"ASSESSMENT OF THE EFFECT OF ADVANCED PLATELET RICH FIBRIN (A-PRF) ON PROLIFERATION, MIGRATION AND ATTACHMENT OF HUMAN PERIODONTAL LIGAMENT FIBROBLASTS – AN IN-VITRO STUDY."

By

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KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI, KARNATAKA

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ABBREVATIONS

SR. NO.	ABBREVATION	FULL FORM
1.	PDL	Periodontal ligament
2.	PRP	Platelet rich plasma
3.	PRF	Platelet rich fibrin
4.	L-PRF	Leukocyte platelet rich fibrin
5.	CGF	Concentrated growth factor
6.	A-PRF	Advanced platelet rich fibrin
7.	PRGF	Platelet rich in growth factor
8.	PCs	Platelet concentrates
9.	PDGF	Platelet derived growth factor
10.	TGF – 1	Transforming growth factor 1
11.	IGF-1	Insulin like growth factor 1
12.	VEGF	Vascular endothelial growth factor
13.	EGF	Epidermal growth factor
14.	FGF	Fibroblast growth factor
15.	VWF	Von Willebrand factor
16.	EMD	Enamel matrix derivative
17.	MTT	3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide
18.	EDTA	Ethylenediamine tetraacetic acid

19.	FBS	Fetal bovine serum
20.	DMEM	Dulbecco's modified eagle medium
21.	DMSO	Dimethyl sulfoxide
22.	CO_2	Carbon dioxide
23.	RBC	Red blood cell
24.	Units of measurement ml µm µL cm	 Millilitre Micro-metre Micro-litre Centimetre
25.	rpm	Rotations per minute
26.	TSP-1	Thrombospondin
27.	OPG	Osteoprotegerin
28.	ALP	Alkaline phosphatase
29.	HGF	Hepatocyte growth factor
30.	CTGF	Connective tissue growth factor
31.	ELISA	Enzyme linked immunoassay

ABSTRACT:

Backgroud: Platelet concentrates have been proposed as a therapeutic tool to enhance and promote cellular and molecular mechanisms during wound healing and periodontal regeneration. Recently, the use of Advanced platelet rich fibrin (A-PRF) has shown to be more potent than the standard PRF in terms release of growth factors and the architecture of their fibrin network. The architecture of A-PRF matrix with even distribution of cells and growth factors can enhance the activity of different cell lines over a definite period of time and provide cell availability for periodontal tissue repair and regeneration.

Objective: The aim of the study was to evaluate the effect of Advanced platelet rich fibrin (A-PRF) on the proliferation, migration and attachment of cultured human PDL fibroblasts.

Materials and Methods: Human PDL fibroblasts were cultured from freshly extracted premolar teeth planned for orthodontic extraction. MTT assay was used to assess the proliferation rate of PDL cells cultured in Group 1 (DMEM+10%FBS) and Group 2 (DMEM+A-PRF). Cell migration was assessed using in-vitro scratch assay. ImageJ software measured the distance of wound closure at 0hour, 24hours and 48 hours. Cell attachment assay was computed by counting the viable cells using haemocytometer under a microscope with tryphan blue staining.

Statistical analysis: Comparison between and within the groups was done using one way ANOVA and pair wise comparison was done suing Tukey’s post-hoc test for proliferation and migration assay. Mean and standard deviation of attached cells was compared using Student t-test for attachment assay**.**

Results: The proliferation rate of PDL fibroblasts exposed to A-PRF was statistically significant at 48 hours (p= 0.018) and at 24 hours the activity was significant in Group 1 (p=0.02). Complete wound closure was seen in Group 2 at 24 hours, when compared to Group 1 which was noted at 48 hours. The difference was statistically significant (p=0.00). The attachment of PDL fibroblasts on root planed dentin specimens for Group 2 was 135.8% more than Group 1 which was highly significant (p=0.00).

Conclusion: A-PRF showed a stimulatory effect on proliferation, migration and attachment of PDL cells in-vitro.

Keywords: Platelet concentrates, Advanced-platelet rich fibrin (A-PRF), PDL fibroblasts, wound healing.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPHS:

INTRODUCTION:

Periodontitis, a chronic inflammatory disease causes destruction of both hard and soft tissues of the tooth [(1)]. Reducing the tissue inflammation, preventing further attachment loss and regeneration of the lost periodontium remains to be the ultimate goal of periodontal therapy [(2)]. Rehabilitation of the epithelial seal, formation of new cementum with attachment of connective tissue fibres and revamping of alveolar bone level determines a successful outcome of periodontal regeneration [(3)].

The restoration of diseased tissues, requires a sequential and uneventful healing for maintaining the structure and function of the periodontium [(4)]. The general principle of healing consists of cellular and molecular events occurring between different cell types and the extra-cellular matrix [(5)]. The cells that occupy the perivascular space of the periodontium have an inherent differentiation potential into its cell types like the cementoblasts, osteoblasts and fibroblasts which prepares a favourable environment for periodontal regeneration [(6)].

The fibroblasts in the connective tissue of the periodontium have a central role in the remodelling phase of wound healing which encourages the regeneration of collagen fibres and connects the cementum to gingival connective tissue and alveolar bone [(7)].

Process of wound healing is regulated and exhilarated by biologically active substances mainly the growth factors. These agents balance the key cellular processes such as mitogenesis, cell differentiation and metabolism. Platelets released from the blood clot are an autogenous source of growth factors with a central role during the initial wound healing phase [(8)].

The role of platelets in primary hemostasis during inflammation and tissue regeneration is attributed to its property to release cytokines and growth factors stored in the α-granules [(9)]. Growth factors liberated by platelets such as platelet-derived growth factors (PDGFs), transforming growth factor-β (TGF-β), as well as insulin-like growth factor-I, are mitogenic proteins linked to formation of collagen, bone and early wound closure [(10)].

Platelet concentrates (PCs) are autologous extracts, obtained after processing a whole blood sample, through centrifugation. PCs namely "Platelet-rich plasma (PRP) and Platelet-rich fibrin (PRF)" based on the concept of cell therapy provide autologous growth factors within a fibrin matrix [(11)]. The 1st generation of PCs are "platelet-rich plasma (PRP) and plasma rich in growth factors (PRGF)" [(11)]. The 2nd generation of platelet concentrate, PRF, introduced in France by Choukroun and colleagues in 2001, overcame the drawbacks associated with PRP preparation that mandates use of anticoagulants to avoid blood coagulation [(12)].

Use of PRF is simple, requires neither anticoagulant nor bovine thrombin or calcium chloride. The whole blood collected is centrifuged without any additives [(13)]. PRF has shown the tissue regenerative potential and is also a scaffold carrier for mesenchymal cells [(14)]. The PRF concentrate is able to form a 3-dimensional fibrin network of platelets and leukocytes (including monocytes, lymphocytes, and granulocytes) that act as scaffold during early phases of wound healing [(15)].

The crosstalk between cells and fibrin matrix prompts a gradual growth factor release and promotes better wound healing during the initial phase [(15)]. The three-dimensional fibrin network of PRF would simulate the extracellular matrix in relation to its structure thereby creating an environment for the cells to function optimally thus allowing more efficient cell migration and proliferation [16].

The first delivered PRF preparation called L-PRF centrifuged with a relative centrifugation force of 708g for 12mins [17]. Recently, the "Low-speed centrifugation concept" has modified the process of PC preparation [18]. It is presumed that, decreasing the centrifugation speed (G-force) might boost the number of platelets along with leukocytes in the PRF matrix [19]. Besides having a porous structure, this matrix permits additional room for easy release of platelets and immune cells thereby resulting in greater sustained delivery of growth factors [20].

Of the more recent protocols, is preparing A-PRF & A-PRF+ membranes (Max RCF: 276g for 14 minutes and 208 g for 8 min respectively) [21]. A-PRF is reported to have a significant higher delivery of various growth factors, like the PDGF, TGF-β1, EGF, VEGF, and insulin-like growth factor (IGF), compared to L-PRF and PRP [22]. The centrifugation protocol for A-PRF showed a more interfibrous space, loose structure and more cells within the fibrin rich clot [20].

Present literature substantiates the use of both PRP & PRF in modulation of cell activities like differentiation, proliferation, migration of various cell types such as dermal pre-keratinocytes, human gingival fibroblasts, osteoblasts in a specific manner [23]. A systematic review of in-vitro studies examined the effect of PRF on cell proliferation, migration, differentiation, inflammation, and osteoclastogenesis of various cells types and confirmed PRF as an active source of biologic agents that can facilitate wound repair and regeneration [24]

The intention of any regenerative process is to initiate new PDL on the root cemental surface, with connective tissue fibres inserted on the tooth root produced by PDL fibroblast cells [4,8]. The regenerative events necessitate the connective tissue cells to migrate and attach to the wounded site so as to restore and repair the tissues [5,6,7]. There are very few invitro studies available on the effect of A-PRF on the cells that actively participate in periodontal repair and regeneration during wound healing events [19,25,26].

Thus, the current study aims to assess the effect of A-PRF on proliferation, migration, and attachment of PDL fibroblasts *in vitro*.

AIM AND OBJECTIVES

AIM OF THE STUDY:

Assessment of the effect of Advanced-platelet rich fibrin (A-PRF) on proliferation, migration and attachment of human periodontal ligament fibroblasts.

OBJECTIVES OF THE STUDY:

1. To assess the proliferation of human periodontal fibroblasts treated with and without Advanced Platelet Rich Fibrin (A-PRF).

2. To assess the migration of human periodontal fibroblasts treated with and without Advanced Platelet Rich Fibrin (A-PRF).

3. To assess for the attachment of human periodontal fibroblasts on root specimens treated with and without Advanced Platelet Rich Fibrin (A-PRF).

4. To compare the proliferation, migration and attachment of human periodontal fibroblasts treated with and without Advanced Platelet Rich Fibrin (A-PRF).

REVIEW OF LITERATURE

The goal for treating periodontitis is not only limited to cease the disease progression, but also aims towards regenerating the lost periodontium due to disease. Literature reviews the use of various biomaterials and recently, platelet derived products have gained popularity in the field of periodontics due to their growth factor release which actively participates in wound healing, repair and regeneration.

