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Comparative Evaluation of Crestal Bone Height in Implants with or without Platelet Rich Fibrin- A Clinical and Radiographic Study

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ABSTRACT

Background: PRF is a second generation of platelet derivative that favours soft tissue regeneration, diminishes crestal/vertical bone loss, when used with implants. Preservation of peri-implant bone is one of the key factors in the process of tissue repair and regeneration for a successful implant therapy. The study aimed to determine the crestal bone changes around dental implants placed with PRF as compared to those placed without PRF.

Methods: A randomized control clinical trial was conducted to study changes in crestal bone height at peri-implant sites. 20 edentulous sites- 10 implant sites with PRF (test) & 10 implants without PRF (control). Clinical parameters- plaque index, gingival index, bleeding on probing, peri-implant probing depth, Wasserman's mobility index & Radiographic parameters using Cone Beam Computed Tomography were measured at baseline, 3 months & 6 months. Using SPSS software version 21, the intragroup comparison was done using Repeated Measures ANOVA. The intergroup comparison was obtained using the unpaired t-test.

Results: Intragroup comparison showed significant improvement in clinical & radiographic parameters in both the groups. In intergroup comparison, BWIP (Buccal bone width from implant platform) & BW4IP (Buccal bone width 4mm from implant platform) showed significantly better results & more crestal bone gain in Group A (PRF) than the Group B (non-PRF).

Conclusions: Advent of PRF benefited implant aesthetics and stability. Implants along with PRF had less crestal bone changes & significantly more gain in buccal bone width than the control sites

INTRODUCTION

A successful implant has the ability to osseointegrate with the bone bed in the host, to support a prosthesis and sustain occlusal stresses during function.^[1] The mechanism of osseointegration is closely related to biomaterials. i.e. materials designed to be implanted into the living system to substitute, or regenerate tissues and tissue functions.^[2]

Platelet rich fibrin is an autologous healing biomaterial and second generation of platelet derivatives.^[3] Platelets release osteogenic cytokines, such as platelets-derived growth factor, insulin-like growth factor-1 & 2, angiogenic cytokine i.e. vascular endothelial growth factor. It has great potential for bone and soft tissue regeneration.^[4] The letter number of ethical committee clearance for plan of thesis was DIRDS/2019/629.

MATERIALS & METHODS

The study sample consisted of 20 edentulous sites in patients selected from the out-patient department of Periodontology and Oral Implantology of Dasmesh Institute of Research and Dental Sciences, Faridkot, Punjab. After complete medical and dental history, a thorough clinical and radiographic examination and blood investigations were done. The study protocol gained ethical approval from the institutional review committee. Verbal and written informed consent taken from all the selected subjects. Patients maintaining good oral hygiene, within the age group of 18 to 70 year with single or multiple missing teeth were included. Any acute infection, systemic disease or conditions such as osseous metabolic disorders, severe alveolar bone loss, chronic smokers and alcohol abusers, radiation therapy, untreated periodontal disease, any systemic or local medication that might interfere with the peri-implant healing process were excluded from the study. Patients with edentulous sites which were indicated for implant placement were selected as per the inclusion criteria. A total of 20 implants were placed under this study. The implant sites were randomly divided into two groups having 10 implant sites in each group: Group A - Osteotomy site for implants treated with PRF (Test), Group B - Osteotomy site for implants treated without PRF (Control).

Surgical Procedure

Group A: Figure 1 shows pre-operative photo in case of group A. After effective local anaesthesia (2% lignocaine with 1:1,00,000 adrenaline) under strict aseptic conditions, a mid-crestal horizontal incision was given on the edentulous span using surgical blade no. 15 and full-thickness mucoperiosteal flap was elevated and crestal bone was exposed. Marking drill was used to mark the osteotomy site. The pilot drill was used till the desired length depending upon the size (i.e. length and diameter) of the implant to be placed. Osteotomy site was prepared by using sequential drills (as per the manufacturer's protocol) followed by placement of an implant and the cover screw (Figure 2). PRF membrane was prepared by taking 10 ml blood sample from antecubital vein & transferred it to plain glass tube (without anticoagulant). Glass tube was placed in the centrifuge machine and rotated at 3000 revolutions per minute for 10 minutes similar to Choukroun's protocol that leads to formation of three layers: topmost layer- platelet poor plasma (PPP), middle layer- PRF clot, bottom layer- red blood cells (RBC) (Figure 3). The PRF clot was squeezed between gauze piece to obtain the PRF membrane (Figure 4). This membrane was placed over the surgical site after implant placement (Figure 5). The flaps were approximated using the simple interrupted loop sutures with non-resorbable 3-0 Mersilk sutures (Figure 6). Healing abutment was placed 3 months post-operatively and peri-implant probing depth was measured 6 months post-operatively (Figures 7 & 8)



