Background: Lack of collateral blood supply and the low compliance environment of pulp makes its healing, a challenge. Vascular endothelial growth factor (VEGF) is a primary angiogenic growth factor. Knowledge of dose and time-dependent expression of VEGF from platelet concentrates, namely platelet-rich fibrin (PRF) and PRF matrix (PRFM), along with vital pulp can aid in developing strategies to improve the outcome of vital pulp therapy and regenerative procedures. Hence, the aim of this study was to compare VEGF kinetics of PRF, PRFM, and dental pulp.

Materials and Methods: The PRF, PRFM, and vital dental pulp were placed in culture media for a week; the supernatant was collected from these samples at days 1, 4, and 7. VEGF-A expression was evaluated using ELISA and compared with the weight of the sample so as to quantify the release of VEGF-A per milligram of sample.

Results: PRF exhibited maximum VEGF-A release on day 4 and was sustained till day 7. In contrast, PRFM and dental pulp showed no significant release of VEGF-A till day 7. However, on day 7, there was a rapid increase in VEGF-A expression from dental pulp that was comparable to PRF. On comparing the release of VEGF-A per milligram of tissue, pulp exhibited the maximum values.

Conclusion: Among the platelet concentrates, differential expression of VEGF-A was superior in PRF. The use of PRF in partial pulpitis should be explored in order to restore pulp vascularity and hasten pulpal healing.

Keywords: Dental pulp; growth factors; platelet-rich fibrin; platelet-rich fibrin matrix; regenerative endodontics; vascular endothelial growth factor

INTRODUCTION

The dental pulp is a unique organ encased within the hard dentinal walls. Due to which it is difficult to regenerate pulp tissue without collateral supply except from the apical root end. In a clinical scenario, the severity of inflammation is the determining factor for the onset of repair and regeneration, possibly triggered and mediated by signals released from injured dental pulp cells. This process of regeneration necessitates undeterred neoangiogenesis, which is controlled by the microenvironment. The process of pulpal wound healing and subsequent regeneration thus requires the facilitation of growth factors and scaffolds from an external source. Scaffolds may act as a vehicle for growth, reconstruction, and enhancement of vascularity through angiogenesis and facilitate the recruitment of stem cells by cell homing.[1] The key regulator for physiologic angiogenesis is vascular endothelial growth factor – A release from platelet-rich fibrin, platelet-rich fibrin matrix, and dental pulp at different time intervals.

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factor (VEGF) which is currently receiving much attention. Dentin matrix releases VEGF mediated by the osteoblastic precursor cells and exerts a significant role in regulating the fate of progenitor cells. Various techniques have been explored to harness the properties of VEGF by electrostatic binding to synthetic and natural polymers. However, they are expensive with restricted biocompatibility.

Platelet-rich fibrin (PRF) developed by Choukroun et al. is a bioscaffold that is well documented in regenerative procedures and is known to release high quantities of growth factors such as transforming growth factor-β, platelet-derived growth factor-AB, or VEGF and matrix proteins such as thrombospondin-1, fibronectin, and vitronectin. In the field of facial plastic surgery, another variant, PRF matrix (PRFM), is gaining popularity as a rapid and inexpensive method of enhancing healing. Its rigid nature offers excellent handling properties and easy placement in constricted areas such as the pulp chamber or the tooth apex.

The precise mechanism and rate of release of growth factors from these concentrates remain unclear. The process of neoangiogenesis begins as early as day 3 postinjury, which may be upregulated by VEGF release. The endodontic application of platelet concentrates for augmenting the process of dentin regeneration through growth factor release needs to be explored. While studies focus on the release of growth factor from different platelet concentrates, there is no clarity on the impact of VEGF kinetics of these concentrates on the pulp. It would be interesting to compare the growth factor release from platelet concentrates with that of the dental pulp. Therefore, this study aims to quantify and compare VEGF release by PRF, PRFM, and dental pulp at different time intervals.

MATERIALS AND METHODS

Ethical statements
The protocol of the study was accepted by the Institutional Ethics Committee and implemented in accordance with 1975 Helsinki Declaration amended in 2000. Informed consent was obtained from all participants before the start of the study.

Sample collection and preparation
Sample collection for PRF and PRFM was done from 15 healthy consenting volunteers, 9 males and 6 females, with an average age of 21 years. Participants with systemic diseases, patients on any drugs affecting hematological parameters, and pregnant and lactating women were excluded. Pulp samples were obtained from healthy volunteers with intact teeth and vital pulp, indicated for extraction for orthodontic or prosthodontic reasons, with no recent history of anti-inflammatory or antibiotic medication, and with no evidence of periodontal or periapical pathology.

