DOI: 10.7860/JCDR/2024/69728.19251

Original Article

Dentistry Section

Vitronectin Levels in Leukocyte-platelet Rich Fibrin, Injectable-platelet Rich Fibrin, and Serum: A Cross-sectional Study

ANIRUDH B ACHARYA[1], ADITI LOKHANDE[2], SWETHA ACHARYA[3], MIHIR KULKARNI[4], SRINATH THAKUR[5]

ABSTRACT

Introduction: Platelet-rich Fibrin (PRF) is an autologous platelet concentrate preparation containing several proteins that aid in healing. Vitronectin is one of these proteins that has not been quantified in all types of PRF. Various protocols have been suggested to alter the yield of different components of PRF to enhance wound healing. Hence, it is beneficial to know the vitronectin levels in PRF.

Aim: To detect, estimate, and compare the levels of vitronectin in two PRF protocols and serum.

Materials and Methods: The present cross-sectional study conducted in the Department of Periodontics at the SDM College of Dental Sciences and Hospital. Dharwad, Karnataka, India from January 2019 to June 2020 involved 12 systemically and periodontally healthy volunteers. Blood was obtained from each volunteer to collect and prepare serum, Leukocyte-PRF (L-PRF), and injectable-PRF (i-PRF), respectively. Three distinct samples-supernatant, exudate, and clot-were collected and categorised into seven groups (L-PRF supernatant, L-PRF exudate, L-PRF clot, i-PRF supernatant, i-PRF exudate, i-PRF clot, blood serum) that were assayed for levels of vitronectin. The data were statistically analysed using the independent t-test, one-way Analysis of Variance (ANOVA), and Newman-Keuls Post-hoc procedures.

Results: The mean age was 24.92±2.57 years. Vitronectin was detected and estimated in all the samples. Vitronectin levels ranged from 64.09±0.04 ng/mL to 64.20±0.21 ng/mL. One-way ANOVA applied to test between and within groups was significant (p=0.049). A statistically significant difference was observed only between L-PRF exudate and serum (p=0.05).

Conclusion: The comparable levels of vitronectin in L-PRF and i-PRF observed in present study suggest that vitronectin in these two PRF protocols may aid wound healing.

Keywords: Blood platelets, Blood protein, Enzyme-linked immunosorbent assay, Glycoprotein, Wound healing

INTRODUCTION

Wound healing and tissue regeneration are intricate processes involving cellular and extracellular processes, and molecular signaling [1]. Evidence regarding all aspects of these mechanisms is in continuing areas of research, but it is understood that platelets play a pivotal role. Platelet concentrates/platelet gels {thrombin-activated autologous Platelet-rich Plasma (PRP)} as topical applications (sometimes in combination with grafting biomaterials) have been employed in periodontal therapy [2]. Platelets contribute to healing because they contain growth factors that influence the cell cycle, induction, differentiation, collagen production, vascularisation, and cell recruitment at the wound site [3], thereby being the rationale for using preparations containing platelets to boost wound healing, repair, and regeneration [4]. These growth factors encompass a group of polypeptides with low molecular weights such as Platelet-derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF), and Insulin-like Growth Factor (IGF) that have properties contributing to cellular functions in wound healing and regenerative mechanisms [5,6]. Therefore, platelet concentrates like PRP and PRF are being routinely applied in periodontics and implant dentistry.

The use of PRP was first reported by Whitman DH et al., showing pronounced advantages because it could enhance osteoprogenitor cells in bone [7]. However, PRP posed antigenic risks as it required the use of bovine thrombin. PRF, introduced by Choukroun J et al., is now a widely used platelet concentrate [8]. It contains an autologous fibrin matrix and is more advantageous than PRP because it is strictly autologous, easy to prepare, and does not require biochemical handling of the blood [9]. Various proteins and polypeptides present in PRF, and their concentrations are found to vary compared with serum or plasma. These substances have various physiological roles and can be hypothetically harvested differentially via variations in PRF preparation protocols. Vitronectin is a serum protein with multifunctional capabilities, primarily produced in the liver, and has been defined as an S-protein (a circulating 75 kDa glycoprotein) [10]. It is homologous to adhesive proteins like fibrinogen, fibronectin, and von Willebrand factor, which are pro-coagulant, interacting with platelets and the vessel wall. Vitronectin is an important matrix-associated regulator of blood coagulation because it can bind heparin, plasminogen, Plasminogen Activator inhibitors (PA), and Thrombinantithrombin III (TAT) complexes [11]. Vitronectin is derived from serum, readily binds proteoglycans in the extracellular matrix, and is found in tissues [12].

