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Comparative *ex-vivo* evaluation of the expression of Vascular Endothelial Growth Factor (VEGF) in Leukocyte and Platelet-Rich Fibrin (L-PRF), injectable Platelet-Rich Fibrin (i-PRF), & Stored Whole blood: An ELISA based observational study

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Abstract
Introduction: Platelets have a vital role in haemostasis, healing and secretion of growth factors. The biologically active peptides in platelets significantly modulate proliferation of human pluripotent stem cells including periodontal ligament cells, thus, aiding regeneration. Vascular Endothelial Growth Factor (VEGF) is an angiogenic growth factor that promotes healing.
Objective: Evaluation of expression of VEGF levels in Leukocyte and Platelet-Rich Fibrin (L-PRF), injectable Platelet-Rich Fibrin (i-PRF, and stored whole blood.
Methodology: Autologous blood was collected in sterile/citrated vacutainers by drawing peripheral blood samples from 10 healthy subjects. Each sample was divided into 3 groups (Stored Whole Blood, L-PRP & i-PRF). The samples were processed for preparation of Platelet Concentrates *viz* Leukocyte and Platelet-Rich Fibrin (L-PRF), injectable Platelet-Rich Fibrin (i-PRF) as per Choukroun's protocols. Quantitative assessment of expression of VEGF was carried out with ELISA. Comparative statistical analysis was performed using SYSTAT-13 software.
Results: Quantitative analysis of VEGF revealed higher levels in Leukocyte and Platelet-Rich Fibrin (L-PRF), injectable Platelet-Rich Fibrin (i-PRF) compared to those of stored whole blood groups.
Conclusion: Leukocyte and Platelet-Rich Fibrin (L-PRF), injectable Platelet-Rich Fibrin (i-PRF) having increased expression of VEGF can be preferentially applied in regenerative periodontal treatment protocols.

Keywords: Angiogenic, platelet-rich fibrin, growth factors, Vascular Endothelial Growth Factor (VEGF)

Introduction
Periodontitis is a chronic inflammatory condition involving the tooth-supporting structures, resulting in progressive loss of connective tissue attachment and alveolar bone which ultimately results in tooth loss. Periodontal therapy aims at averting the disease progression and regeneration of lost tissues. Growth factors have a significant role in the regulation of biological events of regeneration & wound repair of nearly all tissues including the periodontium [1].
Periodontal wound healing involves complex interactions amid the periodontal cells *viz*, epithelial cells, gingival fibroblasts, periodontal ligament cells, and osteoblasts. During wound healing, disruption of vasculature leads to fibrin formation, platelet aggregation, and release of several growth factors from platelets that promote tissue repair, angiogenesis, inflammation, and immune response [2]. The fibrin, fibronectin & vitronectin secreted by platelets, act as connective tissue matrix and adhesion molecules for efficient cell migration. Platelets contain biologically active peptides and the binding of these proteins within a developing fibrin mesh or to the extracellular matrix creates chemotactic gradients which favour stem cell recruitment, stimulate cell migration, differentiation, and regulation of biological events of regeneration & promoting wound repair [3].

Autologous platelet concentrates such as Platelet Rich Plasma (PRP) and Platelet Rich Fibrin (PRF) are widely used to improve/accelerate tissue repair aiding wound healing and in regenerative periodontal procedures. Platelet-Rich Fibrin (PRF), a second-generation platelet concentrate introduced by Choukron *et al*. in 2001, contains a fibrin-rich meshwork of concentrated platelets, growth factors, leukocytes & cytokines [4]. Platelets have a crucial role in maintaining vascular integrity & regulating hemostasis and wound healing. The alpha-granules in platelets upon activation, during injury or inflammation of tissues, secrete growth factors *viz* Platelet-Derived Growth Factor (PDGF), Insulin-Like Growth Factor-1 (IGF-1), Epidermal Growth Factor (EGF), Vascular-Endothelial Growth Factor (VEGF), Transforming Growth Factor-β (TGF-β), which initiate wound healing by attracting and activating macrophages, fibroblasts, and endothelial cells and cytokines associated with the healing and regeneration [5]. VEGF is an angiogenic signaling protein involved in both vasculogenesis (the de novo formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature). Myriad actions and effects of VEGF are produced by different isoforms of VEGF, That modulate/trigger varied biological processes. Understanding of VEGf has raised more questions about this complicated, many-faceted—growth factor/cytokine/morphogen/survival factor/permeability factor—protein! Which truly is an ENIGMA to regenerative therapists.

