ORIGINAL RESEARCH

Effect of Calcium Silicate Cements on Vascular Endothelial Growth Factor Release from Platelet-rich Fibrin and its Architectural Changes

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Abstract

Aim and objective: The purpose of this study was to evaluate and compare the changes in the release of vascular endothelial growth factor (VEGF) from platelet-rich fibrin (PRF) as well as the changes observed in the fibrin architecture of PRF when layered with mineral trioxide aggregate (MTA), Biodentine, and Theracal.

Materials and methods: Platelet-rich fibrin was obtained from eight volunteers to form PRF membrane (PRFm) and divided into four groups of control (PRF alone), PRF with MTA, PRF with Biodentine, and PRF with Theracal by layering the PRFm over with these materials. Four samples of each group were prepared. Release of VEGF was estimated using enzyme-linked immunosorbent assay (ELISA) at 1 hour and 5 hours. PRFm in contact with the materials was analyzed under a scanning electron microscope to observe the fibrin architecture.

Results: A significantly higher amount of VEGF was released from the Theracal group as compared to control (PRF only), Biodentine, and MTA. The fibrin architecture of the Biodentine group was more similar to that of the control group at both 1 hour and 5 hours.

Conclusion: Theracal could be a suitable material to be used along with PRF for endodontic use wherever indicated.

Clinical significance: The results show an increased release of VEGF from PRFm when layered with Theracal. PRFm used in procedures like revascularization and pulpotomy may therefore be layered with these dental materials to enhance the regeneration and create a biocompatible seal.

Keywords: Platelet-rich fibrin, Regenerative endodontics, Scanning electron microscopy, Vascular endothelial growth factor.

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Introduction

Platelet-rich fibrin (PRF) first described by Choukroun et al.[1] is referred as the second-generation platelet concentrate. Activated platelets are rich in growth factors like platelet-derived growth factor, transforming growth factors (TGF-β), insulin-like growth factor, epithelial growth factor, and vascular endothelial growth factor (VEGF).[2] Evidence from both *in vivo* and *in vitro* studies have shown their stimulatory effects on the mesenchymal cells implicating a vital role in wound repair and tissue regeneration.[3] Due to the osteogenic and regenerative potential of PRF, its use has been established for various procedures in dentistry including pulp capping, apexogenesis, post-extraction sockets, and periodontal surgeries.[4] Revascularization procedure is frequently used in cases of immature necrotic teeth as an alternative treatment modality to conventional root canal treatment. It aims at creating aseptic root canal space using antibiotics, disinfecting solutions and induction of bleeding for blood clot formation that leads to homing of stem cells. This is followed by placement of a scaffold-like PRFm over the blood clot for effective packing of a biocompatible material to seal the blood clot. Mineral trioxide aggregate (MTA) has been the preferred material for sealing in such cases owing to its established biocompatibility and great sealing ability. Other popular restorative materials used in dentistry include various calcium silicate-based materials like Biodentine, calcium-enriched mixture, and Theracal-LC and all of them have been studied and proven to possess a stimulatory effect on the dental pulp with regard to the reparative dentin formation. These materials could also be used in conjunction with platelet concentrates for various endodontic procedures like apexogenesis and pulp capping.[5] TheraCal LC (Bisco Inc., Schamburg, IL, USA) is a light-cured resin-modified calcium silicate material indicated for use in direct and indirect pulp therapy. The material shows superior biocompatibility and is reported to be less cytotoxic than other resin-based light-cured liners due to release of more calcium ions compared to others.[6] Association of MTA, Biodentine, and TGF release has been reported in various studies.[7,8] However, there is not much evidence on the correlation of these endodontic materials with VEGF. Besides the inherent ability to induce growth factor release, pH is another factor that has been shown to influence the release of growth factor from a membrane.[9]

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Therefore, this study intended to evaluate and compare the changes in the release of VEGF from PRF as well as the changes observed in the fibrin architecture of PRF under the influence of different calcium silicate cements; MTA, Biodentine, and Theracal.

Materials and Methods

The study was conducted in ITS Dental College, Hospital and Research Center, Greater Noida, India. Ethical clearance was obtained from the institutional ethical committee (Ref. No. ITSDCGN/PRIN/L/2020/009). Eight healthy volunteers (American Society of Anesthesiologist physical status; ASA 1) were randomly selected. They were informed about the nature of the study and a written informed consent was obtained from them.

