	Mean	SD	*P*
MTT	Control	94.13	4.794	<0.001
	L-PRF	208.75	17.052	
	I-PRF	249.88	22.184	
VEGF	Control	0.094	0.006866	<0.001
	L-PRF	0.133	0.006497	
	I-PRF	0.139	0.008211	
TGF-BETA	Control	0.076	0.001069	<0.001
	L-PRF	0.131	0.007019	
	I-PRF	0.138	0.007964	
IL-8	Control	0.067	0.002748	<0.001
	L-PRF	0.120	0.007110	
	I-PRF	0.121	0.009920	
IL-6	Control	0.056	0.001069	<0.001
	L-PRF	0.106	0.003563	
	I-PRF	0.117	0.010637	
ALP	Control	66.75	3.770	<0.001
	L-PRF	155.88	6.312	
	I-PRF	173.63	6.022	
ARS	Control	16.00	2.449	<0.001
	L-PRF	31.38	1.923	
	I-PRF	35.63	3.623	

VGEF: Vascular endothelial growth factor, TGF: Transforming growth factor, ALP: Alkaline phosphatase, IL: Interleukin, I-PRF: Injectable platelet-rich fibrin, L-PRF: Leukocyte-rich platelet-rich fibrin, SD: Standard deviation, ARS: Alizarin red S, MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

Supplementary Table 2: *Post hoc* comparison of the above values

Variable	Group 1	Group 2	Difference	*P*
MTT	Control	L-PRF	−114.625*	0.000
	Control	I-PRF	−155.750*	0.000
	L-PRF	I-PRF	−41.125*	0.000
VEGF	Control	L-PRF	−0.038750*	0.000
	Control	I-PRF	−0.044500*	0.000
	L-PRF	I-PRF	−0.005750	0.272
TGF-BETA	Control	L-PRF	−0.055875*	0.000
	Control	I-PRF	−0.062500*	0.000
	L-PRF	I-PRF	−0.006625	0.104
IL-8	Control	L-PRF	−0.053500*	0.000
	Control	I-PRF	−0.054750*	0.000
	L-PRF	I-PRF	−0.001250	0.936
IL-6	Control	L-PRF	−0.050875*	0.000
	Control	I-PRF	−0.061000*	0.000
	L-PRF	I-PRF	−0.010125*	0.014
ALP	Control	L-PRF	−89.125*	0.000
	Control	I-PRF	−106.875*	0.000
	L-PRF	I-PRF	−17.750*	0.000
ARS	Control	L-PRF	−15.375*	0.000
	Control	I-PRF	−19.625*	0.000
	L-PRF	I-PRF	−4.250*	0.015

VGEF: Vascular endothelial growth factor, TGF: Transforming growth factor, ALP: Alkaline phosphatase, IL: Interleukin, I-PRF: Injectable platelet-rich fibrin, L-PRF: Leukocyte-rich platelet-rich fibrin, ARS: Alizarin red S, MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)