The literature has reviewed the use of PRP and PRF and its effect on different cell lines i.e. mesenchymal stem cells [14], epithelial cells [27], human gingival fibroblasts [28], PDL fibroblasts [27,29] and osteoblasts [30] wherein, both the platelet concentrates have shown to enhance the activity of gingival, PDL fibroblast and osteoblasts but suppresses the growth of epithelial cells. Under the guidance of certain biologic clues, the resident cells of the periodontium mainly fibroblasts actively participate in tissue repair and remodelling thus promoting tissue regeneration.

Patricio C. Smith, Christopher McCulloch, Constanza E. Martinez, Jorge Martínez (2019) in their review article have described the role of periodontal fibroblasts during wound healing and its function in restoring the structure of periodontal tissues. Wound closure is regulated by epithelial cells and connective tissue cells. It was stated that the role of gingival and periodontal fibroblasts in healing and repair of tissues is crucial and begins by organising and synthesizing the collagen fibres of connective tissue promoting attachment. Thus, concluding that the cell populations are needed for regeneration and the actively functional fibres will restore the periodontium. [4]

The culture and identification of PDLSCs (PDL stem cells) was demonstrated by ***Wenjun Zhu and Min Liang (2015)*** in their review article and stated that PDLSCs possess the characteristics of MSCs. Type I collagenase and dispase are chosen over trypsin and EDTA as enzyme digest methods and has shown higher proliferation rate and stronger differentiation activity of PDLSCs. The culture media affects the biologic features of PDLSCs and those cultured in α- MEM (α minimum essential medium) had higher proliferation and osteogenic potential than those cultured in DMEM (Dubecco's modified eagle's medium). Cumulative data suggests that the sequential use of different types of growth factors seems to be effective for the differentiation of PDLSCs which makes them a guaranteed tool for periodontal regeneration. (6)

Sarita Dabra and Preetinder Singh (2011) reviewed the role of growth factors, their mode of action and molecular signalling pathways in periodontal pathologies. Growth factors participate in tissue repair by regulating proliferation, differentiation and mitotic activity of cells and extracellular matrix synthesis. The differentiation of PDL fibroblasts in specific tissue cell type is enhanced in the presence of these signalling molecules. The availability of growth factors greatly improves the predictability of tissue engineering for regeneration. Overall, the use of GFs will populate the PDL space, root and bone surfaces with desired cell population required for formation of new attachment apparatus. (31)

The factors influencing wound healing post periodontal surgery was described by ***Giuseppe Polimeni, Andreas V. Xiropaidis & Ulf M. E. Wikesjo (2006)***. The review compiled the current scientific evidence and stated that the biologic and clinical factors mainly the cells from PDL, primary wound closure, space maintenance and wound stability together contribute to successful periodontal regeneration. The periodontal wound healing and regeneration of the lost periodontium is not only influenced by the biomaterial used but also depends on the technique used to achieve periodontal regeneration. (5)

Role of platelet concentrates in PDL regeneration.

A systematic review by ***F J Strauss et al 2019*** summarized the in-vitro studies available in the literature to evaluate the efficiency of PRF on various cell lines in terms of cellular proliferation, migration and differentiation. The review included 53 studies published till December 2018. The study found that the autologous source of growth factors i.e. PRF greatly promotes cell migration, proliferation and differentiation of PDL cells into tissue specific cell type. This enhanced activity provides early recruitment of cells in the wound area and facilitates tissue repair and regeneration. (24)

A study by ***Kanyawat Rattanasuwan et al (2018)*** used the 1st generation of platelet concentrate i.e. “platelet rich plasma (PRP)”. The effect of PRP on PDL fibroblasts was evaluated focusing on the proliferation, migration and attachment parameters. The use of various concentrations of PRP mainly 5% and 10% was able to stimulate the activity of PDL fibroblasts because of the release of various growth factors from PRP. The study concluded that 10% of PRP modulated PDL cell proliferation, migration and attachment at a greater rate than 5% of PRP. (32)

Julia Etulain (2017) described the role of platelets as modulators of the physiopathological process of inflammation and tissue regeneration. The growth factors released from platelets are responsible for this function and promote revascularization by induction of proliferation and differentiation of endothelial cells, repairs the connective tissue by activation of fibroblasts and causes differentiation of mesenchymal cells into tissue specific cell types. Platelet-rich plasma (PRP), a reservoir of platelets and growth factors, is activity involved in differentiation of various cell types thus promoting tissue repair and regeneration. Thus, PRP is a promising regenerative biomaterial which hastens tissue recovery after surgery. (33)

A study by ***Eduardo Anitua, Maria Troya and Gorka Orive (2013)*** evaluated the effect of autologous platelet rich in growth factor (PRGF) on proliferation, migration of PDL fibroblasts and release of growth factors. PRGF has a stimulating effect on PDL cell proliferation, migration, adhesion and provides a mixture of growth factors prepared from the patient's own blood. This technology amplifies the expression of endogenous growth factors like the VEGF, TSP-1, CTGF, HGF and pro-collagen type I to the wound area. The availability of these agents delivered by PRGF is said to modulate and enhance the healing by facilitating interaction between the cells and the extracellular matrix which is necessary for proper functioning of the periodontium thus contributing to regeneration. (2)

A study by ***Y-C Chang and J-H Zhao (2011)*** examined the effect of PRF on periodontal ligament fibroblasts (PDLFs). The natural fibrin framework of PRF prevents proteolysis of growth factors and facilitates its release for a longer period of time. Proliferation of PDLFs was enhanced with upregulation of phosphorylated extracellular protein kinase (p-ERK) expression. Also, expression of OPG and ALP activity was enhanced which indicates decreased osteolytic activity and formation of new bone formation. (29)

An in-vitro study by ***Chung-Hung Tsai, Shih-Ya Shen, Jiing-Huei Zhao, Yu-Chao Chang (2009)*** examined the biologic effects of PRF on gingival fibroblasts, PDL cells, oral epithelial cells and osteoblasts over a 3-day culture period. The transition of cell proliferation in a cell-type specific manner was observed in the study. With no cytotoxicity towards periodontally related cells, the growth factors released from PRF had shown to increase the proliferation of gingival fibroblasts, osteoblasts and PDL cells and inhibiting the epithelial cell proliferation. This might prevent the interference of epithelium on the root surfaces for new attachment and promote PDL regeneration. [27]

David M. Dohan et al (2006) in their retrospective analysis acknowledged the biochemical properties and fibrin technologies of fibrin adhesives, concentrated PRP (cPRP) and PRF. Differences in these 3 generations of surgical additives lie in their gelling mode where, fibrin adhesive and cPRP use bovine thrombin or calcium chloride for fibrin polymerization and PRF polymerises naturally and slowly during centrifugation. The different mode of polymerization influences the mechanical and biologic properties of the final fibrin matrix. PRF membrane with weak thrombin concentrates, slow polymerization mode and a 3-dimensional organization consisting of equilateral junctions gives great elasticity to the fibrin matrix which supports cytokine enmeshment and cellular migration better than cPRP. [11]

Optimised platelet rich fibrin: Advanced platelet rich fibrin (A-PRF)

The evolution of the second generation of PRF is brought about by modifying the centrifugation force by decreasing the rpm and increasing the time. The optimised PRF obtained named as advanced platelet rich fibrin (A-PRF) has a centrifugation cycle of 1500rpm for 14 minutes. A-PRF has two forms which differs based on centrifugation cycle i.e. A-PRF (centrifuged at 1500rpm for 14minutes) and A-PRF+ (centrifuged at 1500 for 8 minutes) [19,26]. This change in centrifugation force has led to an even distribution of cells throughout the A-PRF matrix as compared to PRF with more release of growth factors [20].

Effect of A-PRF, a newer platelet derived product was evaluated by S***haram Ghanaati et al (2014)*** and compared to the standard PRF (S-PRF). Change in the centrifugation force seen with A-PRF resulted in increased number of platelets. Along with this, A-PRF clot showed more neutrophilic granulocytes which could contribute towards monocyte differentiation into macrophages. A-PRF would contribute towards both soft and hard tissue regeneration with their growth factor release by promoting cell-based tissue engineering for periodontal regeneration. [20]

Luciano P et al 2019 in their study evaluated the activity of gingival and PDL fibroblasts using A-PRF+, FGF and L-PRF in terms of proliferation, cell viability and cellular migration in wound healing culture plates. The proliferation of fibroblasts was greater for A-PRF+ and L-PRF. However, A-PRF showed 19.6% more activity than L-PRF and 42.5% more than FGF group. The study used ImageJ software to measure the wound closure at 24 hours. The results suggested the activity of L-PRF and A-PRF+ to be more pronounced as compared to FGF group and negative control which was statistically significant ($p<0.01$ for both groups). The authors concluded that their results would help to select the best PRF for periodontal tissue repair and regeneration. [26]

A study by ***Masako Fujioka-Kobayashi et al 2017*** *used v*arious types of PRF i.e. A-PRF, “A-PRF+ and L-PRF”. The cellular response of gingival fibroblasts and growth factor release was evaluated for 10 days. The result of their study reported that the cellular activity was highest for both A-PRF and A-PRF+ group as compared to L-PRF. Compared to control group, the cell migration in L-PRF group increase by 200% and both A-PRF+ and A-PRF group showed increased activity by 300%. But the difference between A-PRF and A-PRF+ was not significant. Moreover, the results showed more collagen type 1 content from cells exposed to A-PRF+ suggesting that optimising the centrifugation force of PRF would lead to promising regenerative outcomes. (19)

In-vitro study by ***Eizaburo Kobayashi et al (2017)*** demonstrated the growth factor release from PRP, PRF and A-PRF was compared over a period of 10 days. ELISA was used to assess and quantify the growth factors such as PDGF-AA, PDGF-AB, PDGF-BB, TGF-β1, VEGF, EGF and IGF at 15mins, 60min, 8hours, day 1, day 3 and day 10^{th}. A significantly higher amount of growth factor release was from A-PRF as compared to PRP and PRF. Among all the growth factors, PDGF-AA was found to be highest in all PCs with lower levels of EGF and IGF. With these findings it was noticed that the newer platelet concentrate A-PRF showed gradual release of growth factors for over 10 days and proved clinically beneficial for regenerative procedures. (22)

An in-vitro study by ***Hideo Masuki et al (2016)*** compared the pro-inflammatory cytokine and growth factor contents in PRP, PRGF, A-PRF and CGF by determining the blood cell count for platelet concentrates and ELISA for determining growth factor and cytokine levels. The findings showed that A-PRF and CGF preparations consisted of TGF-β1, PDGF-BB, VEGF, IL- 1β, IL-6 at a higher concentration than PRP. The benefits of A-PRF and CGF over PRP makes them capable for inducing angiogenesis, promoting wound healing with tissue regeneration. (34)

MATERIALS AND METHODOLOGY:

SOURCE OF DATA

Ethics statement

The study protocol was approved by the Research and Ethical Committee of KLE V.K Institute of Dental Sciences, KLE University, Belagavi.

