Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

Sartaz Rahman[1]*, Gayathri GV[2] and DS Mehta[3]

[1]*Postgraduate Student, Department of Periodontics, Bapuji Dental College and Hospital, Davangere, Karnataka, India*
[2]*Professor and Head of the Department, Department of Periodontics, Bapuji Dental College and Hospital, Davangere, Karnataka, India*
[3]*Professor, Department of Periodontics, Bapuji Dental College and Hospital, Davangere, Karnataka, India*

*Corresponding Author: Sartaz Rahman, Postgraduate Student, Department of Periodontics, Bapuji Dental College and Hospital, Davangere, Karnataka, India.

Abstract

Objective: Advanced platelet rich fibrin (A-PRF) is a biopolymer which releases growth factors to facilitate tissue regeneration and healing. Rapid degradability and poor mechanical properties have hindered its use solely as a GTR membrane. Treatment with crosslinking agents modify the density of the biopolymer fibres and improve their stability. In the present study we assess the crosslinking effect of Glutaraldehyde (GLUT) on de¬gradation time and mechanical properties of A-PRF membrane.

Methodology: For this study, Fifteen human A-PRF membrane samples were prepared. Out of which 9 samples were treated with 0.1% GLUT for 10 minutes (experimental group) and the remaining 6 were untreated (control group). The release of GLUT from treated samples was evaluated by means of a spectrophotometer. Biodegradability of A-PRF samples of both the groups were evaluated by using human plasmin and mechanical properties by using a universal testing machine under standard ambient conditions.

Result: The release of GLUT from experimental group was found to be minimal. The control group samples degraded within 30 - 32 days whereas the GLUT- treated samples degraded within 50 - 53 days. Additionally, the experimental group samples revealed better mechanical properties than the control group.

Conclusion: Our result suggest that, crosslinking with GLUT effectively prolongs the degradation time and improves the mechanical properties of the A- PRF membrane thus opening the possibilities of its use alone as a GTR membrane.

Clinical Relevance: The result support the use of glutaraldehyde crosslinked A-PRF membrane as a torchbearer for the search of chairside, cost effective and optimal GTR membrane for periodontal regeneration.

Keywords: *A-PRF; Glutaraldehyde; Crosslinking*

Abbreviations

ERT: Endogenous Regenerative Therapy; GLUT: Glutaraldehyde; A-PRF: Advanced Platelet Rich Fibrin; GTR: Guided Tissue Regeneration; ADMA: Acellular Dermal Matrix Graft

Introduction

The main objective of periodontal therapy is to achieve the regeneration of lost supporting structures of teeth due to periodontitis [1]. Currently there is an increased focus on endogenous regenerative techniques. (ERT) for predictable periodontal regeneration. ERT includes patient derived platelet concentrates, growth factors and fibrin scaffolds which assist in recruitment of progenitor stem cells from neighbouring tissues [2].

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2329

Advanced Platelet-rich fibrin (A-PRF) is a third generation platelet and autologous cicatricial matrix used as a biomaterial, which releases growth factors to facilitate tissue regeneration and promotes hard and soft tissue healing [3]. Its compressed membranous form has been cited to be used as an absorbable barrier membrane in guided tissue regeneration (GTR) treatment [2]. Studies suggest that the adjunctive use of PRF in combination with barrier membranes are effective not only in the treatment of intrabony defects in chronic periodontitis but also used to provide good soft tissue coverage over the immediate implants [3,4].

Though PRF membrane doesn't degrade immediately after its application due to slow remodelling of its strong fibrin matrix but looses its structural integrity within 7 days similar to non crosslinked collagen membrane [5]. Barrier membranes are expected to remain intact atleast for 3 - 4 weeks at the implanted sites for progressive regeneration and integration of periodontal tissues [6]. The major shortcomings of A-PRF include, rapid degradability and poor mechanical strength. Thus, A-PRF membrane alone has not been popularized in GTR treatment as it cannot maintain space for tissue regeneration and prevent epithelial cell migration for sufficient periods of time [7].

