Original Research Paper

Science

CYTOTOXICITY TESTING OF A NOVEL CROSSLINKED PRF (C-PRF) DEVELOPED FOR DENTAL PULP TISSUE ENGINEERING PURPOSE

Dr. Suman Kar*	M.D.S.; M.Phil, Ph.D.(Scholar) Associate. Prof. ADCH (Jamshedpur), Jharkhand, India *Corresponding Author
Dr. R. R. Paul	M.D.S.; Ph.D. Prof. Emeritus JISU(Kol), ,India
Dr. H. D. Adhikari	M.D.S.; Ex-Prof and HOD of RADC(Kol),India
Dr Ankita Das	Ph.D. (IIEST) Shibpur, India
Dr. Jaydeep Samanta	M.D.S. Associate professor, HIDSAR, Haldia,WB,India
Dr. Swarupananda Bera	M.D.S.; Prof. ADCH (Jamshedpur), Jharkhand,India
Dr. Debjyoti Karmakar	M.D.S.; Ast. Prof.; MMCH(Malda),WB,India

ABSTRACT

Background- Whole pulp amputation followed by pulp space disinfection and filling with an artificial material causes loss of significant amount of dentin leaving a non-vital and weakened tooth. Regenerative endodontics with its emerging field of modern tissue engineering has demonstrated promising results using stem cells associated with scaffolds and responsive molecules. [1] Introduction- PRF was recognized as the "second generation" of this family of biomaterials. [6] PRF being tested in pulp tissue engineering by different research groups showed mixed results. (7,8) Research studies have shown that the interactions between the cells and their niche are closely related to physicochemical properties of the scaffolding materials [9, 10]. As PRF is a fragile gel its physical character needs to be improved by cross linking and thereby more longer period of liberation of its growth factors and delayed disintegration in physiological system. Aims and Objectives- Aim of our study was to prepare a very economical and autologous biomaterial for pulp tissue engineering by crosslinking of PRF with tannic acid. Our objective was to detect cytotoxic effect of tannic acid in PRF. Methods and Materials- We followed Choukroun et al. protocol to prepare PRF samples from whole venous blood collected from donors. PRF samples were then cross-linked in freshly prepared TA solution in dapendish for 10 minutes at room temperature. Concentrations of TA 1 wt% was used for preparing samples. After crosslinking, the gels were washed with normal saline for 5 min. to ensure that all excess TA was removed. The viability of cells cultured on the scaffolds was assessed through MTT assay (EZcountTM MTT cell Assay Kit, HiMedia, Mumbai, India). Observations- Both MTT Assay and Phalloidine staining showed favourable results of no clear cytotoxic effects of C-PRF. Conclusion- Based on the results of the cell viability analysis it can be concluded that none of the tannic acid crosslinked PRF created any clear cytotoxicity in the MC3T3 cells. So, C-PRF can safely be used as scaffold for dental pulp or similar tissue engineering purposes.

KEYWORDS : Platelet rich fibrin (PRF), Crosslinked PRF(C-PRF), Tannic acid, Crosslinking, Cytotoxicity testing

INTRODUCTION:

Dental pulp is a soft connective tissue containing blood vessels and nerves to sustain its own physiological functions and those of the adjacent tissues. It is also a residence for cells of different types, including odontoblasts populated at the dentin surface and fibroblasts distributed throughout the extracellular matrix (ECM) of the pulp. The ECM is also rich in terms of collagens (collagens type 1: 56%, type 3: 41%, type 5: 2%) and noncollagenous (chondroitin 4- and 6-sulfate: 60%, dermatan sulfate: 34%, keratan sulfate: 2%, and glycosaminoglycans as proteoglycans) [1]

Regeneration potential of dental pulp will certainly avoid complications associated with the endodontically treated tooth. Endodontically treated tooth leads to considerable structural deformations due to removal of part of enamel, dentin, and pulp. Such deformations may result in tooth fracture and trauma as the postoperative tooth becomes deceased and brittle. Because of the lost pulpal sensation and inability of the tooth to detect microbial challenges, it can also be more susceptible to re-infections [2].

