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Expression of Vascular Endothelial Growth Factor Using Platelet Rich Fibrin (PRF) and Nanohydroxyapatite (nano-HA) in Treatment of Periodontal Intra-Bony Defects - A Randomized Controlled Trial

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ABSTRACT

The study aims to assess the concentration of vascular endothelial growth factors (VEGF) with platelet rich fibrin (PRF) biomaterial, while using it separately or in combination with nanohydroxyapatite (nano-HA) for treating intra-bony defects (IBDs) using radiographic evaluation (DBS-Win software). Sixty patients with IBD (one site/patient) and chronic periodontitis were recruited randomly to test either autologous PRF platelet concentrate, nano-HA bone graft, a combination of PRF platelet concentrate and nano-HA, or alone conventional open flap debridement (OFD). Recordings of clinical parameters including probing depth (PD), gingival index (GI), and clinical attachment level (CAL) were obtained at baseline and 6 months, post-operatively. One-way analysis of variance (ANOVA) was used to compare four groups; whereas, multiple comparisons were done through Tukey's post hoc test. The results showed that CAL at baseline changed from 6.67 ± 1.23 to 4.5 ± 1.42 in group I, 6.6 ± 2.51 to 4.9 ± 1.48 in group II, 5.2 ± 2.17 to 3.1 ± 1.27 in group III, and 4.7 ± 2.22 to 3.7 ± 2.35 in group IV after 6 months. The most significant increase in bone density and fill was observed for IBD depth in group III that was recorded as 62.82 ± 24.6 and 2.31 ± 0.75 mm, respectively. VEGF concentrations were significantly increased at 3, 7, and 14 days in all groups. The use of PRF with nano-HA was successful regenerative periodontal therapy to manage periodontal IBDs, unlike using PRF alone. Increase in VEGF concentrations in all group confirmed its role in angiogenesis and osteogenesis in the early stages of bone defect healing.

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Abbreviations: CaP, Calcium phosphate; CAL, Clinical attachment level; DFDBA, Demineralized freeze-dried bone allograft; ELISA, Enzyme-linked immunosorbent assay; GCF, Gingival Clavicular Fluid; GI, Gingival Index; IBD, Intra-Bony Defect; Nano-HA, Nanohydroxyapatite; ANOVA, One-way analysis of variance; OFD, Open flap debridement; PRF, Platelet rich fibrin; PRP, Platelet rich plasma; PPP, Platelet poor plasma; PD, Probing depth; Rpm, Revolutions per minute; SPSS 20[®], Statistical Package for Social Science; SD, Standard Deviation; VEGF, Vascular Endothelial Growth Factor.

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1. Introduction

Periodontists have been experimenting different modalities to regenerate aggressive periodontitis along with various success degrees. Periodontal surgery has yielded significant outcomes, and platelet rich fibrin (PRF) is one of them. Clinically accepted responses result due to porous hydroxyapatite (HA) bone grafting material, if filled in periodontal intra-bony defects depth (IBDs) (Anitha et al., 2017). PRF is a platelet and leukocyte preparation that concentrates several polypeptide growth factors and; thus, has the likelihood in regenerative treatment for periodontal defects (Pradeep et al., 2017).

Periodontitis is an inflammatory situation in terms of microbial plaque that results in periodontal tissue destruction and osseous defects in alveolar bone (Bayani et al. 2017). PRF comprises of platelets, leukocytes, circulating stem cells, and cytokines based on a composition of a fibrin matrix (Liu et al. 2019). PRF fabricates into bioabsorbable fibrin scaffolds comprising of several growth factors

after deriving from human venous blood (Wang et al., 2016). It further provides a gel with integrity, osteogenic potential and bioactivity (Shahsavari-Pour et al. 2018). In fact, perioperative drug delivery is a routine alternative treatment due to recombinant or exogenous growth factors in different surgical fields such as orthopedic surgery, plastic surgery, burn surgery, and oral surgery (Yamakawa and Hayashida, 2019; Mendes et al. 2018; Kawase et al. 2015; Ved et al. 2018).

Nanohydroxyapatite (nano-HA) represents a significant class of bone graft materials due to its enhanced osseointegrative properties. Schnettler et al. (2004) found that n-HA stimulates osteoblast activity with new bone formation; whereas, n-HA forms a strong bond with newly deposited bone. n-HA material differed from microcrystalline hydroxyapatite biomaterials due to its chemical composition. Natural bone resembles to the chemical composition of $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ and 1.67 ratio of calcium and phosphate respectively (Mendes et al. 2018).

Bone mineral content and particle size are helpful in accelerating substitution by vital bone (Strietzel et al. 2007). The use of PRF provides a promising regenerative procedure. PRF resembles a fibrin network that enhances cell migration, proliferation, and cicatrization (Toffler et al. 2009). PRF exhibit many advantages over platelet rich plasma (PRP), as a second-generation platelet concentrate. PRF preparation process creates a gel-like matrix that release, a relatively constant concentration of growth factors over a period of 7 days (Dohan et al. 2006; Carroll et al. 2005). PRF is not a membrane, however it is autogenous and can be easily manipulated into a membrane (Hafez et al. 2015).