A study was aimed at the modification of biopolymers to achieve an idealized combination of mechanical properties, geometry and surface chemistry by subjecting it to crosslinking treatments. These modified biopolymers provided resistance against enzyme-dependent degradation while increasing the strength of the biopolymer membranes by modifying their crosslinking densities.[8] Introduction of intrafibriller and interfibriller chemical bonds during crosslinking causes mechanical strengthening of collagenous tissue [9].

Various physical agents like Rose Bengal dye, white light/ultraviolet radiation and chemical agents like glucose, ribose, glyceraldehyde, glutaraldehyde, genipin etc have been used as crosslinking agents for collagen degradation evaluation [10,11]. Among the chemical agents, Glutaraldehyde (GLUT) is the most commonly used crosslinking agent in clinical use for fixing collagenous tissues. GLUT is fairly inexpensive, easily available and has the ability to cross-link quickly over large distances with a wide array of amino groups [8,11-13].

Significant increase in corneal rigidity and resistance to enzymatic degradation of porcine heart valves was observed following crosslinking by glutaraldehyde [14,15]. Glutaraldehyde vapour treatment has enhanced the mechanical property, retarded the degradation and maintained the bioactivity of electrospun fibrinogen scaffold [8].

We hypothesized to improve on the shortcomings of PRF to use it as GTR membrane. Therefore, in this study, we treated A-PRF membrane with Glutaraldehyde and tested its degradability and mechanical properties *in vitro*.

Materials and Methods

Fifteen systemically healthy volunteers within the age group of 20-35 years of either sex having platelet count within normal limits (1.5 to 3 lakhs) were chosen. Subjects who are smokers and/or on anticoagulant therapy were excluded.

The study design and consent forms for all the procedures performed with human subjects were approved by the institutional ethical committee (Ref. No. BDC/Exam/291/2018-19) at Bapuji Dental College and Hospital, Davangere, Karnataka, India, which is in accordance with Helsinki declaration of 1975 as revised in 2008. The purpose of the study was explained to the volunteers verbally and written consent to participate in the study was obtained prior to commencement of the study.

PRF preparation

A-PRF samples were prepared according to the protocol developed by Ghanati., *et al* [16]. 10 ml of venous blood was collected from the volunteers using Vacutainer™ tubes (Japan Becton, Deckinson and Company, Tokyo Japan) and transferred to A-PRF™ tubes (Zhejiong Gongdong Medical Technology Co., Ltd, China) which were immediately centrifuged by A-PRF12 system (A-PRF-12; Dragon Laboratory Instruments Ltd. Beijing, China) at 1500 rpm for 14 minutes. Following centrifugation, three parts were obtained in the tube: a red blood cell base at the bottom, acellular plasma as a supernatant (platelet-poor plasma), and the PRF clot in between. The clot was removed from the PRF tube and then membrane was prepared by compression in an A-PRF expression box.

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2330

Glutaraldehyde crosslinking of the PRF

Three A-PRF membranes were crosslinked with 10 ml of 0.1% GLUT solution, in phosphate buffer solution at a pH of 7.4 for 10 minutes at room temperature (Figure 1).
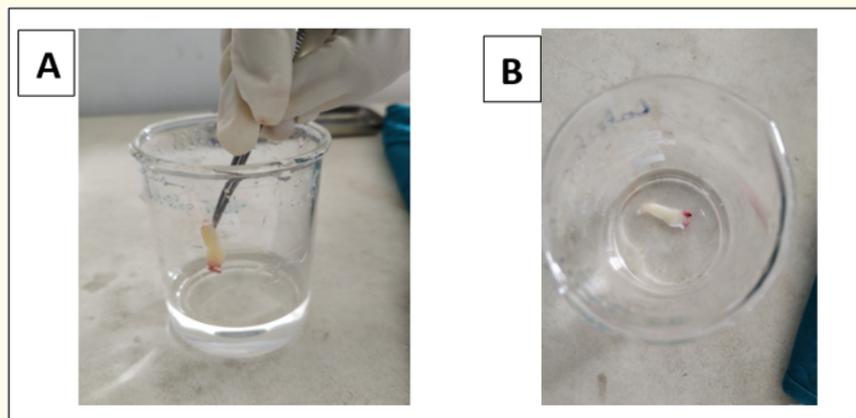

Figure 1: A&B- PRF membrane kept in 0.1% GLUT at 7.4 pH, 10 mins for crosslinking.