The study followed a double blinding protocol where the volunteers were unaware of the type of material their sample would be tested with. Two investigators (SS, Laboratory technician) analyzing the samples for VEGF samples using enzyme-linked immunosorbent assay (ELISA) and PRFm under scanning electron microscopy (SEM) were blinded to the group of materials. Investigators (BR, AM) manipulating and preparing the samples could not be blinded.

Blood Sample Collection

Twenty milliliters of peripheral blood were obtained using a syringe. It was then transferred to vacutainer tubes without anti-coagulants by dividing into four equal parts of 5 mL each.

PRFm Preparation

Centrifugation of collected blood samples was done at 3,000 rpm. The PRF membrane (PRFm) was obtained by separating PRF from the red blood cell base and compressing it with a sterile gauze piece. Three separate groups were made for the experiment along with one control group.

Group I: PRFm (PRFm alone).
Group II: PRFm + MTA (White)–Angelus.
Group III: PRFm + Biodentine (Septodont, Saint Maur-des Fosses, France).
Group IV: PRFm + Theracal LC (Bisco).

Manipulation of the dental materials was done according to the manufacturer's instructions and compacted into 2-mL Eppendorf tubes up to 0.5-mL mark followed by compaction of PRFm over each of the experimental materials (groups II, III, and IV). Control group I contained PRFm alone.

Grouped samples contained in tubes were freed from exudates by vortexing and the supernatant fluid obtained was collected with the use of self-adjustable micropipettes at 1- and 5-hour time interval followed by immediate centrifugation for 15 minutes. pH strips were used for the estimation of pH in the supernatants from each group at 1- and 5-hour interval. After this, the supernatant fluid was stored and frozen at −80°C until the determination of the growth factors.

Quantitative assessment of VEGF from the stored fluid samples of all groups was assessed at 1 and 5-hour interval using Human VEGF ELISA kit (Chongqing Biospes Co., Ltd). The testing was done as per the instructions provided with the kit. The density of yellow precipitate formed after reagent dilution was analyzed by measuring the optical density absorbance at 450 nm using a microplate reader, and then the concentration of VEGF was calculated.

Sample Preparation for Scanning Electron Microscopy

PRFm was freshly prepared from new blood samples obtained from healthy volunteers. Ten milliliters of blood were withdrawn from an individual to obtain PRF and ultimately prepare PRFm. Two samples from each group under study were prepared for SEM analysis. The membrane was put in a sterile Petri dish. Prepared PRFm specimens were layered with different experimental materials. Approximately 150 mg of each material was manipulated, applied, and allowed to set over the membrane. A total of eight samples were prepared and the specimens were then kept in a humidifying chamber for 5 hours. The materials were removed carefully from the PRFm and these treated PRFm specimens were then sputter-coated with nanogold particles (gold coating thickness 4–8 nm) using a sputter-coated unit-Polaron SC7640 (UK). Carl Zeiss EVO 40 at 20 kV (Cambridge UK) unit was used for the SEM analysis of the samples. Samples were visualized under different magnifications ranging from 1,000 to 10,000× for visualizing the surface architecture.

Statistical Analysis

Intergroup comparisons were performed using the Kruskal–Wallis test (*post hoc* with Bonferroni correction) in SPSS version 21 (IBM Inc., Chicago). Bonferroni test was applied to make adjustments during group comparisons since our sample size was less and to avoid data being incorrectly deemed significant. The p value < 0.05 was considered to be statistically significant.

Results

Vascular Endothelial Growth Factor Quantification

At 1 hour interval, ELISA testing of the samples yielded a significantly high ($p < 0.05$) VEGF release in group III (PRF with Theracal) with a mean of 0.9002 pg/dL as compared to control group I, where the mean release of VEGF was noted to be 0.2316 pg/dL. Mineral trioxide aggregate and Biodentine group also showed increased release of the growth factor after 1 hour interval, although the results were statistically insignificant ($p > 0.05$).

At a 5-hour interval, Theracal consistently showed a higher level of VEGF release estimated to be 0.8662 pg/dL. Higher release of growth factor was also observed in Biodentine group (0.8729 pg/dL) at 5 hours as compared to PRF alone (0.0708 pg/dL) (Table 1 and Fig 1).

pH Assessment

The pH assessment of the fluid samples was done at 1- and 5-hour interval using pH strips. The pH of PRF samples alone remained constant (pH = 7) while there was a negligible decrease in the pH of samples in the experimental groups over time, however, the difference was statistically insignificant ($p > 0.05$). All the three experimental groups maintained an alkaline pH with a mean of 9.5 ± 0.8.