Informed consent was obtained from patients who volunteered to give blood sample for preparation of A-PRF. The study was conducted in the Department of Periodontics, KLE'S V K Institute of Dental Sciences, Belagavi utilizing the outpatient department facilities. The laboratory procedures were undertaken at KLE'S Dr. Prabhakar Kore's Basic Scientific Research Centre (BSRC), Belagavi.

Primary cultures of human PDL fibroblast cells were prepared from freshly extracted premolar teeth. Sufficient numbers of cells were obtained by subsequent propagation. The study was carried out on PDL fibroblast cells from third to fifth passages. The cells were then exposed to two different groups for the in-vitro experiment:

Group 1 (CONTROL): PDL fibroblasts exposed to 200ul DMEM and 10% foetal bovine solution (without A-PRF).

Group 2 (TEST): PDL fibroblast exposed to 200ul DMEM along with an aliquoted solution of 2ul of DMEM and A-PRF

INCLUSION CRITERIA

1. Age between 18 – 30 years
2. Teeth indicated for orthodontic extraction.
3. Teeth without any pathology like caries, periapical lesions etc.

EXCLUSION CRITERIA

1. Patients with systemic disease like diabetes mellitus
2. Patient under medication such as aspirin, anticoagulant therapy, antibioticsor any other medications which have an effect on platelets.
3. Patients with blood disorders like thrombocytopenia
4. Tobacco use
5. Cancer of hematopoetic system.
6. Lactating mothers, pregnant women

ARMAMENTARIUM

1. 15ml sterile bottle
2. Kidney tray
3. Surgical gloves
4. BP blade no # 22 and handle
5. Toothed tissue forceps
6. Plain tissue forceps
7. Glass petridish
8. Cell culture T flask
9. Syringe with needle (2ml and 5ml)
10. Micropipette
11. Pipette tips
12. Micro centrifuge tubes
13. Micropipette stand
14. 24 well culture plate
15. 96 well culture plate
16. Syringe filters 0.2µm
17. Neubauer's counting chamber
18. Blood collection plain tubes
19. CO_2 incubator
20. Centrifugation machine
21. Vertical laminar air flow
22. Light Microscope

TABLE 1: CHEMICAL REAGENTS USED

SR.NO	REAGENTS	COMPANY
1.	Dulbecco's Modified Eagle's Medium (DMEM)	Gibco life technology
2.	0.25% Trypsin	Gibco life technology
3.	Penicillin (10,000 Units/mL) + streptomycin (10,000 ug/mL)solution	Hyclone
4.	Gentamycin	Laborate Pharmaceuticals India Ltd.
5.	Amphotericin	Chandra BhagatPharma Pvt. Ltd
6.	Fetal Bovine Serum	HIMEDIA
7.	Phosphate Buffer Saline	HIMEDIA
9.	MTT Reagent	HIMEDIA
10.	Dimethylsulfoxide (DMSO)	Fischer Scientific (Qualigens)
11.	0.4% Tryphan blue	HIMEDIA

FIGURE 1: CHEMICAL REAGENTS USED

FIGURE 2: ARMAMENTARIUM

FIGURE 3: LIGHT MICROSCOPE

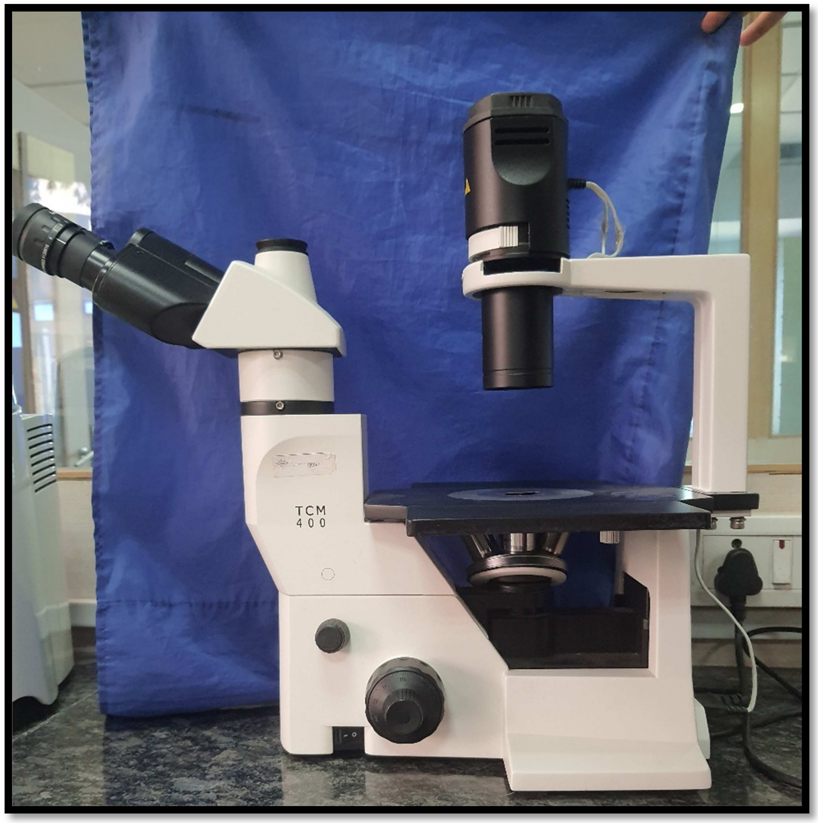
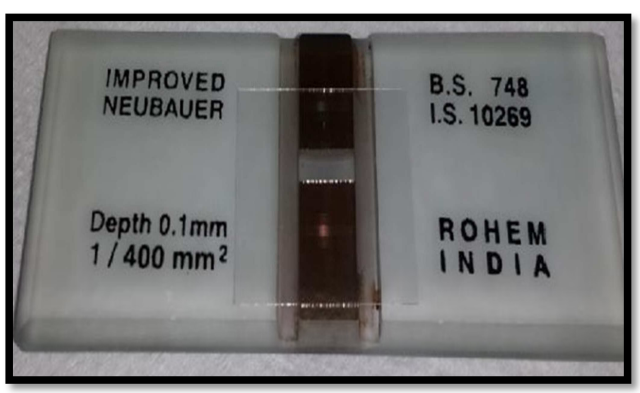


FIGURE 4: SPECTROPHOTOMETER

FIGURE 5: CENTRIFUGE MACHINE

FIGURE 6: CO_2 INCUBATOR

FIGURE 7: NEUBAEUR'S CHAMBER USED FOR CELL COUNTING



METHOD OF COLLECTION OF DATA

Preparation of advanced-platelet rich fibrin (“A-PRF”)

10ml of fresh human venous blood was drawn from healthy patients and collected in two separate 5ml glass-coated plastic tubes with no anti-coagulants and immediately centrifuged at 1500rpm for 14 minutes. The three distinct layers were seen after centrifugation. The upper part was the acellular plasma, the middle buffy coat layer comprised of the fibrin clot and the bottom part was the RBC layer. The fibrin clot was separated from the bottom layer by using a sterile blade. The A-PRF clot was then gently pressed into a membrane using sterile gauze.

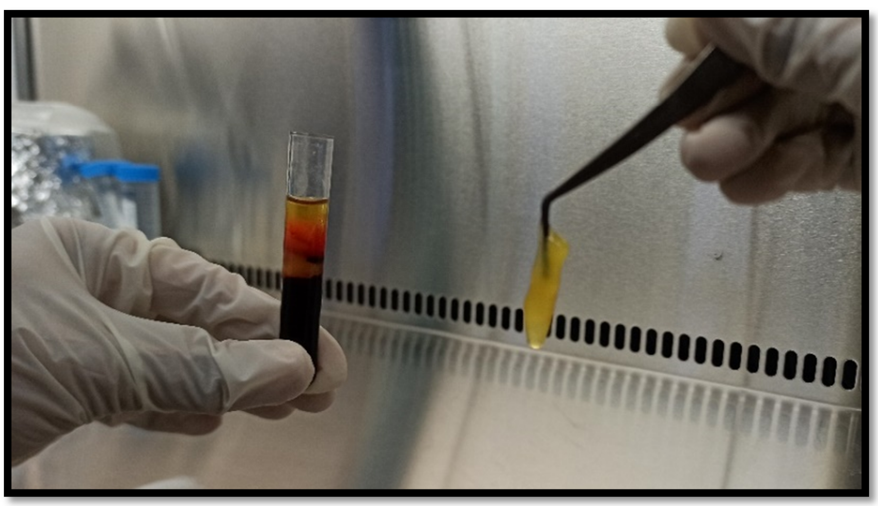
FIGURE 8: COLLECTION OF BLOOD SAMPLE

5ml OF VENOUS BLOOD DRAWN FROM HEALTHY SUBJECT

FIGURE 9: PREPARATION OF A-PRF

(A) Blood Transferred to A Plain Tube Without Any Anticoagulants

(B) **Obtained A-PRF Membrane**



(C) COLLECTION OF A-PRF MEMBRANE

PDL-fibroblasts isolation and Cell-Culture.

Human PDL-fibroblast cells were cultured from the premolars extracted for orthodontic reasons. After extraction, tooth was placed in DMEM supplemented with 0.5 μL/ml of amphotericin, 200 μL/ml penicillin G, 200 μL/ml streptomycin and washed with PBS. Under the vertical laminar air-flow the tooth was removed from the bottle and transferred to a sterile glass petri dish. The tooth was first washed with PBS in the petri dish and then transferred to another petri dish to which 2ml of DMEM was added. The tissue over the mid-root surface was scrapped with a BP blade no. 22 attached to its handle and macerated into smaller portions.

The obtained tissue was then seeded with a micropipette into a 24 well plate to which DMEM supplemented with 0.5μL/ml of amphotericin B, 200μL/ml penicillin G, 200gL/ml streptomycin. This plate was then placed in the CO_2 incubator at 37^oC in a humidified atmosphere containing 5%CO_2 and allowed to incubate. The media was changed every 2 to 3 days and the cells were sub-cultured on acquiring sufficient confluency.