Figure 1: Pre-operative (Group A)

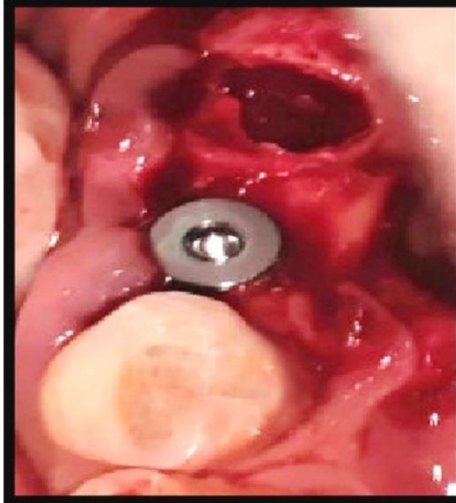


Figure 2: Implant Placement (Group A)



Figure 3: PRF prepared by Centrifugation (Group A)



Figure 4: Separation of PRF from the Plasma & RBCs (Group A)

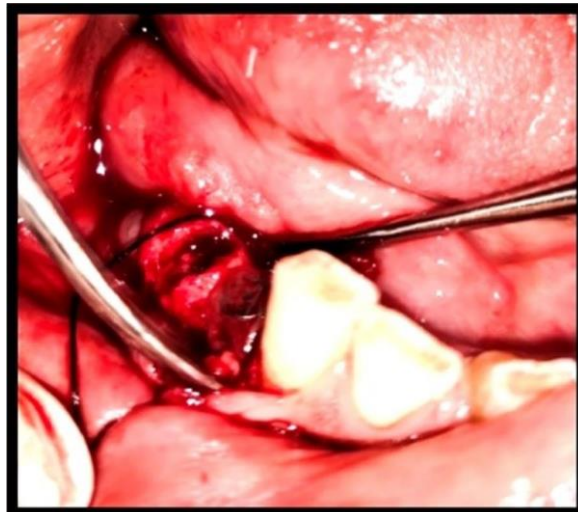


Figure 5: PRF placed over implants (Group A)



Figure 6: Sutures placed (Group A)

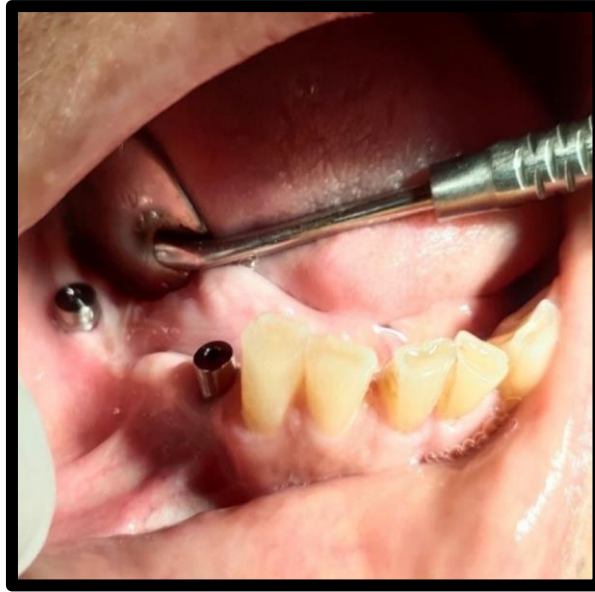


Figure 7: Healing abutment placed 3 months post-operatively (Group A)



Figure 8: Peri-implant probing depth at 6 months (Group A)

Group B: Following similar surgical procedure of an implant placement without the use of PRF (Figures 9-13).