Considering the role of vitronectin in wound healing and regeneration, it is of interest to quantify this polypeptide in PRF. Also, an awareness of any particular PRF protocol that demonstrates an optimal concentration of vitronectin will be of clinical utility, clarifying its potential contribution to periodontal outcomes. Evidence exists regarding the measure of vitronectin in blood serum [12], but to the best of our knowledge, no investigations have reported the quantity of vitronectin in all PRF protocols. Therefore, the present study aimed to investigate PRF and blood serum with the objectives of detection, estimation, and comparison of vitronectin in L-PRF, i-PRF, and blood serum.

MATERIALS AND METHODS

The cross-sectional study was conducted in the Department of Periodontics at the SDM College of Dental Sciences and Hospital, Dharwad, Karnataka, India, from January 2019 to June 2020. The study protocol was in accordance with the Declaration of Helsinki of 1975 and its subsequent revisions in 2013. Ethical clearance was

obtained from the Institutional Ethical Committee (IRB No. 2018/P/PERIO/68), and informed written consent was obtained from all participants before their enrollment in the study.

Inclusion and Exclusion criteia: Anamnesis, medical/dental/periodontal examinations, and blood collection were carried out on 12 volunteers (an equal number of males and females) with the inclusion criteria of being between 18-30 years of age and being systemically and periodontally healthy. Exclusion criteria included volunteers taking antibiotics, anti-inflammatory drugs, or other medications (such as anticoagulants, antiplatelets, antiresorptives) that could influence the study outcomes, those with active lesions or infections, pregnant or lactating women, and tobacco users.

Sample size: A convenient sample of 12 subjects was recruited for the study.

Study Procedure

The procedure for sample collection, extraction, and preparation of the different PRF protocols was followed based on earlier reports [8,13-15]. Blood samples were collected by drawing 15 mL of blood from the antecubital fossa of each participant, out of which L-PRF, i-PRF, and blood serum were prepared, each with 5 mL of blood. A 5 mL blood sample was collected in a dry glass-coated tube and kept motionless for 30 minutes to provide enough time for it to coagulate complete coagulation. The tube was then centrifuged at 3000 rpm for 15 minutes to obtain the blood serum. To prepare Choukroun J's PRF [8,13-15], 5 mL of blood was taken in a glass-coated vacutainer and subjected to centrifugation (Choukroun PRF Duo Quattro® System-Full System, Nice, France) at 3000 rpm for 10 minutes to obtain L-PRF. The remaining 5 mL of blood was taken in another glass-coated vacutainer and centrifuged at 700 rpm for 3 minutes to obtain i-PRF.

After the processing of the PRF, distinct samples were collected, including a supernatant representing acellular plasma or Platelet-poor Plasma (PPP), an exudate derived from the PRF clot corresponding to the liquid confined within the fibrin mesh, and the PRF clot. To collect the exudate, the PRF clot was kept in a sterile metal cup for 10 minutes to allow for the slow release of serum from within [13]. All collected samples were stored in Eppendorf's collection tubes at -80°C. The collected supernatant, exudate, and clot, both from L-PRF and i-PRF, along with the serum, were divided into seven groups, namely, blood serum, i-PRF exudates, i-PRF clot, L-PRF supernatant, L-PRF exudates, and L-PRF clot, all of which were assayed for levels of vitronectin using a commercially available Enzyme-linked Immunosorbent Assay (ELISA) kit {detection range: 3-200 ng/mL; sensitivity: 1-10 ng/mL; Krishgen Biosystems (Kinesis Dx), Mumbai, India}. The samples were tested as per the manufacturer's instructions at the concerned laboratory.

STATISTICAL ANALYSIS

The data were statistically analysed using statistical software (IBM-Statistical Packages for Social Sciences (SPSS) version 23.0, Armonk, NY, USA). The Kolmogorov-Smirnov test was applied for normality. Based on the normality of the distribution, appropriate parametric tests were used, including the independent t-test, one-way ANOVA, and Newman-Keuls Post-hoc procedures for comparisons. The probability value was set at $p \leq 0.05$.

