Title
Assessment of the phenotypic effects of Platelet Rich Fibrin on Mesenchymal Stem Cells derived from Minced Pulp

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Publication Date
2020

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Assessment of the phenotypic effects of Platelet Rich Fibrin on Mesenchymal Stem Cells derived from Minced Pulp

A thesis submitted in partial satisfaction of the

requirement for the degree Master of Science

in Oral Biology

by

Gautam Mahendra Shirodkar

2020

ABSTRACT OF THE THESIS

Assessment of the phenotypic effects of Platelet Rich Fibrin on Mesenchymal Stem Cells derived from Minced Pulp

by

Gautam Mahendra Shirodkar

Master of Science in Oral Biology

University of California, Los Angeles, 2020

Professor Mo K. Kang, Chair

Abstract:

Our aim is to investigate the effects of autologous platelet-rich fibrin (PRF) on Mesenchymal Stem cell derived from Minced Pulp (MP-MSCs). We first developed a mouse model of PRF to study the phenotypic effects of PRF in cultured cells. We obtained PRF from the blood and prepared PRF-enriched culture media. The phenotypic effects of PRF on MP-MSCs were determined by assessing the changes in cell proliferation, differentiation and immunophenotypic profiling. The mRNA levels of ALP, OCN, DMP1 and DSPP were determined by qRT-PCR. It was found that PRF increased the proliferation capacity of MP-MSCs and reduced the cell doubling time. With PRF exposure, the MP-MSCs were able to retain their immunophenotypic characteristics defining them as MSCs, as the cells expressing surface markers CD105, CD146

and CD73 were higher. MP-MSCs were able to undergo osteogenic differentiation in the presence of PRF and the mRNA levels of OCN was significantly increased in the presence of PRF. To assess the odontogenic differentiation of cells in response to PRF, we prepared dentin-slice model in which we cultured MP-MSCs embedded in PRF. Histological sections of the dentin slice model revealed that there was increase in the cellularity of the pulp tissue along the edges of the pulp tissue. Based on our findings, PRF can act as a source of growth factors cell proliferation, migration and differentiation.

The thesis of Gautam Mahendra Shirodkar is approved.

Wei Chen

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Mo K. Kang, Committee Chair

University of California, Los Angeles

2020

Acknowledgement

I would like to sincerely thank my mentor **Dr. Mo Kang** for his guidance throughout my training at UCLA School of Dentistry. His support, mentorship and guidance has been instrumental in my educational and career development, and I will always be grateful to him.

I would also like to thank **Dr. Wei Chen** for his support with my educational pursuits and his invaluable inputs and feedback in the development of my postgraduate project.

I would like to thank my lab members and dear friends - **Abdullah Alshaikh**, **Chaehwan Lee, Jaeyoung Lee and Dhruvi Patel** for working alongside me, helping me learn and develop new skills and sharing their expertise with me in order to help me complete my postgraduate work.

I would like to thank my family, *Aaie, Baba and Aajie* for their unconditional love and support. Without them, I would not be where I am today, and I will always be indebted to them. Special gratitude goes to my fiancé, Sayali, for trusting in me, encouraging and supporting me through these years. Last but not the least, my best friends - Sanjay and Saurabh who have always been my major source of support.

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1. Introduction

Dental caries, trauma are common causes of the dental pulp exposure, infection or necrosis. Depending of the extent of carious involvement or fracture of a tooth, different treatment approaches are employed. Typically, if caries or trauma involves the dental pulp and if the tooth is restorable, endodontic treatment is commonly done. Persistent research in improving treatment outcomes from endodontic therapy has increased the success rate, yet few drawbacks still exist. One potential problem is that the teeth receiving the root canal therapy lose their vitality and there is lack of nutrition from the dental pulp. In the case of a developing tooth with immature apices, the physiological growth and development of root is arrested. Furthermore, during the process of a root canal treatment, dentin is lost during the preparation of the root canal; this leads to thinning of the root canal wall and thus reduces its fracture resistance. The regeneration or replacement of the dental pulp tissues affected by caries, trauma, or infectious diseases is expected to solve many dental problems. Newer scientific advances emerging from regenerative techniques aim at improving the treatment modalities in the current endodontic techniques, thereby improving the treatment options for the very patients we serve.

According to the glossary of Endodontic terms, regenerative endodontics is defined as - Biologically-based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex. Regenerative procedures aim at regeneration of the pulp-dentin complex, regenerate the lost coronal dentin and continue the physiological growth of the developing root apices and maintain the tooth in its healthy state.

Regenerative endodontics applies the concept of the of tissue engineering - stem cells, scaffold and bioactive growth factors in the canal space to regenerate the pulp tissue damaged by

infection, trauma or developmental anomalies[1]. The concept of tissue engineering technology has proven to be an effective method in the pulp/dentin regeneration. The stem-cell-based approach has demonstrated that pulp/dentin tissues can be regenerated in the emptied root canal space with blood supply[2,3,4,5]. To identify a suitable cell population for this goal, many odontogenic stem cells, including dental pulp stem cells(DPSCs)[6], periodontal ligament stem cells(PDLSCs)[7], stem cells from human exfoliated deciduous teeth (SHED) [8] , and stem cells from apical papilla (SCAP)[9], and some non-odontogenic stem cells, including adipose-derived stem cells (ADSCs)[10], bone marrow mesenchymal stem cells (BMSCs)[10,11], embryonic stem cells (ESCs)[12], neural crest cells (NCCs)[13] have been selected for screening. Studies have demonstrated that all odontogenic stem cells have a certain degree of multipotency in vitro and form pulp-dentin complexes combined with scaffold materials, in vivo. Among these cell types, postnatal DPSCs have the most potential as stem cells for endodontic tissue regeneration[14,15,16]. The transplantation of DPSCs with tooth slices or scaffold materials in vivo has demonstrated that DPSCs can differentiate into odontoblasts and further form the structure of pulp-dentin complexes[14,3]. As one of the key components required for pulp regeneration is an ideal scaffold which provides space for cells to grow, proliferate and differentiate. An ideal scaffold should be biocompatible, non-allergic, biodegradable and should not produce toxic byproducts. Various types of synthetic scaffolds are being used in regenerative procedures which includes hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder[6,14], Hydrogels[17,18] Polydioxanone II[19], Collagen[20], Poly(L-Lactic acid) PLLA[21]. These materials have been shown to promote growth and differentiation of mesenchymal stem cells, however, these are non-autologous and have certain limitation when it comes to availability, technique sensitivity, physical and chemical properties, and biodegradability.

Platelet Rich Fibrin (PRF) was first reported by Choukron et al. in 2001. It is a three-dimensional protein gel obtained by the direct centrifugation of autologous peripheral blood and is thus considered an autologous graft[22]. The main advantages of PRF is that it contains host immune defense cells (leukocytes) which act to fight infection. Furthermore, PRF was initially developed with high centrifugation speeds allowing a fibrin clot to form which may be utilized as a three-dimensional scaffold to further speed the healing of bone and gingival tissues[22]. PRF is reported to be non-toxic or non-allergic, it is easy and inexpensive to prepare and carries no risk of cross-infection or associated ethical issues[23]. In addition, as a scaffold material, PRF can release cytokines and growth factors that promote tissue regeneration. PRF can therefore not only utilize its mechanical effects to postpone platelets, but can also chemically associate with small molecules, such as various cytokines present in the circulation and glucose glycosaminoglycans, resulting in slow and sustained cytokine release during PRF degradation[24]. Together, this suggests that PRF may be the ideal material for tissue regeneration. The PRF matrix can also promote endothelial cell proliferation and angiogenesis, promoting healing at wound sites[25].

Previous studies from our lab have proposed pulp tissue grafting for regeneration of the dental pulp. Direct transplantation of the minced dental pulp allows us to bypass the in vitro cell expansion of DPSCs. We demonstrated that human minced pulp tissue can yield MSCs and these cells retained the odontogenic and osteogenic differentiation capacities. In the pulp tissue grafting model, pulp tissue and MP-MSCs will be a part of the recipient tooth and might undergo time-dependent changes[26].

In the present study we hypothesize that Platelet Rich Fibrin (PRF) acts a reservoir of growth factors and can provide the necessary framework for the MP-MSCs to proliferate and

differentiate so as to regenerate the pulp-dentin complex. The main objective of the study is to understand the effects of PRF on proliferation and differentiation of MP-MSCs.

2. Materials and Methods

2.1 Sample collection and primary culture of MP-MSCs

Freshly extracted human mature non-carious teeth were collected from the Oral Surgery Clinic at UCLA School of Dentistry. The age of the patient ranged between 12-21 years. After extraction, the teeth were stored in sterile Falcon tubes containing a-MEM medium (Invitrogen, Carlsbad, CA), 3% Antibiotic-Antimycotic (Life Technologies, CA). The sterile tubes were kept on ice until being transferred to the biosafety cabinet for dental pulp isolation, which was completed on the same day of extraction.