Significant and continuous stimulation and proliferation of dermal pre-keratinocytes, maxillofacial osteoblasts, gingival fibroblasts, and pre-adipocytes is induced by PRF (Ehrenfest, et al. 2009). This revealed a strong differentiation of osteoblasts and initiated mineralization process in PRF concentrate detected through light and scanning electron microscopy. Chang and Zhao (2011) conducted a retrospective study to investigate the use of PRF with synthetic bone graft for IBDs. The results depicted that IBDs were associated with reduced clinical attachment gain and probing depth (PD). Recently, PRF with alloplast was applied to treat perio-endo combined IBD to improve clinical parameters, increase periapical bone density, and increase gingival thickness over 6-month period (Su, and Chang, 2015). Another study by Castro et al. (2017) conducted a systematic review and meta-analysis study about regenerative potential of PRF during different periodontal surgery. The results showed that there is positive impact of PRF on periodontal plastic surgery, furcation invasion, and IBDs.

Vascularization is among the important events of bone healing (Hollinger et al. 1999). Vascular endothelial growth factor (VEGF) are produced by various types of cells and include macrophages, keratinocytes, tumor cells, platelets, and renal mesenchymal cells (Chintalgattu et al 2003). The functions of VEGF are not confined to vascular endothelial system; rather, it plays a major role in normal physiological functions including; hematopoiesis, bone formation, and wound healing (Yancopoulos et al. 2000; Gerber et al. 1999). Enhancement in chemotaxis of mesenchymal stem cells, along with differentiation and proliferation of osteoblasts through indirect effect on osteoprogenitor cells depends on VEGF that increases bone formation (Giannobile, 1996; Keramaris et al. 2008).

VEGF is an important biomarker in determining the progression of periodontal disease as it is a key factor that leads to its progression (Prapulla, Sujatha and Pradeep, 2007). As it has been observed that in case of periodontitis the levels of VEGF in gingival clavicular fluid (GCF) increases, which then decrease in concentration when the effective treatment is provided. (Prapulla, Sujatha and Pradeep, 2007). The healing properties of PRF provided by the previous studies regarding VEGF is limited. Also, evaluation of

periodontal healing with the use of PRF in combination with nano-HA in IBD is not investigated, thoroughly. Therefore, this study aims to assess the level of VEGF with PRF concentrate when used alone or in combination with nano-HA in treatment of IBDs. The study has the hypothesis that the use of PRF with nano-HA is effective in regenerative periodontal therapy in managing periodontal IBDs as compared to using PRF alone.

2. Materials and methods

2.1. Study subjects

Patients in this study were selected from the Department of Periodontology of the Faculty of Dentistry, King Abdulaziz University, Saudi Arabia, from February 2015 and November 2016. A total of 60 non-smoking patients diagnosed with severe chronic periodontitis were recruited, among which 33 were males and 27 were females aged between 27 and 48 years. The subjects for this study included patients with moderate or advanced stage of periodontal disease. Patients were included if they had: (1) no systemic diseases; (2) better compliance to plaque control instructions; (3) all vital teeth; (4) at least one interproximal IBD; $\text{PD} \geq 6$ mm and clinical attachment levels (CAL) $\geq 3-4$ mm after 3 weeks of initial therapy; and (5) IBDs with minimum depth of 3 mm detected through diagnostic periapical radiographs. Patients were excluded if they were pregnant and presented with inadequate compliance of oral hygiene maintenance schedule.

The flowchart describing recruitment of participants in the study is illustrated in Fig. 1. Process of randomization was used for assigning patients to different groups that were provided with different treatments. Similar to the study of Thorat and Baghele (2017), statistical power analysis software was used to divide 60 intra-bone defects into 4 groups. It was determined that 6 patients per group were needed to achieve 80% power with 95% confidence interval (CI); however, 15 patients were there in each group in the present study:

- Group I was treated with PRF concentrate and open flap debridement (OFD).
- Group II was treated with nano-HA bone graft and OFD.
- Group III was treated with OFD and PRF concentrate was treated with nano-HA bone graft.
- Group IV was treated with OFD alone.

2.2. Pre-surgical therapy and grouping

Full mouth scaling was included in initial periodontal therapy using ultrasonic instruments under local anesthesia. Periodontal conditions were reexamined after 4 weeks of the initial treatment. Treatments were only provided to the patients having full-mouth dental plaque score of < 1 . However, patients with persistence of an interproximal site with $\text{PD} \geq 6$ mm, $\text{CAL} \geq 3-4$ mm, and interproximal IBDs of ≥ 1 mm were suggested for surgical treatment. The IBDs among the participants were measured after OFD, to check IBD depth and remaining number of walls.

Before undergoing surgical treatment, measurements were taken for CAL, gingival index (GI) and baseline data PD (Polson et al. 1980). A calibrated periodontal probe was used to take these measurements as it contained William's markings to the nearest millimeter. The calculations included deepest point of baseline defects.