RESULTS

Total 12 volunteers were included in the study, with an equal number of males (n=6) and females (n=6) providing the required blood samples. The mean age was 24.92±2.57 years. A summary of vitronectin levels in the seven groups is presented in [Table/Fig-1], where the mean values ranged from 64.09±0.04 ng/mL to 64.20±0.21 ng/mL, revealing comparable levels of vitronectin amongst all groups. One-way ANOVA applied to test between, and within groups showed significance between the seven groups (p=0.049).

Groups	n	Mean±SD (ng/mL)
i-PRF supernatant	12	64.16±0.07
i-PRF exudates	12	64.19±0.06
i-PRF clot	12	64.15±0.05
L-PRF supernatant	12	64.14±0.06
L-PRF exudates	12	64.09±0.04
L-PRF clot	12	64.10±0.05
Blood serum	12	64.20±0.21

[Table/Fig-1]: Summary of levels of vitronectin in the seven groups.
i-PRF: Injectable platelet rich fibrin; L-PRF: Leukocyte platelet rich fibrin: SD: Standard deviation; n: Numbers

On pair-wise comparison of vitronectin by Newman-Kuels' multiple post-hoc procedures of the seven groups, a statistically significant difference in-vitronectin concentration between L-PRF exudate and blood serum was observed (p=0.050) [Table/Fig-2]. The level of vitronectin was quantitatively higher in the blood serum group when compared with the other groups, as shown in [Table/Fig-3].

Comparison of vitronectin levels between males and females by independent t-test was not statistically significant, implying that there is no variation in-vitronectin concentrations between males and females in the seven groups, as shown in [Table/Fig-4].

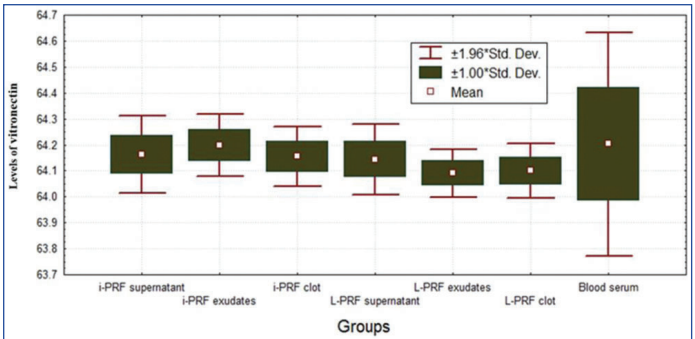
DISCUSSION

The clinical use of platelet-rich concentrates is based on the premise that they have beneficial effects on wound healing and regenerative potential. In present investigation, the aim was to detect, quantify, and compare the levels of vitronectin in L-PRF, i-PRF, and blood serum of healthy volunteers. Vitronectin was detected, and its concentration was estimated in blood serum, i-PRF exudates, i-PRF clot, L-PRF supernatant, L-PRF exudates, and L-PRF clot obtained from all the samples. The influence of gender and age of these study subjects as a source of bias on vitronectin levels was minimised because of the equal number of males and females in each group and within the specified range of 18-30 years as a reflection of the prime age of health.

Vitronectin has been quantified in the serum of healthy controls [12,16-19]. In present study's samples, the levels of vitronectin in

Groups	i-PRF supernatant	i-PRF exudates	i-PRF clot	L-PRF supernatant	L-PRF exudates	L-PRF clot	Blood serum
Mean±SD (ng/mL)	64.16±0.07	64.19±0.06	64.15±0.05	64.14±0.06	64.09±0.04	64.10±0.05	64.20±0.21
i-PRF supernatant	-						
i-PRF exudates	p=0.386	-					
i-PRF clot	p=0.855	p=0.545	-				
L-PRF supernatant	p=0.896	p=0.555	p=0.793	-			
L-PRF exudates	p=0.427	p=0.111	p=0.419	p=0.413	-		
L-PRF clot	p=0.431	p=0.129	p=0.379	p=0.286	p=0.840	-	
Blood serum	p=0.583	p=0.903	p=0.644	p=0.605	p=0.050*	p=0.133	-

[Table/Fig-2]: Pair-wise comparison of vitronectin in the seven groups by Newman-Keuls Post-hoc procedures.
i-PRF: Injectable platelet rich fibrin; L-PRF: Leukocyte platelet rich fibrin: SD: Standard deviation; n: Numbers; *statistically significant



[Table/Fig-3]: Comparison of vitronectin (ng/mL) in the seven groups.