After thoroughly washing the teeth with Phosphate Buffered Saline, the attached gingival tissue was removed. The teeth were inspected to check for closed apices, fractures, caries or external resorption. Once the teeth were confirmed healthy, then they were used for experimental purposes. With the help of a wire cutter, the teeth were cracked open at the cemento-enamel junction. With sterile tweezer, the pulp was carefully retrieved and placed onto a 60mm petri dish with primary culture medium for at least 30 minutes. Using sterile micro-scissors, the pulp was minced into fine pieces ($<1\ mm^{3)}$ and transferred to a new 48 well plate with fresh primary culture medium. The primary culture medium was made using a-MEM medium containing 20% fetal bovine serum (FBS) (Invitrogen), 15 mg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA), and 20 mmol/L L-glutamine (Invitrogen). Dishes were incubated in 80% humidity, 5% carbon dioxide atmosphere at 37 degrees Celsius. When 80% confluency was reached, the finely minced dental pulp tissues were transferred to new dish with fresh primary culture medium. MP-MSCs were then maintained in basal medium, α-MEM (Invitrogen,

Carlsbad, CA) with 10% FBS (Invitrogen) and 5 μg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA).

2.2 Primary culture of mouse Gingival Mesenchymal Stem Cells (mGMSCs)

The palatal gingiva of the mice was harvested using standard aseptic protocol. After separation of the gingiva, the tissue was washed with PBS and was minced into fine pieces. The tissue was digested with a solution of 3 mg/ml collagenase (type I) supplemented with 4 mg/ml dispase (both from Sigma-Aldrich) in 2 ml α-minimum essential medium (Invitrogen, Carlsbad, CA) for 60 min in a cell incubator. Then, the cells were passed through a 70-μm strainer to obtain single-cell suspensions. The cells were plated into 60-mm plates and cultured in α-MEM (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen) and 5 μg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA) at 37°C in a humidified atmosphere of 5% CO2 and 95% air to obtain passage 0 (P0) single-cell-derived clones. Cell cultures at P3 were used for the following in vitro study.

2.3 Obtaining Platelet Rich Fibrin

The mice were deeply anesthetized using Isoflurane prior to blood sample collection procedure. The mice will be placed in dorsal recumbency once the animal has reached an appropriate plane of anesthesia. An appropriately 27-gauge needle attached to a syringe was insert beveled up at a 30-40° angle through the diaphragm, with syringe parallel to the midline of the mouse. The insertion of the needle was slightly left of and under the sternum, directed toward the mice head. After retraction of the plunger to create a vacuum inside the syringe, the needle was advanced until blood flash appears in needle hub until a sufficient amount of blood was collected. Approximately 1ml of blood was collected from each mouse and was collected in an

Eppendorf tube. The mice will be euthanized immediately upon completion of blood collection by Cervical dislocation.

Immediately after blood collection, it was centrifuged at 2700g for 12 mins at 4°C. PRF was separated from the tube carefully using cotton pliers and was soaked on a sterile gauze. It was used to make PRF-enriched media containing α-MEM (Intvitrogen, Carlsbad, CA) with 5% FBS (Invitrogen) and 5 μg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA). We standardized the PRF-enriched media was incubating PRF obtained from 1ml of blood with 1ml of media.

2.4 MTT Cell Proliferation assay

$1x10^4$ MP-MSCs from P3 were seeded in 96-well plates; the cells in the experimental group were treated with PRF-enriched media and the control group was treated with basal media. The MTT assay was used to investigate cells proliferation assay, (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) on Day 3, Day 5 and Day 7. The MTT Reagent (ATCC) was then added following manufacturer instructions. After incubation for 2-4 hours, the Detergent Reagent (ATCC) was added and mixed gently. Following incubation for 4-24 hours, the absorbance was measured with Synergy ^{TM} Microplate Reader at 570 nm. The results were later analyzed statistically. The experiment was performed in triplicates and repeated 3 times.

2.5 Immunophenotypic Characterization of MP-MSCs in the presence of PRF

Cells were pelleted and resuspended in flow buffer which consists of PBS with and 1% BSA. The Cells were incubated with different concentrations of antibody according to the manufacturer's method. The antibodies against CD105, CD146, CD73 were used for the experiment.

2.6 Cell Migration assay

Cells were seeded in transwell inserts with 8 μm pores (Corning, NY) and the well of the cell culture plate were filled with a-MEM with 1% FBS. After 24 hours and 48 hours, the non-migrated cells inside the insert were removed with a cotton swab and cells migrated outside the insert were stained with 1% crystal violet.

2.7 Odontogenic differentiation assay

MP-MSCs from different tissue passages were seeded in 6-well plates at $3x10^5$ per well. Before they reached 80% confluency, odontogenic differentiation was induced using odontogenic induction medium, which is basal medium conditioned with 100 μmol/L L-ascorbic acid 2-phosphate (Sigma, St Louis, MO), 9mmol/L KH2PO4, 10 mmol/L β-glycerolphosphate, and 9.8 nmol/L dexamethasone (Sigma, St Louis, MO). Control group cells were maintained in basal medium. The medium was changed every two days.

At day 3, 7 and 14, the cells were stained with Alkaline Phostphatase (ALP) Staining Kit (Sigma, St. Louis, MO). Cells were first washed with Dulbecco's phosphate buffered saline (DPBS) (ThermoFisher), they were then fixed with 1:2.5 acetone/citrate for 30 seconds. Following fixation, cells were washed with distilled water for three times. Mixed 1 mL of Fast Violet B Salt with 1 mL of sodium nitrite solution for 2 minutes. Then, 1 mL of Naphtol AS-MX phosphatase alkaline solution was added with 48 mL of distilled water. Then, 2 mL of the mixture was added to each well. The cells were incubated for 10 minutes in the dark. Each group was seeded in triplicates and the experiment was repeated 5 times. The results are then observed under the microscope and photographs or the wells are taken.

Equivalent number of cells were seeded in a 6-well plate for Alizarin Red Staining. Triplicate of each cell passage were seeded for control group that were maintained in basal medium. Odontogenic induction medium was used to maintain the induction group for 14 days. Both medias were changed every two days. At day 14, cells were washed with DPBS and fixed using 10% naturally buffered formalin for 30 minutes. The samples were later washed with sterile water before staining with 40 mM Alizarin red (pH 4.2) for 45 minutes at room temperature with gentle rotation. To remove Alizarin Red staining, cells were rinsed for five times with distilled water. The results are then observed under the microscope and photographs or the wells are taken. Each group was seeded in triplicates and the experiment was repeated 3 times.

2.8 Reverse transcription and quantitative real-time PCR (qPCR)

One group of MP-MSCs from P3 treated with PRF-enriched media and the other group was treated with basal media were allowed to reach 70-80% confluency before total RNA was isolated using TRIzol reagents (Invitrogen). Chloroform was added and the samples were centrifuged for 15 minutes. The aqueous layer containing RNA was preserved. Isopropanol was added to allow formation of RNA precipitate. Total RNA samples were then washed with ethanol twice. NanoDrop Spectrophotometer (ThermoFisher Scientific) was used to measure total RNA and assess its quality. Amplification of cDNA was then completed with SYBR Green I Master Mix (Roche), utilizing the LightCycler 480 II real-time PCR system (Roche), following manufacturer's instructions. Thermal conditions were set at 95°C at 10 minutes (initial denaturation), followed by 45 cycles of 95°C for 10 seconds. Lastly, 58°C for 45 seconds and 72°C for 10 seconds (denaturation). The internal control used was GAPDH and all samples were triplicated. Second derivative of the Cq value was determined by comparing the gene of interest

to GAPDH for fold-differences of amplification as recommended by manufacturer's protocol (Roche). The experiment was repeated twice.

2.9 Dentin Slice Model

The cemento-enamel junction of the tooth was cut horizontally with diamond bur to obtain dentin slices with 1 mm thickness. The dentin slices were left in 70% alcohol overnight and autoclaved before use. The pulp space of the tooth was filled with PRF gel and minced pulp tissue was place in the PRF gel and in contact with the tooth. These dentin slices were placed in primary media for 3 weeks. No minced pulp tissue was placed in the control group. The dentin slices were washed with PBS before fixation with 10% natural buffered formalin for 24 hours. Tissues were further washed with sterile water and embedded in paraffin. Five nanometer sections were made with a microtome and tissue were mounted onto slides. Histological slides were placed in a conventional oven to for deparaffinization. They were later rehydrated with xylene washing for two times, followed by serial-dilution of ethanol (100% twice, 95% twice, and 70% once). For hematoxylin and eosin staining, slides were washed with running tap water for 5 minutes, stained with hematoxylin for 2.5 minutes. Slides were subsequently rinsed with 95% ethanol and stained with eosin for 1 minutes. Moreover, slides were washed with running tap water then dehydrated with serially diluted ethanol (70% once, 95% twice, and 100% three times). They were finally washed with xylene for three times before mounting. H&E staining was also performed using Sigma-Aldrich staining kit as the protocol recommended.

