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Characterization of Leukocyte-Platelet Rich Fibrin, a Novel Biomaterial

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CHARACTERIZATION OF LEUKOCYTE-PLATELET RICH FIBRIN, A NOVEL BIOMATERIAL

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

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Acknowledgment

I would like to dedicate my master project to my wife Zeena and son Faisal who have supported and encouraged me throughout this journey. I would like to also thank Suyog Yoganarasimha.

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Abstract

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Autologous platelet concentrates represent promising innovative tools in the field of regenerative medicine and are successfully used in oral surgery. Several commercial systems exist that generate various forms of platelet concentrates including Platelet-rich plasma (PRP) and Platelet-rich fibrin (PRF). The alpha- granules of entrapped platelets release a variety of peptide growth factors that promotes healing. Usually PRP is a suspension that can be injected into the site of injury or used as a gel with the addition of thrombin (PRP-gel). In contrast Choukroun's L-PRF is a dense fibrin based biomaterial enriched with platelets and growth factors. The physical state of these natural biomaterials especially L-PRF permits manual handling and suturing onto the tissue bead to improve healing. However, our knowledge about the mechanical characteristic of L-PRF is quite limited and a good understanding of material properties will enable expansion of

current clinical applications. This study demonstrates the techniques to identify L-PRF's mechanical properties (uniaxial tensile testing and suture retention strength); morphology (scanning electron microscope); biological stability and cytocompatibility.

Objectives: This paper is intended to provide insights into basic attributes of L-PRF including its mechanical properties (uniaxial tensile testing and suture retention strength); morphology by scanning electron microscopy; biological stability and cytocompatibility.

Results: The mechanical properties were evaluated in two modes: uniaxial tensile testing and suture retention strength test. The results demonstrate viscoelastic behavior of L-PRF. Even though the elastic modulus is low (0.47 MPa), the membrane is tough (energy to break, 5 N.mm) and is capable of undergoing significant deformation (217%). Data from suture retention testing, an indicator of the ability of the membrane to be sutured to the tissues, suggested a significantly tough and deformable material (modulus-0.2 MPa, strain-140% and energy to break-3.2 N.mm) in L-PRF. One of the limitations of fibrin products in regenerative medicine is its short biological life. Made from endogenous fibrin, L-PRF is susceptible to enzyme degradation and undergoes fibrinolysis. In order to evaluate the resistance of L-PRF to enzyme-mediated degradation, fresh L-PRF was subjected to trypsin treatment (0.01%) and incubated at 37^{o}C. We observed complete degradation of L-PRF within three days. Genipin crosslinking of L-PRF membranes decreased degradation by almost 60%. The ability of L-PRF membranes to support cell growth was evaluated by culturing mouse calvarial osteoblasts on crosslinked and uncrosslinked membranes. Uncrosslinked scaffolds underwent degradation to various levels while the genipin crosslinked membranes retained their structure and supported cell growth.

Conclusion: Based on these findings, it is clear that L-PRF is a novel biomaterial with unique attributes: predictable preparation from autologous blood, simplicity of protocol, defined architecture, impressive mechanical properties and abundance of growth factors from activated platelets. The blood is allowed to clot under physiological conditions with no exposure to anti-coagulants, exogenous thrombin and calcium chloride. All of these characteristics make L-PRF promising biomaterial for applications in regenerative medicine.

INTRODUCTION

The use of blood and blood-derived products to seal wounds and improve healing in different clinical situations started with fibrin glues, which are mainly fibrinogen concentrates. Addition of platelets to fibrin glue not only improved their strength but also promoted neoangiogenesis and regeneration. These benefits are attributed to the release of a variety of peptide growth factors from the alpha-granules of platelets upon activation[1]. Platelet concentrates (PC) were seen as a practical way to deliver growth factors[2] and was strongly driven by commercial interests rather than research characterization[3]. In fact, PCs are difficult to characterize because unlike homogenous and defined pharmacological preparations, they are a potpourri of signaling molecules and blood cells (platelet and leukocytes) entrapped within a fibrin matrix. Different commercial and proprietary preparations yield a variety of PC that are different in cellular composition, growth factor recovery and kinetics of release[4].

It is important to realize that in most oral surgeries, platelet-rich plasma (PRP) preparations are used as a gel in open surgical wounds and not as platelet suspensions. In these situations, the gelation is induced by the addition of thrombin, calcium chloride, batroxobin or other agents and directly placed in the site of injury[5]. Due to rapid activation, fibrinogen polymerization is often incomplete and results in friable fibrin gels with very little mechanical strength. In addition, injectable PRP gels undergo rapid fibrinolysis[6,7].