Figure 9: Pre-operative (Group B)

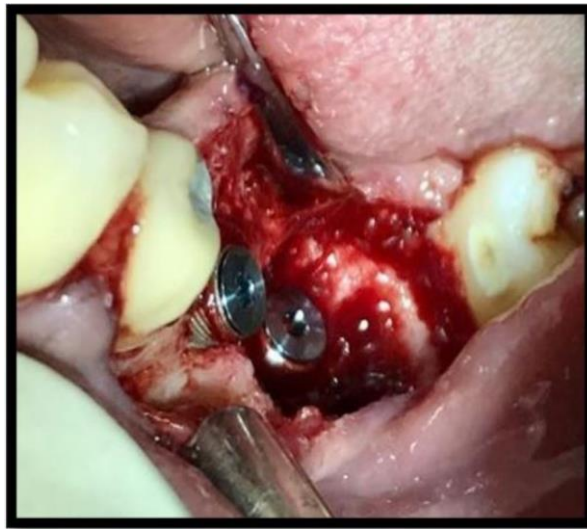


Figure 10: Implant placement (Group B)



Figure 11: Sutures placed (Group B)



Figure 12: Healing Abutment placed 3 months post-operatively (Group B)



Figure 13: Peri-implant probing depth at 6 months (Group B)

Post-operative care

After the surgical procedure, antibiotics (Tab. Augmentin 625 mg thrice daily for 5 days) and non-steroidal anti-inflammatory drugs (NSAIDs) (Combination of Paracetamol 500 mg & Diclofenac sodium 50 mg thrice daily for 3 days) were prescribed to all the subjects. Post-operative instructions were given to the patient.

Post-operative measurements were recorded at baseline, 3 months & 6 months. Clinical parameters considered were Plaque Index (PI) (Silness&Løe, 1964)^[5], Gingival Index (GI) (Løe&Silness, 1963)^[5], Bleeding on Probing (BOP), Peri-implant Probing Depth (PPD), Mobility Index (Wasserman et al. 1973) ^[6]. Radiographic parameters ^[7] considered using Cone Beam CT (NewTom version 10.1) were BHIP (buccal bone height from the implant platform), BWIP (buccal bone width at the implant platform), BW4IP (buccal bone width 4 mm below the implant platform measured along the implant length), LHIP (lingual bone height from the implant platform), LWIP (lingual bone width at the implant platform), BWBIC (buccal bone width at the first bone to implant contact), LWBIC (lingual bone width at the first bone to implant contact), MHIP (mesial bone height from the implant platform), DHIP (distal bone height from the implant platform).

Statistical analysis

The data thus obtained was tabulated and analysed statistically using the Statistical Package for Social Sciences (SPSS) software version 21. The intragroup comparison for the different time intervals was done using Repeated Measures ANOVA. The intergroup comparison for the difference of mean scores between two independent groups was done using the unpaired/independent t-test. The level of the significance for the present study was fixed at 5%.

RESULTS

Twenty edentulous sites were recruited for this study using strict inclusion and exclusion criteria. Both males and females in the age group 18 to 70 years (mean age- 54 years) were randomly distributed into two groups having 10 edentulous sites in each. At the baseline (Implant surgery), 3 months and 6 months interval, the clinical parameters and radiographic parameters were recorded in all sites of Group A (PRF) and Group B (Non-PRF).

Intragroup comparison

In group A & group B, the mean difference in scores of clinical as well as the radiographic parameters was statistically significant from baseline to 3 months & 6 months and from 3 months to 6 months ($p=0.001$). In group A, significant decrease in scores of clinical parameters was seen. In group B, significant increase in scores of plaque index, gingival index, bleeding on probing, peri-implant probing depth was seen. Mobility index scores decreased significantly in both the groups.

While observing radiographic parameters over a period of 6 months, more positive results and less crestal bone loss was recorded within the group-A (PRF) than group-B (Non-PRF).

Intergroup comparison- There was a significant difference ($P < 0.05$) in clinical parameters in group A compared to group B.

The mean difference of plaque index, gingival index, peri-implant probing depth & mobility index score between the Group A and Group B from baseline to 3 months and 6 months was found to be statistically significant.

The mean difference of bleeding on probing between two groups was non-significant at the baseline but statistically significant at 3 and 6 months.