2.10 Statistical analysis

Statistical analyses were performed with an unpaired Student's t test for 2 groups using Prism 6 software (GraphPad Software, La Jolla, CA, USA). A value of $p<0.05$ was considered as a statistically significant difference. The graphs represent mean values and include SD unless stated otherwise.

3. Results

3.1 Establishing a mouse model of Platelet Rich Fibrin

Platelet-rich fibrin (PRF) is a new generation of platelet concentrates which can be obtained from whole blood and does not involve any biochemical handling, like treatment with anti-coagulants. In the current study, we have used mouse blood which was obtained via cardiac puncture.

After centrifuging the blood at 3500 rpm for 12 mins at 4°C in an Eppendorf tube, three distinct layers were seen (Fig 1): the bottom layer was made up of Red Blood Corpuscles (RBCs), the top layer was Acellular plasma and the middle layer was Platelet Rich Fibrin (PRF) (Fig 1A). It was a gel like matrix and approximately 0.1 – 0.2 ml of PRF gel was obtained for 1ml of blood. After obtaining the gel like matrix, the plasma fluid was squeezed out using a sterile gauge and the RBCs were cleaned from the PRF. (Fig 1B)

Hence, using the standard protocol for obtaining PRF from human blood, we were able to obtain PRF from mouse blood. We used the PRF enriched media to perform all in vitro experiments.

3.2 Exposure of cultured MP-MSC cells to PRF enhanced the cell proliferation potential

MSCs were obtained from minced dental pulp when the tissues were maintained in primary media in 2-3 weeks. The obtained cells were maintained in basal culture media and used

for experiments. All experiments were performed with MSCs from early passage cells (P_{1-3}) and triplicated within each experiment. Also, every experiment was repeated for three times.
The palatal gingiva from mice was harvested aseptically and was subjected to tissue digestion using dispase and collagenase. 3 days onwards clusters of cells were seen adherent to the culture plate. The cells have spindle-shaped morphology and were plastic adherent. The cells were detached and passed so as to disrupt the clusters and provide space for the cells to grow. The cells grew after detachments and continued to display growth and proliferation.

The effect of PRF on cell proliferation was assessed using MTT assay. It is a colorimetric assay which uses MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and is based on the conversion of MTT into formazan crystals by living proliferating cells, which determines mitochondrial activity. The cell proliferation rate of MP-MSCs treated with PRF enriched media was compared with the proliferation rate of MP-MSCs treated with basal media. Statistically significant difference was seen when the proliferation rate of the two groups were compared on day 3,5 and 7 (Fig 2A). The MP-MSCs treated with PRF enriched media showed higher proliferative capacity on each day. Similar results were obtained from MTT assay when GMSCs obtained from the mouse gingiva were treated with PRF. There was statistically significant difference in the proliferation rate of the two groups were compared on day 3,5 and 7 (Fig 2B).

The population doubling is to the total number of times the cells in the population have doubled since their primary isolation in vitro. This is an estimate rounded off to the nearest whole number. The doubling time required by the MP-MSCs when treated with PRF was lower than the time required by MP-MSCs when treated with basal media (Fig 2C). The doubling time was steady in the case of PRF, however, it kept fluctuating in the control group. The population doubling units were higher when the cells were treated with PRF. This means that cell count was

higher in the PRF treated group. This data suggests that PRF lead to an increase in the proliferation rate of MP-MSCs.

3.3 Exposure of cells to PRF increased the number of cells expressing mesenchymal stem cell markers.

To characterize the immunophenotype of MP-MSCs in the presence of PRF, we measured the expression level of cell surface marker by flow cytometry. CD105, also known as endoglin, is a type I membrane glycoprotein that functions as an accessory receptor for TGF-beta superfamily ligands. CD146 is identified as an endothelial cell marker with a role in cell-matrix interaction and angiogenesis. CD73 is also identified as one of the markers of the mesenchymal stem cells. We performed flow cytometric analysis on MP-MSCs treated with PRF and analyzed the CD146, CD105 and CD73 expression. There was an increase in the expression of these markers. This data suggested that PRF led to an increase in the stemness of the cells. (Fig 3)

3.4 PRF enhanced the migration capacity MP-MSCs.

MSCs are characterized with their ability to migrate as they are desired to move into tissue to perform their roles. Therefore, sufficient migration capability of MG-MSCs is important for successful tissue regeneration. Migration ability MP-MSCs in the presence of PRF was evaluated by Transwell assay (Fig 4). There was increase in the number of migrated cells in the PRF group as compared to the control group. This suggests MP-MSCs can migrate towards PRF and can proliferate to further undergo differentiation.

3.5 PRF maintains the osteogenic differentiation potential of MP-MSCs.

To investigate the effect of PRF on the osteogenic differentiation capacities of MP-MSCs, ALP staining was performed as ALP is an early marker for osteoblasts. Staining was performed on Day 0, 3, 7 and 14. The staining intensity was higher on Day 14 as compared to day 3 and day 7 (Fig 5A).

Alizarin Red Staining is used to measure the mineralization and it stains free calcium and certain calcium compounds. The intensity red staining for calcium compounds increased over a period of time and was higher on Day 14 (Fig 5B). Quantification ARS absorbance reveled a statistically significant difference between the staining intensity on Day 7 between the cells treated with PRF and the untreated cells (Fig 5C). This experiment was also performed on mouse GMSCs. The mGMSCs were able to undergo osteogenic induction from as reveled for ARS staining for Day 3 (Fig 5D). The quantification ARS absorbance reveled a statistically significant difference between the staining intensity on Day 7 and 14 between the cells treated with PRF and the untreated cells. The absorbance was higher for than the human MP-MSCs (Fig 5E). Hence, our data suggest that PRF maintains the mineralization potential of MP-MSCs.

3.6 mRNA expression patterns of genes with odontogenic differentiation.

To study the odontogenic differentiation capacity, we measured the mRNA expression of four well-known genes involved in dentinogenesis. The mRNA expressions of OCN, the markers for early stage of dentinogenesis, were significantly higher in the PRF treated group than the basal media group (Fig 6B). In particular, mRNA expression of OCN increased significantly from day 7. The mRNA expression of DMP1 and DSPP, markers for late stage of dentinogenesis also increased from day 7. There was no significant difference between the mRNA expression of ALP, DMP1 and DSPP when the cells were treated with PRF, however, the PRF did not alter the

expression of these genes. This data suggests that MP-MSCs are capable of undergoing odontogenic differentiation in the presence of PRF.

3.7 Minced pulp tissue shows evidence of cell migration towards the periphery of the tissue in the presence of PRF.

We established a dentin slice model using minced pulp tissue and PRF (Fig 7). Primary data obtained from these dentin slices after H&E staining revealed PRF tissue which was seen as a fibrous meshwork. Increased cellularity was observed in the human dental pulp tissue. Increased concentration of cellularity in the periphery of the minced pulp tissue rather than the center was noted at serial tissue passages (FIG 8A, B, C, D) However, PRF did not show abundant cellularity (FIG 8E).

4. Discussion

The aim of regenerative endodontics is to replace damaged endodontic structures: pulp connective tissue with its vascularization, its innervation and its peripheral dentin. The modality of conventional endodontic treatment involves removal of all damaged pulp tissues from teeth and their replacement by inert material after adequate disinfection. For many years, this drastic treatment has been considered as the unique treatment without alternative strategies. Stem cells, growth factors and scaffold are important factors for regeneration and hence our study aims at understanding the effects on Platelet Rich Fibrin on the stem cells. Previously, it was demonstrated that MSCs derived from minced pulp have the potential to regenerate the dental pulp tissue.[26.]

PRF is a promising scaffold for endodontics regeneration as it can provide a scaffold and source of growth factors for the MP-MSCs to proliferate. We were able to obtain PRF gel from

mice blood in a similar pattern like PRF is obtained from human blood although the quantity of blood obtained from mice blood was very low. To study the effects of PRF on MP-MSCs, we prepared a PRF enriched media as described previously[27]. We first compared the effects of PRF on the proliferation rate of MP-MSCs. Previous studies have shown that PRF induces a more gradual and sustained release of growth factors[28]. It has been shown that within the first 10 min, up to 70% of growth factors are released to the surrounding environment with nearly 100% released within the first hour[28]. These growth factors, despite being released at early time points, have a significant influence on the cellular behavior of many cell types[29,30,31]. Our data from MTT assay with MP-MSCs reveled that the growth factors present in PRF were able to act upon the cells and there was a significant increase in the proliferation rate of MP-MSCs. To understand if the effect on human cells from growth factors released from mice PRF was valid, we performed MTT assay on gingival MSCs isolated from mouse palatal gingiva. We were able to see similar results, and this validated our finding that PRF increased the proliferation rate of MP-MSCs. PRF also decreased the time required by the cells to multiple and this could be owed to the presence of growth factors from PRF. Higher migratory phenotype is one of the major characteristics of MSCs because of the nature of MSCs that must move to wound tissue to perform their roles[32]. Compared to cell-based therapy where cultured MSCs are transplanted, the source of MSCs in tissue-based therapy is the migrating cells from the transplanted tissues. Therefore, sufficient migration capability of MG-MSCs is important for successful tissue regeneration. Our data suggested that MP-MSCs showed higher migration towards PRF and this suggests that tissue-based therapy using minced tissue may also have sufficient therapeutic efficiency when supplemented with PRF matrix. PRF also led to an increase in the markers associated with MSCs. There was an increase in the cells expressing CD73, CD105 and CD146.