In contrast, the processes of blood coagulation (fibrinogen polymerization), platelet enrichment and activation occur simultaneously in the preparation of L-PRF. The coagulation cascade is triggered when whole blood contacts the walls of a dry glass tube and continues throughout the centrifugation process. This results in the formation of a mechanically-strong blood clot (L-PRF) that can be surgically handled and used.

Even though L-PRF has been shown to have dense fibrin structure and delayed growth factor release profile[8], detailed mechanical characterization of these membranes are lacking. This is significant gap in knowledge, given the popularity of these membranes in clinical practice as well as its potential to be used as a biomaterial. Current study focusses on the protocol for deriving L-PRF as well as methods that can be employed to study its mechanical properties. This data is intended to serve as baseline for ongoing studies investigating the viscoelastic properties of this interesting natural biomaterial.

MATERIALS AND METHODS

Protocol:

All blood-drawing procedures should be done by licensed and certified professionals. Only one human subject was used for all experiments, therefore, IRB approval was not required. Special precautions regarding informed consent and protecting participant identification need to be followed. All experiments listed in this protocol involve handling of human blood and/or blood products and appropriate personal protective equipment need to be worn at all times. The waste should be considered as biohazard and disposed of according to regulations.

Preparation of L-PRF:

10ml of fresh blood samples were collected in glass tubes without anticoagulants from healthy volunteers (A). The samples were immediately centrifuged at 400g for 15 minutes using a table top centrifuge Hettich EBA 20 Supplied by Intra-Lock. The L-PRF clots were collected between the red corpuscles at the bottom and acellular plasma at the top of the tube (B). Thereafter they were gently compressed to form membranes (C).

Uniaxial tensile testing:

The mechanical properties of the L-PRF were characterized by uniaxial tensile testing. L-PRF membranes (n=6) were punched into "dog bones" (2.75mm wide at their narrowest point with a gauge length of 7.5mm), thickness of each sample was measured and tested on MTS Bionix 200 testing system with a 50N load cell (MTS systems corp.). The samples were stretched at a rate of 10.0mm/min. Elastic modulus, energy to break, and strain at break were calculated and recorded by MTS test works 4.0.

Suture retention strength:

L-PRF membranes (n=3) were cut into rectangular samples measuring (10x25mm), thickness of each sample was measured prior to testing. A pinhole was made in the center of the sample using a stainless steel orthodontic ligature wire of 220μm thick. The ligature wire was passed through the pinhole forming a loop and was then fixed to the tensile testing machine (MTS systems Corp.). The opposite edge of the sample was fixed to the bottom jaw of the testing machine[10]. The ligature wire was pulled at a rate of 10.0mm/min. Energy to break, Elastic modulus and strain at break were calculated and recorded by MTS test works 4.0.

Morphological examination:

The surface morphology of L-PRF was studied by scanning electron microscopy (SEM). They were punched into discs using a 10mm biopsy punch (Acu-punch) and placed in 24 well plate for further processing. Samples were washed with PBS and fixed with 2.5% glutaraldehyde (in PBS) for 20 minutes and dehydrated in ethanol solutions of 50%, 70%, 80%, 90% and 100% for 5 minutes each. Followed by drying with 100% hexamethyldisilazane (HMDS) (Sigma Aldrich) for 3 minutes; excess HMDS was removed and samples aerated overnight[11]. They were mounted on stubs and sputter coated with platinum. Morphology was examined with JEOL LV 5610 SEM operating at an acceleration voltage of 20KV.

Cross-linking percentage with ninhydrin:

The cross-linking percentage of L-PRF and genipin cross-linked L-PRF were determined using the ninhydrin assay. To prepare genipin cross-linked L-PRF, membranes were soaked in 4ml of 1% genipin solution in 6 well culture plate for 48 hours and subsequently washed with PBS[12,13]. Both samples were punched into discs using a 10mm biopsy punch and heated with 1ml of 2 % (w/v) ninhydrin for 15 minutes at 100^{o}C, the solution was cooled to room temperature and diluted with 1.5ml of 50% ethanol. Thereafter, 200µl aliquots were pipetted into a 96-well plate and the absorbance was analyzed in a Bio-Tek Synergy 2 micro plate reader at 570nm. A Glycine standard (1mM-0.031mM) curve was also generated to establish the relationship

between free amino acid concentration (FAA) and absorbance. Cross-linking percentage was determined using the formula below[12].