DBS-Win software was used to assess the radiographic measurements. This software is a part of the recently introduced vista scan system (Durr Dental, Germany). The risk of radiation dose in digital radiography to CBCT patients was minimized using vistas-

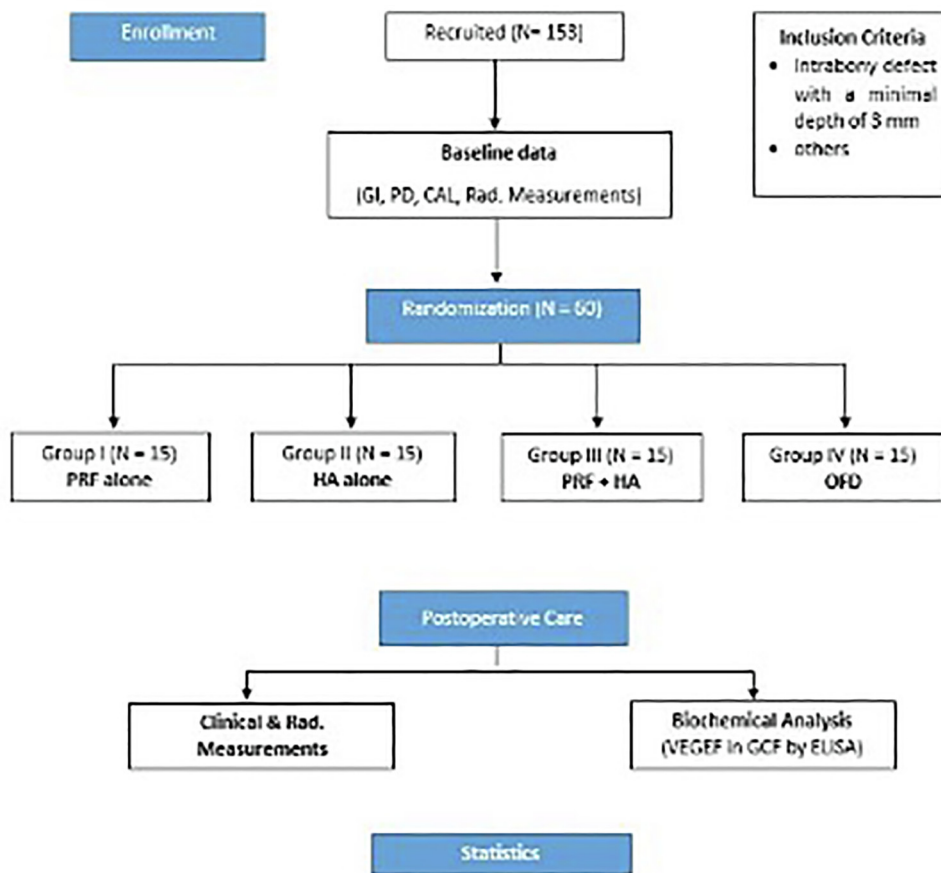


Fig. 1. Recruitment of the participants.

can system and DBS-Win software (Durr Dental, Germany). The radiographs were taken using film holding device and paralleling technique to minimize errors and to obtain reproducible images (Inocêncio Faria et al. 2013).

The gray value (0) was assigned to black and value 256 was assigned to white to calculate the mean gray value in each region of interest. A parallel line was drawn towards the roof surface and measure the bone density to obtain the linear density measurements. A line was drawn midway in the alveolar process of selected defect and extended to the level of the apex of the root from the apex of the alveolar crest. This is the portion of the alveolar bone that is extended beyond the periphery of the socket present to the interproximal side. The recording of grey level along each line was obtained at the beginning, middle, and end of the line (Fig. 2). Fig. 3A shows that the mean average density (grey level) along the line was obtained by taking average of the three readings. Whereas, Fig. 3B shows the difference between values of IBD and the distance from the selected alveolar crest to the base of defect to calculate bone fill at baseline and 6 months.

All surgeries were performed by two expert operators (MB and MS), following the same technique. One calibrated masked examiner recorded all clinical measurements, who was not involved in the study. The pre- and post-operative parameters were recorded by expert operators, as blinded for the examiners. Calibration exercise helped in assessing the reproducibility of intra-examiner two separate days, with at least difference of 48 h. Calibration was considered valid, only if $\geq 90\%$ of the recordings were reproduced within difference of 1.0 mm.

2.3. PRF preparation

The PRF preparation was guided according to the protocol developed by Choukroun et al. (2001). Around 5 ml intravenous blood around by venipuncture of the antecubital vein was collected in a sterile 6 ml vacutainer tube without anticoagulant before undergoing surgery. Overall, the PRF supports loads, has double tendency of stretching under tension, mechanically resistant, and retains surgical sutures effectively distorts itself substantially before tear. The production technique of PRF is simple which requires just blood sample, and table centrifuge. The procedure of blood collection and transfer speed of the centrifuge determine the success of this technique.