Osteogenic differentiation capacity of stem cells is more important for pulp regeneration than the other types of differentiation capacity because of the similarity between odontoblast and osteoblast. Dentin and bone produced by odontoblasts and osteoblasts are very similar in composition, and the protein expressions in the process of producing the mineralized matrix are also very similar, such as OCN and ALP[33]. We performed ALP staining and quantified the mRNA expression levels of ALP. There was no significant difference in the ALP expression when the cells were treated with PRF. After inducing mineralization in MP-MSCs over a period of 14 days, there was increase in the mineral deposits in the PRF group. This experiment was also performed with mGMSCs and similar results were obtained.

While understanding the limitations of our study where we have used PRF from mice blood and used it for experiments with human pulp tissue, we basically tried to establish a new experimental model. To further confirm if the effects of mouse PRF were valid, we isolated gingival MSCs from mouse and performed few experiments using these cells. We were able to find similar results as seen with human MP-MSCs. Further experiments with human PRF are necessary to confirm our findings.

Revascularization is a technique by which bleeding is induced in the pulp canal space which aids in regeneration of the dental pulp. However, there were numerous drawbacks to this procedure. Studies revealed that there was ingrowth of periodontal connective tissue instead of pulpal connective tissue[34]; another study showed cementum and bone-like tissue[35,36]. It is also reported that there is breakdown of the blood clot in the pulp canal space, leaving no scaffold for new tissue to grow into the empty canal space[37]. Histologic studies of revascularized teeth in animal showed that the tissue growing in the pulp space was fibrous soft connective tissue[38]. Hence the use of a PRF as a scaffold along with minced pulp tissue graft proves to be a very promising treatment approach for regenerative endodontics. PRF can be a source of growth

factors and can also be a scaffold for the cells to grow and differentiate. This will limit the growth of ectopic tissues like bone, cementum and lead to the growth of tissues required for pulp regeneration.

There are still many challenges in the application of Minced pulp for dental pulp regeneration. However, for those patients with wisdom teeth or whose teeth have to be extracted for orthodontic purposes, autologous pulp could be obtained from the extracted fresh teeth. Meanwhile, the use of PRF could reduce the cost of dental pulp regeneration by avoiding the application of commercial exogenous growth factors. Thus, the present investigation paves the way for further studies to regenerate dental pulp in clinical settings by utilizing autologous or allogeneic cells in combination with autologous PRF.

In conclusion, this study provides new insight into dental pulp regeneration using PRF. Our results show that PRF can act as a source of growth factors cell proliferation, migration and differentiation. Intracanal transplantation of the minced pulp and PRF constructs could effectively promote the regeneration of dentin-pulp-like structures.

5. Figures and Legends

Figure 1. Obtaining PRF from mouse blood

(A) Three distinct layers were seen: the bottom layer was made up of RBCs, the top layer was acellular plasma and the middle layer was Platelet Rich Fibrin (PRF).

(B) About 0.1 – 0.2 ml of PRF was obtained from 1ml of blood

(C) Schematic representation of the preparation of PRF-enriched media

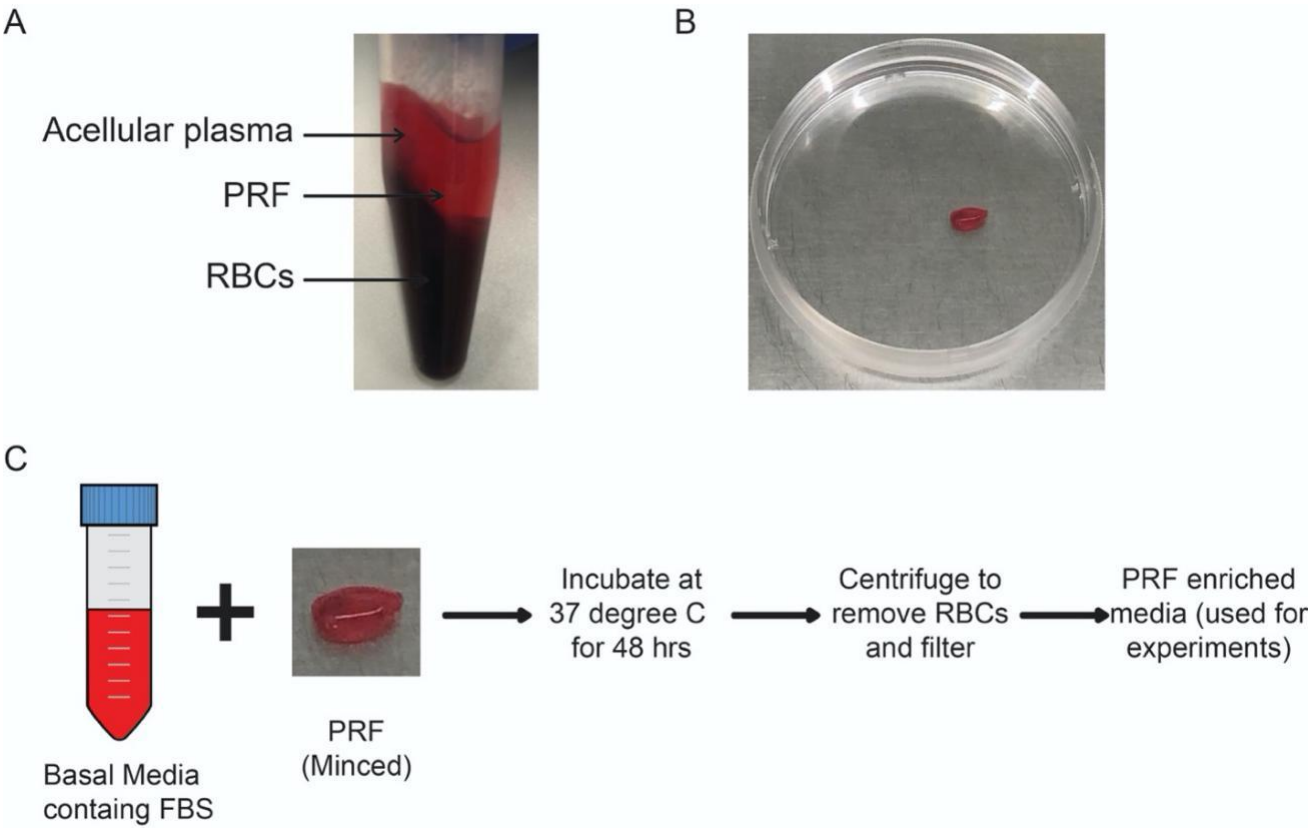


Figure 2: PRF increased the cell proliferation potential of MP-MSCs.

(A) MTT Assay for cell proliferation comparing the proliferation rate of MP-MSCs in the presence and absence of PRF.

(B) MTT Assay for cell proliferation comparing the proliferation rate of mGMSCs in the presence and absence of PRF.

(C) Comparison of population doubling time and population doubling units of MP-MSCs in the presence and absence of PRF.

* $p<0.05$, Data are expressed as the means ± SD of 5 determinations.