$$Concentration\ of\ free\ NH_2Groups\ = \left(\frac{FAA\ Conc\ \times\ NH_2\ molecular\ weight}{sample\ weight}\right)$$

$$Degree\ of\ Crosslinking$$

$$= \left(\frac{(free\ NH_2\ Groups)PRF - \ (free\ NH_2\ Groups)G - PRF}{(free\ NH_2\ Groups)PRF} \times 100\right)$$

Biodegradation with trypsin:

L-PRF and genipin cross-linked L-PRF (n=3) were placed in 500µl of 0.01% trypsin and incubated at 37^{o}C for 3 days, the trypsin solution was refreshed every 24 hours to ensure enzyme activity. Samples were weighed at day 1 prior to enzyme application and at day 3. The difference in start and end weight represents enzymatic degradation.[14]

MTS cell proliferation assay:

To study cell proliferation on crosslinked and uncrosslinked clots, viable cells were determined by using the colorimetric MTS assay. The principle being that, metabolically active cells will react with tetrazolium salt in the MTS reagent to produce a soluble formazan dye that can be spectrophotometrically read at 490nm. MC3T3s (mouse calvarial preosteoblasts) were

trypsinized and the number of cells were counted with a hemocytometer. $4x10^5$ cells in a suspension of 300µl were seeded into 10mm cloning rings placed on the top surface of clots in 24 well plate. Cloning ring were removed after 24hours of cell culture. On Day 4 MTS assay was carried out. The culture medium (αMEM) was changes at a frequency of 3 days. The cellular constructs were rinsed with PBS thrice for a duration of 5 minutes followed by incubation with 20% MTS reagent in serum free media for 2h. Thereafter, 200µL aliquots were pipetted into a 96-well plate and the samples were analyzed in a Bio Tek Synergy 2 micro plate reader at 490nm.

RESULTS

The mechanical properties were evaluated in two modes: uniaxial tensile testing and suture retention strength test. The results demonstrate viscoelastic behavior of L-PRF. Even though the elastic modulus is low (0.47 MPa), the membrane is tough (energy to break, 5 N.mm) and is capable of undergoing significant deformation (217%, Figure 5). Data from suture retention testing, an indicator of the ability of the membrane to be sutured to the tissues, suggested a significantly tough and deformable material (modulus-0.2 MPa, strain-140% and energy to break-3.2 N.mm) in L-PRF.

The Scanning Electron Microscope image of the L-PRF clot at different sections (top, middle and bottom) layer is illustrated in **Figure 6**. As can be seen, the top left portion is composed predominantly of fibrin network with no cells. The top right is enriched with platelets with various degree of activation and degranulation. The lower left shows a buffy coat with numerous leukocytes. The lower right has a mixture of leukocytes and red blood cells entrapped within a fibrin matrix.

Degradation of L-PRF membranes following incubation in 0.01% trypsin. All L-PRF membranes disintegrated completely in trypsin within 3 days while genipin crosslinked L-

PRF were 60% more stable. This shows that chemical crosslinking can be a viable strategy to improve the longevity of L-PRF membranes when placed in vivo **(Table 1).**

The ability of L-PRF membranes to support cell growth was evaluated by culturing mouse calvarial osteoblasts on crosslinked and uncrosslinked membranes. Uncrosslinked scaffolds underwent degradation to various levels while the genipin crosslinked membranes retained their structure and supported cell growth (Table 2).

DISCUSSION

Autologous platelet concentrates are promising in the field of regenerative medicine[15] because of the abundance of growth factors. However, these preparations often lacked a defined structure that makes surgical manipulation very difficult. Many times, the suspensions and gels are not retained effectively at the site of delivery, resulting in unpredictable outcomes. L-PRF represents a huge advance in the evolution of platelet concentrates in that it is essentially a firm fibrin membrane with entrapped platelets. These solid membranes possess excellent handling characteristics, and can be securely sutured at an anatomically desired location during open surgeries. However, its physical and biological properties are relatively unknown.

The L-PRF will form consistently when steps described above are strictly adhered to (Figure 1). One of the important considerations in generating a good L-PRF membrane is the time delay between blood draw and centrifugation. The success of L-PRF technique entirely depends on the speed of blood collection and immediate transfer to the centrifuge[16], usually within a minute. It is impossible to generate a well-structured L-PRF clot (with its specific cell content, matrix architecture and growth factor release profile), if blood harvesting is prolonged and not homogenous; a small incoherent, friable mass of fibrin with unknown content is formed instead.