Biomaterials for tooth regeneration are expected to provide a homelike environment for cells, be biocompatible and biodegradable, allow functionality for a variety of cells, be clinically applicable, and possess multiple structural characteristics due to well-orchestrated hierarchical structures and functions of dental tissues. However, research studies have shown that the interactions between the cells and their niche are closely related to physicochemical properties of the scaffolding materials. [3,4]

PRF in now been widely used in dentistry in various clinical regenerative situations. Although this regenerative modality still remains unfamiliar to many clinicians, the evidence supporting its use has accumulated over the years, demonstrating its ability to improve tissue regeneration. The combination of PRF with regenerative therapy has been shown to be most promising for periodontal regeneration of intrabony and furcation defects, as well as soft tissue root coverage of gingival recessions. Evidence from the medical literature suggests that PRF is able to decrease infection following tooth extraction and may further limit dimensional changes of alveolar ridge following tooth loss. Nevertheless, ease of use of PRF, combined with its low cost and autologous source, makes it an ideal biomaterial worth further investigation across a variety of surgical procedures in dentistry [5].

Studies comparing different materials with PRF used in

VOLUME - 12, ISSUE - 04, APRIL - 2023 • PRINT ISSN No. 2277 - 8160 • DOI : 10.36106/gjra

coronal pulpotomy techniques in context of pulp tissue regeneration have proved some promising results [6,7] As PRF compressive strength is very less, it was assumed that cross-linking PRF will improve its compressive strength and better suit for pulp tissue engineering purposes.

Tannic acid (TA, C76H52O46), a naturally occurring water-soluble polyphenolic extract, has been approved as a safe compound by the US Food and Drug Administration (FDA), which made it a safe excipient/active additive in food, drink, and pharmaceutical formulations. (8-10) TA is abundantly available, as it is commonly present in plant leaves (e.g., green tea), fruit skins, vegetables, nuts, red wine, coffee, and wood bark. TA (penta-m-digalloyl glucose) contains a unique hydrolyzable structure of tannin with a glucose moiety core. (11) The hydroxyl groups of this glucose are esterified with five digallic acids. (12) TA exhibits antioxidant, antimicrobial, antiviral, and anti-inflammatory properties. (13) TA's broad range of bioactivities makes it a potent and viable pharmaceutical additive molecule for various indications.

AIMS AND OBJECTIVES

Aim of our study was to test cytotoxicity of our novel tannic acid crosslinked PRF (C-PRF)

METHODS AND MATERIALS

PREPARATION OF PRF

The protocol followed is that of "Choukroun et al.[14] Written consent was taken previously from the donors. The PRF preparation protocol is very simple. Around 10 ml of whole venous blood is collected in sterile glass tubes of 10 ml capacity without anticoagulant. Another 10 ml tube with normal saline was taken as counterbalance in centrifugal machine. The tubes were then placed immediately in a centrifugal machine [Fig-] and are immediately centrifuged at 2700 r.p.m. for 12 min. Three distinct layers were appeared in the tubes: Lower red layer containing red blood cells, upper straw colored platelet poor plasma and in the middle segment the turbid fibrin clot PRF [Fig-]. We can obtain the PRF extracting the matrix from the tube with forceps and removing the red clot. The success of this technique depends entirely on the blood collection and the transfer speed in the centrifuge.

PREPARATION OF THE NOVEL CROSSLINKED PRF (C-PRF)

Tannic acid (TA) powder was purchased from Sigma–Aldrich (Bangalore, India). PRF was prepared freshly as described above. PRF samples were crosslinked in freshly prepared TA solution in dapendish for 10 minutes at room temperature. TA was used at a concentration of 1 wt% for preparing samples. After crosslinking, the gels were washed with normal saline for 5 min. to ensure that all excess TA was removed.

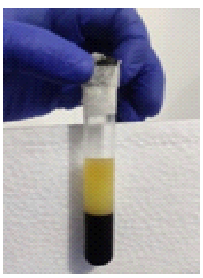
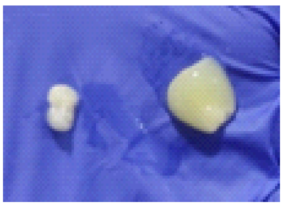


Fig.- PRF **PRF in TA soln.**



Crosslinked PRF in left Uncrosslinked PRF in right

CYTOTOXICITY TESTING:

Cell Culture:

For biological assays, the scaffolds (PRF, C-PRF) were seeded with MC3T3 at a concentration of 5x104 cells/ml and cultured as described for each experiment.