Platelet-rich fibrin preparation (Group 1)
Five milliliters of blood was drawn from the right antecubital vein by venipuncture and immediately centrifuged on a tabletop centrifuge (Remi Centrifuge CE, Remi Elektrotechnik Limited, Vasai, India) at 400 g for 10 min. This process caused blood to separate into three basic fractions: a bottom layer of red blood cells (RBCs), a middle layer of PRF gel, and a top layer of platelet-poor plasma (PPP) [Figure 1a]. The PRF gel was separated from the bottom RBC layer using sterile stainless steel scissors, while the PPP was discarded. Subsequently, PRF was transferred into an Eppendorf tube with Dulbecco’s Modified Culture Media (DMEM, Gibco-Invitrogen).

Platelet-rich fibrin matrix preparation (Group 2)
Five milliliters of blood was collected in a similar manner, and PRP was obtained by centrifugation at 1100 g for 6 min, which was transferred, under sterile conditions, into a calcium chloride-containing glass bottle (0.25 mL CaCl₂ 1M, Diagnostica Stago, USA). This was gently swirled and centrifuged at 4500 g for 25 min at 25°C, using a tabletop centrifuge (Remi Centrifuge CE, Remi Elektrotechnik Limited, Vasai, India). PRFM, a translucent, yellow-white layer at the bottom, was recovered and immediately transferred into an Eppendorf tube with DMEM media [Figure 1a].

Dental pulp (Group 3)
Pulp tissue was obtained from teeth before extraction. 1.8 ml of 2% lignocaine hydrochloride (Xylocaine, Zydus Healthcare Ltd., Ahmedabad, India) was administered to prevent vasoconstriction and subsequent hypoxia of pulp tissue. Following disinfection with 2% chlorhexidine gluconate (Asep RC, Anabond Stedman Pharma Research (P) Ltd., Chennai, India), standard access cavity preparation was performed under rubber dam isolation, and pulp tissue was extirpated in toto with a sterile barbed broach and immediately transferred into an Eppendorf tube with DMEM media.

Storage of samples and conditioned media
The samples were transferred in a six-well culture dish and stored in 5 ml DMEM media supplemented with streptomycin/ampicillin antibiotics and placed in cell culture incubator at 37°C with CO₂. The supernatants were collected at 1-, 4-, and 7-day intervals and stored at −80°C for growth factor quantification. The culture dishes were replenished with 5 ml of DMEM media at each interval.

Growth factor assay
ELISA kit (Diaclone SAS, Besancon, France) was used as per the manufacturers’ instructions to quantify VEGF-A released from
Group 1, Group 2, and Group 3 at days 1, 4, and 7. 100 µl each of sample and assay diluents was added into antibody precoated 96-well plates and incubated for 2 h at room temperature. The wells were washed with wash buffer solution and incubated with peroxidase-conjugated polyclonal antibodies to VEGF. After washing, an enzyme-substrate solution was added and incubated for 30 min. Stop solution was added and further incubated for 30 min. The absorbance value of each well was read on a spectrophotometer (PerkinElmer-UV-NIR, EnSight HH3400) using 450 nm as the primary wavelength and optionally 630 nm as the reference wavelength (610 nm to 650 nm). The detection limit for VEGF was 9 pg/ml. Standard and samples were evaluated in triplicate, and five independent experiments were conducted for each platelet concentrate and pulp samples.

Statistical analysis
Statistical analysis of the data was performed using SPSS software (SPSS20, SPSS Inc., Chicago, IL, USA). Descriptive statistics expressed as mean and standard deviation were derived. ANOVA test was used for comparison among the groups.

RESULTS
The PRF expressed significantly high VEGF (3644.17 ± 4097.25 pg/ml and 87.03 ± 99.76 pg/mg) compared to that of the dental pulp and PRFM at day 1. Similarly, PRF showed maximum release at day 1, peaking at day 4, along with a gradual release lasting until day 7 [Graph 1a]. These findings were found to be in accordance with previous studies by Dohan et al.[9] VEGF promotes chemotaxis along with the growth and differentiation of human pulp cells into odontoblasts.[10] Along with other growth factors inherent in PRF, this may synergistically contribute to odontogenesis. Previous studies have shown that recombinant VEGF promoted angiogenesis and increased the microvessel density of dental pulp.[11] However, it would be more feasible to harness autologous growth factor in PRF, eliminating the possibility of an immune reaction from an expensive recombinant factor, where even the concentration of recombinant VEGF remains questionable.

PRFM did not show a high release of VEGF-A in our study [Graph 1a and b] which is in contrast to the study by Lucarelli et al. PRFM at the ultramorphological level shows no cells, however, it has a dense fibrin network.[6] Lack of expression of VEGF in PRFM could be because PRFM does not express VEGF.