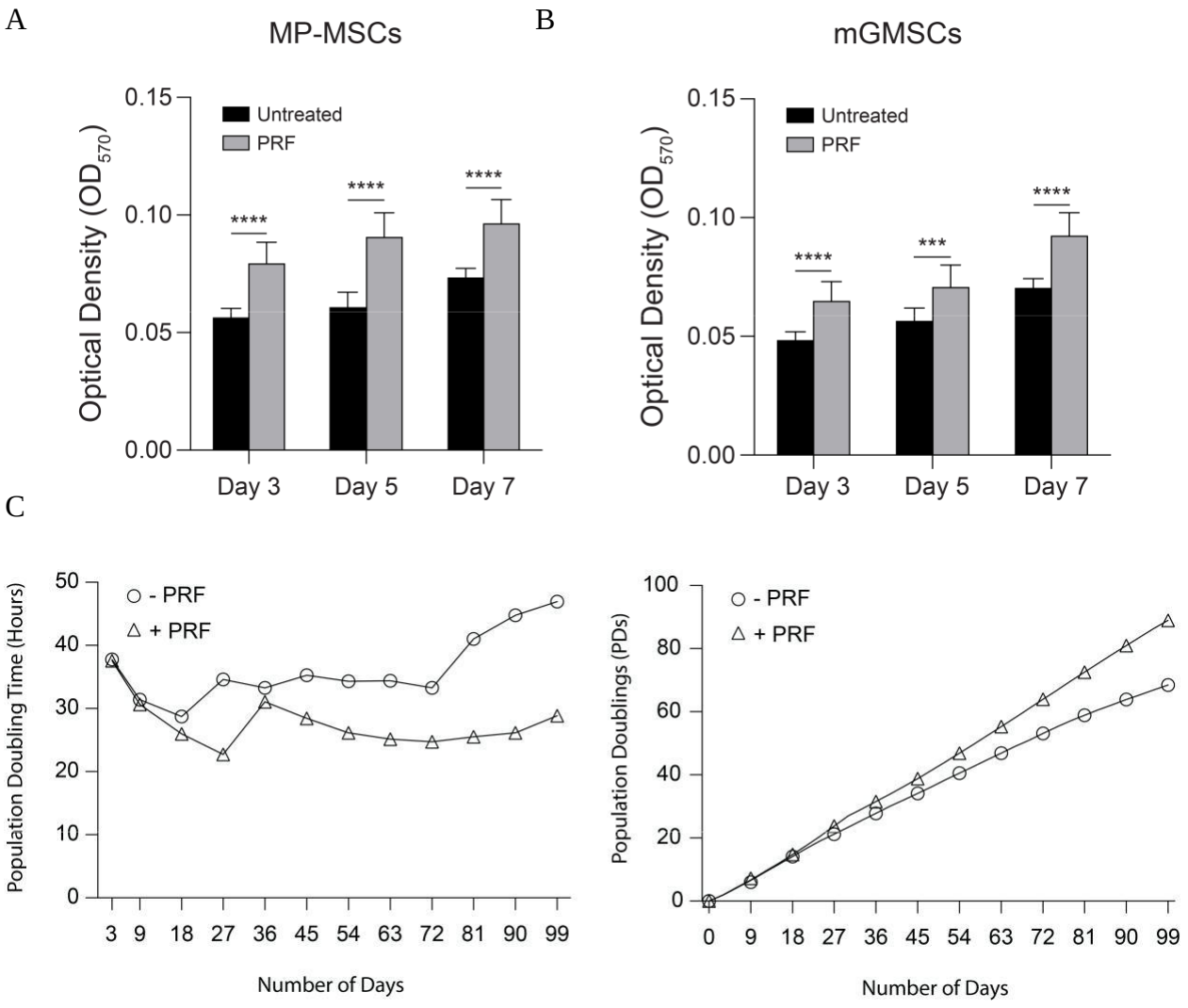


Figure 3: Immunophenotypic profile of MP-MSCs in the presence of PRF.

Flow cytometry analysis was performed to measure the expression of cell surface markers of MP-MSCs in the presence of PRF.

(A) Negative control FITC and PE; (B) CD73; (C) CD146 and (D) CD105

Graph pattern of each cell surface marker.

(B) A graph to compare the expression level of surface markers of each cell line.

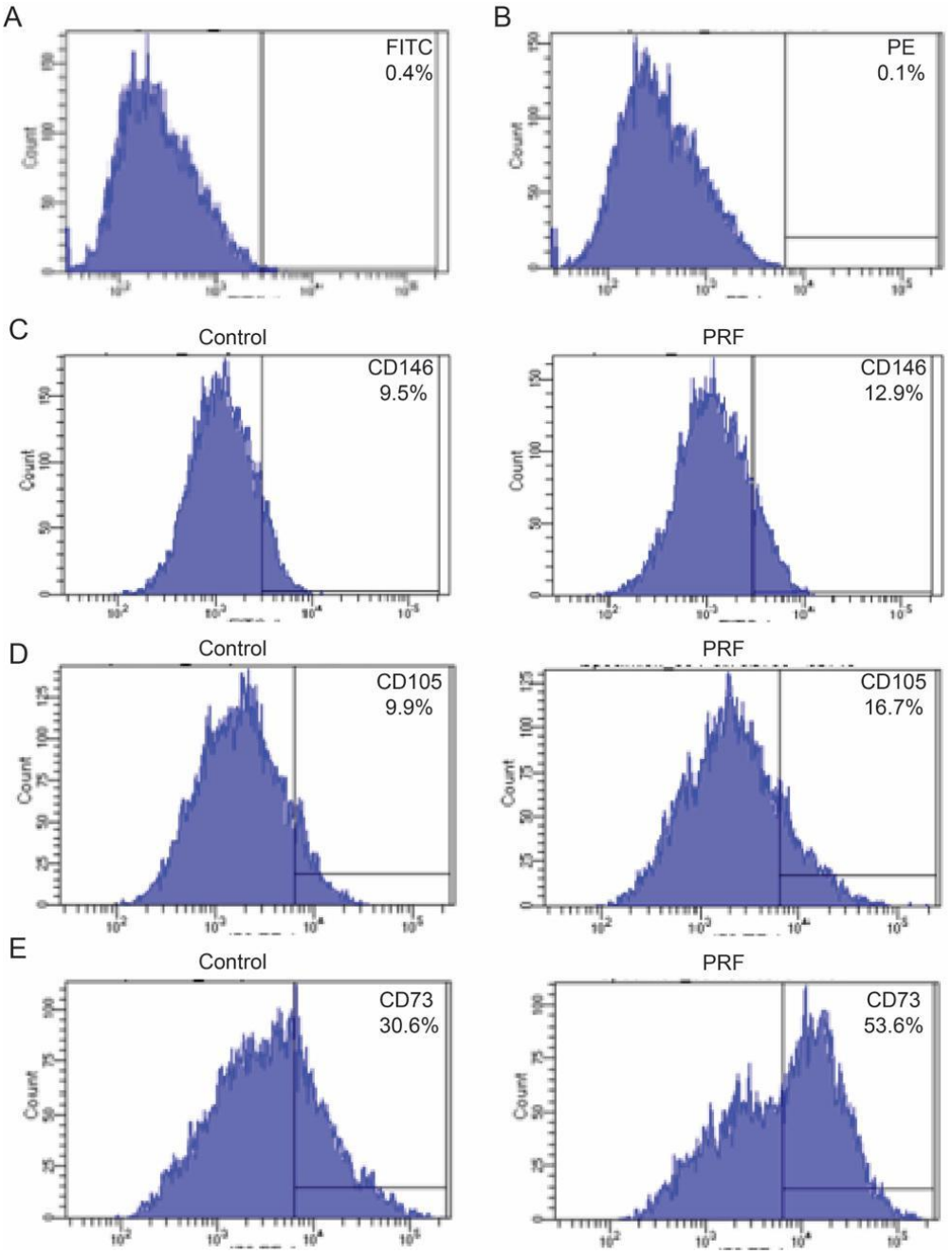


Figure 4.

Migration capacity of MP-MSCs in the presence of PRF.

The migration capacities of MP-MSCs were measured uding transwell migration assay after 1 day and 2 days of cell seeding.

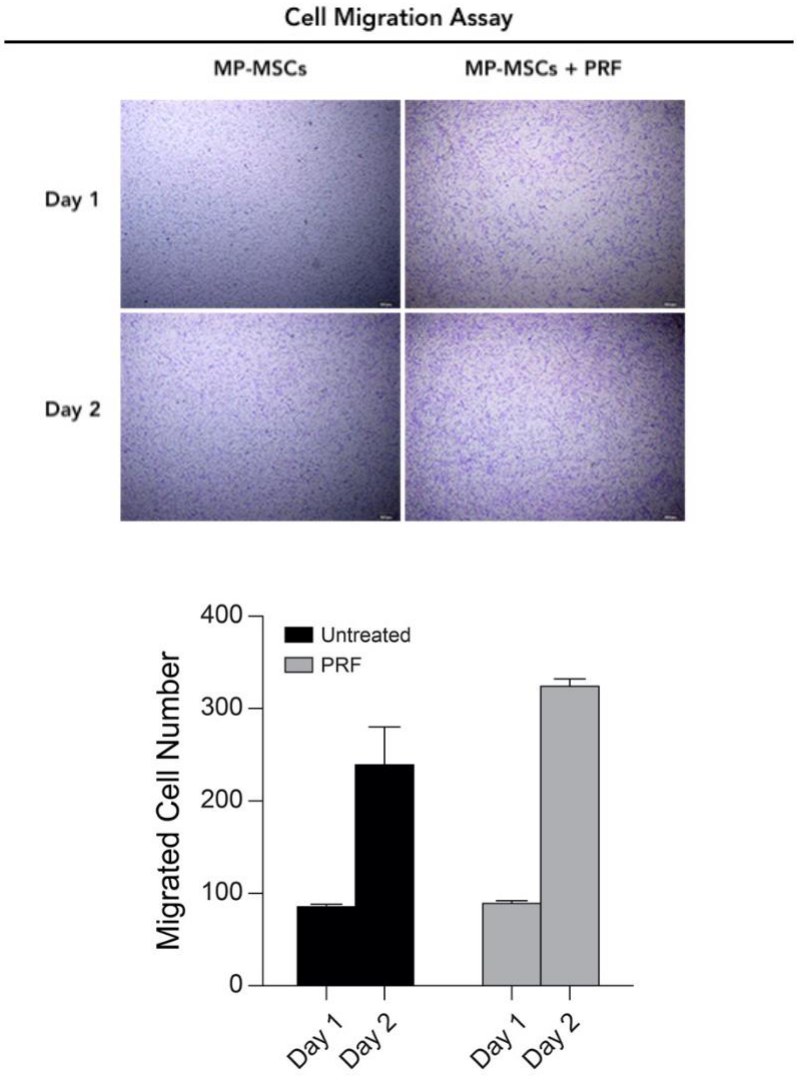


Figure 5: Osteogenic/odontogenic differentiation capacity MP-MSCs when treated with PRF

(A) Alkaline phosphatase (ALP) staining after treatment of osteogenic induction media or basal media for 3, 7 and 14 days.