SEM Analysis Results

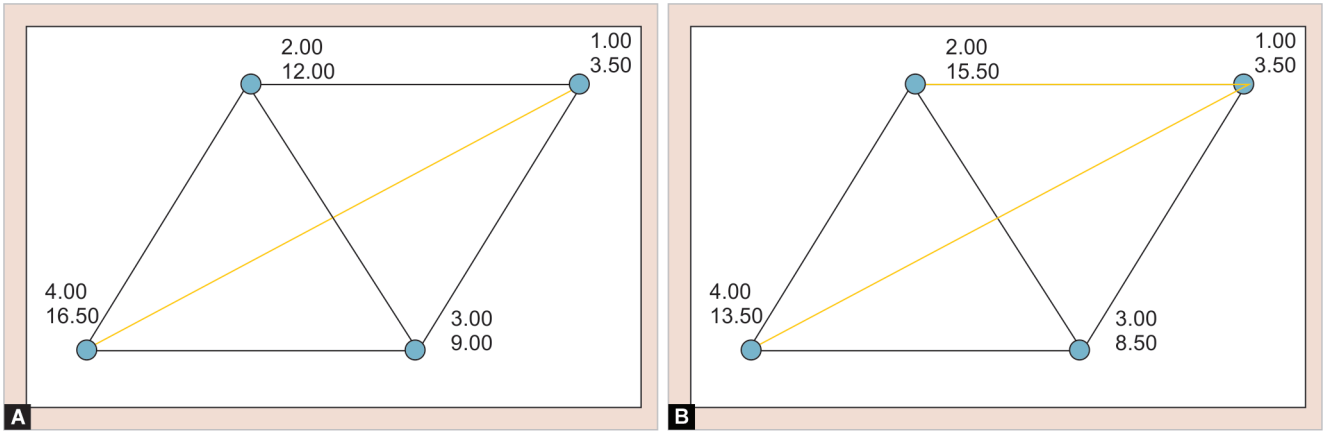
Control

Fibrin borders were seen to be clearly delineated at 1,000, 2,000, and 10,000× magnification with minimal pore structure between them. Fibers appeared with gentle curves and lateral co-aggregation (Figs 2A to C).

Table 1: Intergroup comparisons of mean VEGF values at 1 and 5 hours

		VEGF levels at 1 hour		
Group	*n*	*Mean (SD) (units = pg/dL)*	*Comparison group*	*p value*[a]
PRF	4	0.2316 (0.0098)	PRF + Biodentine	$p > 0.05$
			PRF + MTA	$p > 0.05$
			PRF + Theracal	$\mathbf{p < 0.05}$*
PRF + Biodentine	4	0.3557 (0.00816)	PRF + MTA	$p > 0.05$
			PRF + Theracal	$p > 0.05$
PRF + MTA	4	0.3448 (0.00607)	PRF + Theracal	$p > 0.05$
PRF + Theracal	4	0.9002 (0.000953)	–	–
VEGF levels at 5 hours				
PRF	4	0.0708 (0.000263)	PRF + Biodentine	$\mathbf{p < 0.05}$*
			PRF + MTA	$p > 0.05$
			PRF + Theracal	$\mathbf{p < 0.05}$*
PRF + Biodentine	4	0.8729 (0.0108)	PRF + MTA	$p > 0.05$
			PRF + Theracal	$p > 0.05$
PRF + MTA	4	0.3710 (0.00834)	PRF + Theracal	$p > 0.05$
PRF + Theracal	4	0.8662 (0.00895)	–	–

VEGF, vascular endothelial growth factor; PRF, platelet-rich fibrin; MTA, mineral trioxide aggregate; GIC, glass ionomer cement; SD, standard deviation; pg/dL, picograms per deciliter
$p < 0.05$—Statistically significant
*Significant
[a]Kruskal–Wallis test with Bonferroni correction



Figs 1A and B: Pairwise comparisons between groups at: (A) 1 hour; (B) 5 hours

Mineral Trioxide Aggregate

Ill-defined strands were appreciated at 2,000× magnification. However, at 10k magnification, strands showed dense and clumped architecture. Platelet inclusions were found randomly scattered along with residual particles of MTA (Figs 2D to F).

Biodentine

At 10,000× magnification, dense fibrin strands could be appreciated with clear delineation of borders. Appearance was similar to that shown with MTA at higher magnification. Fibers appeared more or less straight or gently curved at lower magnifications of 1,000 and 2,000× (Figs 2G to I).

