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Influence of varying preparation protocols on the release of biologic mediators in platelet rich fibrin membrane: A novel periodontal treatment practice

(Influencia de diversos protocolos de preparación en la liberación de mediadores biológicos en la membrana de fibrina rica en plaquetas: Una nueva práctica de tratamiento periodontal)

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Resumen(español)

La concentración de plaquetas y la presencia de factores de crecimiento varían en diferentes protocolos de preparación, ya que cada uno de estos métodos tiene características técnicas (disparidad de tubos, fuerza de centrifugación, enzima inductora de gel) que afectan acumulativamente la cantidad y la cinética de liberación de factores de crecimiento derivados de plaquetas. El presente estudio se selecciona para evaluar la liberación de mediadores biológicos en membranas de fibrina ricas en plaquetas cuando se varían los protocolos de producción. El propósito de este estudio fue examinar la influencia de diferentes protocolos de preparación, como la velocidad y el tiempo de centrifugación, en la liberación de mediadores biológicos como PDGF - BB (factor de crecimiento derivado de plaquetas - BB), VEGF-A (factor de crecimiento endotelial vascular A) y TGF-β (factor de crecimiento transformante β) en la membrana de fibrina rica en plaquetas. El estudio estima la cantidad de factores de crecimiento como el factor de crecimiento derivado de plaquetas (PDGF-BB), el factor de crecimiento transformante beta (TGF-β) y el factor de crecimiento endotelial vascular A (VEGF-A) en la fibrina rica en plaquetas bajo la influencia de diversos protocolos de preparación como la velocidad y el tiempo de centrifugación utilizando la técnica de ensayo inmunoabsorbente ligado a enzimas (ELISA).

Palabras clave(español)

Periodontología, plaquetas, ELISA, odontología, fibrina.

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Abstract(english)

The platelet concentration and the presence of growth factors varies in different preparation protocol like each one of these methods has technical characteristic (disparity of tubes, centrifugation force, gel-inducing enzyme) cumulatively affecting the amount and kinetics of release of platelet derived growth factors. The present study is selected to evaluate the release of biological mediators in platelet rich fibrin membrane when the production protocols are varied.The purpose of this study was to examine the influence of varying preparation protocols like centrifugation rate and time on the release of biologic mediators like PDGF - BB (platelet derived growth factor - BB), VEGF-A (vascular endothelial growth factor A) and TGF-β (transforming growth factor β) in the platelet rich fibrin membrane. The study estimates the amount of growth factors like platelet derived growth factor (PDGF-BB), transforming growth factor beta (TGF-β), and vascular endothelial growth factor A (VEGF-A) in platelet rich fibrin under the influence of varying preparation protocols like centrifugation rate and time using enzyme linked immunosorbent assay (ELISA) technique.

Keywords(english)

Periodontology, platelet, ELISA, dentistry, fibrin.

Introduction

Periodontal disease is one of the major dental diseases that affect human population worldwide. Periodontal disease is a chronic inflammatory condition caused by a plethora of etiologic factors that is characterized by destruction of the periodontal tissues resulting in loss of connective tissue attachment, alveolar bone and formation of pathological pockets around the diseased teeth.

Periodontal regeneration entails the replacement of tissues lost by disease or injury with physiologically equivalent engineered tissues. Various procedures and agents that have been used to attain regeneration and new attachment includes open flap debridement, root conditioning, the use of bone grafts and bone substitutes, guided-tissue regeneration, coronally positioned flaps, and a combination of these.Platelet concentrates for surgical use are tools of regenerative medicine designed for the local release of platelet growth factors into a surgical or wounded site, in order to stimulate tissue healing or regeneration. Platelets contain a variety of protein molecules like signaling, membrane proteins, cytoskeleton regulatory proteins, cytokines, and other bioactive peptides that play a crucial role not only in hemostasis but also in initiating and regulating basic aspects of wound healing.1Platelet rich fibrin (PRF), the second generation platelet concentrate represents a novel measure in the therapeutic concept with elementary processing and absence of artificial biochemical modification like the use of bovine thrombin.3 PRF is prepared by immediate centrifugation of whole blood without anticoagulant with the formation of three biological phases like a coagulated red cell layer at the bottom, a rigid and elastic PRF gel as intermediate layer and a supernatant serum. Rapid activation of coagulation cascade and synthesis of thrombin take place during centrifugation thus inducing fibrin formation and platelet activation. The crux of PRF synthesis lies in an attempt to accumulate platelets and release cytokines in a fibrin clot yielded by a natural polymerization process during centrifugation with slow release of growth factors and matrix glycoproteins during or more than 7days 4 . The fibrin matrix present in PRF is flexible, elastic and very strong consisting of weak thrombin concentrations which entails equilateral junctions. These connected junctions permit the establishment of a fine and flexible network capable of supporting cytokines and cellular migration that occurs resulting in an increase in the lifespan of these cytokines as their release and use will occur at the time of initial cicatricle matrix remodeling. The platelet concentration and the presence of growth factors varies in different preparation protocol like each one of these methods has technical characteristic (disparity of tubes, centrifugation force, gel-inducing enzyme) cumulatively affecting the amount and kinetics of release of platelet derived growth factors.5 The present study is selected to evaluate the release of biological mediators in platelet rich fibrin membrane when the production protocols are varied.The purpose of this study was to examine the influence of varying preparation protocols like centrifugation rate and time on the release of biologic mediators like PDGF - BB (platelet derived growth factor - BB), VEGF-A (vascular endothelial growth factor A) and TGF-β (transforming growth factor β) in the platelet rich fibrin membrane. The study estimates the amount of growth factors like platelet derived growth factor (PDGF-BB), transforming growth factor beta (TGF-β), and vascular endothelial growth factor A (VEGF-A) in platelet rich fibrin under