(B) Alizarin red staining after treatment of osteogenic induction media on Day 0, 3, 7 and 14 in MP-MSCs in the presence or absence of PRF.

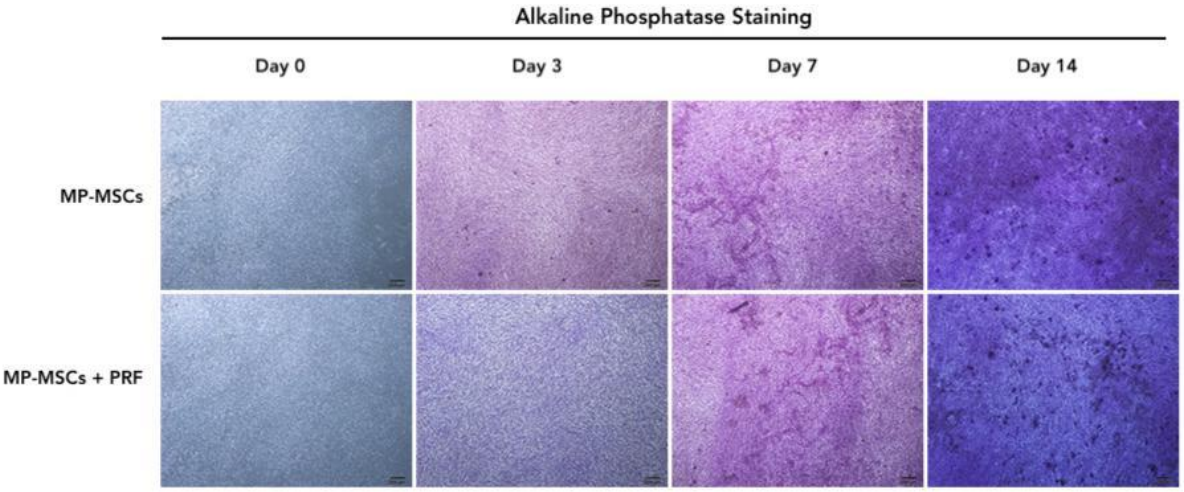
(C) ARS absorbance measured by spectrophotometer with 405nm wavelength. * $p<0.05$, Data are expressed as the means ± SD of 3 determinations.

(D) Alizarin red staining after treatment of osteogenic induction media on Day 0, 3, 7 and 14 in mGMSCs in the presence or absence of PRF.

(D) ARS absorbance measured by spectrophotometer with 405nm wavelength. * $p<0.05$, Data are expressed as the means ± SD of 3 determinations.

(E) Magnified microscope view of dense mineralization area of MP-MSCs and mGMSCs.

A



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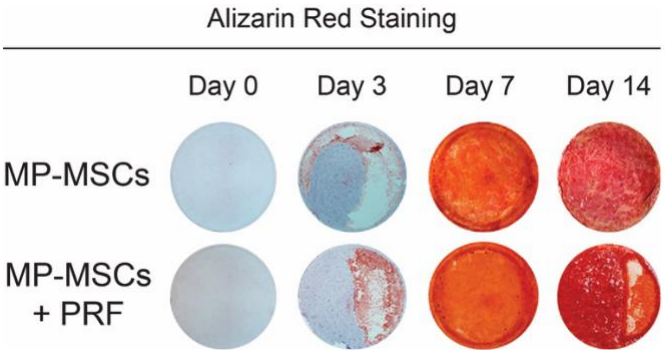
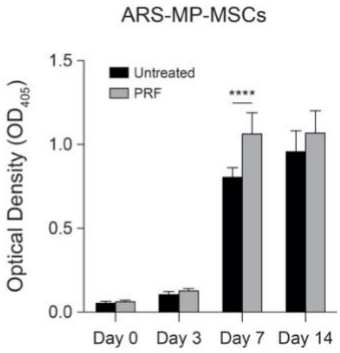
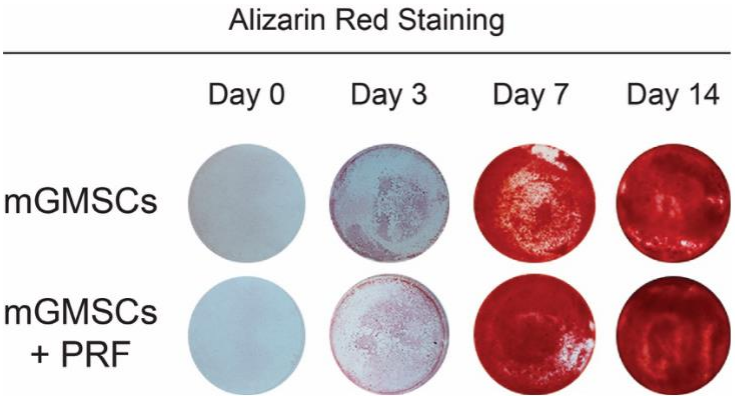

Alizarin Red Staining
Day 0
Day 3
Day 7
Day 14
MP-MSCs
MP-MSCs + PRF

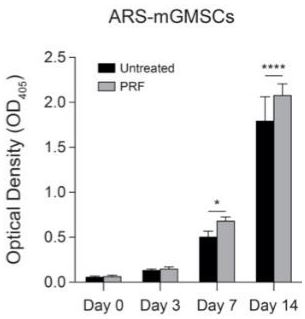
C


ARS-MP-MSCs
Untreated
PRF
****
Optical Density (OD405)
1.5
1.0
0.5
0.0
Day 0
Day 3
Day 7
Day 14

D


Alizarin Red Staining
Day 0
Day 3
Day 7
Day 14
mGMSCs
mGMSCs + PRF

E


ARS-mGMSCs
Untreated
PRF
****
*
Optical Density (OD405)
2.5
2.0
1.5
1.0
0.5
0.0
Day 0
Day 3
Day 7
Day 14

E

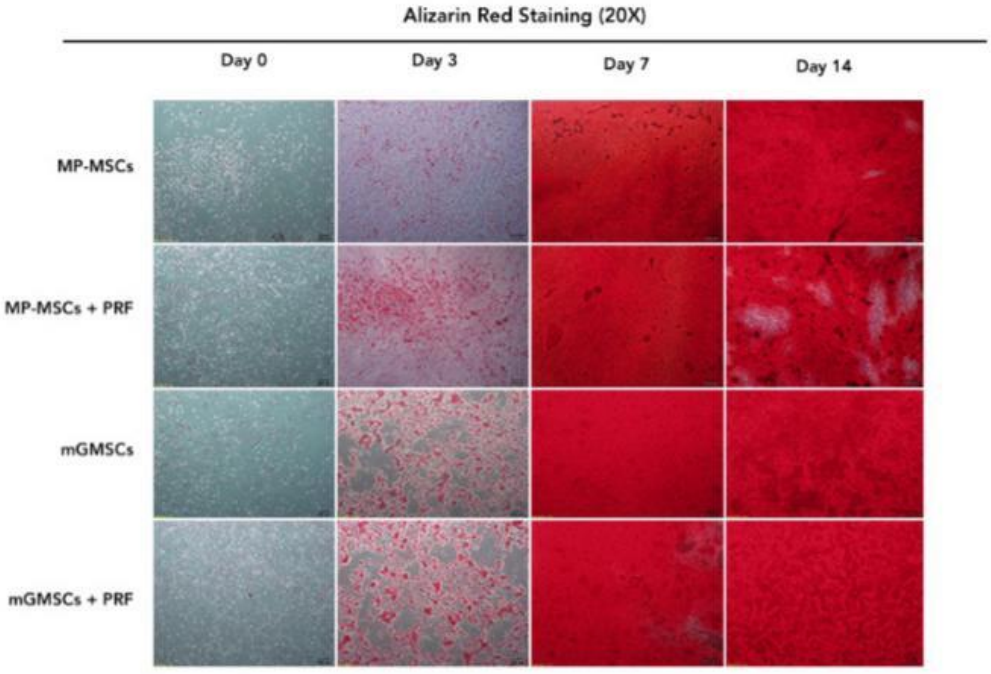
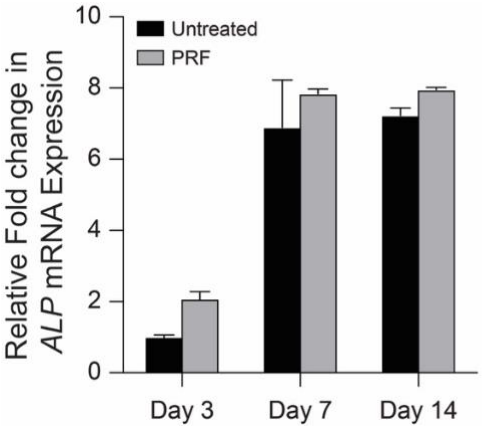

Alizarin Red Staining (20X)
Day 0
Day 3
Day 7
Day 14
MP-MSCs
MP-MSCs + PRF
mGMSCs
mGMSCs + PRF

Figure 6: mRNA expression patterns of genes associated with osteogenic/odontogenic differentiation.