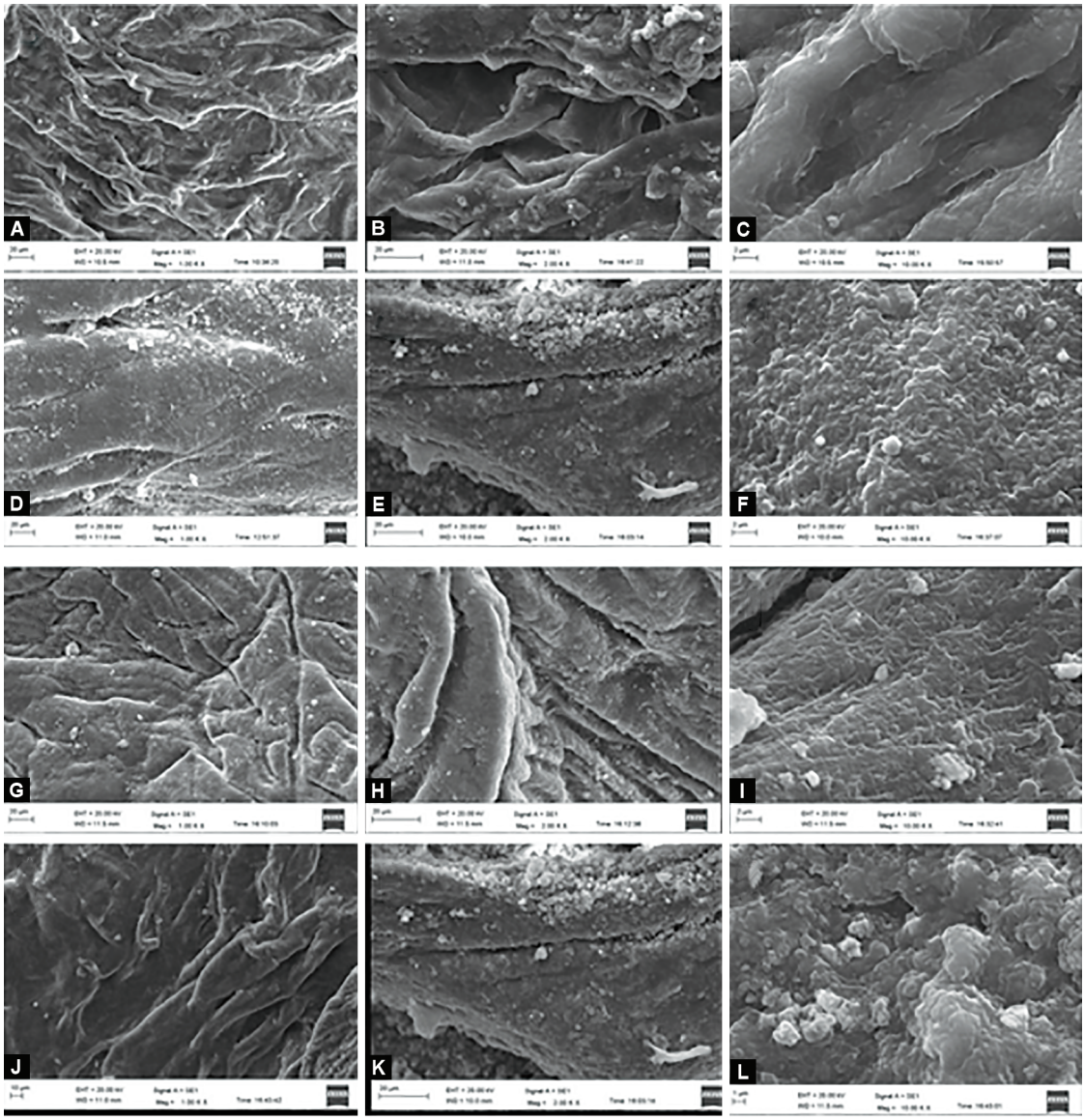
Theracal

The architecture appeared similar to that depicted by MTA and Biodentine group at all magnifications. At 10,000×, lateral aggregation of fibers and reduced pore formation was noted. Residual particles of theracal material were seen scattered throughout (Figs 2J to L).

Our results showed that the amount of VEGF release from PRF was highest when the newer calcium-based silicate cement-Theracal was placed over it and the architectural integrity of the membrane remained similar to that of MTA and Biodentine.