CBCT parameters- BWIP, BW4IP intergroup comparison results were significant. More positive outcome in terms of improvement in buccal bone width was observed within the group A (PRF) than the group B (Non-PRF). While other parameters such as BHIP, LHIP, BWBIC, LWBIC, MHIP, DHIP intergroup comparison results were non-significant between group A and group B.

INTER GROUP						
	Groups	At Baseline	3 months	6 months	% change at 3 months	% change at 6 months
Plaque Index	Group A	0.96±0.18	0.40±0.13	0.23±0.04	57.30±13.96	75.04±8.42
	Group B	0.41±0.12	1.08±0.31	1.26±0.23	-185.17±105.75	-238.33±135.52
	P value				0.001 (Sig)	0.001 (Sig)
Gingival Index	Group A	0.62±0.24	0.46±0.12	0.36±0.13	12.88±45.43	22.00±80.35
	Group B	0.42±0.18	0.72±0.19	0.69±0.29	-102.74±95.75	-107.62±123.57
	P value				0.001 (Sig)	0.012 (Sig)
Bleeding on Probing	Group A	0.80±0.42	0.20±0.48	0.10±0.31		
	Group B	0.60±0.31	0.70±0.48	0.90±0.51		
	P value	0.562 (Non-Sig)	0.021 (Sig)	0.001 (Sig)		
Peri-implant Probing Depth	Group A	4.10±1.19	1.30±0.48	1.10±0.31	64.66±19.24	70.50±14.55
	Group B	4.00±0.66	2.50±0.73	1.90±0.69	35.83±17.48	50.66±18.98
	P value				0.001 (Sig)	0.001 (Sig)
Mobility	Group A	2.60±0.69	1.10±0.32	1.00±0.01	54.16±21.24	59.16±9.97
	Group B	1.90±0.74	1.60±0.32	1.30±0.48	15.00±25.39	26.66±48.93
	P value				0.001 (NS)	0.001 (Sig)

Table 1: Intergroup comparison of clinical parameters

INTRA GROUP					
	Groups	At Baseline	3 months	6 months	P value
Plaque Index	Group A	0.96±0.18	0.40±0.13	0.23±0.04	0.001(Sig)
	Group B	0.41±0.12	1.08±0.31	1.26±0.23	0.001(Sig)
Gingival Index	Group A	0.62±0.24	0.46±0.12	0.36±0.13	0.001(Sig)

	Group B	0.42±0.18	0.72±0.19	0.69±0.29	0.001(Sig)
Bleeding on Probing	Group A	0.80±0.42	0.20±0.48	0.10±0.31	0.001(Sig)
	Group B	0.60±0.31	0.70±0.48	0.90±0.51	0.001(Sig)
Peri-implant Probing Depth	Group A	4.10±1.19	1.30±0.48	1.10±0.31	0.001(Sig)
	Group B	4.00±0.66	2.50±0.73	1.90±0.69	0.001(Sig)
Mobility	Group A	2.60±0.69	1.10±0.32	1.00±0.01	0.001(Sig)
	Group B	1.90±0.74	1.60±0.32	1.30±0.48	0.001(Sig)