Groups	Male (n=6) Vitronectin Mean±SD (ng/mL)	Female (n=6) Vitronectin Mean±SD (ng/mL)	p-value
i-PRF supernatant	64.16±0.09	64.16±0.06	0.971
i-PRF exudates	64.22±0.06	64.17±0.05	0.203
i-PRF clot	64.15±0.07	64.15±0.04	0.927
L-PRF supernatant	64.14±0.08	64.14±0.06	0.907
L-PRF exudates	64.11±0.04	64.07±0.03	0.113
L-PRF clot	64.09±0.06	64.10±0.03	0.841
Blood serum	64.22±0.27	64.18±0.17	0.787

[Table/Fig-4]: Comparison of vitronectin levels in males and females in the seven groups by independent t-test.
i-PRF: Injectable platelet rich fibrin; L-PRF: Leukocyte platelet rich fibrin: SD: Standard deviation; n: Numbers

blood serum were higher than i-PRF supernatant, i-PRF exudates, i-PRF clot, L-PRF supernatant, L-PRF exudates, and L-PRF clot but comparable. Boyd NA and co-workers investigated [12] the serum concentration of vitronectin (mg/liter) in health and various disorders such as liver and renal diseases, systemic lupus erythematosus, rheumatoid arthritis, etc., using radial immunodiffusion assay, due to which a dependable comparison with the current study cannot be made. Serum vitronectin concentrations in healthy controls (198.70±46.47 µg/mL) were compared with coronary artery disease (347.74±231.10 µg/mL) in another study [16]. The serum levels of vitronectin in the healthy controls aged 56.5±8.4 years of the aforementioned cited investigation were higher than the present study's younger-aged participants. The report by Teschler H et al., has shown serum vitronectin as 58.4±11.1 ng/mL in healthy controls which is relatable to the serum vitronectin concentrations of present study [17]. The aforesaid study compared healthy controls with hypersensitivity pneumonitis that had serum vitronectin levels 593.2±134.6 ng/mL. Other studies also showed variable serum vitronectin concentrations in health when compared to present study possibly due to different technical aspects [18,19]. It is to be noted that most of these previous studies compared serum vitronectin in health with a variety of diseases and no conclusion can be reached, in general, as to whether serum vitronectin concentration in health is higher, or lower than in disease. Also, there is a variation in healthy serum vitronectin concentrations among those reports. Therefore, attempting to compare present investigation's vitronectin serum concentrations in health with the literature for a definitive estimate is difficult, but the understanding is that it is measurable in serum.

The reasons for evaluating vitronectin in L-PRF and i-PRF are because the L-PRF clot contains the majority of the platelets and leukocytes from the primary blood harvest, with a three-dimensional distribution and a strong fibrin architecture; this solid biomaterial is in an activated form and cannot be injected [20], and i-PRF can release increased concentrations of several growth factors and facilitate greater fibroblast migration and expression of PDGF, TGF-β, and collagen [21]. Hence, it is relevant to have data regarding vitronectin in PRF to ascertain its availability in clinical procedures when using this platelet concentrate. The extensive study by Dohan Ehrenfest DM et al.,[20] estimating the amounts of TGF-β1, PDGF-AB, VEGF, TSP-1, fibronectin, and vitronectin in L-PRF, Plasma Rich In Growth Factors (PRGF), and Plasma Poor in Growth Factors (PPGF) at different time intervals has provided valuable data, concluding that vitronectin is the only protein to be released completely after four hours (in L-PRF, vitronectin ranged from 925±105 ng at 0-20 minutes to 10±4 ng at 5-7 days; vitronectin at 0-20 minutes in PRGF and PPGF was 245±76 ng and 426±156 ng, respectively). These measured values were obtained after subjecting the L-PRF to culture media before ELISA quantification. When compared with these estimates, the L-PRF vitronectin concentration was lower in the present investigation, which may be attributable to the difference in methodology. It has been observed that vitronectin in L-PRF clot exudate along with fibronectin may encourage fibrin adhesion and formation when combined with liquid PRGF [22]. This should add stimulus to in-depth analyses of vitronectin in PRF research.