The prepared solution was centrifuged for 10 min at 3,000 revolutions per minute (rpm) in the centrifugation machine. There is formation of platelet poor plasma (PPP) at the top, structured fibrin clot in the middle of the tube, and red corpuscles at the bottom, if blood centrifugation is conducted immediately after its collection. Coagulation cascade is initiated during the centrifugation process, when the blood meets the test tube wall. After removing PPP from the top, a sterile tweezers and scissor were used to separate from red corpuscles base at the bottom of the test tube. The middle layer (PRF) was placed in a sterile dish after its removal.

2.4. Surgical procedure

Each patient was included in one of the four groups by selecting sealed envelopes that contained a paper labeled “OFD + PRF”,

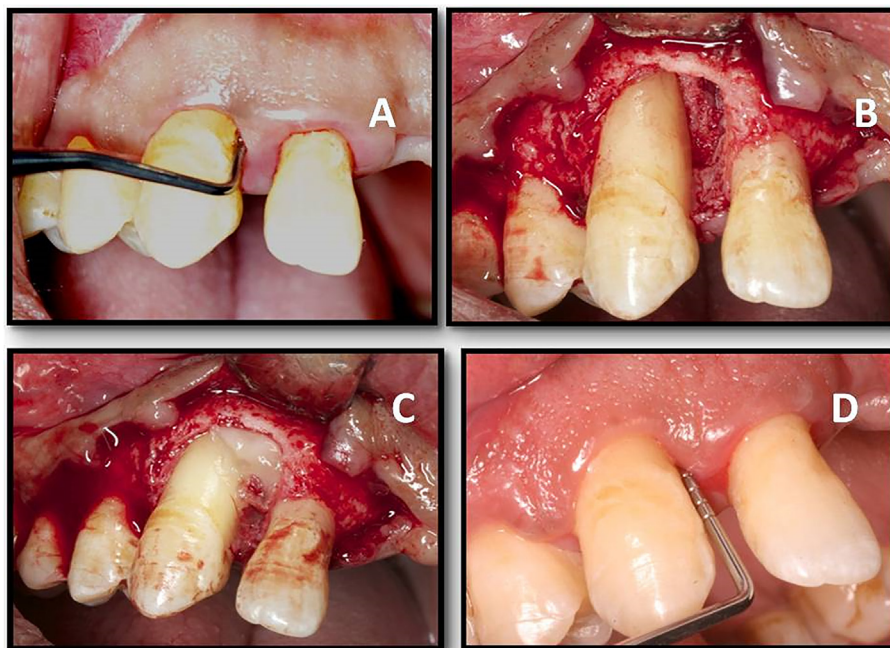


Fig. 2. A) Preoperative clinical photograph of 38 years old male patient showing 10 mm PPD mesial to upper right canine; B) Clinical photograph after flap reflection and subgingival debridement. An IBD was present mesial to upper right canine; C) Clinical photograph shows the IBD was filled with minced PRF; D) Six months postoperatively showing a reduction in PD of 3 mm.

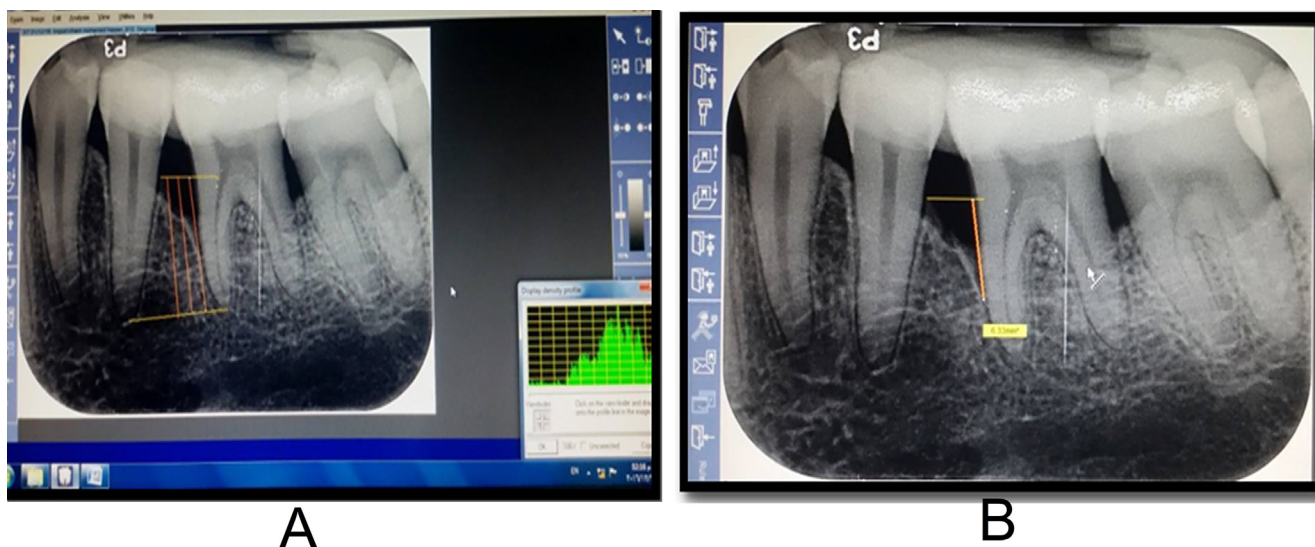


Fig. 3. A: Measurement of the bone density using vistascan software B: Measurement of IBD (The distance from the alveolar crest to the base of the defect) using vistascan software.