It has been accepted that mechanobiological interactions between cells and extracellular matrix (ECM) have a critical influence in all aspects of cell behavior including migration, proliferation and differentiation[17,18]. L-PRF, a unique type of blood clot, is formed under specific circumstances and is comprised of complex, branched network of fibrin. L-PRF functions as a provisional ECM that is turned over into functional tissue during healing. Being subjected to mechanical forces, successful healing outcomes are dependent on the structural integrity of L-PRF and hence elucidating their physical properties is important. We performed uniaxial tensile testing (to identify the intrinsic material properties) and suture retention testing (to identify the failure characteristics) on fresh L-PRF. Unlike PRP gel or clotted blood that does not have a defined structures, L-PRF resembles dense connective tissue with superior handling characteristics. We report an elastic modulus of 0.470 MPa (SD=0.107) for L-PRF membranes and stretch twice its initial length before failure (strain of 215%). These data match with published literature[19,20] who reported low stiffness (1-10 MPa) and high strain (up to 150%) before breaking. The difference in the values can be due to the use of fibrin network compared to the use of AFM analysis of single fibrin fiber in the above mentioned studies.

Suture retention strength is a surgically important parameter of graft materials and it is defined as the force necessary to pull a suture from the graft or cause the wall of the graft to fail. Our experiments used straight-across procedure (as defined by ANSI[21]). The force required to the pull the ligature wire through the L-PRF of 3.23 N.mm (SD=0.329). Overall, we found the L-

PRF to be mechanically tough, capable of supporting loads and the ability to stretch twice as much on tension and retains sutures quite well (deforms significantly before tearing).

The lack of stability and structural integrity of L-PRF in biological environments is a major limitation in its use in tissue engineering. We sought to address this issue by chemically crosslinking L-PRF using genipin. Unlike gluteraldehyde which is associated with toxicity, genipin is a naturally occurring biodegradable molecule with low cytotoxicity. After genipin treatment, the membranes were significantly stable in trypsin and supported cell proliferation over 4 days. However, only 20% of L-PRF was crosslinked with genipin (determined by ninhydrin assay). This data suggests that while chemical crosslinking is a viable strategy, other alternatives need to be explored.

Based on these findings, it is clear that L-PRF is a novel biomaterial with unique attributes: predictable preparation from autologous blood, simplicity of protocol, defined architecture, impressive mechanical properties and abundance of growth factors from activated platelets. The blood is allowed to clot under physiological conditions with no exposure to anti-coagulants, exogenous thrombin and calcium chloride. All of these characteristics make L-PRF promising biomaterial for applications in regenerative medicine.

One of the clinical issues to deal with in the application of L-PRF is the heterogeneity in the quality of platelets and blood components. At present, very little is understood about L-PRF

generated from patients with coagulation disorders or patients on medications that affect blood clotting (heparin, warfarin or platelet inhibitors). Answers to these questions will undoubtedly improve our understanding of healing as well as contribute to advance the field of personalized medicine.

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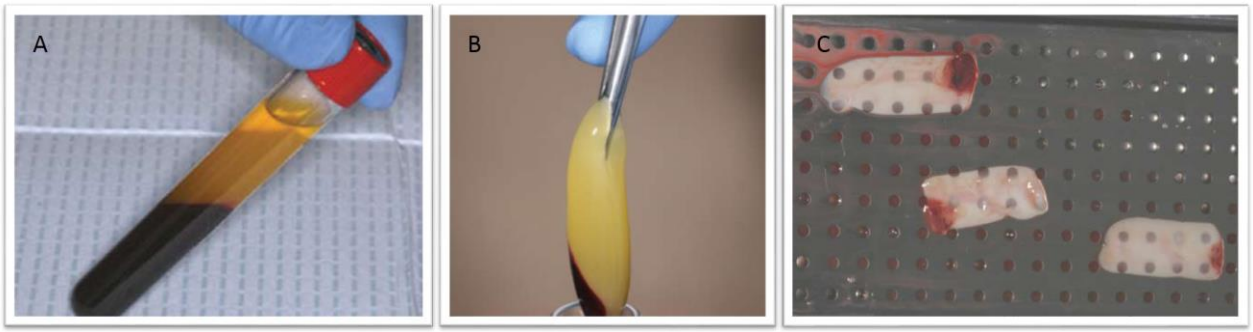
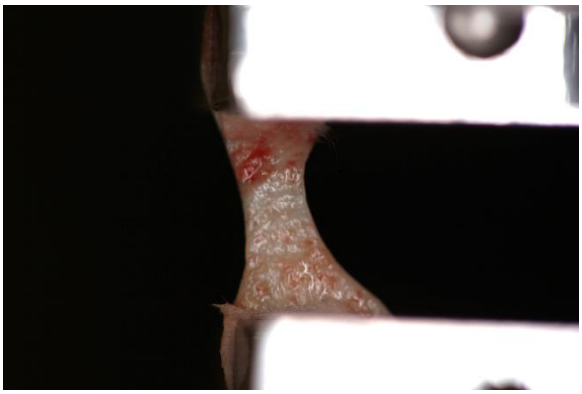
Figure 1: Stages of PRF preparation