However, due to the paucity of data in the literature, present study cannot make a direct comparison of vitronectin quantification in L-PRF and i-PRF, but some of the other proteins in PRF of healthy individuals that have been investigated are taken into consideration for a quantitative perspective of PRF proteins. The concentrations of TGF-β1, PDGF-AB, PDGF-BB, VEGF, Bone Morphogenetic Protein-2 (BMP-2), and Fibroblast Growth Factor-2 (FGF-2) released by liquid fibrinogen were evaluated with ELISA by Serafini G et al., [23]. Fibronectin was estimated in a PRGF preparation [24]. Kobayashi E et al., compared growth factors PDGF-AA, PDGF-AB, PDGF-BB, TGF-β, VEGF, EGF, and IGF in PRP, PRF, and advanced-PRF (A-PRF) [13]. The quantification of vitronectin was not done in any of these reports. The present study's results indicate similar levels of vitronectin in L-PRF and i-PRF. This adds credence to present investigation's data, which will contribute to the literature and help in future studies.

Limitation(s)

The small sample size, not having included other related proteins like fibronectin and thrombospondin, and the release kinetics of vitronectin from L-PRF and i-PRF not having been evaluated, are some of the limitations of present study.

CONCLUSION(S)

In present investigation, vitronectin was detected and quantified in L-PRF and i-PRF. The vitronectin concentrations in L-PRF and i-PRF are comparable. This may be the first report that has measured vitronectin in i-PRF. The present study could also infer that the supernatant and the exudate from the clots, which are often discarded, might have clinical relevance for use in periodontal/oral surgical procedures. Further exploration of vitronectin is strongly advocated to enhance the understanding of its yield and behaviour concerning other components of PRF and different PRF protocols.

REFERENCES

[1] Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, et al. Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part I: Technological concepts and evolution. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;101(3):e37-44.
[2] Bhanot S, Alex JC. Current applications of platelet gels in facial plastic surgery. Facial Plast Surg. 2002;18(1):27-33.
[3] Kiran NK, Mukunda KS, Tilak Raj TN. Platelet concentrates: A promising innovation in dentistry. Journal of Dental Sciences and Research. 2011;2(1):50-61.
[4] Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost. 2004;91(1):04-15.
[5] Anitua E, Sánchez M, Orive G, Andía I. The potential impact of the preparation rich in growth factors (PRGF) in different medical fields. Biomaterials. 2007;28(31):4551-60.
[6] Lind M. Growth factor stimulation of bone healing. Effects on osteoblasts, osteomies, and implants fixation. Acta Orthop Scand Suppl. 1998;283:02-37.
[7] Whitman DH, Berry RL, Green DM. Platelet gel: An autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. J Oral Maxillofac Surg. 1997;55(11):1294-99.