The present study was conducted to evaluate the levels of Vascular Endothelial Growth Factor (VEGF) in platelet concentrates & stored blood. The objective was to quantitatively evaluate & compare the expression of Vascular Endothelial Growth Factor (VEGF) levels in Leukocyte and Platelet-Rich Fibrin (L-PRF), injectable Platelet-Rich Fibrin (L-PRF), & Stored Whole blood using Enzyme Linked Immunosorbent Assay (ELISA).

Materials and Methods

The Study was conducted in the Department of Periodontology of a Tertiary Care Dental Centre, in collaboration with Department of Laboratory Sciences & Molecular Medicine, of a tertiary care Heath Centre of Armed Forces, amongst the patients attending the central Out-Patient Department (OPD) of the facility. Patients/individuals in good Systemic health, with no contraindications for periodontal surgery & good plaque control, within an age range of 18-55 years were selected/recruited for the study. Exclusion criteria comprised of participants with history (past 6 months) of antibiotic prophylaxis or Steroid therapy/presently under antibiotic medication, suffering from the chronic systemic diseases/condition, smokers, pregnant and lactating mothers. Necessary approval from the institutional ethical committee was obtained for the *ex-vivo* study. Individuals/patients were informed about the details/ benefits and protocol of the study and a written informed consent was obtained from the participants.

Blood collection: For the study, autologous blood (5ml x 03 samples each) was collected in sterile/citrated vacutainers (BD Vacutainer® blood collection tubes) by drawing peripheral venous blood samples (Figure 1a) with a 21-gauge needle (BD Vacutainer® Eclipse™ blood collection needle) from 10 systemically healthy and willing subjects, aged 18-55 years as:

- with anti-coagulant citrate dextrose for the Stored whole blood group (Group-A),
- without anticoagulants using Plastic vacutainer for the injectable platelet-rich fibrin (i-PRF) group (Group-B) &
- without anticoagulants using conventional vacuum plain glass tube for Leukocyte- &
- Platelet-Rich Fibrin (L-PRF) group (Group-C) respectively.

The samples were processed/centrifuged in REMI R 8C PLUS centrifuge (REMI Laboratory Instruments, Mumbai, Maharashtra, India) as per Choukroun's protocol for preparation of Platelet Concentrates *viz* injectable platelet-rich fibrin (i-PRF), @700rpm for 3min, Leukocyte and Platelet-Rich Fibrin (L-PRF) @2700rpm for 12 min. PPP was derived from the supernatant remaining after L-PRF preparation. Further the Whole blood sample was processed/centrifuged @3000rpm for 15min* after 24 hrs and plasma & buffy coat was extracted and stored in Eppendorf tubes. (Figure 1) (Table-1)