(A) Expressions of osteogenic/odontogenic genes: Osteocalcin (OCN) Alkaline Phosphatase (ALP) when MP-MSCs were treated with and without PRF.

* $p<0.05$, Data are expressed as the means ± SD of 3 determinations.

A



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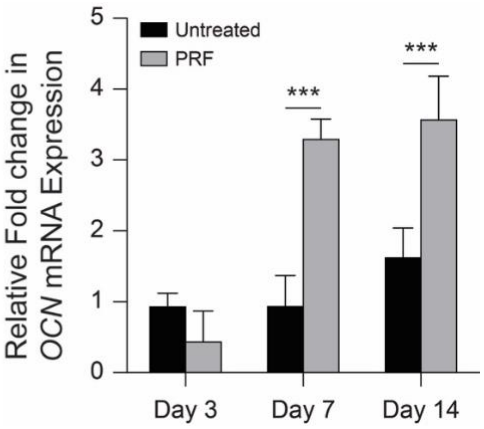

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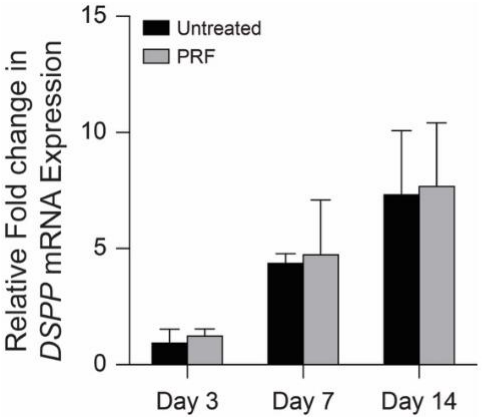


D

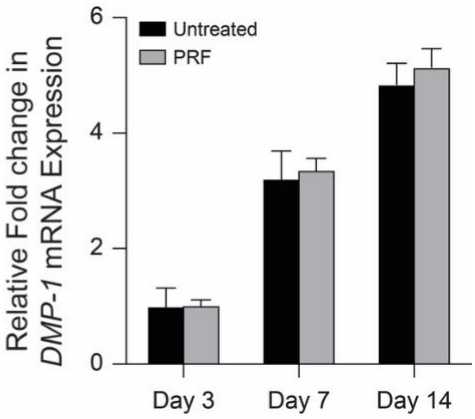


Figure 7: Dentin Slice Model

(A) and (B) Dentin slice about 1 mm in thickness. The pulp space is filled with PRF and minced pulp tissue is placed in contact with PRF and dentinal walls.

(C) Dentin slice with PRF tissue only.

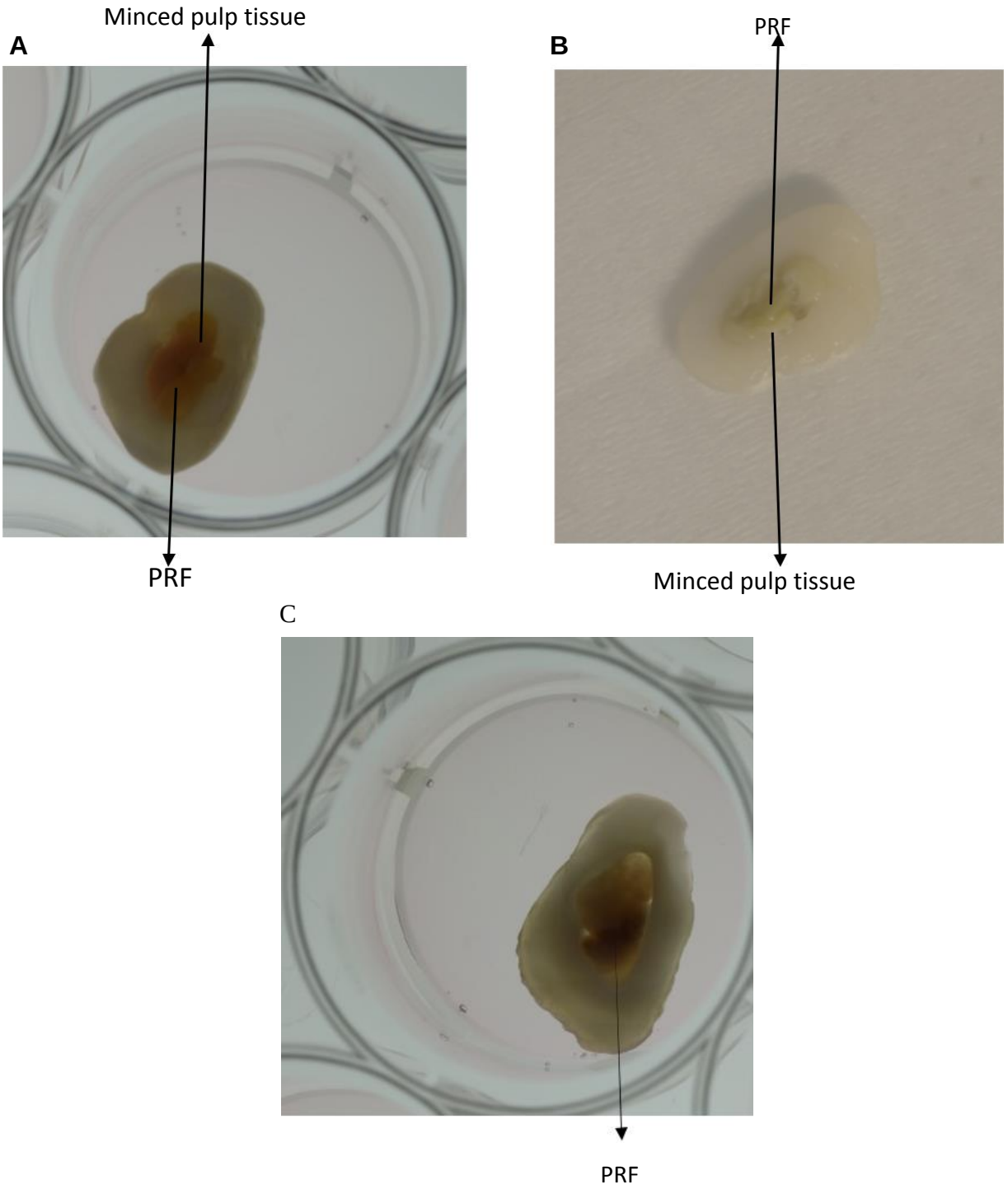


Figure 8. Minced pulp tissue shows evidence of cell migration towards the periphery of the tissue in the presence of PRF.