[8] Choukroun J, Adda F, Schoeffler C, Vervelle AP. An opportunity in paro-implantology: PRF. Implantodontie. 2000;42:55-62.
[9] Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc Natl Acad Sci U S A. 1974;71(4):1207-10.
[10] Hodies R. Preparation from human serum of an alpha-one protein which induces the immediate growth of unadapted cells in vitro. J Cell Biol. 1967;32(2):297-308.
[11] Kost C, Stüber W, Ehrlich HJ, Pannekoek H, Preissner KT. Mapping of binding sites for heparin, plasminogen activator inhibitor-1, and plasminogen to vitronectin's heparin-binding region reveals a novel vitronectin-dependent feedback mechanism for the control of plasmin formation. J Biol Chem. 1992;267(17):12098-105.
[12] Boyd NA, Bradwell AR, Thompson RA. Quantitation of vitronectin in serum: Evaluation of its usefulness in routine clinical practice. J Clin Pathol. 1993;46(11):1042-45.
[13] Kobayashi E, Flückiger L, Fujioka-Kobayashi M, Sawada K, Sculean A, Schaller B, et al. Comparative release of growth factors from PRP, PRF, and advanced-PRF. Clin Oral Investig. 2016;20(9):2353-60.
[14] Su CY, Kuo YP, Tseng YH, Su CH, Burnouf T. In vitro release of growth factors from platelet-rich fibrin (PRF): A proposal to optimize the clinical applications of PRF. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108(1):56-61.
[15] Shah R, Triveni MG, Thomas R, Mehta DS. An update on the protocols and biologic actions of platelet rich fibrin in dentistry. Eur J Prosthodont Restor Dent. 2017;25(2):64-72.
[16] Yaghoubi A, Ghojazadeh M, Abolhasani S, Alikhah H, Khaki-Khatibi F. Correlation of serum levels of vitronectin, malondialdehyde and Hs-CRP with disease severity in coronary artery disease. J Cardiovasc Thorac Res. 2015;7(3):113-17.
[17] Teschler H, Pohl WR, Thompson AB, Konietzko N, Mosher DF, Costabel U, et al. Elevated levels of bronchoalveolar lavage vitronectin in hypersensitivity pneumonitis. Am Rev Respir Dis. 1993;147(2):332-37.
[18] Yalçındağ A, Uzun A, Nilüfer Yalçındağ F, Delibaş N. Serum vitronectin levels in patients with Behçet's disease. Inflamm Res. 2012;61(11):1241-46.
[19] Leavesley DI, Kashyap AS, Croll T, Sivaramakrishnan M, Shokoohmand A, Hollier BG, et al. Vitronectin--Master controller or micromanager? IUBMB Life. 2013;65(10):807-18.
[20] Dohan Ehrenfest DM, Bielecki T, Jimbo R, Barbé G, Del Corso M, Inchingolo F, et al. Do the fibrin architecture and leukocyte content influence the growth factor release of platelet concentrates? An evidence-based answer comparing a pure platelet-rich plasma (P-PRP) gel and a leukocyte- and platelet-rich fibrin (L-PRF). Curr Pharm Biotechnol. 2012;13(7):1145-52.
[21] Miron RJ, Fujioka-Kobayashi M, Hernandez M, Kandalam U, Zhang Y, Ghanaati S, et al. Injectable platelet rich fibrin (i-PRF): Opportunities in regenerative dentistry? Clin Oral Investig. 2017;21(8):2619-27.
[22] Lollobrigida M, Maritato M, Bozzuto G, Formisano G, Molinari A, De Biase A. Biomimetic implant surface functionalization with liquid L-PRF products: In vitro study. Biomed Res Int. 2018;2018:9031435.
[23] Serafini G, Lopreiato M, Lollobrigida M, Lamazza L, Mazzucchi G, Fortunato L, et al. Platelet Rich Fibrin (PRF) and its related products: Biomolecular characterization of the liquid fibrinogen. J Clin Med. 2020;9(4):1099.
[24] Bayer A, Wijaya B, Möbus L, Rademacher F, Rodewald M, Tohidnezhad M, et al. Platelet-released growth factors and platelet-rich fibrin induce expression of factors involved in extracellular matrix organization in human keratinocytes. Int J Mol Sci. 2020;21(12):4404.

PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Restorative Dentistry, College of Dental Medicine, University of Sharjah, Sharjah, United Arab Emirates.
2. Consultant; Private Practitioner (Periodontics), Bengaluru, Karnataka, India.
3. Research Scholar, Department of Oral Pathology, JSS Dental College, Mysuru, Karnataka, India.
4. Associate Professor, Department of Periodontics, SDM College of Dental Sciences and Hospital, Dharwad, Karnataka, India.
5. Former Professor, Department of Periodontics, SDM College of Dental Sciences and Hospital, Dharwad, Karnataka, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Anirudh B Acharya,
Assistant Professor, Department of Restorative Dentistry, College of Dental Medicine, University of Sharjah, Sharjah, United Arab Emirates.
E-mail: abacharya@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jan 22, 2024
- Manual Googling: Feb 10, 2024
- iThenticate Software: Feb 24, 2024 (5%)

ETYMOLOGY: Author Origin

EMENDATIONS: 6

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jan 20, 2024**
Date of Peer Review: **Feb 13, 2024**
Date of Acceptance: **Feb 26, 2024**
Date of Publishing: **Apr 01, 2024**