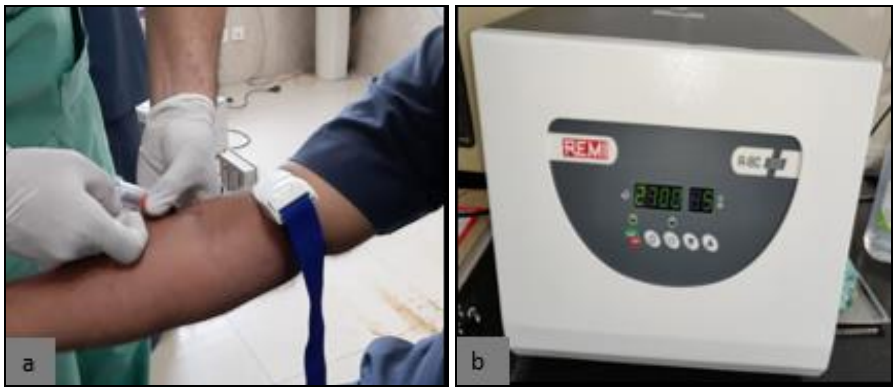


Fig 1: (a) Collection of blood in sterile test tube. (b) REMI R 8C PLUS centrifuge

Platelet-Rich Fibrin (PRF) preparation: Platelet Concentrates (Leukocyte and Platelet-Rich Fibrin (L-PRF) & injectable platelet-rich fibrin (L-PRF),) were prepared as per Choukroun's protocols. 5ml blood volume in glass vacutainer was centrifuged in REMI R 8C PLUS centrifuge (REMI Laboratory Instruments, Mumbai, Maharashtra, India) @ 2700 rpm for 12 min. Post centrifugation a gel was obtained having three layers *viz*; uppermost layer of Platelet Poor Plasma, the intermediate layer of Fibrin Clot and the lowermost layer of concentrated Red Blood Cells. The supernatant remaining after LPRF preparation comprising of acellular Plasma /Platelet poor plasma (PPP) was extracted and stored in Eppendorf tubes and assessed for growth factor levels (Group D). The fibrin clot/gel so obtained was

compressed in-between sterile glass slabs, membrane obtained was relocated in Eppendorf tubes for the assessment of growth factors [6]. Remaining 5ml blood volume was simultaneously centrifuged at low speed @700 rpm for 3 min in accordance with the low-speed centrifugation concept (LSCC), in specified plastic tubes (prevents the activation of coagulation cascade in contrast to the glass tubes) during centrifugation. After centrifugation, the blood is separated into a yellow orange upper phase (injectable platelet-rich fibrin (i-PRF), and a red lower phase (red cell fraction), i-PRF is collected using a micropipette by controlled aspiration of the upper fluid phase (Figure 2) [7, 8].

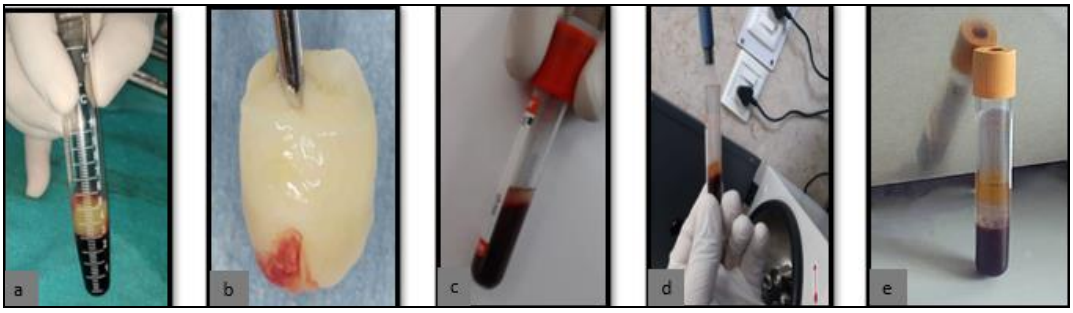


Fig 2: (a) Test tube with the three separate layers (b) Collection of L-PRF (c & d) Collection of i-PRF (e) Collection Stored blood sample Serum