“OFD + nano -HA”, “PRF + nano-HA”, or “OFD”. The surgeon opened each envelop before surgery and dictated the treatment assigned to that specific patient. Buccal and lingual sulcular incisions were made and mucoperiosteal flaps were elevated after administrating local anesthesia. Greater than 1 mm of the alveolar bone extended beyond the defect margin towards one or two teeth distally and medially was exposed by raising mucoperiosteal flaps on the facial and lingual/palatal aspects of each involved site. Ultrasonic instruments were used to carry out meticulous defect debridement and root planning. There was no performance of osseous re-contouring.

Reassessment of morphology and depth of IBDs was done during surgery, by recording the number of bony walls. The recording showed that minimum depth of selected defects was 1 mm. This measurement was taken from the most coronal point of the bony walls that was that surrounded the defect to the deepest point. The method described above was used for preparing PRF gel (Autologous Platelet Gel) for the patients of group I. PRF acted as a membrane as it covered the defect like GTR membrane. The representative PRF gel was prepared from centrifugation of 5 ml of whole blood. In group II, the defect was filled by n-HA (1 and 100 nm¹). A sterile dish was used to mix graft with sterile saline,

which was then added in small increments condensed with an instrument, until the defect was filled.

In group III, the n-HA was condensed in the defect then the IBD was covered by PRF membrane. PRF membrane was extended above the periphery of defect in the lingual and buccal direction. While, the defects of group IV were only treated by OFD, in which flap was repositioned and adapted in order to achieve maximum flap adaptation. The flaps were sutured with absorbable polyglycolic acid 4–0 sutures in an interrupted manner to place the flap margins at their original level.

2.5. Postoperative care

All the patients were instructed to rinse their teeth with 0.12% chlorhexidine gluconate (Antiseptal, Kahira Co. for Pharm. and Chem., IND Cairo-ARE) twice daily for two minutes for two weeks after surgery. They were also recommended to do gentle brushing with a soft toothbrush. The sutures were removed after 2 weeks of surgery. Furthermore, the patients were instructed to resume their normal mechanical oral hygiene measures after one month of surgery that included; flossing, and brushing using a soft toothbrush. Recall appointments were carried out at 3, 7, and 14 days for collection of GCF samples. Later, for the first month, there were recall appointments every week, which were reduced after one month. After one month, recall appointments were conducted on monthly basis for professional prophylaxis and oral hygiene reinforcement. The quantitative changes in the defects were evaluated by obtaining radiographic measurements that were reassessed at 6 months of the therapy. Clinical measurements were also obtained at 6 months due to unavailability of patients after three months; although, the tissue healing was almost completed by this time.

2.6. Biochemical analysis

The GCF samples were obtained from all the studied groups at the time of surgery before elevation of periodontal flap, and then after 3, 7, and 14 days after surgical intervention. The periopaper strips were placed by collecting GCF in the selected defect gently to touch the foundation of gingival sulcus. Each paper strip was left for 30 s and then stored at -70°C in a sterile Eppendorf tube until assayed. A commercially available enzyme-linked immunosorbent assay (ELISA) kit (VEGF Enzyme Immunoassay, Sunlong Biotech Co., Ltd) was used to assay VEGF concentrations according to the instructions of the manufacturer.

2.7. Statistical analysis

The data were entered into Microsoft Excel and then coded and uploaded on SPSS 20[®] (Statistical Package for Social Science). The data were presented in the form of mean and standard deviation (SD). One-Way Analysis of Variance (ANOVA) was used to compare the four groups clinically, radio-graphically and bio-chemically; whereas, Tukey's post hoc was used for conducting multiple comparisons. Paired *t*-test was used to evaluate the effect of time on each group (baseline and six months after). The statistically significant value for each test was set at $P \leq 0.05$.

2.8. Ethical considerations

The study followed the Code of Ethics of the World Medical Association (Declaration of Helsinki). All the patients were explained about the aim of study and the proposed treatment before recruiting them in the study. If the patient agreed then he/she was asked to sign a consent form approved by the local ethics committee of King Abdul-Aziz University. This form was in

accordance with the guidelines published by the CONSORT group and the World Medical Association's Declaration of Helsinki, (proposal number 043–16). The Clinical Trial Registration No. of this study was NCT02810548.

3. Results

Table 1 shows the characteristics of patients in four groups indicating that age and gender were similar in all the four with (P -value ≤ 0.05) Moreover, there was insignificant difference between all the four groups for GI at baseline and six months. There was an insignificant percent change in GI among the four groups at the 6 months.