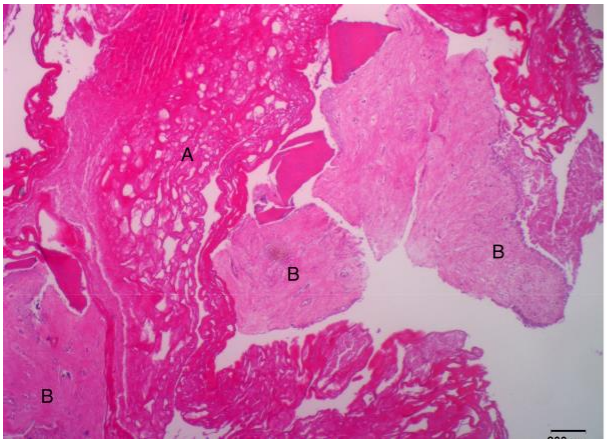
(A) and (B) H&E sections of dentin slices: A is PRF tissue and B is minced pulp tissue. PRF tissue is seen as a fibrous meshwork. Pulp tissue shows increased cellularity at the periphery. (10X magnification, Scale: 100 um)

(C) 20X magnified image of the pulp tissue showing increased cellular content at the periphery. (Scale: 100 um)

(D) 20X magnified image of the pulp tissue along the dentin walls. (Scale: 100 um)

(E) and (F) H&E section of dentin slices with PRF tissue only. (Scale: 100 um)

A



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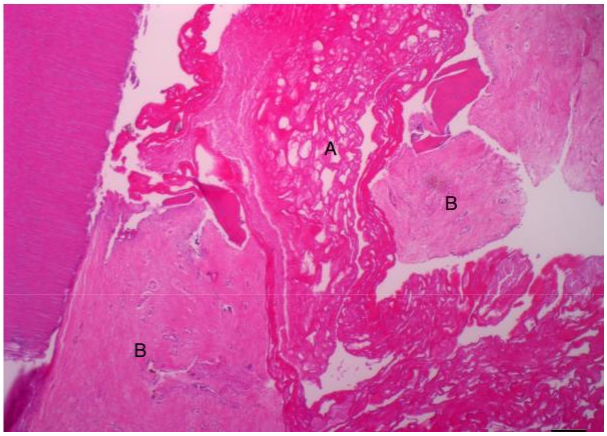

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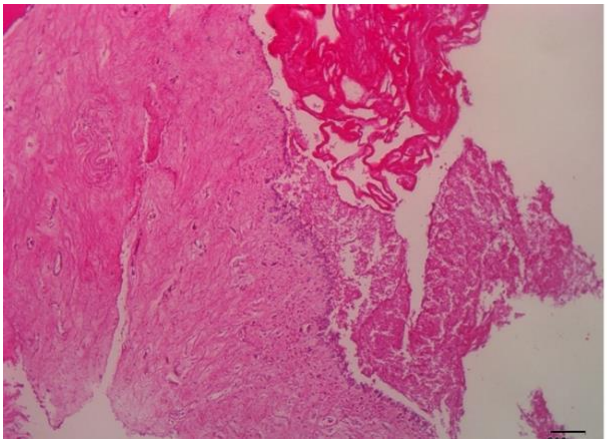

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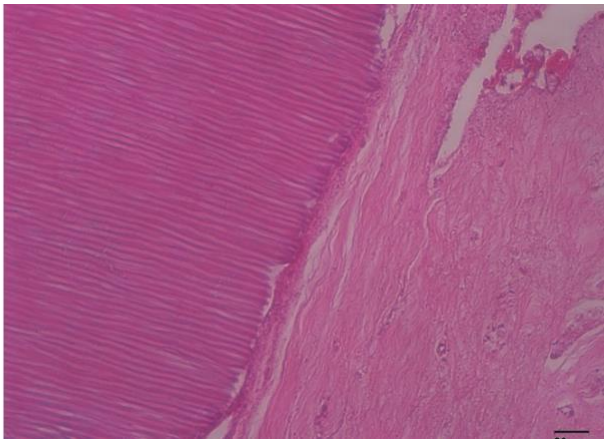
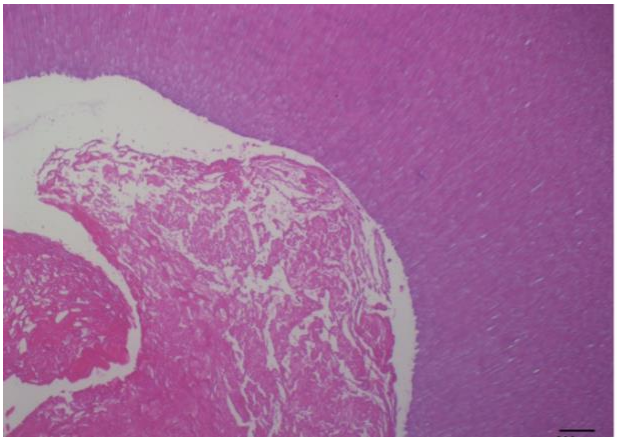


E



F

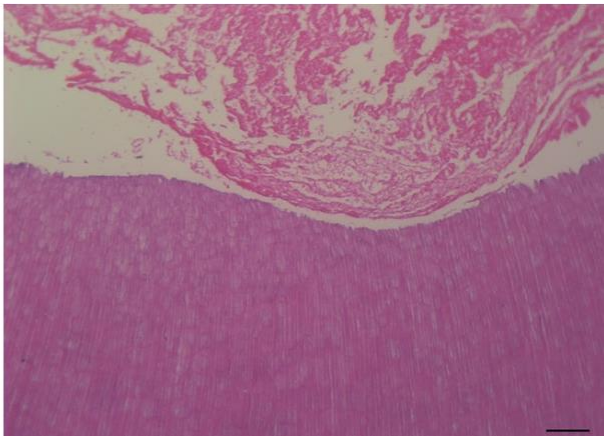
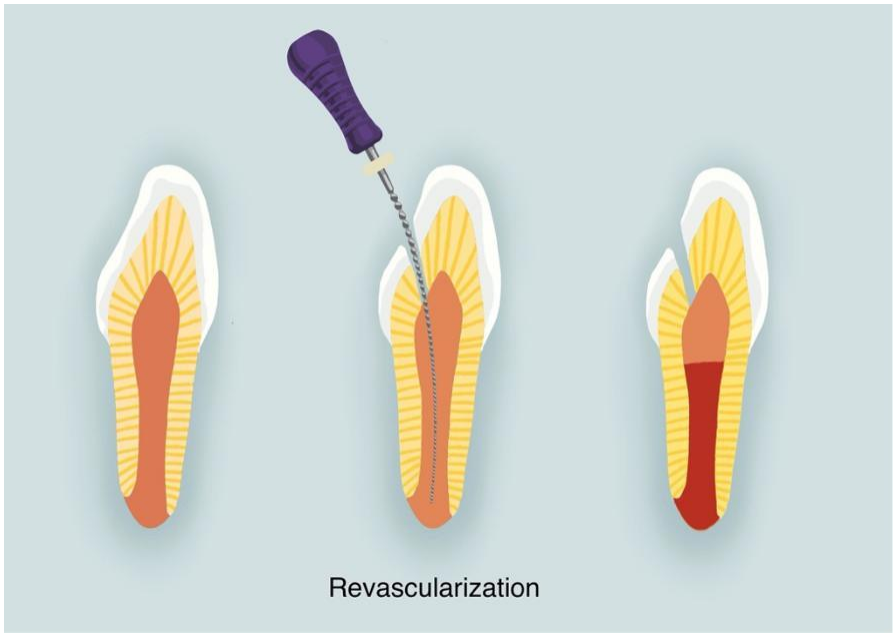


Figure 8. Proposed model using Minced pulp and PRF

(A) Regenerative Endodontic treatment - Revascularization procedure.

(B) Regenerative Endodontic treatment using Minced dental pulp and PRF.

A



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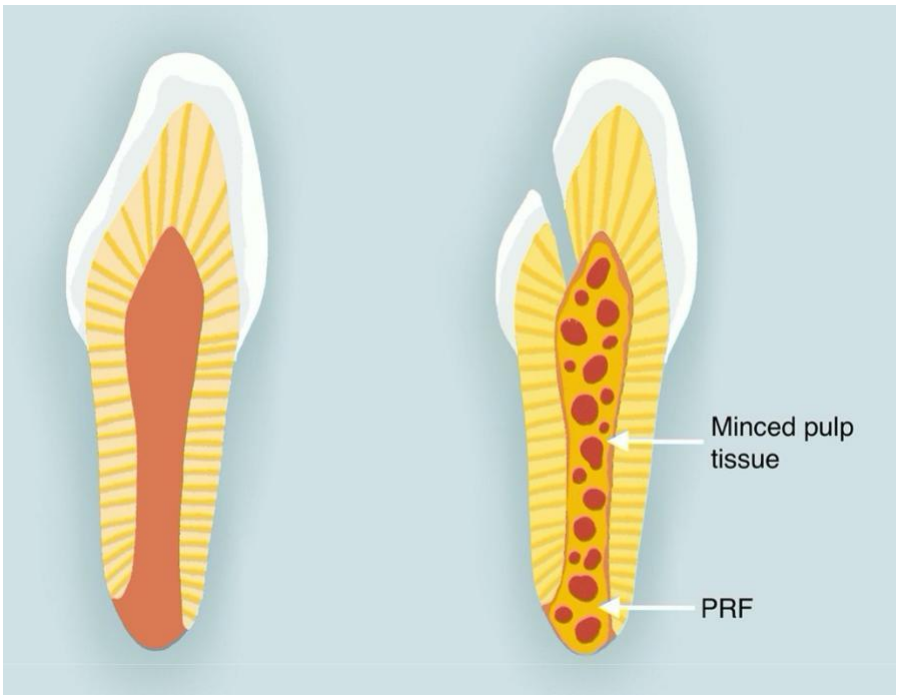

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