Glutaraldehyde release test [17]

The release of GLUT from crosslinked PRF membranes were evaluated by means of a spectrophotometric method (UV1600; Shimadzu, USA) Three GLUT crosslinked PRF membranes were immersed in 3 ml phosphate buffer solution in separate beakers with pH of 7.4 at 37°C. They were then transferred to 7 ml of a 0.1M glycine solution at room temperature. After 24 hours the glycine solution (release buffer) from each test sample was collected and its absorbance at 436 nm was measured using spectrophotometer. GLUT concentration released by the samples in the glycine solution were determined by comparison with a standard calibration curve. The test was continued for 2 weeks to evaluate further GLUT release from the samples (Figure 2).
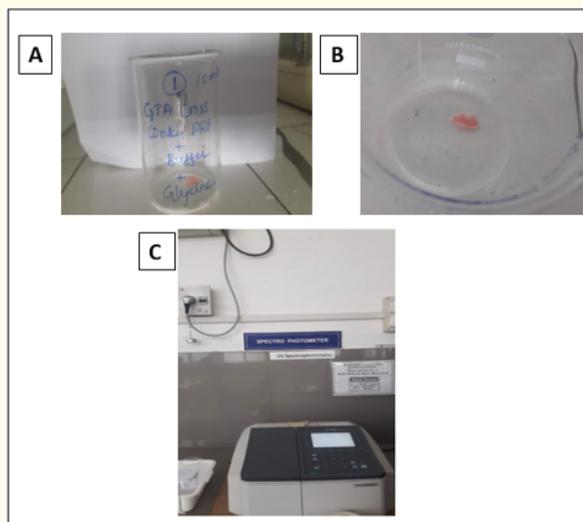

Figure 2: A- GLUT crosslinked PRF membranes immersed in phosphate buffer. B-Membranes transferred to 0.1M glycine. C- The release of GLUT evaluated by a spectrophotometer.

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2331

Accelerated degradation *in vitro* test [18]

Six A- PRF membrane disks of equal diameter (0.8 mm) were prepared by using a biopsy punch (Medsor impex, India) and kept in 24-well plates (upper row containing crosslinked samples and the lower row containing glutaraldehyde uncrosslinked samples) Fibrin is known to be degraded by plasmin *in vivo*. Hence the PRF membrane discs were incubated in a CO_2 incubator with media containing Hank's balanced salt solution (HBSS) supplemented with human plasmin (2 microgram of plasmin per ml of HBSS) (Sigma-Aldrich, UK). The media was replenished once in every 48 hours and change in their appearance was photographed (Figure 3). The test was repeated two additional times independently and the degradation time of all six samples were observed.
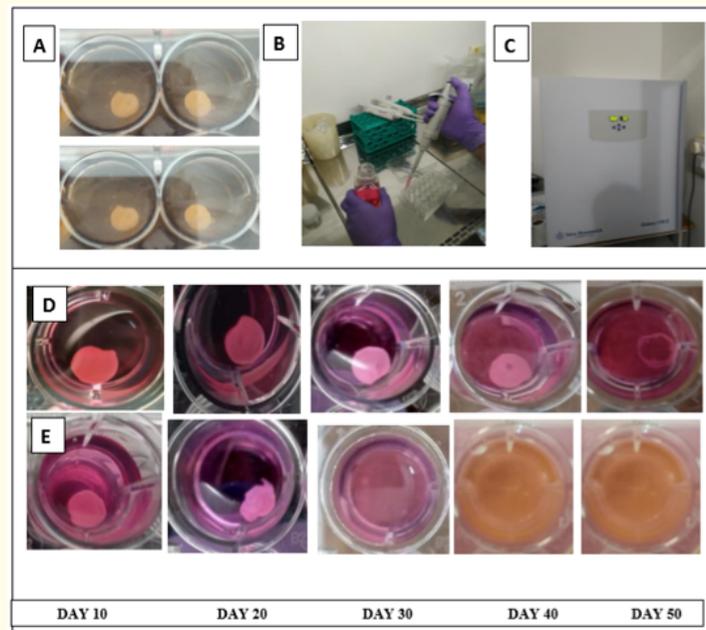