Cell viability:

The viability of cells cultured on the scaffolds was assessed through MTT assay (EZcountTM MTT cell Assay Kit, HiMedia, Mumbai, India). After 1 and 5 days culture, MTT solution was added to each well along with serum-free cell culture media. Cells were incubated for another 4 h at 37 °C and 100 μl of solubilization buffer was added to the well plates. The well plates were placed on a shaker for 10 min. The scaffolds were discarded and 100 μl of the supernatant from each sample group was pipetted into a fresh 96-well plate. The optical density at 570 nm and 670 nm was measured using an UV-V in multi-plate spectrophotometer (ThermoScientificTMMultiskanTMGO Microplate Spectrophotometer, Finland). The experiment was performed in triplicate and percentage cell viability was calculated by using the following formula:

$$\%\,cell\ viability = \frac{(Abs_{test} - Abs_{blank})}{(Abs_{control} - Abs_{blank})} \times 100$$

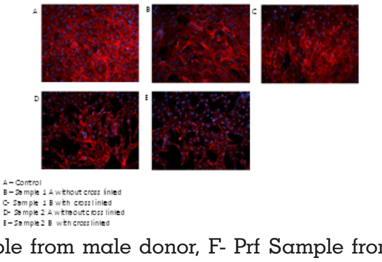
where, Abs_{test}, Abs_{blank} and $Abs_{control}$ signify absorbance of the sample, blank and control at 570 nm.

Phalloidine staining:

After culturing for 5 days, the cell seeded scaffolds were washed in PBS (pH 7.4) and fixed in 4% paraformaldehyde for 15 min. After washing with PBS, the scaffolds were incubated with 50 μg/ml rhodamine-conjugated phalloidin (Invitrogen, CA, USA) for 90 min and counterstained with 1 μg/mL of DAPI for 1 min. Cell images were acquired through inverted fluorescence microscope (Nikon Eclipse T U, Japan) equipped with 20x objective. The blue excitation wavelength was set at 340-380 nm which emitted at 435-485 nm. Similarly for red, the excitation wavelength was set at 512-552 nm which emitted at 565-615 nm.

RESULTS

Sample	Abs 570	Abs 570	Abs 570	Abs 670	Abs 670	Abs 670	Abs 570-670	Abs 570-670	Abs 570-670	Avg 570-670	Test-Blank	% cell viability
Blank	0.046	0.048	0.045	0.043	0.046	0.043	0.003	0.002	0.002	0.002333	0	
Control	0.419	0.412	0.415	0.105	0.125	0.198	0.314	0.287	0.217	0.272667	0.270333	100
M	0.194	0.195	0.195	0.055	0.051	0.058	0.139	0.144	0.137	0.14	0.137667	50.92478
MC	0.167	0.227	0.229	0.059	0.055	0.056	0.108	0.172	0.173	0.151	0.148667	54.99383
F	0.17	0.173	0.179	0.064	0.061	0.064	0.106	0.112	0.115	0.111	0.108667	40.19729
FC	0.183	0.186	0.184	0.065	0.067	0.066	0.118	0.119	0.118	0.118333	0.116	42.90999



— Prf Sample from male donor, F- Prf Sample from female donor, MC- Crosslinked Prf Sample from male donor FC- Crosslinked Prf Sample from female donor. Both MTT Assay and Phalloidine staining showed favourable results of no clear cytotoxic effects of C-PRF.

DISCUSSION:

A second generation platelet concentrate, or PRF, is frequently referred to as Choukroun's PRF. The PRF is made up of blood components that aid in faster wound healing. A three dimensional immune concentrate with a specific composition might be thought of as PRF. It contains a variety of growth factors, including insulin like growth factor, platelet derived growth factor, and transforming growth factor 1, which all demonstrate a variety of potent local features, including cell migration, attachment, proliferation, and differentiation. In the context of tissue engineering, it has been demonstrated to be an effective biomaterial for pulp regeneration. PRF functions as an inter-positional and therapeutic biomaterial [15].