Once the cell growth was 60-70% confluent by about 4-6 weeks, it was subjected to the process of trypsinisation. The medium was removed from the wells completely and the cell layer was washed with PBS. To allow the cells to detach from the plate, 0.05% trypsin in EDTA buffer was added to the wells and was allowed to stand for 2-3 minutes. The cell count was determined by using Neubauer's cell counting chamber. Dilutions were prepared to get the required cell density. Cells were then seeded in 96 well plates and were allowed to attach to the plate overnight. PDL fibroblasts were then subjected to A-PRF to perform the proliferation, migration and attachment assays.

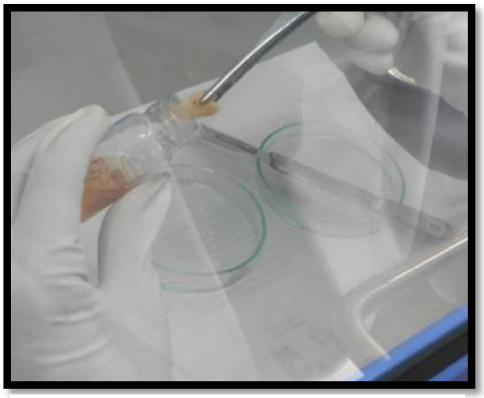
FIGURE 10: COLLECTION OF TOOTH SAMPLE

(A) Bottle containing PBS

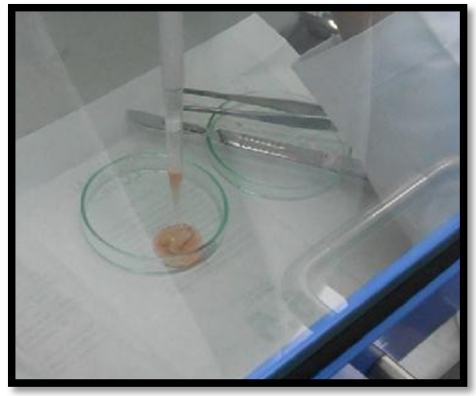
(B) Freshly extracted tooth

(C) Tooth transferred to PBS

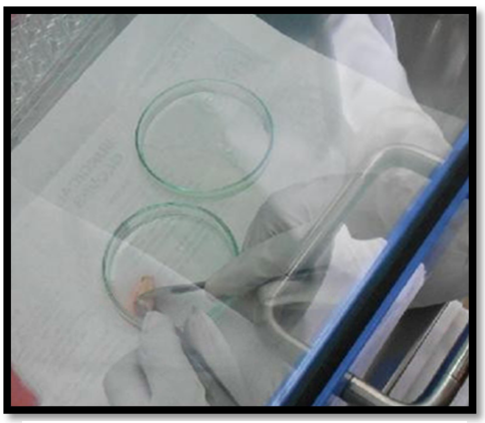
FIGURE 11: HARVESTING OF PDL FIBROBLAST CELLS



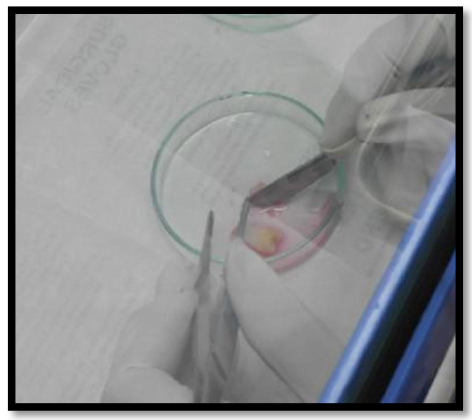
(a) Extracted tooth taken into the petridish



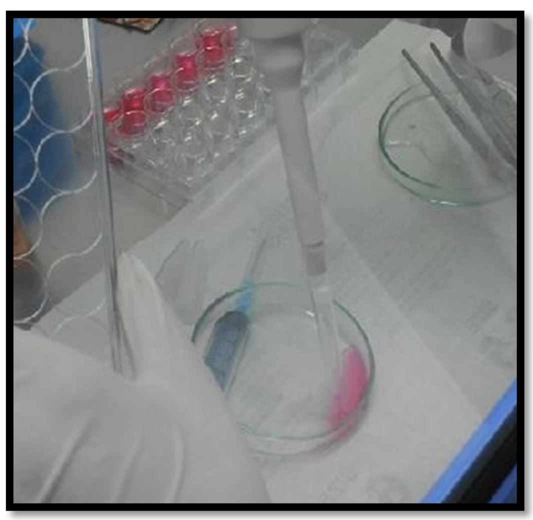
(b) 2ml of DMEM media added onto the tooth



(c) Scraping of PDL tissue from the mid-root section



(d)Tissue is minced in to small fractions



(e) Tissue along with media aspirated and added to 24 well plate

FIGURE 12: CULTURED PDL FIBROBLASTS UNDER MICROSCOPE

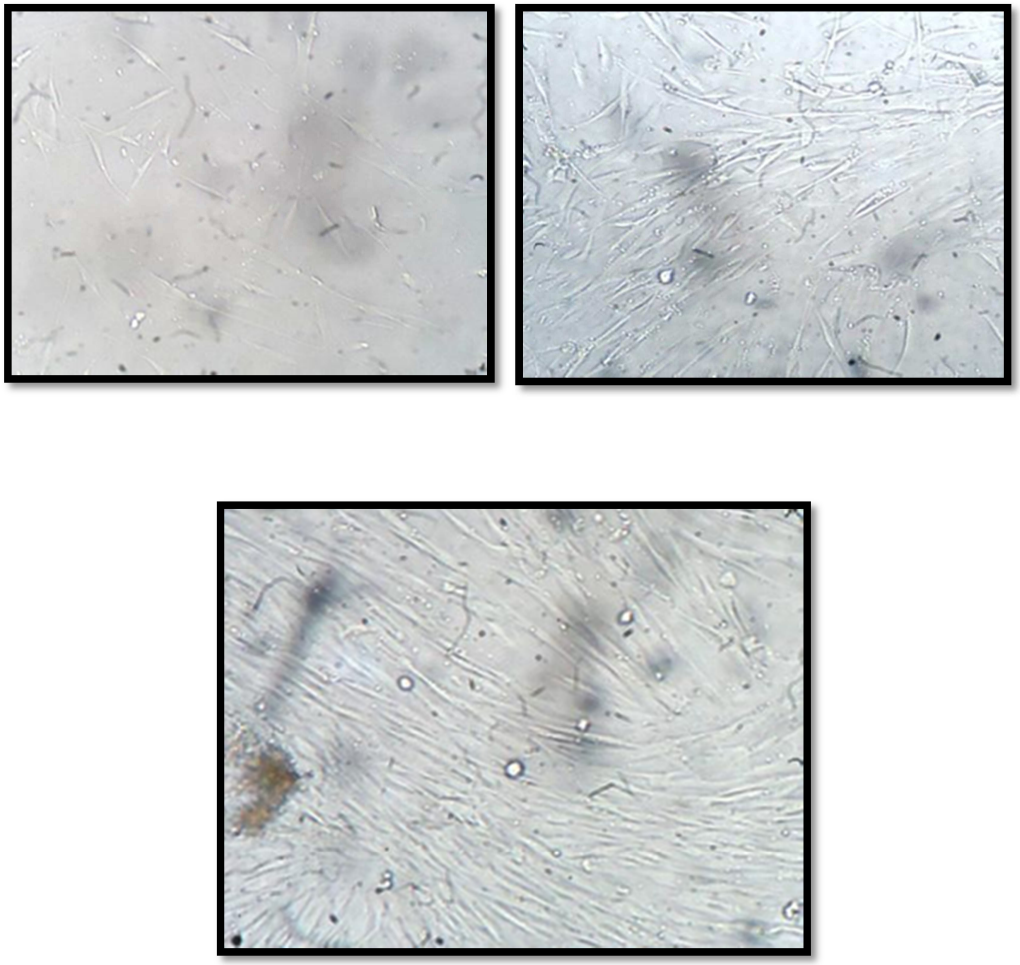
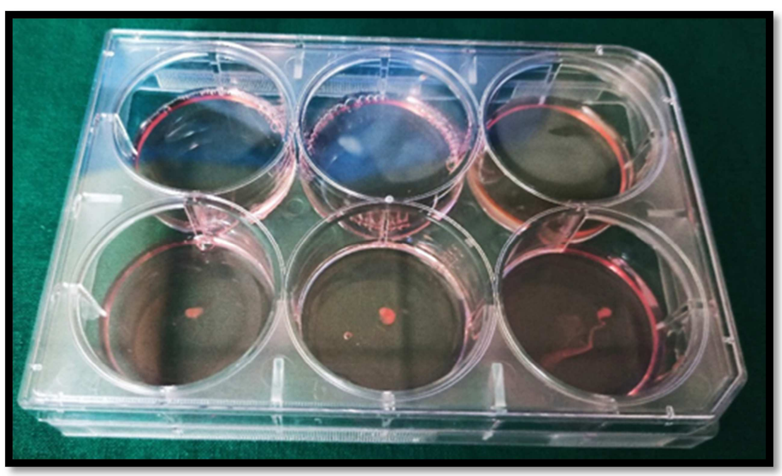


FIGURE13: EXPOSING PDL FIBROBLASTS TO A-PRF



1. **CELL PROLIFERATION:**

Cell proliferation of the PDL fibroblast cells was determined by MTT assay.

PRINCIPLE OF MTT ASSAY:

MTT assay is a colorimetric assay which determines cell proliferation. The reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase is evaluated in this assay. MTT that enters the cells is taken up by the mitochondria and is reduced to an insoluble, coloured (dark purple) formazan product. The crystals formed are then solubilized with an organic solvent (i.e. DMSO). The solubilised formazan is measured spectrophotometrically at 570 nm. The amount of colour produced is directly proportional to the number of viable cells. Reduction of MTT takes place only in metabolically active cells the level of activity is a measure of the viability of the cells the amount of colour produced is directly proportional to the number of viable cells.

PROCEDURE: [32]

The cells were seeded at a concentration of 1 x 10^5 cell/well in 96 well micro titre plate and were allowed to attach overnight. The cells were treated with A- PRF appropriately diluted with DMEM media and the control group was treated with DMEM and 10% FBS media. The plate was then kept for incubation for 5 days in CO_2 incubator at 37^oC in a humidified atmosphere containing 5%CO_2. "20µl of 5 mg/ml MTT reagent was added to wells after 5 days and the plate was kept for 4hr incubation in dark place at room temperature. (Aluminium foil was used as MTT reagent is photosensitive). The supernatant was carefully removed without disturbing the precipitated Formazan crystals and 200µl of DMSO was added to dissolve the crystals formed. The optical density was measured using spectrophotometer at a wavelength of 570 nm. The proliferation effect of A-PRF was assessed by spectrophotometric determination of colour change due to conversion of MTT into "Formazan blue" by living cells".