Figure 3: AA-GLUT crosslinked membranes kept on the top row and uncrosslinked membranes on the botton row in a 24 well plate. B- Hank's balanced salt solution (HBSS) supplemented with human plasmin (2 microgram of plasmin per ml of HBSS) added. C- Incubation in CO2 incubator. D-. The GLUT- treated samples degraded within 50- 53 days E- Control group samples degraded within 30-32 days.

Mechanical tests [19]

A specially designed plexiglass mold was fabricated to make the fibrin specimens identical in size, volume, and figure. The thickness of the mold was 2 mm and the width was 2 mm in the narrow middle part and 6 mm in the larger ends. The total volume of the mold was 104 mm^3.

The mechanical properties of the three GLUT crosslinked samples and three uncrosslinked membranes were measured using a desktop universal testing machine (Tinius Olsen h50KS, India) with maximum load cell capacity of 500N under standard ambient conditions of $25 \pm 3°C$ and $50 \pm 25\%$ RH (Relative Humidity). The larger ends of the dog-bone shape specimen were held with the clips of the machine without any tension such that, the initial apparent gauge length (the distance between clamp faces) was set to 10 mm for all the samples. Tensile loading was applied at a cross head speed of 2 mm/min; the maximum load at specimen failure was recorded and tensile strength was calculated using following formula: $S = F/A$, where F is maximum force (N) and A is unit area (m^2). Stress-strain curve was recorded with test Xpert II software simultaneously. Stiffness of the specimen (Young's modulus of elasticity) was obtained by stress/strain and the total area under the curve designated as toughness of the specimens (Figure 4).
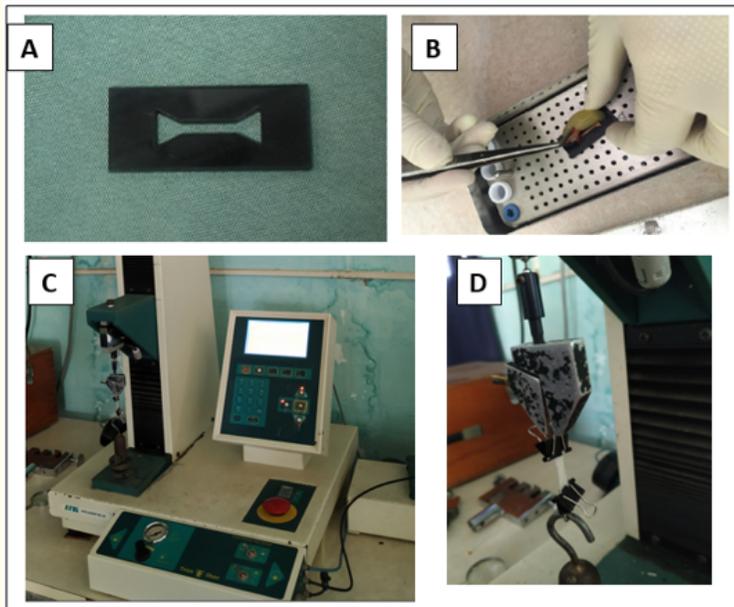
Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2332

Figure 4: *Plexiglass mold was fabricated. B- All Samples compressed in plexiglass mold C- The larger ends of the dog-bone shape specimen held by clips with initial apparent gauge length of 10 mm D- Tensile loading applied at a cross head speed of 2 mm/min*

Results

Glutaraldehyde release test

After 24 hours of storage in buffer solution, the crosslinked A-PRF membrane released an amount of GLUT corresponding to 0.083%. No further GLUT release was observed following further storage of samples in buffer solution for upto 2 weeks (Table 1).