Table 2: Intragroup comparison of clinical parameters

INTERGROUP						
Parameters	Groups	At Baseline	3 months	6 months	% change at 3 months	% change at 6 months
BHIP	Group A	-2.25±1.81	-1.56±1.74	-0.38±2.51	24.14±28.91	77.46±123.39
	Group B	-0.49±2.21	-2.42±2.38	-1.84±1.48	49.02±305.53	-87.35±294.45
	P value				0.801 (NS)	0.110 (NS)
BWIP	Group A	0.25±0.47	0.44±0.55	0.94±0.98	-161.88±205.02	-727.50±101.96
	Group B	0.89±0.64	0.26±0.51	0.19±0.31	57.50±55.33	35.00±284.84
	P value				0.004 (Sig)	0.001 (Sig)
BW4IP	Group A	1.92±0.95	2.54±2.27	2.38±1.80	-19.39±70.82	-32.24±108.18
	Group B	1.59±0.41	2.00±1.19	1.47±0.97	-40.07±133.49	2.45±85.41
	P value				0.004 (Sig)	0.043(Sig)
BWBIC	Group A	6.54±2.67	6.73±2.51	6.03±2.72	-4.65±5.71	5.28±21.38
	Group B	5.23±2.33	5.61±2.53	5.46±2.64	-7.48±8.96	-3.48±8.43
	P value				0.411 (Sig)	0.244 (Sig)
LHIP	Group A	-2.10±1.86	-1.48±1.85	-0.17±1.71	39.53±49.63	67.21±159.02
	Group B	-1.02±1.53	-0.42±1.16	-1.16±1.77	66.35±112.73	-49.74±96.48
	P value				0.500 (Sig)	0.062 (Sig)
LWIP	Group A	0.27±0.45	0.88±0.66	0.93±1.09		
	Group B	0.48±0.67	0.76±0.62	0.54±0.65		
	P value	0.427 (NS)	0.682 (NS)	0.345 (NS)		
LWBIC	Group A	6.26±2.74	6.67±2.66	5.97±2.78	-13.00±22.87	2.00±28.85
	Group B	4.94±1.78	4.96±2.17	5.05±2.44	-0.13±201.3	-2.04±27.94
	P value				0.189 (NS)	0.998 (NS)
MHIP	Group A	-3.82±2.67	-2.39±3.02	-0.71±1.74	41.78±51.29	90.60±70.07
	Group B	-2.18±3.23	-1.09±3.40	-0.39±1.70	48.25±109.97	92.05±66.25
	P value				0.868 (NS)	0.963 (NS)
DHIP	Group A	-5.01±2.91	-3.39±2.55	-2.61±2.48	35.28±23.69	47.10±27.27
	Group B	-1.32±2.95	-1.94±3.12	-1.46±2.69	-15.36±121.12	74.36±100.92
	P value				0.211 (NS)	0.420 (NS)

Table 3: Intergroup comparison of Radiographic parameters

INTRA GROUP					
Parameters	Groups	At Baseline	3 months	6 months	P value
BHIP	Group A	-2.25±1.81	-1.56±1.74	-0.38±2.51	0.001 (Sig)
	Group B	-0.49±2.21	-2.42±2.38	-1.84±1.48	0.001 (Sig)
BWIP	Group A	0.25±0.47	0.44±0.55	0.94±0.98	0.001 (Sig)
	Group B	0.89±0.64	0.26±0.51	0.19±0.31	0.001 (Sig)

BW4IP	Group A	1.92±0.95	2.54±2.27	2.38±1.80	0.001 (Sig)
	Group B	1.59±0.41	2.00±1.19	1.47±0.97	0.001 (Sig)
BWBIC	Group A	6.54±2.67	6.73±2.51	6.03±2.72	0.001 (Sig)
	Group B	5.23±2.33	5.61±2.53	5.46±2.64	0.001 (Sig)
LHIP	Group A	-2.10±1.86	-1.48±1.85	-0.17±1.71	0.001 (Sig)
	Group B	-1.02±1.53	-0.42±1.16	-1.16±1.77	0.001 (Sig)
LWIP	Group A	0.27±0.45	0.88±0.66	0.93±1.09	0.001 (Sig)
	Group B	0.48±0.67	0.76±0.62	0.54±0.65	0.001 (Sig)
LWBIC	Group A	6.26±2.74	6.67±2.66	5.97±2.78	0.001 (Sig)
	Group B	4.94±1.78	4.96±2.17	5.05±2.44	0.001 (Sig)
MHIP	Group A	-3.82±2.67	-2.39±3.02	-0.71±1.74	0.001 (Sig)
	Group B	-2.18±3.23	-1.09±3.40	-0.39±1.70	0.001 (Sig)
DHIP	Group A	-5.01±2.91	-3.39±2.55	-2.61±2.48	0.001 (Sig)
	Group B	-1.32±2.95	-1.94±3.12	-1.46±2.69	0.001 (Sig)

Table 4: Intragroup comparison of Radiographic parameters

DISCUSSION

Platelet rich fibrin (PRF) was first used by Choukroun et al. (2000) in France and belongs to second generation of platelet concentrates.^[8] Fibrin, which is the activated form of a plasma molecule called “Fibrinogen” is a soluble fibrillary molecule and is massively present in the plasma, also in the platelet alpha granules. It aids in platelet aggregation during homeostasis and the fibrin matrix also has the property of angiogenesis.^[9] A simplified processing technique, not requiring biochemical blood handling, makes PRF superior to platelet rich plasma. The platelet and leukocyte cytokines are gradually released during fibrin matrix physiological resorption. This gradual release of cytokines regulates the inflammatory phenomena within the wounded tissues. However, the mechanical function of PRF was also considered. This technique mimics the natural coagulation process, produces an inexpensive and simple bioactive membrane. The PRF membranes allow early wound protection and aid in primary soft tissue closure.^[10]