The proliferation activity of PDL fibroblasts was assessed in the following groups:

Group 1: PDL fibroblasts treated with DMEM + 10% FBS

Group 2: PDL fibroblasts treated with DMEM + A-PRF

Negative control: PDL Fibroblasts treated with only DMEM.

The proliferation activity of PDL fibroblasts was compared to a standard group i.e. DMEM media only which served as a negative control and the effects of Group 1 and Group 2 were compared with standard group.

The relative proliferation activity of PDL fibroblasts in Group 1 and Group 2 were calculated as percentage of control. The Optical density (OD) of negative group was taken as 100%. The results were calculated as follows: [Graph 1]

$$\frac{\text{(OD of groups)}}{\text{(OD of negative control)}} \times 100$$

PROLIFERATION ASSAY [32]:

Proliferation of human PDL fibroblasts will be assessed using the MTT assay [5]

Cultured PDL fibroblasts will be trypsinised and $2x10^9$cells will be seeded in the 96-well tissue culture plates

The cells will be maintained in 200µl of DMEM solution supplemented with 10% FBS for 24h at 37degree C.

After incubation the fibroblasts will be washed with phosphate buffer saline (PBS) and then prepared for

GROUP 1 (CONTROL)

PRF containing PDL fibroblasts supplemented with 200ul DMEM and 10% foetal bovine solution

GROUP 2 (TEST)

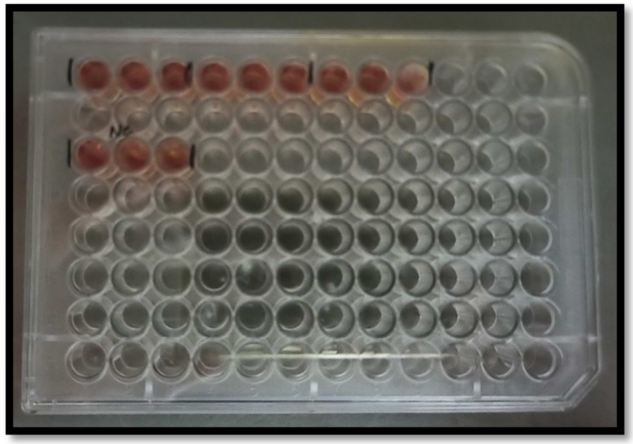
Containing PDL fibroblasts supplemented with 200ul DMEM added to it will be aliquoted solution of 2ul of DMEM and A-PRF

These two groups will be incubated for a period of 48/72h

After incubation, the culture media will be discarded and 100ul of MTT assay solution will be added to each group and will be incubated for 4h

After incubation the cells will be washed with PBS and will be transferred to a spectrophotometer with absorbance measured at 540nm

FIGURE 14: MTT ASSAY FOR CELL PROLIFERATION.



Cells seeded for proliferation assay in 96-well culture plates.

Culture plate covered with aluminium foil as MTT reagent is photosensitive

Colour change noticed 4 hours after exposing to MTT reagent

2. CELL MIGRATION

In-vitro scratch test was performed to detect migration of the PDL fibroblast cells.

Principle

This in-vitro test monitors the closure of the wound area by creating “a new artificial gap, so called ‘‘scratch’’, on a confluent cell monolayer. The cells on the periphery of the artificial wound will move towards the open wound area to close the ‘‘scratch’’ till new cell to cell contacts are formed again. The fundamental steps involve creation of a ‘‘scratch’’ on the cell monolayer, recording of images at the beginning and regular intervals during cell migration”, and comparing the images for assessment of the rate of cell migration.

In the present study, the closure of the wound was measured in µm in the following groups at 0hour, 24hrs and 48hrs

Group 1: PDL fibroblasts treated with DMEM + 10% FBS

Group 2: PDL fibroblasts treated with A-PRF

Procedure (35)

The cells seeded at a concentration of $1x10^5$cells/well in a 96- well culture plate were allowed to attach overnight. Using a sterile micropipette tip, a scratch was made in each well. DMEM and FBS media were added to the control wells. Separate wells were treated with A-PRF membrane.

The scratch was then observed under the light microscope and an area was marked beside one part of the scratch and a picture was taken. The culture plate was incubated in 5% CO_2, at 37°C. Pictures of the same marked area were taken at 0hr, 24hrs and 48hrs. The width of the wound was measured in μm using the ImageJ software.

<u>Measuring the wound closure in μm using ImageJ software.</u> [36]

To set a measurement scale, a line was drawn between two points of known distance. Three predetermined locations were selected from where the measurements were recorded for all the images. Taking into consideration the angle and the length, a horizontal line is drawn between the two points at the three predetermined locations. The obtained value is transferred to a data window where measurements for each particle was recorded. The mean value of the obtained three values was taken for statistical analysis.

MIGRATION ASSAY [35]:

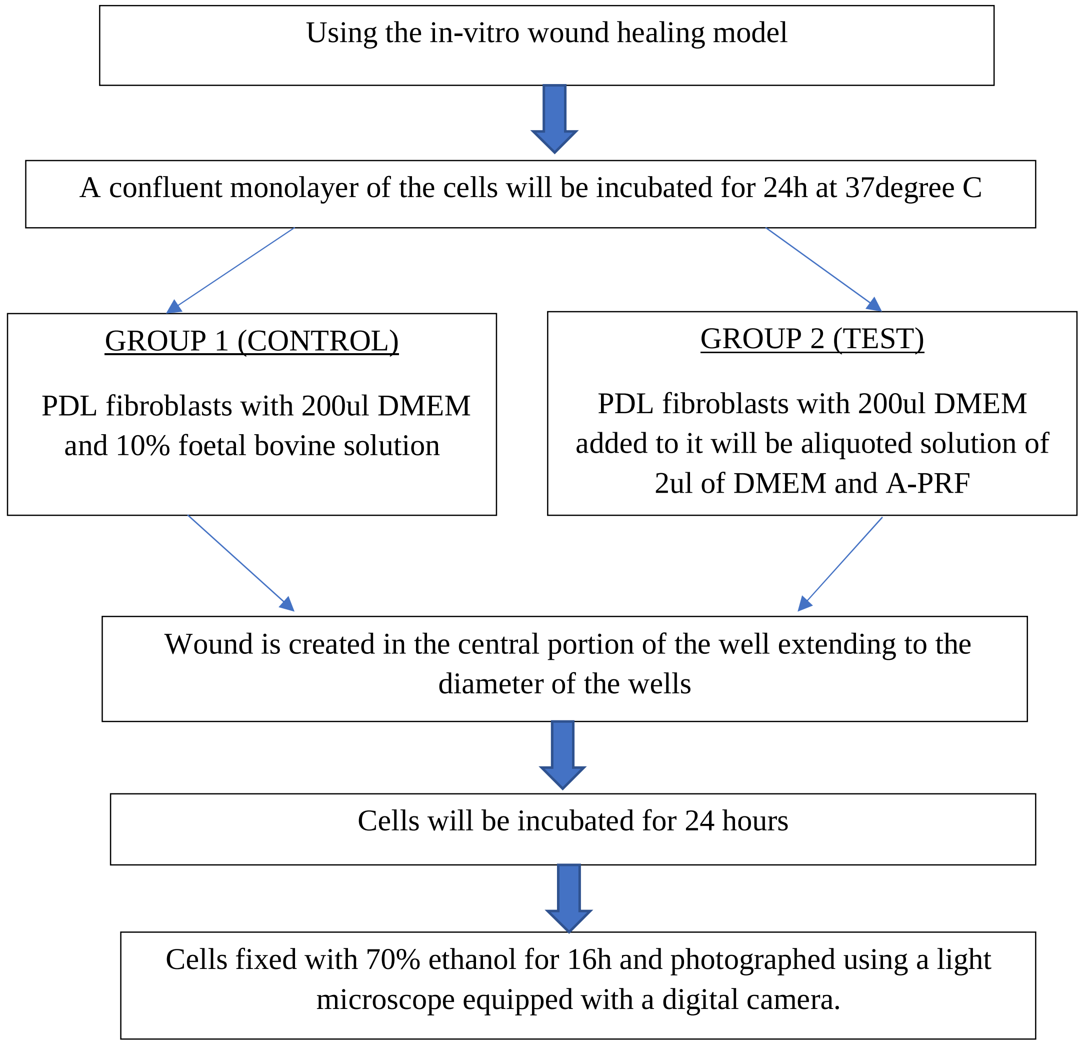
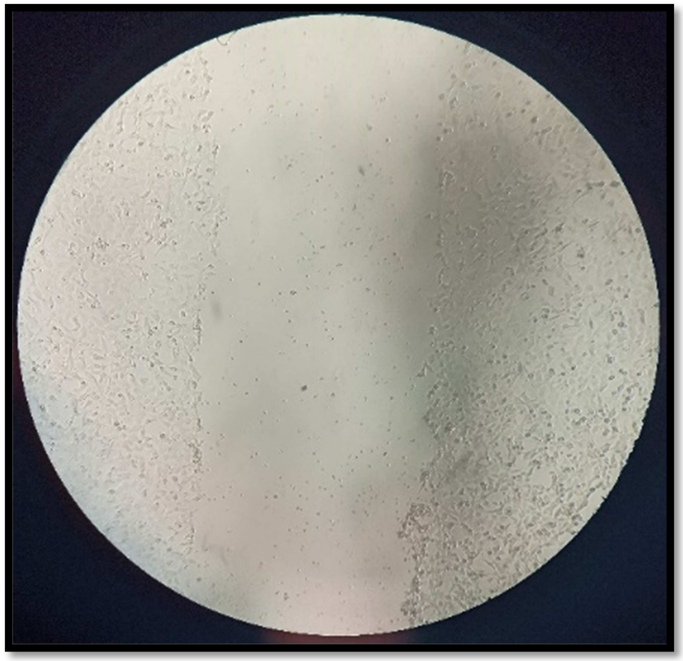
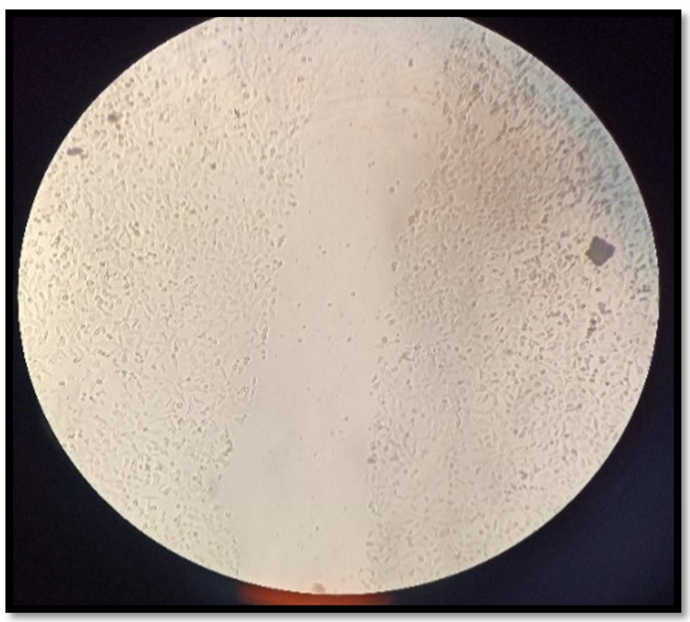