	Groups	No. of samples	Mean ± SD	P value
Tensile strength	1	3	.16 ± 0.20	0.005*
	2	3	.07 ± 0.01	
Modulus of elasticity	1	3	.11 ± 0.01	0.643
	2	3	.11 ± 0.01	
Toughness	1	3	.20 ± 0.03	
	2	3	.10 ± 0.02	0.016*

Table 1: *Mechanical test results.*

*Group 1: GLUT Crosslinked Samples; Group 2: Control Samples. *-Statistically significant*

Accelerated degradation *in vitro* test

The GLUT crosslinked A-PRF membrane disks were degraded in a time-dependent manner and completely digested within 50 - 53 days of incubation. In contrast, the uncrosslinked A-PRF membrane disks initially turned the HBSS cloudy and then showed complete digestion between 30 - 32 days of incubation.

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2333

Mechanical test

The GLUT crosslinked and uncrosslinked membranes were examined by a tensile test and were characterized by three parameters: (1) Tensile strength (2) Young's modulus of elasticity (3) Toughness. The results of all tests for two groups are summarized in table 1. Tensile strength of the crosslinked group with mean value of 0.16MPa was significantly higher than the uncrosslinked group with mean value of 0.07 Mpa (P value= 0.005). The crosslinked group was slightly stiffer than uncrosslinked group but was not statistically significant (P = 0.643). Toughness of crosslinked group was significantly higher than uncrosslinked group (P = 0.016).

Discussion

PRF is an autogenic biomaterial containing a large number of growth factors and a dense fibrin network. [20] It is prepared by a specific centrifugation protocol by Ghanati [16] through contact activation, which facilitates intrinsic coagulation cascade without biochemical modification [21]. This protocol enhances natural and slow polymerization of blood clot to facilitate higher percentage of fine and flexible equilateral fibrin junctions, which provide greater elasticity and support the enmeshment of intrinsic cytokines and growth factors. It has stimulant potential on cell proliferation, migration chemotaxis and serves as a healing matrix [22-24].

PRF has been clinically used for the management of Grade II furcations [25] in improving gingival biotype and root coverage [26,27] in socket augmentation [27] and for sinus lift procedure [28,29]. Open flap debridement (OFD) with PRF has shown better reduction in probing depth and gain in clinical attachment level than the OFD alone [30,31]. Studies that have investigated the combined use of PRF and bone substitutes have revealed better clinical outcomes than bone substitutes alone [32-35].

Here an attempt was made to overcome the limitation of the use of PRF as a GTR membrane by crosslinking. This effectively controls the biodegradation rate and improves the tensile properties of the membrane.

Glutaraldehyde is one of the strongest crosslinking agent with highest number of cross links. Prolonged exposure to high concentration of GLUT lead to incomplete cross linking due to rapid initial polymerization at the surface of fibres. This prevents access of GLUT molecule to the core region for initiation of crosslinking either by creation of nucleation sites or by steric hindrance, which facilitates the consumption of large amount of free glutaraldehyde to cause high toxicity. Higher concentration of glutaraldehyde showed marked alteration in cell morphology and reduction in cell viability. At low concentration, the initial polymerization was slower and intramolecular cross links were formed in reconstituted collagen fibres in a time dependent manner. Penetration of GLUT molecule accessibility to the reactive group was faster in room temperature than in cold [36]. Treatment with 0.1% of GLUT successfully increased the biomechanical strength of human and porcine sclera [10]. Hence 0.1% GLUT was used for crosslinking of A-PRF at room temperature for 10 minutes.

Though patients on anticoagulant therapy have been known to produce quite normal PRF clots but the centrifugation time needs to be prolonged to obtain better PRF membranes. Coagulation and hemostatic processes have been found to be impaired by smoking thus affecting the coagulation-fibrinolysis cascade and impairing normal PRF clot formation [37,38]. Thus, both these groups of patients were excluded from the study.

PRF box helped to obtain a relatively uniform size and thickness of the PRF membrane from the clot. Converting these membranes to discs of 0.8 mm radius, by the use of a biopsy punch eliminated the inconsistency in tissue sampling and the concomitant problem of irregular diffusion of glutaraldehyde [39].