DISCUSSION
Lack of collateral circulation and the low compliant environment makes the healing of pulp extremely challenging. During inflammation, pulp is vulnerable to inflammatory cytokines which alter the microcirculation.[7] VEGF, a proangiogenic factor, is generally upregulated in pulpitis, however, if the inflammation persists, it becomes downregulated and pulpal vasculature decreases to almost one-third the normal.[8] A potential scaffold containing angiogenic growth factors would be a promising tool to enrich pulpal vasculature. Hence, this study was undertaken to assess the rate of VEGF expression from biological scaffolds such as PRF and PRFM and was compared with the dental pulp.

PRF releases growth factors like VEGF by activation of platelets, and in the current study, PRF showed maximum release at day 1, peaking at day 4, along with a gradual release lasting until day 7 [Graph 1a]. These findings were found to be in accordance with previous studies by Dohan et al.[9] VEGF promotes chemotaxis along with the growth and differentiation of human pulp cells into odontoblasts.[10] Along with other growth factors inherent in PRF, this may synergistically contribute to odontogenesis. Previous studies have shown that recombinant VEGF promoted angiogenesis and increased the microvessel density of dental pulp.[11] However, it would be more feasible to harness autologous growth factor in PRF, eliminating the possibility of an immune reaction from an expensive recombinant factor, where even the concentration of recombinant VEGF remains questionable.

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not have leukocytes and has only the fibrin component. This absence of leukocytes in PRFM may prove to be beneficial in stimulating reparative response, as the role of leukocytes is controversial in differentiation of stem cells. PRFM is a rigid material compared to PRF, aiding in ease of handling during compaction of the bioactive material in regenerative endodontic procedures. There are limited studies with regard to release of VEGF-A from PRFM, and this requires further research. Dohan Ehrenfest et al. observed that platelet concentrate technologies are not dependent on the number of platelets, rather on how platelets, leukocytes, fibrin, and growth factors are interlinked in the final product.[12] In our study, we conducted protein estimation and measured the weight of each sample in order to standardize the samples of different mass. ELISA procedure was performed to quantify the VEGF expression, which is a gold standard.[3]

The present study witnessed a significant difference in the expression of VEGF-A from dental pulp on day 7 compared to days 1 and 4 [Graph 1a and b]. Dental pulp cells usually start proliferating from 3 to 7 days.[13] Hence, it could be implied that there is underlying cell proliferation during this time leading to the expression of VEGF. The results are in accordance to a study conducted by Matsushita et al.[10] VEGF expressed by pulp works autocrine directly on the pulpal cells and facilitates chemotaxis, cell growth, and cell differentiation. In pulp cells, two receptors, namely VEGF R1 and VEGF R2, are present. VEGF R2 mainly mediates this action, together with AP-1 transcription factors and c-fos protein.[14] There was a substantially higher release by dental pulp relative to PRF by taking into account the release of VEGF-A in units of pg/mg of tissue. Following a pulpal injury, VEGF presence in high concentrations can facilitate the reparative and regenerative response of the tissue.[1] In deep carious lesions showing reversible pulpitis, an angiogenic supplement may reverse the inflammatory process. PRF can be used as a therapeutic material to facilitate a regenerative response since it released VEGF-A as much as the dental pulp. This growth factor will cause the proliferation of human pulp cells and their division into odontoblasts, thereby helping to restore damaged pulp and dentin [Figure 1b].[15] Since the pulp is covered by hard tissue and has no collateral circulation, pulpal injury may lead to inflammation and failure to properly treat may lead to necrosis. Human pulp cells will, therefore, have an ability to counteract this vulnerability through VEGF production.[16] The production and release of multiple growth and differentiation factors from PRF, upon its activation, form the basis of its properties. These growth factors are critical for the regulation and enhancement of wound healing process by mediating mitogenesis, chemotaxis, differentiation, and metabolism, which are essential for pulp tissue repair.[17-19] Our findings suggest that therapeutic intervention with autologous growth factors which mimic the growth factor kinetics of pulp, at initial inflammatory stages of day 1–7, could control inflammatory cellular processes and promote angiogenesis in inflamed tissues, thus accelerating healing.

CONCLUSION

It can be concluded that PRF has a potential, promising usage in pulp therapy. Our data strongly suggest that VEGF kinetics in PRF is similar to that of the pulp, which can be harnessed to modulate pulpal inflammation and restore vascularity. Further preclinical and clinical trials are essential to ascertain the prognosis of pulp therapy using PRF.

Pulp showing high VEGF-A expression can prove to be an effective scaffold by itself. Pulp transplantation can be an expected advancement in regenerative endodontics, and accordingly, therapies can be instituted.

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Conflicts of interest

There are no conflicts of interest.

Graph 1: (a) Mean of vascular endothelial growth factor in groups in pg/ml; (b) mean of vascular endothelial growth factor in groups in pg/mg
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