Table 2 illustrates the changes made in PD in all the four groups. The amount of reduction of PD was (2.7 ± 0.89) for group I, (2.4 ± 1.17) for group II, (3.0 ± 0.94) for group III, and (0.90 ± 1.10) for group IV. Group III patients were recorded to have the highest mean percent change in PD between baseline and 6 months (44.78%); whereas, group I showed decrease in PD by 36.99% at 6 months compared to the baseline. The probing pocket depths for all the groups were found to be significant. However, recording of the percentage change in PD was obtained for each group.

The mean CAL for 6 months in group I was 4.5 ± 1.42 , II was 4.9 ± 1.48 , III was 3.1 ± 1.27 , and IV was 3.7 ± 2.35 mm. Insignificant changes were in group IV between baseline (4.7 ± 2.22) and 6 months readings (3.7 ± 2.35). Clinical attachment gain was compared and significant changes have been reported in **Table 2**. At end of the study, group III presented greatest percent change (40.38%) in CAL with attachment gain 2.1 ± 1.04 mm. **Table 2** shows that the CAL at baseline changed from 6.67 ± 1.23 to 4.5 ± 1.42 in group I, 6.6 ± 2.51 to 4.9 ± 1.48 in group II, 5.2 ± 2.17 to 3.1 ± 1.27 in group III, and 4.7 ± 2.22 to 3.7 ± 2.35 in group IV after 6 months.

The changes in measurement of CAL before and after surgery among all the four groups were found to have a significant impact on the CAL. The results recorded a change of 32.53% in group I, 25.76% in group II, 40.38% in group III, and 21.28% in group IV.

Group I (PRF alone), II (nano-HA alone), III (PRF + nano-HA), and IV (OFD) were presented with a mean bone density of 56.83 ± 23.41 , 62.26 ± 24.3 , 62.82 ± 24.6 and 58.9 ± 22.8 , respectively after the operation/surgery (**Table 2**). The percentage increase in BD was insignificant in group I, II, and IV, respectively. Notably, a significant change was observed in bone density between baseline and 6 months for group III, with higher BD gain as compared to the other groups (46.78%).

Table 2 presents the postoperative difference in IBD at baseline and 6 months. There were significant changes in three groups i.e. group I, II and, III. **Table 2** shows that difference in IBD was found to be 2.2 ± 0.09 (4.6 ± 1.39 to 2.4 ± 1.28) in group I, 1.49 ± 0.65 (3.6 ± 1.68 to 2.11 ± 1.53) in group II, and 2.31 ± 1.50 (4.4 ± 1.37 to 2.09 ± 1.62) in group III, from baseline to 6 months, respectively. In group IV, the mean IBD was 3.9 ± 1.72 mm and 2.8 ± 1.25 mm at baseline and 6 months, respectively, with non-significant change in bone fill measurements (1.1 ± 0.57). The results obtained for all four groups showed a significant difference in the IBD depth. The percentage changes were recorded to be 47.83%, 41.39%, 52.5%, and 28.21%, respectively.

VEGF concentrations were significantly increased among the four groups. A significant difference between mean concentrations in group I and group IV and group II and group III was found after comparing the concentrations of VEGF. However, there were no significant differences in VEGF concentrations between group II and group III at 14 days, respectively. Sites treated with nano-HA showed highest increase in concentrations of VEGF at different points, reaching 138.7 ± 0.36 in 14 days (**Table 3**).

Table 1
Age and Sex Distribution among Study.

		Group I	Group II	Group III	Group IV	F	p-value
Age	Mean ± SD	37.6 ± 5.3	40.2 ± 5.9	37.4 ± 4.4	41.8 ± 5.5	2.409	0.077
	Range	28–44	32–47	27–41	36–48		
Sex	Female	7 (46.7%)	6 (40.0%)	8 (53.4%)	6 (40.0%)	0.741	0.863
	Male	8 (53.3%)	9 (60.0%)	7 (46.6%)	9 (60.0%)		

Sig.value = <0.000.

Table 2
Comparison between all groups regarding PD, CAL, Bone density and IBD Depth.

	Baseline (N = 15)	Six months (N = 15)	% change	Difference	p-value
Probing Pocket Depth (PD)					
Group I	7.3 ± 0.88	4.6 ± 1.55	36.99	2.7 ± 0.89	0.000
Group II	7.6 ± 1.05	5.2 ± 1.22	31.58	2.4 ± 1.17	0.000
Group III	6.7 ± 1.77	3.7 ± 1.67	44.78	3.0 ± 0.94	0.000
Group IV	6.6 ± 0.95	5.7 ± 1.05	13.64	0.90 ± 1.10	0.020
Clinical Attachment Level (CAL)					
Group I	6.67 ± 1.23	4.5 ± 1.42	32.53	1.17 ± 1.21	0.000
Group II	6.6 ± 2.51	4.9 ± 1.48	25.76	1.7 ± 1.03	0.032
Group III	5.2 ± 2.17	3.1 ± 1.27	40.38	2.1 ± 1.04	0.003
Group IV	4.7 ± 2.22	3.7 ± 2.35	21.28	1.0 ± 1.13	0.206
Bone Density					
Group I	48.47 ± 22.51	56.83 ± 23.41	17.25	0.997	0.327
Group II	54.43 ± 13.3	62.26 ± 24.3	14.39	1.095	0.283
Group III	42.8 ± 14.6	62.82 ± 24.6	46.78	2.710	0.011
Group IV	52.8 ± 12.9	58.9 ± 22.8	11.55	0.902	0.375
Intra-bony Defect (IBD) Depth (post-operatively)					
Group I	4.6 ± 1.39	2.4 ± 1.28	47.83	2.2 ± 0.09	0.000
Group II	3.6 ± 1.68	2.11 ± 1.53	41.39	1.49 ± 0.65	0.017
Group III	4.4 ± 1.37	2.09 ± 1.62	52.5	2.31 ± 1.50	0.000
Group IV	3.9 ± 1.72	2.8 ± 1.25	28.21	1.1 ± 0.57	0.055