Till date very limited literature is available regarding the crosslinking of PRF to prolong its degradation. Heat compressed PRF found to be plasmin resistant remained stable for longer than 10 days *in vitro* but *in vivo* animal implantation revealed its degradation about 3 weeks in contrast to control PRF [18]. Similarly in our study 50 - 53 days of *in vitro* degradation may hope us to expect approximately 155 - 160 days for degradation on implantation in the tissues, which is approximately equal to commercially available collagen based

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2334

membranes [40]. In a study, though a low crosslink density was observed but due to presence of thick fibrin fibres and high serum retention, A- PRF showed its superiority over degradation, in contrast to Platelet Poor Plasma Derived Fibrin membrane due to enhanced ratio of volume to surface area [41].

GLUT toxicity is related to its release from the crosslinked material. In this trial only 0.083 wt % GLUT was released after 24 hours and no further release was observed for following 2 weeks. In contrast to this the cumulative GLUT release from gelatin films crosslinked with 0. 1% GLUT solution was about 2 wt % after 1 week and increased upto 9 wt % after 4 weeks.[17] This variation maybe due to a longer duration of crosslinking in the later.

The mechanical properties of PRF is closely related to its degradability [41]. GLUT treated A-PRF samples have shown an enhancement in the mechanical properties with prolonged degradation. Similar results have been observed in ADMA (Acellular Dermal Matrix graft), gelatin, collagen following crosslinking with GLUT [17,42,43].

Amniotic membrane (AM) matrices treated with low concentration GLUT (i.e. < 0.03 mmol GLUT/mg AM) displayed good compatibility with human corneal epithelial cells [44]. Enzyme-loaded erythrocytes treated with GLUT showed more resistance to lysis without inactivating the enzymes that have been entrapped inside them [45]. Successful implantation of periosteal cells over PRF crosslinked by heat compression showed their potential as scaffold for regenerative cells and preservation of its biocompatibility make its application as GTR membrane in periodontal therapy [18].

Here, the obtained GLUT crosslinked PRF membrane was easy to handle, flexible enough to allow close adaptation to defect morphology was easy to cut and shape without sharp edges and was strong enough to conform to the defect shape, yet was able to preserve its structure without perforation. We can expect the crosslinked A-PRF membrane to be used as a GTR membrane as well as a scaffold for periodontal regeneration.

Conclusion

In vitro trials help us to know the properties and characteristics of newer or modified regenerative materials and also facilitate to design and conduct future clinical trials to detect their beneficial effects on tooth supporting tissues. In our study, Gluteradehyde treatment increased mechanical strength and prolonged degradation time with minimal gluteraldehyde release in A- PRF membranes. This result opens up the possibility of its use as a GTR membrane. However the study is still at its primitive stage and its possible cytotoxic impact and ultrastructure needs extensive evaluation for its clinical application in the field of periodontal regeneration.

Conflict of Interest

The authors state that there is no conflict of interest.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards".

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2335

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Bibliography

1. Ripamonti U and Petit JC. "Bone morphogenetic proteins, cementogenesis, myoblastic stem cells and the induction of periodontal tissue regeneration". *Cytokine and Growth Factor Reviews* 20.5-6 (2009): 489-499.

2. Chen FM., *et al.* "A review on endogenous regenerative technology in periodontal regenerative medicine". *Biomaterials* 31.31 (2010): 7892-927.

3. Dohan DM., *et al.* "Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part I: Technological concepts and evolution". *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* 101.3 (2006): e37-e44.

4. Hafez W K., *et al.* "Platelet rich fibrin as a membrane for coverage of immediate implants: Case-series study on eight patients". *Tanta Dental Journal* 12.3 (2015): 203-210.

5. Blumenthal NM. "The use of collagen membranes to guide regeneration of new connective tissue attachment in dogs". *Journal of Periodontology* 59.12 (1988): 830-836.