OPG was taken before surgery for each patient as a preliminary radiograph for the analysis of jaw bone and relevant anatomic landmarks. Intraoral photographs were taken to evaluate esthetics and functional status of the patient. An accurate way to measure the changes in the crestal bone height and width three-dimensionally (3-D) is using high resolution CBCT sections. The present study includes CBCT parameters for buccal, lingual, mesial and distal bone changes immediately after implant surgery, followed by 3-months and 6-months post-operatively. At the time of insertion, both of our groups showed adequate primary stability. Less crestal bone loss was observed in PRF group within 3- and 6-months follow-up. Similarly, Arora et al. (2016) compared immediate implants with and without the use of PRF. They found that immediate implants with PRF lead to acceleration of bone & soft tissue regeneration and reduced peri-implant pain and inflammation.^[4]

The plaque index reduced significantly in the test group (PRF), but significant increase in scores was seen in control group. Contrary to this, Arora et al. (2016) compared two groups- immediate implants with PRF (test) and without PRF (control group), the intergroup comparison of the mean plaque & gingival index was not statistically significant.^[4] Peri-implant probing depth scores significantly decrease in both the groups from baseline to 3 & 6 months but probing depth reduced significantly in the PRF group. The mean decrease in mobility index scores was higher in PRF group than the non-PRF group. Our results are comparable with the study done on the posterior maxillary sites by Öncü and Alaaddinoğlu (2015) showed that application of PRF improves implant stability during early healing period and induce rapid osseointegration.^[11]

Goswami et al. (2009) compared the crestal bone loss along two implant designs and beheld maximum bone loss on the buccal crestal bone.^[12] In our study, TUFFTM (NORIS MEDICAL, Israel) (Ti6Al4V Grade 5) internal-hex implants having three thread zones were used. The lower V-shape thread zone enables self-tapping. The middle zone square type thread is used for compressing cancellous bone and helping achieving maximum bone-implant contact (BIC). The micro thread on the upper zone adds stability and prevents crestal bone loss. An increase in crestal bone level, 0.69 mm in 3 months and 1.87 mm in 6 months was recorded in this study, the low mean crestal bone loss was noticed in PRF group, considering many growth factors in PRF that enhances both soft and hard tissue repair. The results are in accordance with the study by Boora, Rathee and Bhorla (2015) showed statistically significant change in mesial crestal bone level, 0.25 mm in PRF group and 0.57 mm in non-PRF group within three months. However, significantly minimal crestal bone changes were seen in the PRF group than non-PRF group, similar to the results of present study.^[12] Contrary to our study, El Kenawy et al. (2014) described that the mean MBL was 0.8 mm at baseline and 1.0 mm at 3, 6 months and 1.1 mm at 9 and 12 months. But they also concluded that PRF membranes along with DBBM could be considered a valuable option providing important benefits for the patients regarding aesthetics.^[13] The average crestal bone loss was observed in the group B (control) 1.93 mm in 3 months and 0.67 mm in 6 months and the difference was statistically significant. In contrast, Goswami (2009) proclaimed that implants with rough collar (1.42 mm) led lesser crestal bone loss compared to smooth collar (1.53 mm) implants and the difference was statistically significant after 18 months of implant placement.^[14]

In a disparate study design, King et al. (2002) suggested that the micro gap at implant-abutment interface had no significant effect on crestal bone loss. Most of the implants developed crestal bone loss at 1 month as compared to the baseline. So, it's completely normal as crestal bone loss was an early manifestation of wound healing occurring after 1 month of implant placement.^[14] Crestal bone loss is inevitable, despite that, a surgeon must do one's utmost to decrease the changes in crestal bone level. Thereupon, in the present study, PRF as a healing biomaterial was included in the test group to minimize the crestal bone loss.