Sig.value = <0.000.

Table 3
Mean values of VEGF concentrations in the four groups at different intervals vascular epithelial growth factor.

	Baseline	3 days	7 days	14 days
Group I	56.6 ± 0.25	79.2 ± 0.44	101.1 ± 0.27	111.8 ± 0.37
Group II	56.7 ± 0.94	77.5 ± 0.32	128.2 ± 0.54	138.7 ± 0.36
Group III	67.2 ± 1.23	89.3 ± 0.28	111.3 ± 0.32	131.1 ± 0.40
Group IV	66.9 ± 0.88	87.1 ± 0.29	99.1 ± 0.43	109.6 ± 0.24
F-value	669.326	400.763	291.117	488.122
P-value	0.000	0.000	0.000	0.000

Sig.value = <0.000.

4. Discussion

This study has assessed concentration of VEGFs with PRF when used alone or in combination with nano-HA to treat IBDs. The results demonstrated significant improvement in clinical attachment gain and PD among the teeth that were treated with combination of PRF and nano-HA. PRF, known as second-generation platelet, consists of viable platelets and releasing various growth factors; such as epidermal growth factor, VEGF, PDGF, IGF, TGF, and basic FGF (Carroll et al. 2005). Either used alone or in combination with other materials, the growth factors in the local environment were significant as bone grafts for enhanced periodontal regeneration (Panda et al. 2014). The strong fibrin matrix offered by the PRF clot provides a scaffold to carry cells that were impor-

tant to protect growth factors from proteolysis and assist in tissue regeneration. PRF has the advantage of being completely autologous in nature and cost effective (Wu et al. 2012). PRF was further used in several forms such as minced form (fragments), membrane form, PRF plug, and PRF gel (Ramaprabha and Jacob, 2014). In the present study, the minced form was packed inside the IBDs to evaluate its efficacy for using it as a grafting material. The present study showed significant difference in IBD at baseline and after six months in all the groups.

The clinical results demonstrated a high reduction in PPD after six months among four groups. The highest PD reduction was associated with group III (3.0 ± 0.94 mm), followed by group I (2.7 ± 0.89 mm), and then group II (2.4 ± 1.17 mm). The highest CAL gain was associated with group III (40.38%), followed by group I

(32.53%) then group II (25.76%). PRF alone or PRF in combination with n-HA was used to treat PD and clinical attachment gain in both groups. Whereas the radiographic results showed significant changes after six months for groups treated with PRF, n-HA alone or by the combination of PRF and n-HA, as compared to the defects present at baseline (P -value ≤ 0.05). These results were in consistent with the results presented by [Lekovic et al. \(2012\)](#). [Lekovic et al. \(2012\)](#) studied the effects of PRF versus PRF + bone graft on the healing of periodontal IBD using the split mouth design on 17 paired IBD. The values were obtained at baseline and after 6 months, which reduces PPD reduction and increases CAL at 3.35 ± 0.68 mm and 2.24 ± 0.73 mm, respectively. It was suggested that clinical parameters can be significantly enhanced through PRF associated with the human intra-bony periodontal defects.

The properties of PRF are likely to be enhanced as there a reduction was found in the pocket depth, improving the defect fill and CAL. This statement was confirmed by [Shah et al. \(2015\)](#) by comparing the use of PRF and OFD to treat periodontal IBD and followed up for 6 months. The study suggested that PD reduction of PRF group was 3.6 ± 1.48 mm and CAL gain of PRF group was 2.97 ± 1.42 mm after 6 months. However, the use of PRF versus OFD in group I showed that PD at baseline changed from 7.3 ± 0.88 to 4.6 ± 1.55 ; whereas, CAL at baseline changed from 6.67 ± 1.23 to 4.5 ± 1.42 . [Shah et al. \(2015\)](#) focused on demineralized freeze-dried bone allograft (DFDBA) to show that PD reduction was 3.70 ± 1.78 mm and CAL gain was 2.97 ± 1.54 mm. These results showed significance of Platelet-rich fibrin after 6 months. The present study has reported that platelet-rich fibrin can be effectively used for the treatment related to IBDs. Wide range of results regarding potential synergistic effects of PRF combined with various bone graft materials were reported by previous because of bioactive properties and variability of graft materials ([Lekovic et al. 2012](#); [Shah et al., 2015](#); [Choukroun et al. 2006](#); [Yilmaz et al. 2014](#); [Zhang et al. 2012](#)). However, PRF acts as a biological glue for holding the particles together that further allows bone grafting material manipulation.