6. Aurer A and Jorgić-Srdjak K. "Membranes for periodontal regeneration". *Acta Stomatologica Croatica* 39.1 (2005): 107-112.

7. Panda S. "Adjunctive Effect of Autologus Platelet-Rich Fibrin to Barrier Membrane in the Treatment of Periodontal Intrabony Defects". *Journal of Craniofacial Surgery* 27.3 (2016): 691-696.

8. Sell SA., *et al.* "Cross-linking methods of electrospun fibrinogen scaffolds for tissue engineering applications". *Biomedical Materials* 3.4 (2008): 045001.

9. Bailey AJ. "Structure, function and ageing of the collagens of the eye". *Eye* 1.2 (1987): 175-183.

10. Wollensak G and Spoerl E. "Collagen crosslinking of human and porcine sclera". *Journal of Cataract and Refractive Surgery* 30.3 (2004): 689-695.

11. Weadock K., *et al.* "Evaluation of collagen crosslinking techniques". *Biomaterials, Medical Devices, and Artificial Organs* 11.4 (1983): 293-318.

12. Olde Damink LHH., *et al.* "Glutaraldehyde as a crosslinking agent for collagen-based biomaterials". *Journal of Materials Science: Materials in Medicine* 6.8 (1995): 460-472.

13. Speer DP., *et al.* "Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials". *Journal of Biomedical Materials Research* 14.6 (1980): 753-764.

14. Khor E. "Methods for the treatment of collagenous tissues for bioprostheses". *Biomaterials* 18.2 (1997): 95-103.

15. E Starwich., et al. "Chemical composition and biophysical properties of porcine cardiovascular tissues". *Biomaterials Medical Devices and Artificial Organs* 3.3 (1975): 309-318.

16. Ghanati S., *et al.* "Advanced platelet-rich fibrin: a new concept for cell-based tissue engineering by means of inflammatory cells". *Journal of Oral Implantology* 40.6 (2014): 679-689.

17. Bigi A., *et al.* "Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking". *Biomaterials* 22.8 (2001): 763-768.

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2336

18. Kawase T., *et al*. "The heat-compression technique for the conversion of platelet-rich fibrin preparation to a barrier membrane with a reduced rate of biodegradation". *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 103.4 (2015): 825-831.

19. Khorshidi H., *et al*. "Comparison of the mechanical properties of early leukocyte-and platelet-rich fibrin versus PRGF/endoret membranes". *International Journal of Dentistry* (2016).

20. Choukroun J. "Advanced PRF, & i-PRF: platelet concentrates or blood concentrates". *Journal of Periodontal Medicine and Clinical Practice* 1.1 (2014): 3.

21. Choukroun., *et al*. "Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part V: histologic evaluations of PRF effects on bone allograft maturation in sinus lift". *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 101.3 (2006): 299-303.

22. Patel GK., *et al*. "Platelet-rich fibrin in regeneration of intrabony defects: a randomized controlled trial". *Journal of Periodontology* 88.11 (2017): 1192-1199.

23. Ehrenfest DM., *et al*. "Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte-and platelet-rich fibrin (L-PRF)". *Trends in Biotechnology* 27.3 (2009): 158-167.

24. Rodeo SA., *et al*. "The effect of platelet-rich fibrin matrix on rotator cuff tendon healing: a prospective, randomized clinical study". *The American Journal of Sports Medicine* 40.6 (2012): 1234-1241.

25. Sharma A and Pradeep AR. "Autologous platelet-rich fibrin in the treatment of mandibular degree II furcation defects: A randomized clinical trial". *Journal of Periodontology* 82.10 (2011): 1396-1403.

26. Jasser RA., *et al*. "Platelet-Rich Fibrin as a New Approach in Treating Gingival Recession: Systematic Review and Meta-Analysis. *Journal of Dental Health, Oral Disorders and Therapy* 5.2 (2017): 1-12.

27. Anilkumar K., *et al*. "Platelet-rich-fibrin: A novel root coverage approach". *Journal of Indian Society of Periodontology* 13.1 (2009): 50-54.