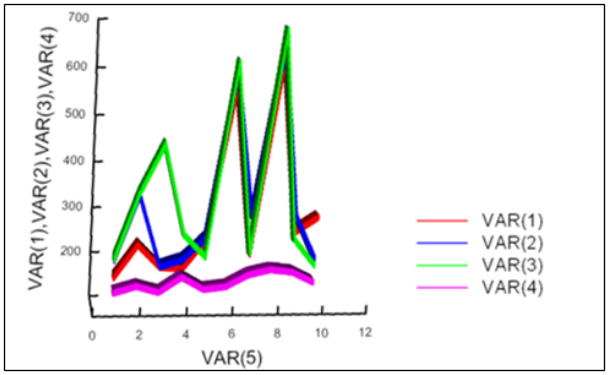
Routine blood testing for CBC was performed using an Automated Blood Cell Counter, Celltac-α MEK-6420P (Nihon Kohden Corporation, Japan) on the specimens obtained from the Whole Blood group (Blue) prepared by centrifugation after mixing 0.1 mL of it with 0.9 mL of a saline solution (Figure 3a). The estimation of number of platelets, red blood cells, neutrophils, lymph cells, and monocytes in the WB of the baseline blood was calculated. The concentrations of Vascular Endothelial blood Growth Factor (VEGF) in Leukocyte and Platelet-Rich Fibrin (L-PRF), injectable platelet-rich fibrin (i-PRF), Stored and Platelet Poor Plasma samples were determined using human VEGF Enzyme-linked Immunosorbent Assay Kit (Bioassay Technology Laboratory (BT Lab) Shanghai Korain Biotech Co., Ltd Shanghai, China). The quantitative analysis of the growth factors was conducted according to the instructions of the manufacturer. (Figure 3)

Fig 3: (a) Automated Blood Cell Counter (b & c) ELISA with Samples and ELISA Reader

Results

Quantitative analysis revealed higher VEGF levels in Platelet-Rich Fibrin (PRF) groups than those of the stored blood group/ PPP group. Mean VEGF level in Group-A (stored blood) was found to be 286.7 ng/ml, for Group-B (inactivated injectable platelet-rich fibrin (i-PRF) 312.0 ng/mL, for Group-C (inactivated Leukocyte and Platelet-Rich Fibrin (L-PRF) 330.8 ng/ml & for Group-D (PPP) was recorded as 134.1 ng/ml. (Table-2)

Statistical Analysis: Comparison of the mean values of VEGF levels released in the samples were analyzed and compared. Results were tabulated and Statistical analysis was done by using SYSTAT-13 software. Paired t-test was performed to compare means of same group under separate scenarios. Post-hoc analysis using Dunn Sidak adjustment was done for adjustment of P value. A level of $P > .05$ was considered significant. Inter group analysis depicted statistically significantly levels of VEGF in L-PRF, i-PRF and stored blood comparison to the PPP group with P value less than 0.05. (Table-3) Comparative graphs were plotted between the variables (Graph-1).



Legend:

Red: Stored Blood

Blue: i-PRF,

Green: L-PRF,

Purple: PPP

Graph 1: Comparison between Stored blood, Platelet concentrates and Platelet poor plasma

Table 1: Sample Groups

Group	Methodology	N
Group A: Stored Blood	3000rpm@15min	10 samples
Group B: Injectable platelet-rich fibrin (i-PRF)	700rpm@3min	10 samples
Group C: Leukocyte and Platelet-Rich Fibrin (L-PRF)	2700rpm@12min	10 samples
Platelet Poor Plasma (PPP)	2700rpm@12min	10 samples

Table 2: VEGF levels post analysis

Sample	Stored Blood	injectable platelet-rich fibrin (i-PRF), (700rpm@ 3min)	Leukocyte and Platelet-Rich Fibrin (L-PRF), (2700rpm@ 12min)	PPP
1	146	185	188	111
2	219	329	334	126
3	169	170	440	112
4	165	186	242	146
5	232	236	192	118
6	570	600	618	124
7	199	282	202	151
8	634	660	688	163
9	250	285	231	157
10	283	187	173	133