FIGURE 15: IN-VITRO SCRATCH WOUND CREATED FOR MIGRATION



GROUP 1



GROUP 2

3. CELL ATTACHMENT

The dentin specimens were prepared from periodontally healthy premolar teeth extracted for orthodontic reasons for attachment of PDL fibroblasts. Collected teeth were root planed using Gracey curettes to remove irregular cementum layer and the fibres.

The dentin specimens were prepared as per the protocol proposed by *Wikesjo et al* [(32)] wherein the middle third of the tooth root area was used for specimen preparation. Tooth specimen measuring 0.5x0.5x0.3 cm were obtained with a diamond cutting bur on micrometer with continuous saline irrigation.

A total of 44 specimens were obtained and immersed in distilled water and was autoclaved before use. The sterilized tooth specimens were placed in the 96-well flat bottom plate. These specimens were then divided into two groups

Group 1: 22 root specimens treated with solution of 2µL DMEM and 10% FBS, allowed to dry at 37 degree C for 6h.

Group 2: 22 root specimens treated with 2µL DMEM and A-PRF, allowed to dry at 37 degree C for 6h.

Following this, 200µl of cell suspension was carefully added over the root specimens and incubated for 24h at 37°C.

The attachment assay was performed according to the protocol given by *Alleyn et al* [(37)]. The number of cells attached to the tooth root specimens were observed using Tryphan blue cell count kit with a haemocytometer under a microscope.

Briefly, the tooth specimen along with attached cell suspension was centrifuged in an eppendroff tube. Following this, the tooth root specimen was removed from the tube. Later, 10-20µL each of 0.4% tryphan blue and cell suspension was added for incubation not more than 3minutes at 37°C. A drop of the tryphan blue-cell mixture was placed in the haemocytometer under the cover slip. The haemocytometer was then staged on the light microscope and focused on the cells.

The principle of tryphan blue staining is based on detection of live cells (which appear as transparent cells) as they contain intact cell membrane and prevents the dye to enter the cell membrane. The dead-cells as they take up the dye, these appear blue under the microscope.

Counting of cells

Focusing on the grid lines of Neuber's haemocytometer under a microscope, using a hand tally counter, the unstained or transparent cells were counted in 1-set of 16 squares. Same was followed for the next 16 corner squares until all the 4-sets of 16 corner squares were noted. The following formula was used for cell counting

$$\text{Cell counting} = \frac{\text{Total number of cells (I. II, III, IV)}}{4} \times 2 \times 10{,}000$$

(2 is the dilution factor for tryphan blue and 10^4 is the number of cells per ml)

Sample size determination for attachment assay.

Sample size estimation is done by using the following formula:

$$n = \frac{2S^2 (Z_\alpha + Z_\beta)^2}{d^2}$$

where,

$S = \frac{S_1 + S_2}{2}$

$Z_\alpha = 1.96$

$Z_\beta = 1.682$

d = 9.260

Power (%) = 95

α error = 5%

β error = 20%

Hence, the sample size was finalized as 22.

S_1: Standard deviation in group 1 S_2: Standard deviation in group 2 d: Mean Difference of standard deviation of group 1 and group 2 n: sample size number Z_α: Alpha error Z_β: Beta error

ATTACHMENT ASSAY [37]:

Tooth specimen preparation:

Extracted tooth will be collected for root specimen preparation

↓

The tooth will be root planed with Gracey curettes

↓

Middle third of the root is used for specimen preparation using diamond cutting bur on micromotor with continuous saline irrigation.

↓

A total of 44 specimen measuring 0.5x0.5x0.3cm will be obtained and immersed in distilled water, autoclaved before use

↓

The sterilized root specimens will be placed into a 96-well flat bottom plate

↓

The specimens divided into two equal groups (of 22 each)

GROUP 1 (CONTROL)
root specimens treated with solution of 2µL DMEM and 10% FBS, allowed to dry at 37 degree C for 6h

GROUP 2 (TEST)
root specimens will be treated with 2µL DMEM and A-PRF, allowed to dry at 37 degree C for 6h

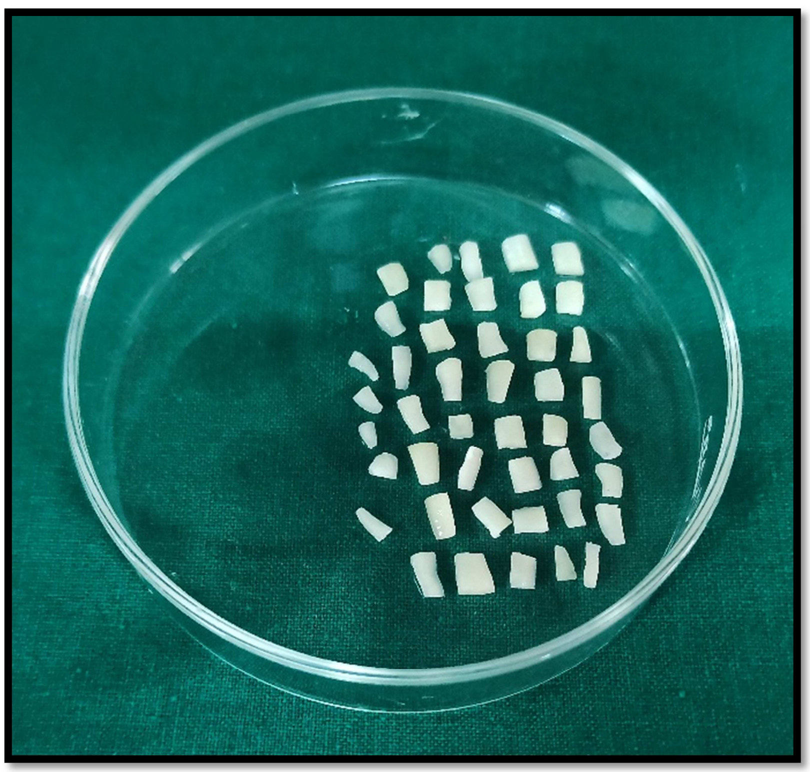
Root sections will be immersed in 200µl of cell suspension and incubated for 24h at 37degree C

↓

Attached fibroblasts will be checked using TRYPHAN blue cell count kit with a hemocytometer.

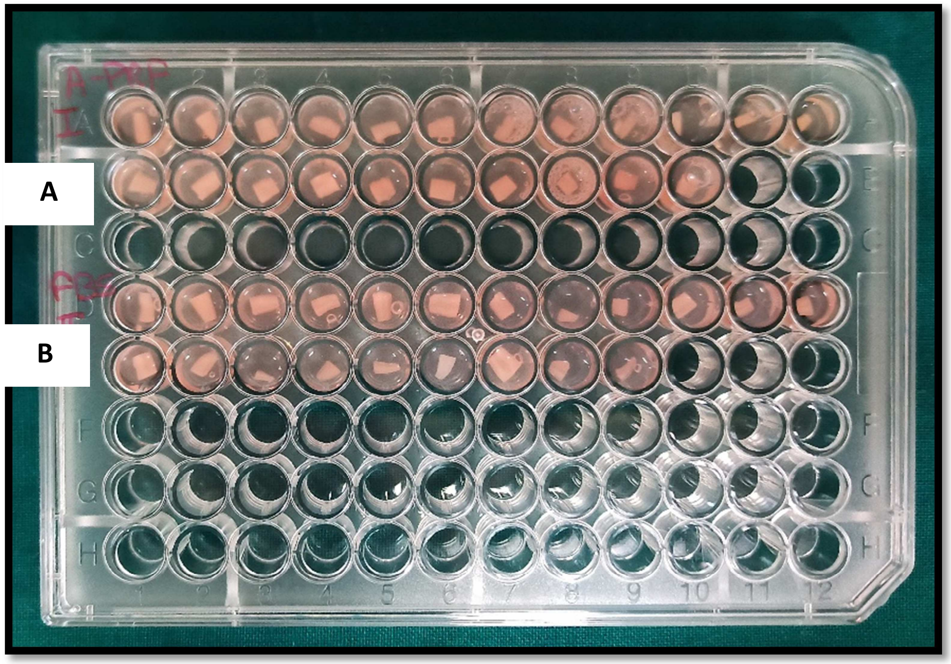
FIGURE 16: PREPARATION OF TOOTH SPECIMEN

Tooth root planed with Gracey curettes



Tooth specimens measuring 0.5x0.5x0.3cm in dimensions for attachment assay

FIGURE 17: SEGGREGATION OF TOOTH SPECIMENS



GROUP A: 22 Root specimens treated with 2μL DMEM and A-PRF, allowed to dry at 37°C for 6h

GROUP B: 22 Root specimens treated with solution of 2μL DMEM and 10% FBS, allowed to dry at 37°C for 6h

STATISTICAL ANALYSIS:

1. Mean and standard deviation was calculated for optical density values for proliferation.
2. Mean and standard deviation was calculated for the distance of wound closure for migration and number of viable cells for attachment.
3. Comparison between and within groups for proliferation and migration was done using ***one way ANOVA.***
4. Pair wise comparison using ***Tukey's post-hoc test*** for proliferation and migration.
5. ***Student t-test*** to compare the number of viable cells for attachment.