28. Hartshorne J and Gluckman H. "A comprehensive clinical review of Platelet Rich Fibrin (PRF) and its role in promoting tissue healing and regeneration in dentistry. Part II: preparation, optimization, handling and application, benefits and limitations of PRF". *International Dental Journal* 6.5 (2016): 34-48.

29. Toffler M., *et al*. "Osteotome-mediated sinus floor elevation using only platelet-rich fibrin: an early report on 110 patients". *Implant Dentistry* 19.5 (2010): 447-456.

30. Ajwani H., *et al*. "Comparative evaluation of platelet-rich fibrin biomaterial and open flap debridement in the treatment of two and three wall intrabony defects". *Journal of International Oral Health* 7.4 (2015): 32-37.

31. Preeja C and Arun S. "Platelet-rich fibrin: Its role in periodontal regeneration". *The Saudi Journal for Dental Research* 5.2 (2014): 117-122.

32. Joseph VR., *et al*. "Clinical effectiveness of autologous platelet rich fibrin in the management of infrabony periodontal defects". *Singapore Dental Journal* 33.1 (2012): 5-12.

33. Elgendy EA and Abo Shady TE. "Clinical and radiographic evaluation of nanocrystalline hydroxyapatite with or without platelet-rich fibrin membrane in the treatment of periodontal intrabony defects". *Journal of Indian Society of Periodontology* 19.1 (2015): 61-65.

34. Gupta SJ., *et al*. "Efficacy of platelet-rich fibrin vs. enamel matrix derivative in the treatment of periodontal intrabony defects: a clinical and cone beam computed tomography study". *International Academy of Periodontology* 16.3 (2014): 86-96.

Comparative Evaluation of Advanced Platelet Rich Fibrin Membrane with and without Glutaraldehyde Crosslinking- A *De Novo In Vitro* Trial

2337

35. Lekovic V., *et al*. "Platelet-rich fibrin and bovine porous bone mineral vs. platelet-rich fibrin in the treatment of intrabony periodontal defects". *Journal of Periodontal Research* 47.4 (2012): 409-417.

36. Cheung DT., *et al*. "Mechanism of crosslinking of proteins by glutaraldehyde III. Reaction with collagen in tissues". *Connective Tissue Research* 13.2 (1985): 109-115.

37. Sammartino G., *et al*. "Prevention of hemorrhagic complications after dental extractions into open heart surgery patients under anti-coagulant therapy: the use of leukocyte-and platelet-rich fibrin". *Journal of Oral Implantology* 37.6 (2011): 681-690.

38. Pretorius E., *et al*. "Smoking and coagulation: the sticky fibrin phenomenon". *Ultrastructural Pathology* 34.4 (2010): 236-239.

39. Kobayashi M., *et al*. "A proposed protocol for the standardized preparation of PRF membranes for clinical use". *Biologicals* 40.5 (2012): 323-329.

40. Bottino MC., *et al*. "Recent advances in the development of GTR/ GBR membranes for periodontal regeneration-A materials perspective". *Dental Materials* 28.7 (2012): 703-721.

41. Isobe K., *et al*. "Mechanical and degradation properties of advanced platelet-rich fibrin (A-PRF), concentrated growth factors (CGF), and platelet-poor plasma-derived fibrin (PPTF)". *International Journal of Implant Dentistry* 3.1 (2017): 17.

42. Bottino MC., *et al*. "Acellular dermal matrix graft: synergistic effect of rehydration and natural crosslinking on mechanical properties". *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 95.2 (2010): 276-82.

43. Charulatha V and Rajaram A. "Influence of different crosslinking treatments on the physical properties of collagen membranes". *Biomaterials* 24.5 (2003): 759-767.

44. Lai JY. "Interrelationship between cross-linking structure, molecular stability, and cytocompatibility of amniotic membranes cross-linked with glutaraldehyde of varying concentrations". *RSC Advances* 4.36 (2014): 18871-18880.

45. Deloach J., *et al*. "Effect of glutaraldehyde treatment on enzyme-loaded erythrocytes". *Biochimica et Biophysica Acta (BBA)-General Subjects* 496.2 (1977): 507-515.