the influence of varying preparation protocols like centrifugation rate and time using enzyme linked immunosorbent assay (ELISA) technique.

Material y methods

This work was done in collaboration between Sree Mookambika Institute of Dental Sciences, Kulasekharam and LMMD (Laboratory medicine and molecular diagnostics) at Rajiv Gandhi Centre of Biotechnology, Trivandrum on October 2014. A total of 10 healthy willing donors were selected from a pool of students at Sree Mookambika Institute of Dental Sciences. Subjects who fulfilled the following inclusion/exclusion criteria were included in the study. The inclusion criteria for the study was, Systemically healthy male volunteers between the age of 18 – 55 yrs.

Selecion criteria. The exclusion criteria were known case of blood disorders, drug intake that interferes with bleeding and clotting mechanisms at least for 3months. 30 ml whole blood was collected from all the 10 donors on the same day at same time in plain centrifuge tubes. The total blood from a volunteer was divided into six equal parts (5 ml each) and was then centrifuged under varying conditions to obtain the PRF (Platelet rich fibrin) clots. The varying conditions were grouped into Group A (8 minutes, 3000 RPM), Group B (10 minutes, 3000 RPM), Group C (12 minutes, 3000 RPM), Group I (2000 RPM, 10 minutes), Group II(3000 RPM, 10 minutes) and Group III (4000 RPM, 10 minutes). The clots obtained were compressed between two glass slides with a 1mm rubber stopper in between them to flush out the fluids and to obtain the PRF membrane of 1mm thickness. The prepared membrane is immediately transferred into 4ml of autoclaved Dulbecco's modified eagle medium and was incubated at 370C for 60 minutes. Then the membranes were transported in liquid nitrogen container to testing labs at Rajiv Gandhi Institute of Biotechnology, Trivandrum, where the samples were stored at -80°C. Growth factor estimation of the PRF membrane was done by ELISA at the LMMD (Laboratory medicine and molecular diagnostics) at Rajiv Gandhi Centre of Biotechnology, Trivandrum. In the present study, 3 major growth factor (PDGF-BB, VEGF-A and TGF-β1) levels in platelet rich fibrin were estimated by ELISA using a commercially available kit Ray Biotech, Inc. The OD value obtained after the ELISA was subjected for statistical analysis.

Statistical analysis. The data was expressed in Mean ± standard deviation. Statistical Package for Social Sciences (SPSS 16.0) version was used for statistical analysis. One way ANOVA was applied for analysis. Post Hoc followed Dunnet test was used to find statistical significance between and within the groups. P value less than 0.05 ($p < 0.05$) is considered to be statistically significant at 95% confidence interval.

Results

The purpose of this study was to identify the changes in the release of biologic mediators like PDGF-BB, VEGF-A and TGF-β under varying preparation protocols. The study is divided into two parts. The first part studies the changes brought about by varying the rate of centrifugation at a constant time in production of PRF. The second part studies the changes brought about by varying the time of centrifugation at a constant centrifugation rate in production of PRF. The growth factors released into the medium was estimated using ELISA. The OD Value obtained was analyzed using statistical analysis method.

Table -1 explains the mean values of PDGF-BB, VEGF-A and TGF-β in ng/ml in PRF. The measured values were lower for PRF in Group-I and Group-III, and the corresponding values for the Group-II were found to be higher.

Table – 2 shows the mean values of different growth factors of groups produced in varying time periods of centrifugation 3000rpm, (Group A: 8 minutes, Group B: 10 minutes and Group C: 12 minutes). The measured values of PDGF-BB, VEGF-A and TGF-β were observed to be lower in the PRF membrane in Group-A and Group-C, and the corresponding values for the Group-B were higher.

The quantity of released growth factors from the PRF membrane in all six groups was significantly

Table-1: Mean values of different growth factors of groups at different RPMS with same time interval

Groups	Types	PDGF-BB (ng/ml) (MEAN±SD)	VEGF-A (ng/ml) (MEAN±SD)	TGF-beta (ng/ml) (MEAN±SD)
Group-I	**2000RPM/10 min**	2.29±0.59	2.01±0.74	20.71±7.76
Group-II	**3000RPM/10 min**	2.64±0.25	2.13±0.80	21.55±5.18
Group-III	**4000RPM/10 min**	2.09±0.51	1.67±0.64	16.71±4.75

Table-2: Mean values of different growth factors of groups at same RPM with different time interval.