RESULTS:

1. **PROLIFERATION (MTT ASSAY)**

TABLE 2: Mean and SD of optical density at 24 hours

GROUPS	MEAN	STANDARD DEVIATION (SD)
GROUP 1	0.555	0.107
GROUP 2	0.493	0.010
NEGATIVE CONTROL	0.368	0.076

Observations:

The mean value of optical density observed at the end of 24 hours in the Group1, Group2 and negative control group were 0.555±0.10, 0.493±0.01, 0.368±0.07 respectively.

TABLE 3: Comparison of optical density at 24 hours by One-way ANOVA.

Sources of variations	Sum of squares	Degree of freedom (df)	Mean square	F-value	p-value
Between groups	0.055	2	0.027	4.648	0.06
Within groups	0.035	6	0.006		
Total	0.09	8			

Observations:

1. One-way ANOVA was applied for comparison between and within groups at 24hours.
2. The difference between and within the groups was not statistically significant.

TABLE 4: Mean and SD of optical density at 48 hours

GROUPS	MEAN	STANDARD DEVIATION (SD)
GROUP 1	0.735	0.237
GROUP 2	0.871	0.219
NEGATIVE CONTROL (NC)	0.368	0.076

Observations:

The mean values of optical density observed at the end of 48 hours in the Group1, Group2 and NC were 0.735±0.237, 0.871±0.219, 0.368±0.076 respectively.

TABLE 5: Comparison of optical density at 48 hours by One-way ANOVA.

Sources of variations	Sum of squares	Degree of freedom (df)	Mean square	F- value	p- value
Between groups	0.407	2	0.204	5.529	0.04*
Within groups	0.221	6	0.037		
Total	0.628	8			

*p<0.05

Observations:

1. One-way ANOVA was applied for comparison between and within groups at 48hours.
2. The difference between and within the groups was statistically significant.

TABLE 6: Pair wise comparison of optical density at 24 hours by Tukey's post-hoc test.

GROUPS	Group1	Group2	NC
Mean	0.555	0.493	0.368
SD	0.107	0.010	0.076
GROUP1	-		
GROUP2	p=0.36	-	
NEGATIVE CONTROL (NC)	p=0.02*	p=0.09	-

*p<0.05

Observations:

1. Tukey's post-hoc test was applied and a statistically significant difference was observed between Group1 and NC at 24hours (p=0.02).
2. Statistically significant difference was not noted between Group1 and Group2 (p= 0.36) and also between Group2 and NC (p= 0.09) at 24hours.

TABLE 7: Pair wise comparison of optical density at 48 hours by Tukey's post-hoc test.

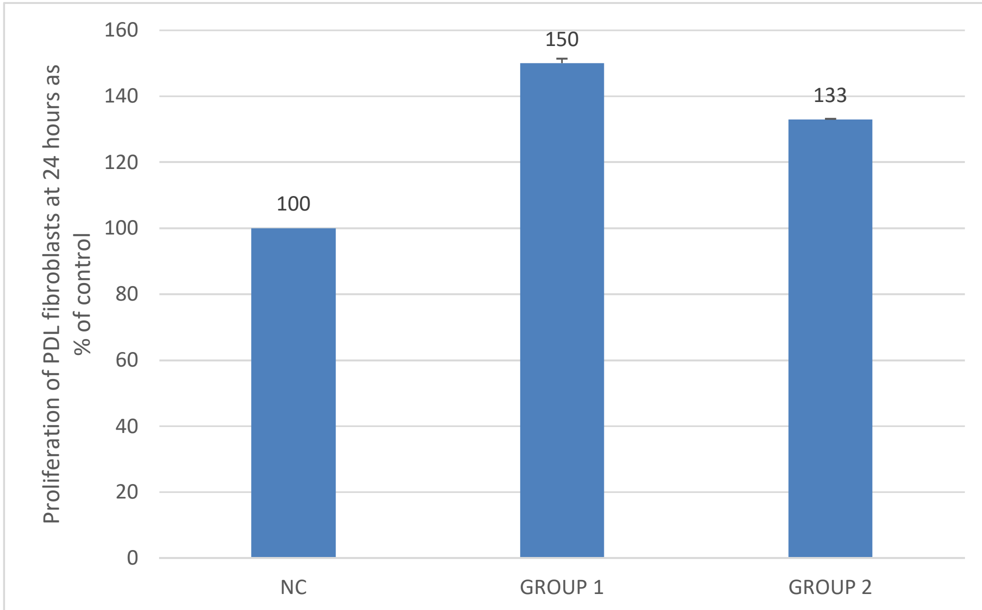
GROUPS	GROUP1	GROUP2	NC
Mean	0.735	0.871	0.368
SD	0.237	0.219	0.076
Group1	-		
Group2	p=0.41	-	
NC	p=0.05	p=0.018*	-

*p<0.05

Observations:

1. Tukey post-hoc test was applied and a statistically significant difference was noted between Group2 and NC at 48hours (p= 0.018).
2. Statistically significant difference was not noted between Group 1 and NC (p= 0.05) and between Group1 and Group2 (p=0.41) at 48hours.

GRAPH 1: Proliferation of PDL fibroblasts at 24 hours.



*p=<0.05

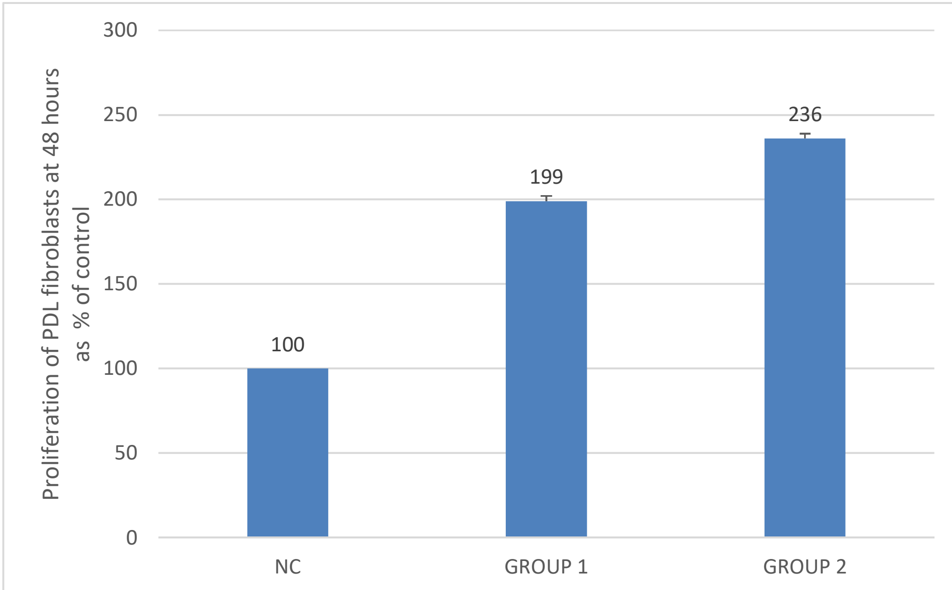
*Denotes statistically significant differences when compared to NC.

Graph 1 depicts the difference seen at 24hours in Group1 and Group2 when compared to the negative control (NC) which was set at 100.

The proliferation rate of PDL fibroblasts at 24 hours increased by 50% for Group1. The proliferation rate of Group2 increased by 33% of that of the NC. At the end of 24 hours, Group1 showed 17% more proliferation than Group2.

Group1 shows statistically significant difference from NC at 24hours. The difference between Group2 and NC and between Group1 and Group2 was not statistically significant. [Table 6]

GRAPH 2: Proliferation of PDL fibroblasts at 48 hours



*p=<0.05

* Denotes statistically significant differences when compared to NC.

Graph 2 depicts the difference seen at 48hours in Group1 and Group2 when compared to negative control (NC) which was set at 100.

The proliferation rate of PDL fibroblasts at 48 hours increased by 99% for Group1. For Group2, the proliferation increased by 136% at the end of 48 hours. The proliferation rate of Group2 increased by 37% compared to Group1.

The difference in proliferation rate for group 2 was statistically significant compared to NC. The difference in proliferation between Group1 and NC and between Group1 and Group2 was statistically not significant. [Table 7]

2. MIGRATION ASSAY (IN-VITRO SCRATCH ASSAY)

TABLE 8: Mean and standard deviation (SD) of wound closure (µm) at 0hr, 24hrs and 48hrs.

Time	**GROUP1**		**GROUP2**	
	Mean	SD	Mean	SD
0hr	94333.33	1604.16	64100.00	4784.35
24hrs	11333.33	1761.63	0.00	0.00
48hrs	0.00	0.00	0.00	0.00

Observations:

The mean distance (µm) of wound closure observed in Group 1 and Group 2

At 0 hour, 94333.33±1604.16 and 64100.00±4784.35 respectively.

At 24 hours, 11333.33±1761.63 and 0.00±0.00 respectively where, 0.00±0.00 indicates complete closure.

At 48 hours, 0.00±0.00 and 0.00±0.00 respectively. [Indicating complete wound closure for both groups]

Table 9: Intra-Group comparison at timely intervals for the distance of wound closure (μm) by one-way ANOVA.

GROUP 1	Sources of variation	Sum of squares	Degree of freedom (df)	Mean of squares	F-value	p-value
	Between groups	15916222222	2	7958111111	4205.696	0.000
	Within groups	11353333.33	6	1892222.222		
	Total	15927575556	8			

GROUP 2	Sources of variation	Sum of squares	Degree of freedom (df)	Mean of squares	F-value	p-value
	Between groups	8217620000	2	4108810000	538.507	0.000
	Within groups	45780000	6	7630000		
	Total	8263400000	8			

*p<0.05

Observations:

One-way ANOVA was applied for intra-group comparison.

Group1 and Group2 showed a statistically significant difference for the distance of wound closure at 0hr, 24hrs and 48hrs for both groups.

Table 10: Comparison of the distance of wound closure (μm) by Student T-test.

Groups	N	Mean	SD	t-Test value	p-value
Group1	9	35222.22	44620.02851	0.756	0.461
Group2	9	21366.67	32139.15058		

Observations:

Student T-test for comparison between the groups, for the distance of wound closure was not statistically significant (p=0.461).

TABLE 11: Pair wise comparison of two groups at timely intervals for the distance of wound closure in micrometre (µm) by Tukey's post-hoc test.