A recent study demonstrated that the hydrogel network based on TA could satisfy demands for a lengthy clinical time as well as physiological capabilities with antioxidant, antibacterial, and anti-inflammatory properties [16]. Also, according to Du et al. research, a hybrid hydrogel made of PEG, quaternized chitosan (QCS), and TA (PEGDA/QCS/TA) was created using chemistry that was inspired by the chemistry of mussels. [17] Several hydrogen bonds between TA and PEG allowed for the introduction of features like anti-swelling, high mechanical strength, antibacterial, and great adhesive qualities to a variety of surfaces. In a different investigation, we discovered that crosslinking with tannic acid improved the physical features of PRF. [18] Genipin (GP), a natural crosslinker, was used by Tatiana C. et. al. to improve the hydrogel's physical properties with the goal of creating a hydrogel with adjustable qualities for tissue engineering applications. Four concentrations of GP were used in two distinct cross-linking procedures to create a stable fibrin gel network of hydrogel. The hydrogels' crosslinking density, mechanical characteristics, swelling, and enzymatic degradation were evaluated for each GP content and fabrication technique. Whereas in method I: Simultaneous gel creation and cross-linking increases the mechanical properties of the gel, in method II: Cross-linking after gel formation produces a high crosslinking and maintains the gel shape for lengthy periods of time. This study supports the use of GP as a suitable fibrin cross-linker at various doses to support L929 cells' ability to survive in vitro for 21 days[21].

However, because genipin is an expensive crosslinker, we experimented with cheaper crosslinkers, like tannic acid, for potential biomedical uses. The biocompatibility of biomedical materials can be revealed via cytotoxicity.[20] Recent research by Lu Denga et al.DC2.4 cells, a cell line of white cell dendrite cells, were used to test the toxicity of various concentrations of TA. As compared to the negative control, TA at concentrations up to 10 g/mL did not significantly affect the viability of DC2.4 cells. In contrast to the negative control, DC2.4 cells began to exhibit considerable toxicity at 50 g/mL or greater concentrations of TA. In spite of the fact that this concentration of TA showed high toxicity in comparison to the negative control, it is noteworthy that the cell viability was maintained at 80.1% in the presence of 50 g/mL of TA. The information on cytotoxicity is significant for guiding the in vivo biological uses of TA. [21] Also, the tannic acid crosslinked samples (1wt %) from male and female donors in our investigation demonstrated no clear cytotoxicity.

CONCLUSION:

Based on the results of the cell viability analysis it can be concluded that none of the tannic acid cross-linked PRF created any clear cytotoxicity in the MC3T3 cells. So, C-PRF can safely be used as scaffold for dental pulp or similar tissue engineering purposes.

REFERENCES

[1] Goldberg, M., and Smith, A. J., 2004, "Cells and Extracellular Matrices of Dentin and Pulp: A Biological Basis for Repair and Tissue Engineering," Crit. Rev. Oral Biol. Med., 15(1), pp. 13–27.

[2] Yuan, Z., Nie, H., Wang, S., Lee, C. H., Li, A., Fu, S. Y., Zhou, H., Chen, L., and Mao, J. J., 2011, "Biomaterial Selection for Tooth Regeneration," Tissue Eng., Part B, 17(5), pp. 373–388

[3] Mintz, B. R., and Cooper, J. A., Jr., 2014, "Hybrid Hyaluronic Acid Hydrogel/ Poly(Varepsilon-Caprolactone) Scaffold Provides Mechanically Favorable Platform for Cartilage Tissue Engineering Studies," J. Biomed. Mater. Res., Part A, 102(9), pp. 2918–2926.

[4] Cameron, A. R., Frith, J. E., Gomez, G. A., Yap, A. S., and Cooper-White, J. J., 2014, "The Effect of Time-Dependent Deformation of Viscoelastic Hydrogels on Myogenic Induction and Rac1 Activity in Mesenchymal Stem Cells," Biomaterials, 35(6), pp. 1857–1868.

[5] Richard J. Miron & Giovanni Zucchelli & Michael A. Pikos & Maurice Salama & Samuel Lee & Vincent Guillemette & Masako Fujioka-Kobayashi & Mark Bishara & Yufeng Zhang & Hom-Lay Wang & Fatiha Chandad & Cleopatra Nacopoulos & Alain Simonpieri & Alexandre Amir Aalam & Pietro Felice & Gilberto Sammartino & Shahram Ghanaati & Maria A Hernandez & Joseph Choukroun Use of platelet-rich fibrin in regenerative dentistry: a systematic review Clin Oral Invest.

[6] Solomon RV, Faizuddin U, Karunakar P, Sarvani GD, Soumya SS. Coronal pulpotomy technique analysis as an alternative to pulpectomy for preserving the tooth vitality in the context of tissue regeneration: a correlated clinical study across 4 adult permanent molars. Case Rep Dent, 2015, 1-12.