[Yilmaz et al. \(2014\)](#) stated that there was a synergetic effect of adhesive property of PRF on accelerating healing of the graft material. These results explained that combination of PRF with β -TCP might be effective in the formation of bone. These results were consistent with the present study as it showed better outcomes of clinical and radiographic parameters after receiving treatment with PRF + nano-HA. The dissolution of hydroxyapatite after grafting might also increase bone formation in the PRF + nano-HA group. It is possible to biodegrade HA scaffolds through dissolution or fragmentation with subsequent phagocytosis by macrophages and also by osteoclast activity ([Müller-Mai et al., 1995](#)). The mimicry of physiological bone processes creates optimal surfaces for colonization with vascular tissue and osteoblasts to favor the biodegradation ([Wenisch et al. 2003](#)). The quality of materials like its surface roughness and size of crystal determines the degree of osteoclast activity on HA scaffolds ([Gomi et al. 1993](#)). The significant increase in the expression of several important markers of osteogenesis like Osteocalcin, Osteonectin, and alkaline phosphatase was affected by nano-HA ([Pilloni et al. 2014](#)). This provides justification for the results of present study showing effective results for group III that was treated with OFD and PRF membrane combined with nano-HA bone graft.

VEGF reflects the role of vascularization within bone healing, facilitating the development of actual osteoid deposition and matrix ([Abdullah, 2016](#)). Importantly, there was an association between the angiogenic and osteogenic cells and signals ([Malhotra and Habibovic, 2016](#)). There is a greater need for vascularization at sites, where bone graft substitutes were used in tissue formation ([Giannoudis et al. 2008](#)). A functioning capillary network provides a migration pathway for endogenous cells when consider-

ing calcium phosphate (CaP) use within a bone defect ([Larsen et al. 2011](#)). Therefore, the coupling of osteogenesis and angiogenesis was essential in the bone-healing environment ([Götz et al. 2012](#)). In the similar context, the present study showed significant increase in the VEGF concentrations among the four groups. Highest increase in concentrations of VEGF was reported at different point (138.7 ± 0.36 in 14 days). Interestingly, this could explain the elevated VEGF concentrations in group IV, where n-HA was used alone as compared to other groups. The cells need to adhere and proliferate for initiating bone formation ([Feng et al. 2011](#)).

Surface roughness is an important consideration when selecting a bone-inducing substrate. According to physical and mechanical principles, a substrate with superficial roughness shows a higher capacity for adhesion, in comparison to the smooth surface ([Thorat et al. 2017](#); [Klein et al. 2013](#); [Yamashita et al. 2009](#)). Considering the results of present study, it was suggested that after six months, PPD was high among the four groups. The highest PD reduction and highest CAL gain was exhibited by the patients in group III. The process of wound healing was considered as a rapid process to study the events of wound healing. It has been observed that new tissue formation indicated periodontal regeneration within 4 weeks following surgical intervention ([Trombelli et al., 1999](#)). However, the maturation of the newly formed tissues may continue for 9–12 months. Therefore, follow-up was carried after 6 months in accordance with other studies which employed similar regenerative techniques for the management of periodontal IBD ([Lekovic et al. 2012](#); [Shah et al. 2015](#)).

The study results are limited because it has evaluated four different modalities in different patient groups that increases the probability of different immune responses among patients. The evaluation of all the four modalities in a single patient with a split mouth protocol would be appropriate and provide authentic results. There were clinical limitations to provide four defects in each patient which fulfilled the selected criteria of 3 mm depth of IBDs. The issue related to immune response and plaque control have been managed by increasing the inclusion and exclusion criteria of selected patients to be generally having good health with placement on postoperative care regime. It is expected that there are no major changes in the bone density after just six months of surgery that could be detected using PRF locally.

5. Conclusion

The study concluded that the use of PRF with nano-HA was successful regenerative periodontal therapy in managing periodontal IBDs as compared to using PRF alone. The combination of PRF + nano-HA showed a beneficial effect, resulting in more bone fill and gain in bone density. Moreover, the results also depicted that group III exhibited highest PD reduction and highest CAL gain. The results of the study suggested that PPD was high among four groups after six months. However, there is a need to draw definitive conclusions about the stability of each therapeutic approach through the evaluation of healing outcome after different treatment modalities in long term studies (PRF alone and/or PRF + bone grafting). Therefore, future studies are recommended to incorporate such factors in the future work. Future studies need to experiment all the four modalities in a single patient with a split mouth protocol. Moreover, the postoperative bone density should be evaluated at 1 year.

6. Consent to participate

Not applicable.

7. Consent for publication

Not applicable.

8. Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

Not applicable.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2020.11.027>.

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