In the present study, conventional implants were placed 1 mm subcrestal, vertical bone height changes were seen more in the non-PRF group than the PRF group. In contrast to this, Veis et al. (2010) showed less vertical bone loss, when implants were placed 1-2 mm subcrestally and platform-switched implants further helps in stabilizing the crestal bone level.^[15] In a systematic review by Annibali et al. (2012) the mean marginal bone loss in platform-switched implants reported was -0.55 mm.^[16]

Radiographically through CBCT, the present study noticed a significant difference in BWIP scores between group A & group B. The group A (PRF) exhibited significant gain in buccal bone width from baseline to 6 months. 0.21 mm gain was seen from baseline to 3 months and 0.50 mm gain from 3-6 months, in total 0.71 mm gain was recorded from baseline to 6 months. This indicates the positive effect of PRF in stabilizing as well as improving the width of buccal bone in delayed implant sites.

In the group B (non-PRF), a significant reduction (0.70 mm) in buccal bone width (BWIP) was seen from baseline to 6 months. The intergroup comparison showed a statistically significant difference between group A & group B. In the study by Senthil (2015) similar reduction in BWIP scores was seen from baseline to 12 months in immediate as well as delayed implant sites while the intergroup difference was non-significant. Also depicting, peri-implant sites with thin buccal bone lost more vertical bone height than sites with thicker buccal bone irrespective of the type of placement.^[17]

A significant gain in BW4IP scores was recorded from baseline to 3 months followed by bone loss from 3 to 6 months in group A well as group B. The intergroup comparison revealed more bone gain from baseline to 3 & 6 months and less bone loss from 3 to 6 months in PRF group than the non-PRF group, leading to a statistically significant difference between group A and group B. A contrasting result was seen in study by Senthil (2015) significant changes were seen in BW4IP scores from baseline to 6 months while the difference was not significant between 6 to 12 months. No difference was seen in between the extraction (test) & healed (control) implant sites.^[17] The main reason for the contrasting results could be the use of PRF in the present study.

In our study, all the implants undergo significant crestal bone height changes within 3 and 6-months follow-up. The PRF (test) group exhibited significant bone gain as LHIP scores at 3 and 6 months follow up, but the intergroup difference was not statistically significant. In group B, a statistically significant gain in lingual bone height from baseline to 3 months, while at 6 months a significant decrease in lingual height was seen. Contrary to this, Goswami (2009) reported significant lingual bone loss from baseline to 18 months of implant surgery.^[21] The present results were found to be statistically significant decrease in MHIP scores in the test group. Mesial bone gain was seen but the intergroup difference was not statistically insignificant. On the contrary, Jang et al. (2008) found bone loss of 0.7 mm after the first year. Mesial crestal resorption ranged from 0.4 mm to 1.2 mm. The current study found a statistically significant decrease in DHIP scores and gain in distal bone height but the intergroup comparison was not statistically significant. On the contrary, Jang et al. (2008) observed that distal crestal resorption ranged from 0.3 mm to 1.3 mm.^[17]

The limitation of the present study could be the small sample size (due to self-funding) in this study. Various implant designs could be introduced as to get better outcomes w.r.t crestal bone height changes.

CONCLUSIONS

The key findings from the study were that Group A (PRF-group) showed significant improvement in clinical as well as radiographic parameters of implant sites than Group B (control group). In PRF group- less crestal bone loss in terms of height and width was seen than the control group. Buccal bone stability should be used as the new standard for determining implant success rather than the periapical bone levels as buccal bone loss precedes the interproximal bone loss. In healed sites, best outcome is seen when the implants are placed greater than 1 mm below the crest and buccal bone width is greater than 2 mm. Advent of PRF is tremendously helpful in implant aesthetics and stability. With the invent of improved implant surfaces and designs, implant survival is not a concern. Success of an implant based on 3D bone stability as well as natural healing biomaterials should be the new future direction. In future, long term studies determining the changes in crestal bone height with respect to implant shoulder with larger sample size can be carried out to obtain more reliable and accurate results.

Ethics Statement and Conflict of Interest Disclosures

Human subjects: Consent was obtained or waived by all participants in this study. Research and Ethical Committee issued approval DIRDS/2019/629. In a meeting of Research and Ethical Committee held on 21.11.2019. The plan of thesis were scrutinized and approval granted. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial

relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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