[7] Suryasowjanya Doranala, Jayaprada Reddy Surakanti, Harikumar Vemisetty, Suraj Reddy Loka, Keerthi Sudireddy, Rajani Punna ; Comparative assessment of titanium-prepared platelet-rich fibrin, EndoSequence root repair material, and calcium hydroxide as pulpotomy agents in permanent teeth with irreversible pulpitis: A randomized controlled trial ;Journal of Conservative Dentististry; Year : 2021 | Volume : 24 | Issue : 6 | Page : 606-610

[8] Hatami, E.; Mu, Y.; Shields, D. N.; Chauhan, S. C.; Kumar, S.; Cory, T. J.; Yallapu, M. M. Mannose-decorated hybrid nanoparticles for enhanced macrophage targeting. Biochem. Biophys. Rep. 2019, 17, 197−207.

[9] Hatami, E.; Bhusetty Nagesh, P. K.; Chowdhury, P.; Elliot, S.; Shields, D.; Chand Chauhan, S.; Jaggi, M.; Yallapu, M. M. Development of Zoledronic Acid-Based Nanoassemblies for BoneTargeted Anticancer Therapy. ACS Biomater. Sci. Eng. 2019, 5, 2343− 2354.

[10] Lublin, A.; Isoda, F.; Patel, H.; Yen, K.; Nguyen, L.; Hajje, D.; Schwartz, M.; Mobbs, C. FDA-approved drugs that protect mammalian neurons from glucose toxicity slow aging dependent on cbp and protect against proteotoxicity. PLoS One 2011, 6, No. e27762.

[11] Orlowski, P.; Zmigrodzka, M.; Tomaszewska, E.; RanoszekSoliwoda, K.; Czupryn, M.; Antos-Bielska, M.; Szemraj, J.; Celichowski, G.; Grobelny, J.; Krzyzowska, M. Tannic acid-modified silver nanoparticles for wound healing: the importance of size. Int. J. Nanomed. 2018, 13, 991−1007.

[12] Buzzini, P.; Arapitsas, P.; Goretti, M.; Branda, E.; Turchetti, B.; Pinelli, P.; Ieri, F.; Romani, A. Antimicrobial and antiviral activity of hydrolysable tannins. Mini-Rev. Med. Chem. 2008, 8, 1179−1187.

[13] Fernandez, O.; Capdevila, J. Z.; Dalla, G.; Melchor, G. Efficacy of Rhizophora mangle aqueous bark extract in the healing of open surgical wounds. Fitoterapia 2002, 73, 564−568.

[14] Choukroun J, Adda F, Schoeffler C, Vervelle A. Une opportunite´ en paro-implantologie: le PRF. Implantodontie 2001;42:55-62. (in French)

[15] Hotwani K, Sharma K. Platelet rich fibrin- a novel acumen into regenerative endodontic therapy. Restorative Dentistry and Endodontics 2014;39:1-6

[16] H. Y. Lee, C. H. Hwang, H. E. Kim and S. H. Jeong, Carbohydr. Polym., 2018, 186, 290–298.

[17] X. C. Du, L. Wu, H. Y. Yan, L. J. Qu, L. N. Wang, X. Wang, S. Ren, D. L. Kong and L. Y. Wang, ACS Biomater. Sci. Eng., 2019, 5, 2610–2620

[18] Dr. Suman Kar; Dr. R. R. Paul; Dr. H. D. Adhikari; Dr. Mousumi Pal; Dr. Pallab Datta; Tarun Shyam Mohan: CHARACTERIZATION OF A NOVEL CROSS LINKED PRF UNDER COMPRESSION INTENDED FOR PULP TISSUE ENGINEERING: VOLUME - 11, ISSUE - 10, OCTOBER - 2022 PRINT ISSN No. 2277 - 8160 DOI : 10.36106/gjra

[19] Tatiana C. Gamboa-Martínez, Victoria Luque-Guillén, Cristina González-García, José Luis Gómez Ribelles, Gloria Gallego-Ferrer: Crosslinked fibrin gels for tissue engineering: Two approaches to improve their properties; J Biomed Mater Res Part A: 103A: 614–621, 2015.

[20] M.C. Gruner, K.P.S. Zanoni, C.F. Borgognoni, et al., Reaching biocompatibility with nanoclays: eliminating the cytotoxicity of Ir(III) complexes, ACS Appl. Mater. Interfaces 10 (32) (2018) 26830–26834

[21] Lu Denga , Yanfang Qia , Zonghua Liua , Yun Xib , Wei Xuea: Effect of tannic acid on blood components and functions Colloids and Surfaces B: Biointerfaces :Volume 184, 1 December 2019, 110505