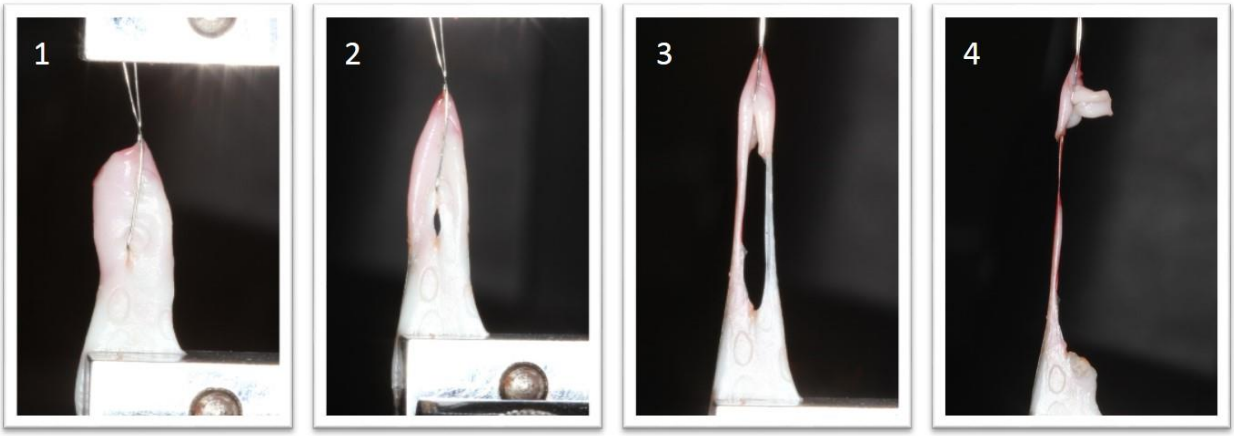
Immediately centrifuged at 2700rpm for 12 minutes **(A)**. PRF clots separated from RBC and PPP **(B)**. Gently compressed to form membranes **(C)**

Figure 2: Uniaxial tensile testing on PRF membrane.



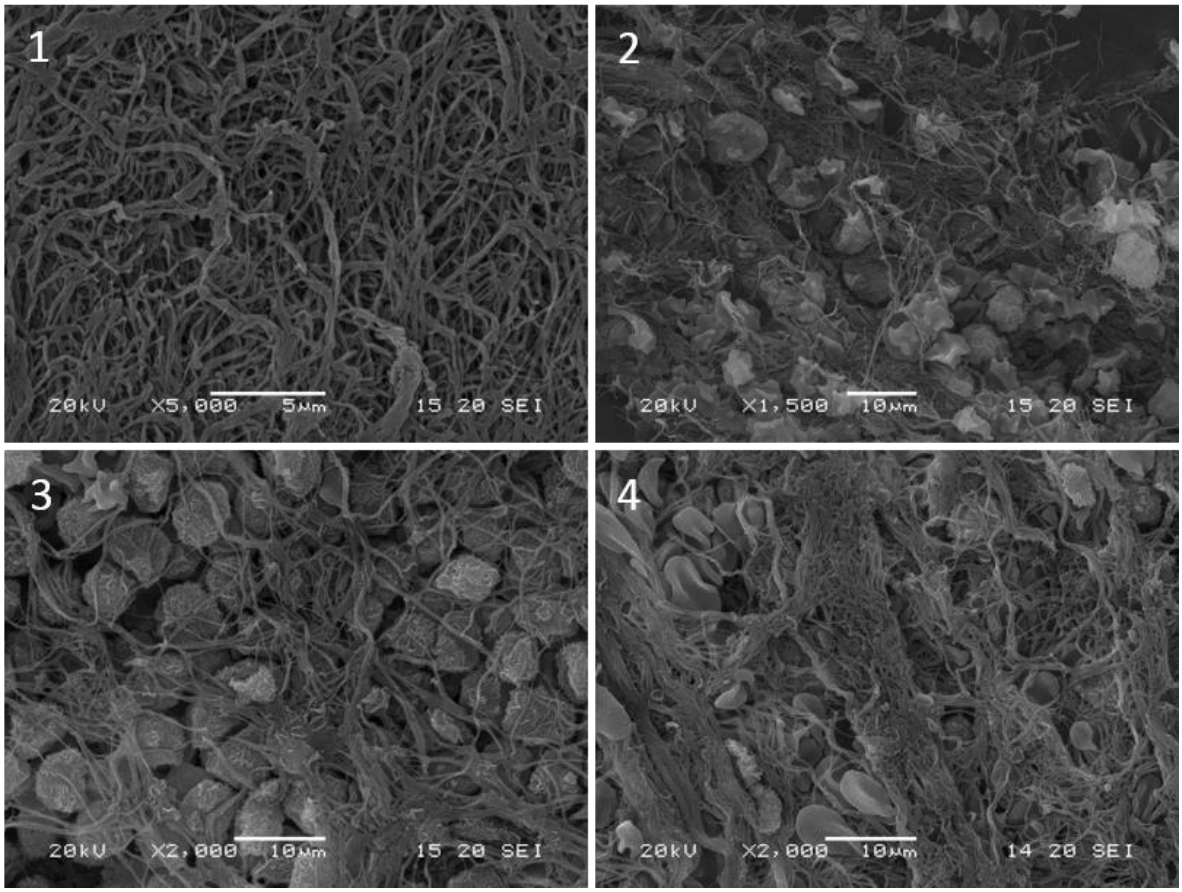
An illustration of the initial rest condition wherein PRF membrane cut into a dog bone is securely held between the grips of tensile testing system.