Discussion

Vascular endothelial growth factor is the most potent angiogenic and vasculogenic factor involved in tertiary dentin formation. Vascular endothelial growth factor has also been identified to induce proliferation and differentiation of human pulp cells into odontoblasts.[10] According to Matsushita et al., VEGF produced by human dental pulp cells acts in an autocrine manner and promotes chemotaxis, proliferation, and differentiation of cells. These facts



Figs 2A to L: (A to C) Control group at 1,000, 2,000, and 10,000× SEM magnification; (D to F) MTA group at 1,000, 2,000, and 10,000× magnification; (G to I) Biodentine group at 1,000, 2,000, and 10,000× magnification; (J to L) Theracal group at 1,000, 2,000, and 10,000× magnification

suggest that VEGF may be a useful growth factor in the repair of damaged pulp and dentin.[11]

According to Su et al.,[4] a continuous and steady release of the growth factors from PRF is seen up to 300 minutes if used immediately after preparation within 1 hour. Dohan Ehrenfest et al.[2] in their study showed that a very high release of VEGF occurred during the first 4 hours post-preparation of PRF and continued slowly till the 7th day.

In our study, we observed that Theracal in conjunction with PRF released the highest VEGF at 1 hour at pH as high as 9, whereas PRF alone produced the minimum amount of VEGF at 1 hour at pH 7. On comparing the VEGF release by Biodentine group to MTA and Theracal group, higher amount of VEGF release was seen with Biodentine as compared to control. The release of VEGF in the control group of PRF was less at 1 and 5 hours.

In a recent study by Youssef et al.,[12] MTA, Biodentine, and Emdogain were seen to increase VEGF release even at 14th day after application as a pulp-capping agent as compared to calcium hydroxide which showed reduction in the VEGF release. Mullaguri et al.[13] conducted a study on TGF and concluded that PRF layered with Biodentine released more amount of TGF-b1 from PRF. Another study by Paranjpe et al. evaluated the effect of MTA on direct contact with human dental pulp cells and showed that MTA was capable of upregulating the release of VEGF from the cells besides promoting odontoblastic activity.[14]

It has been established that the changes in the pH may influence the functioning and architectural integrity of the PRF membrane and the alterations in its thickness, length, porosity, and branching may be assessed to note the changes.[15] Increased lateral aggregation of clots and thickening of fibers has been attributed to calcium ions acting upon fibrinogen.[16] In this study, SEM analysis revealed that experimental groups of MTA, Biodentine, and Theracal had similar effects on the surface architecture of the PRF membrane. This could be because all these three materials belong to the calcium silicate group. It must be kept in mind that with materials having an acidic pH during fibrin polymerization,

architectural changes like thickening and cloudiness may be seen which may cause complete disruption of the fibrin network and hence render them detrimental to be used with PRF.[17] At higher magnification of 10,000×, our observations in the SEM analysis showed a comparable change in the architecture in all groups as compared to control suggesting that all calcium silicate cements might similarly affect fibrin integrity.

Various studies have shown dental pulp stimulatory effects of VEGF besides its ability to promote angiogenesis.[18] The findings of this study might have a beneficial clinical implication as the results showed an increased release of VEGF from PRFm when layered with Theracal. PRFm used in procedures like revascularization and pulpotomy may therefore be layered over with these inductive dental materials to enhance the healing and regenerative outcome. This study also implicates a possible use of Theracal as a coronal sealing material following a regenerative procedure. However, the sealing and solubility properties must be weighed against the bioinductive properties. In a study by Alazrag et al., Theracal showed the least solubility at one week when compared with MTA and Biodentine. In the same study, it was also seen that a higher frequency of marginal gaps was seen with Theracal.[19]

Based on the findings of this study, it is observed that all calcium silicate cements provide a conducive environment for regenerative procedures when used with PRF.

Our study showed that the highest release of VEGF from PRF occurred in the theracal group suggesting that it could be an appropriate material used as a seal over scaffolds used in regenerative endodontic therapy and pulpotomy. Theracal was tested in this study because of its easy manipulation and dispensing in syringe form that could increase operators' ease as well. However, a limitation here was the light-curable nature of the material theracal compared to the conventional settings in MTA and Biodentine.

Considering the limitations of this *in vitro* study with less sample size, further comprehensive *in vivo* research is therefore required to assess its clinical success and applicability.

Conclusion

Vascular endothelial growth factor release is seen more from PRFm in contact with Theracal along with considerable yet minimal disruption of fibrin architecture. Therefore, Theracal may be considered as an alternative dental material to be used in endodontics wherever PRF has indicated as well as a sealing material in regenerative procedures. However, future research with a large sample is required to validate the results further.

Clinical Significance

The results show an increased release of VEGF from PRFm when layered with Theracal. PRFm used in procedures like revascularization and pulpotomy may therefore be layered with these dental materials to enhance the regeneration and create a biocompatible seal.

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