Table 3: Intergroup analysis

Inter group analysis		P_Value
Stored Blood	i-PRF	0.685
Stored Blood	L-PRF	0.749
i-PRF	L-PRF	0.992
Stored Blood	PPP	0.012
i-PRF	PPP	0.053
L-PRF	PPP	0.052

Discussion

Platelet concentrates are extracts of the blood circulating tissue obtained after various processing of a whole blood sample, mostly through centrifugation and not pharmaceutical preparations, containing highly concentrated platelets and abundant growth factors, enabling healing and regeneration of tissues including periodontal regeneration. These Growth factors are expressed at variable intervals during the process of tissue healing, serve as therapeutic agents to promote tissue repair. Tissue engineering and regenerative medicine depend on the relationship between three fundamental elements *viz* Progenitor cells, signaling molecules such as growth factors, morphogenetic proteins, and adhesins. Growth Factors regulate several key cellular processes of mitogenesis, chemotaxis, differentiation, and metabolism [9, 10].

Vascular Endothelial Growth Factor (VEGF) secreted by activated platelets, thrombocytes, macrophages keratinocytes, tumour cells, and renal mesenchymal cells. VEGF is the most potent growth factor critical for angiogenesis of tissues, neo-angiogenesis during the wound healing and also facilitate in maintaining the integrity of endothelial cell lining of the blood vessels and normal physiological functions including; hematopoiesis, bone formation, and wound healing [11].

The sequence of events necessary for periodontal regeneration relies on chemotaxis of mesenchymal stem cells, along with differentiation and proliferation of osteoblasts, for osteogenesis, cementogenesis, and connective tissue formation. The incorporation of recombinant human VEGF into various bone biomaterials has been demonstrated to increase new bone formation, thereby pointing to the fast and potent effects of VEGF [13].

Prapulla, Sujatha, and Pradeep in 2007 observed increased levels of VEGF in gingival clavicular fluid (GCF) in periodontitis which decreases post-treatment and hence can be used as a biomarker in determining the disease progression. Studies in respect to PRF membrane revealed a constant and gradual increase in the release of growth factors attributed to a stronger fibrin architecture entrapping a greater number of leukocytes in the fibrin matrix. Thus, allowing an intense slow release of growth factors from the fibrin matrix [14].

In the present study, growth factor levels in each experimental group were analyzed for expression & pattern of distribution of VEGF. Quantitative analysis revealed statistically significant VEGF levels in L-PRF & i-PRF and stored blood groups compared to compared to PPP group, highlighting its immense regenerative potential. VEGF was also expressed, even though weakly in the supernatant of PRF/Platelet Poor Plasma (PPP) may be utilized in clinical situations rather than discarding the same. Supporting angiogenesis and tissue ingrowth is based on the notion that blood supply is a prerequisite for tissue regeneration. In the dynamic process of healing, Lymphocytes typically arise at day 7, whereas PRF introduces a high number of leukocytes at day 0 thereby speeding the regenerative phase. The regenerative potential of platelet concentrates has been advocated in the field of periodontal therapy that optimizes the biological mechanisms essential for successful new attachment and regeneration [10].

Conclusion

Quantitative analysis revealed statistically significant levels/concentrations of VEGF in PRF than in WB and confirmed a change in the growth factors during the manufacturing process. Stored blood is also a viable option for VEGF that can be utilized in clinical scenarios. The function of platelet concentrate is not the number of leukocytes, but a Biomaterial with enormous potential, which can be utilized as a scaffolding material & a reservoir to deliver certain growth factors at the site of application potently capable of inducing angiogenesis and subsequent wound healing and enhancing tissue regeneration. The study results are suggestive of both objective and theoretical efficacy of applying PRF in clinical settings. For a better understanding of the effect of various growth factors/platelet concentrates on cellular response further studies are required and a standardized procedure that can stabilize the release of growth factors to effectively stimulate the regeneration and tissue healing. Synthetic analogues of growth factors may be the future of regenerative protocols.

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