Figure 3: Photographs of suture retention strength testing.



1. denotes the initial rest condition **2**, **3** & **4** depicts the elongation of the membrane due to the applied tension and PRF's resistance to tear.

Figure 4: SEM Image of different layers of PRF



1 represents the fibrin-rich layer; **2** a zone of enriched platelets with various degree of activation;

3 buffy coat with numerous leukocytes and **4** the red blood cell base.

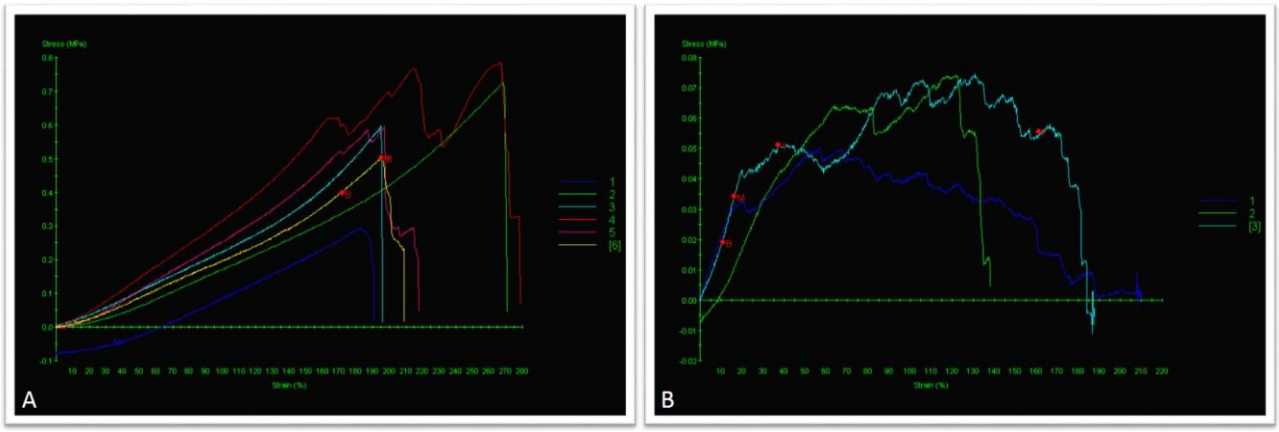
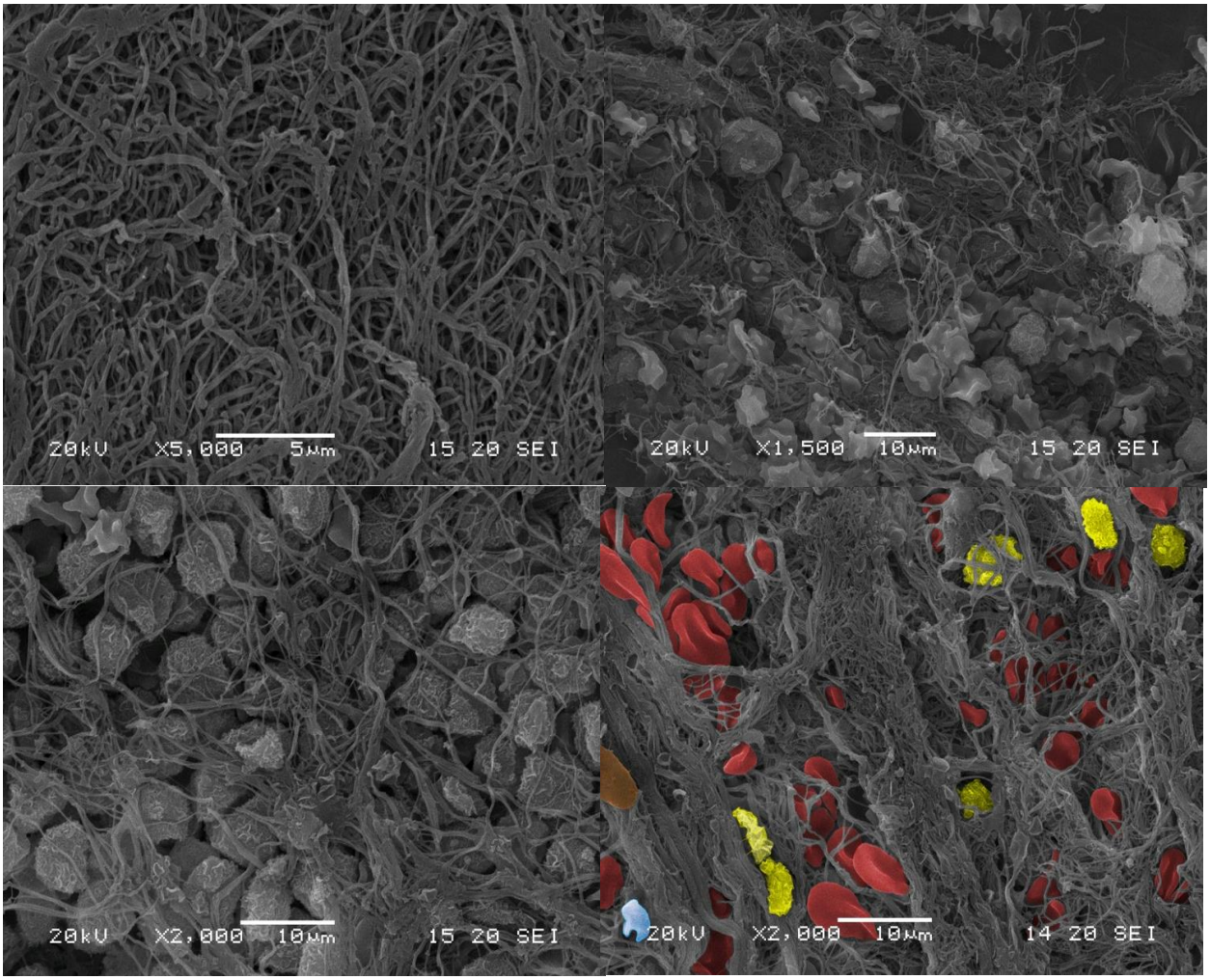