Groups	Types	PDGF-BB (ng/ml) (MEAN±SD)	VEGF-A (ng/ml) (MEAN±SD)	TGF-beta (ng/ml) (MEAN±SD)
Group-A	**3000RPM/8 min**	2.18±0.54	1.65±0.48	17.74±2.30
Group-B	**3000RPM/10 min**	2.64±0.25	2.13±0.80	21.55±5.18
Group-C	**3000RPM/12 min**	2.03±0.69	1.89±0.60	21.49±5.53

higher in Group II and Group B than the other groups. Among the groups themselves, the levels of platelet derived growth factor BB (PDGF BB) observed was found to decrease from 2.64 ± 0.25 ng/ml in Group II to 2.29 ± 0.59 ng/ml in Group I to 2.09 ± 0.51 in Group III and from 2.64 ± 0.25 ng/ml in Group B to 2.18 ± 0.54 ng/ml in Group A to 2.03 ± 0.69 ng/ml in Group C. It was observed that varying the RPM at 2000 and 4000 was disadvantageous for the growth factor recovery. The same negative effect was observed when the time was varied by 8 or 12 minutes. The interpretation of the results suggests that an optimum centrifugation and time for preparing a beneficial PRF was 3000 RPM and 10 minutes.

Discussion

Platelets and fibrin are the two essential biomaterials that plays a pivotal role in the formation of a clot. Hence a better administration technique is required to release biological molecules at essential points of the healing process in order to obtain an optimal wound healing in a fast and finely orchestrated nature. Various researches have indicated that the acceleration of wound healing process requires proper preparation of a product with minimal red blood cell and the platelet concentration that has a 4- to 5- fold increase above baseline count. From the investigation done, it was made clear the ideal PRF preparation protocol where the blood is taken without anticoagulant and immediately softly centrifuged at 3000 RPM for 10 minutes that yields a clot containing almost all the platelets and more than 50% of the leukocytes presenting a strong fibrin architecture and a specific three-dimensional distribution of the platelets and leukocytes. In PRF preparation protocol, without the anticoagulant, the majority of platelets in the sample are activated naturally that triggers a coagulation cascade yielding a fibrin clot with platelets located in the middle, just between the red blood cell layer at the bottom and acellular plasma at the top. The purpose of this study was to examine the influence of varying preparation protocols like centrifugation rate and time on the release of biologic mediators like PDGF - BB (platelet derived growth factor - BB), VEGF-A (vascular endothelial growth factor A) and TGF-β (transforming growth factor β) in the platelet rich fibrin membrane. Platelets are non-nuclear cellular fragments derived from megakaryocytes in the bone marrow, that are specialized secretory elements releasing the contents of their intracellular granules in response to activation. Among the stored mitogenic factors paramount for wound repair are platelet derived growth factor (PDGF) with the -AB and -C isoforms predominating, TGF-β VEGF, b FGF, PDEGF and IGF-1. More than 95% of the presynthesized growth factors are discharged within 1 hour. 2 Platelets synthesize and secrete the growth factors for the remaining 7 days of their life span and once the platelet is exhausted and dies off, the macrophage that has arrived in the region via the vascular in-growth induced by the platelets, assumes the function of wound healing regulation by secreting some of the same growth factors as well as others. Hence, the number of platelets in the blood clot within the wound sets the rate of wound healing. The PRF membrane in the present study was prepared at varying centrifugation rate and time period to determine the optimal preparation protocol for obtaining a massive growth factor release. The centrifugation rate and time were considered variables to be optimized since they could potentially influence the final release of growth factors. Moreover, the effectiveness of PRF is due to the fibrinogen component that may reduce the susceptibility of the endogenous polypeptidic growth factors to proteolytic degradation. It is known that platelet release their growth factors on activation from their alpha granules via active extrusion through the cell membrane but complete growth factors are not released by platelet disruption or fragmentation. Besides, these growth factors are actively extruded through the cell membrane where histones and carbohydrate side chains are added to complete their unique chemistries and make them “active” growth factors. This suggests that the differences in the growth factor release from the PRF membrane is not only dependent on the centrifugation process which is a physical process but also on the molecular mechanism of platelet exocytosis. Platelets contain coated pits and coated vesicles and the proteins

are taken up by the process of receptor-mediated endocytosis which illustrates the fibrinogen uptake by the integrin αIIbβ3. Also the platelet activation causes dramatic cytoskeletal rearrangements that are significant for shape change, adhesion, aggregation, exocytosis and retraction. Microtubule-associated proteins regulate the stability and phosporylation of microtubule reorganization which helps the contractile ring and stress fibres of platelets to involve in exocytosis and retraction. In this regard, therefore, it is critical to define the extent of platelet activation. Unravelling the roles of individual platelet will require culture models as well as the use of microarray systems capable of revealing the response of specific cell types to contact with platelet releasates. Backtracking along signaling pathways and knockout of specific receptors or secreted proteins will help to identify primary response signals.

Conflicts of interest

None to declare

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