Figure 5: Stress-strain curves following mechanical loading of PRF in uniaxial tensile testing mode (A) and Suture retention strength (B). The loading pattern of each sample is represented in different color.



The scanning electron microscope image of the PRF clot at different sections are illustrated (**Figure 6**).

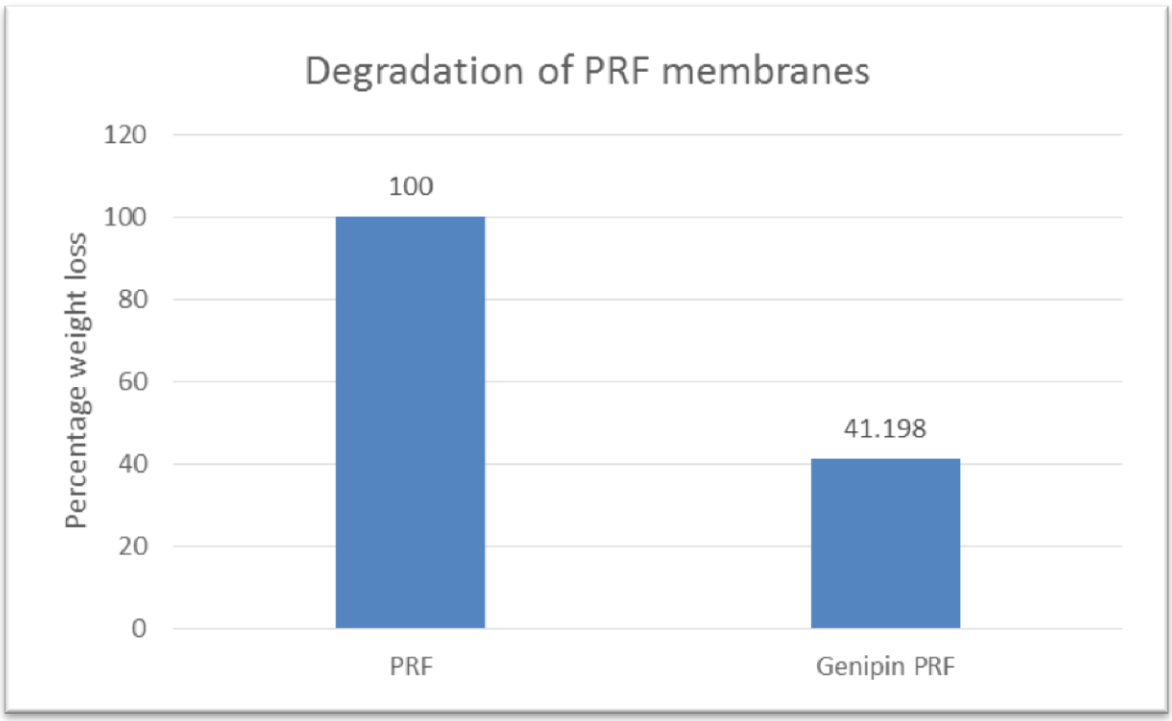


Table 1: Degradation of PRF membranes following incubation in 0.01% trypsin. All PRF membranes disintegrated completely in trypsin within 3 days while genipin crosslinked PRF were 60% more stable. This shows that chemical crosslinking can be a viable strategy to improve the longevity of PRF membranes when placed in vivo.

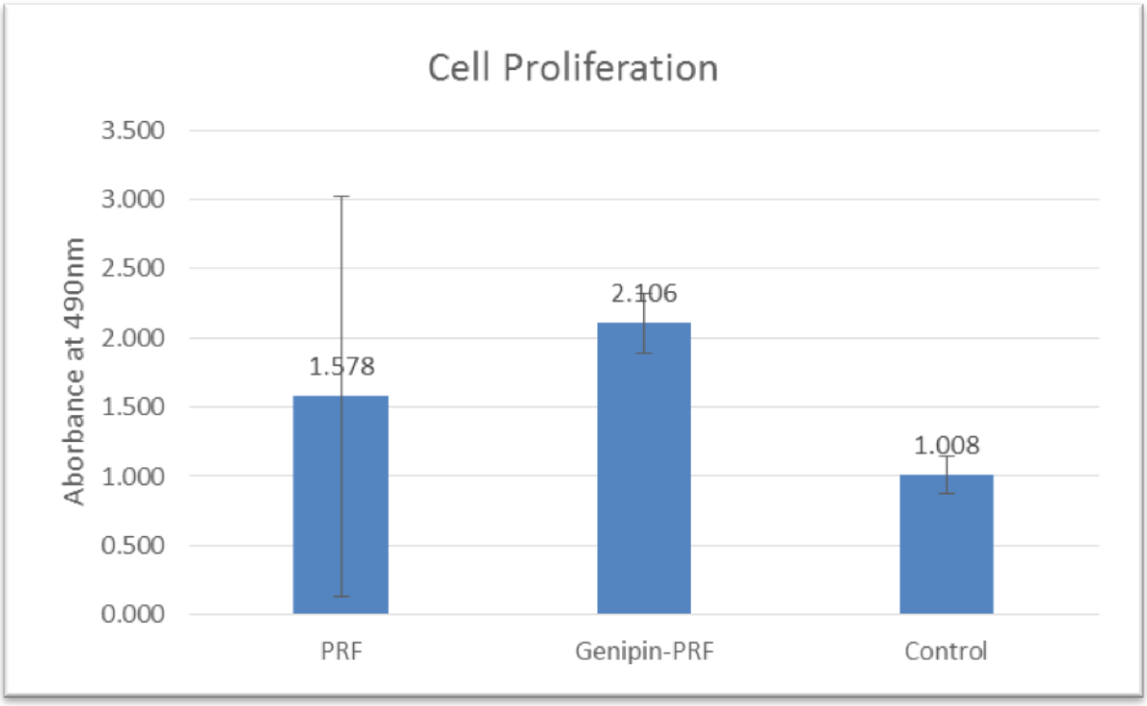


Table 2: Effect of PRF crosslinking on cell viability. Representative images of 4 day culture of MC3T3 cells on uncrosslinked L-PRF (A), genipin-crosslinked L-PRF (B) and tissue culture plastic (C). Uncrosslinked L-PRF degraded in culture to variable extent and showed cell activity similar to plastic. Genipin crosslinked L-PRF maintained their structure and supported robust cell survival. To the left is quantified data from independent experiments with three replicates.