	0hr	24hrs	48hrs	0hr	24hrs	48hrs
	GROUP 1			GROUP 2		
Mean	94333.33	11333.33	0.00	64100.00	0.00	0.00
SD	1604.16	1761.63	0.00	4784.35	0.00	0.00
0hr	-			-		
24hrs	p= 0.00*	-		p= 0.00*	-	
48hrs	p= 0.00*	p= 0.00*	-	p= 0.00*	p= 1.00	-

*p<0.05

Observations:

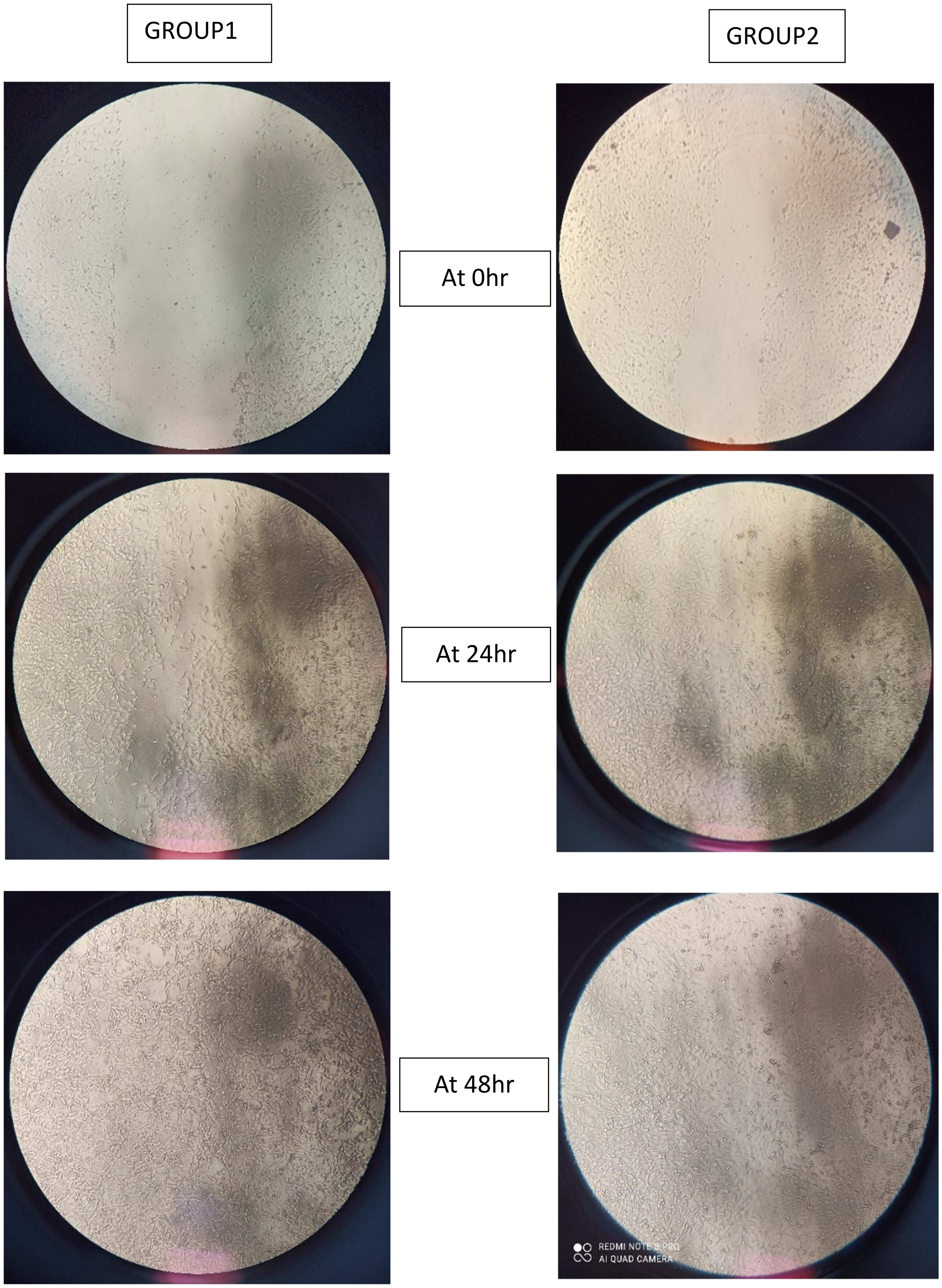
1. Tukey's post-hoc test for Group1 and Group2 showed statistically significant difference at 0hr, 24hrs and 48hrs (p=0.00 for both groups).
2. The difference was not statistically significant within Group2 at 24 and 48 hours (p=1.00).

Graph 3: Comparison of Group 1 and Group 2 for distance of wound closure at timely intervals.

Observations:

1. At 24hrs, Group1 showed significant wound closure and Group2 showed complete wound closure.
2. At 48hrs, Group1 showed complete wound closure.

FIGURE 18: Wound closure seen at 0 hour, 24 hours and 48 hours for Group 1 and Group 2.



3. ATTACHMENT ASSAY

Table 12: Mean and standard deviation (SD) of attached cells by tryphan blue staining in two groups by Student t-Test.

Groups	N	Mean	SD	T- Test value	p-value
Group1	22	92213	5107.6	8.420	0.00*
Group2	22	125276	17695		

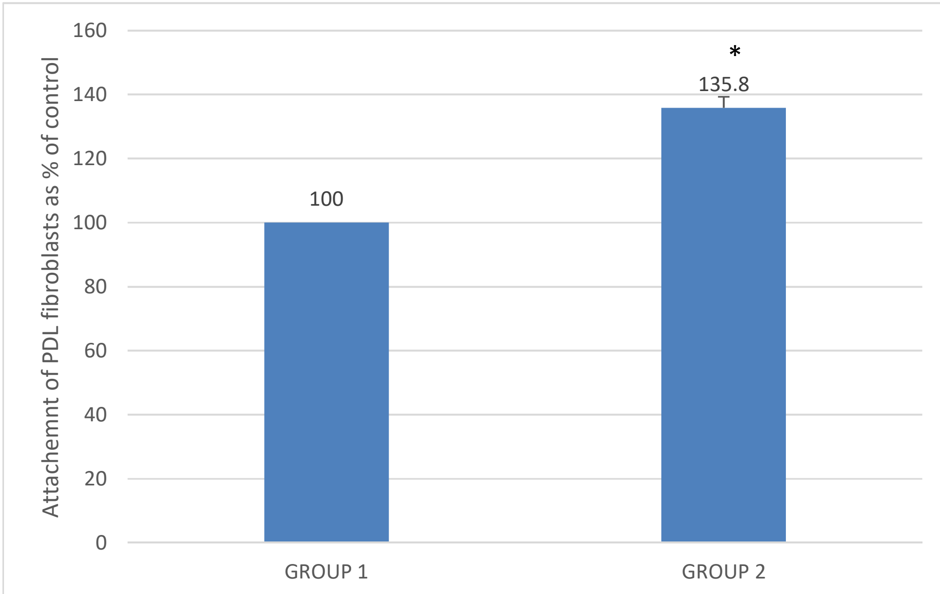
*p<0.05

Observations:

Mean number of cells attached to the tooth specimen in Group1 and Group2 were 92213±5107.6 and 125276±17695 respectively.

A highly statistical significance was observed between Group 1 and Group 2.

Graph 4: Comparison of mean value of number of cells attached in Group1 and Group2.



*Denotes significant differences between groups with $p<0.05$.

Observations:

The attachment in Group 2 showed increase by 135.8% than Group 1.

[Difference in the number of cells attached is expressed in terms of mean ± SD as % of control.]

DISCUSSION:

The connective tissue cells of the periodontium actively participate in wound healing and periodontal regeneration which is dependent on the activation of particular cell types under the influence of certain biologic clues [4]. The proliferation and migration of these resident cells is greatly influenced by the presence of growth factors which alters and favours the microenvironment towards periodontal regeneration [5].

The use of platelet concentrates in regenerative dentistry has proven to be significant because of its inherent property to release growth factors delivered at the defect site. Literature provides evidence to the use PRP [2,38,39] and PRF [27,29] for periodontal regeneration through in-vitro and in-vivo studies. Ease of preparation and release of growth factors over a period of time demonstrates the advantages of PRF over PRP [11,20].

Studies have been conducted on PRF to evaluate for its effect on cell behaviour of PDL fibroblasts. These studies have shown that PRF positively impacts the cellular activity and enhances cellular proliferation, migration, differentiation of human gingival fibroblasts, PDL fibroblasts, osteoblasts [14,27,28,29,40].

The advanced platelet-rich fibrin (A-PRF) is centrifuged at slower speeds (1500 rpm, 14 min) and the modification to centrifugation force has shown to increase the platelet cells and monocyte/macrophage number and cell behaviour [18,20]. Very few studies in the literature have evaluated the effects of A- PRF on PDL fibroblasts [19,25,26].

Therefore, the present study was conducted with aim to assess the effectiveness of A-PRF on proliferation, migration and attachment of human PDL fibroblasts.

Cell Proliferation of PDL fibroblasts:

The present study has compared the proliferation activity of PDL fibroblasts to a standard group i.e. DMEM media only which served as a negative control (NC), and the effects of Group1 (DMEM+10%FBS) and Group2 (A-PRF) were compared to the standard group. Proliferation of PDL fibroblasts was observed using MTT assay [Figure 14].

The activity of the PDL fibroblasts was assessed at 24hrs and 48hrs. At 24 hours, Group1 showed statistically significant (p= 0.024) difference in the proliferation of periodontal fibroblasts than the NC. Whereas, at 24hrs, no statistical difference was noted between Group2 and the NC (p=0.09), and between Group1 and Group2 (p=0.36). [Table 6]

At 48hrs, the difference between Group1 and NC and also between Group1 and Group2, was not statistically significant. However, at 48 hours, Group 2 compared to NC showed a difference with statistical significance activity (p= 0.018). [Table 7]

When the proliferation rate of fibroblasts was compared to that of NC which was set at 100, proliferation of cells in Group1 increased by 50% and in Group2 by 33% at the end of 24hrs. At 24hrs, Group1 showed an increase in proliferation by 17% than Group2. [Graph 1]

At 48hrs, Group1 showed an increase by 99% and Group2 by 136% as compared to the negative control. When noted at 48hrs, Group2 showed an increase in proliferation by 37 % compared to Group1. However, the results did not show a statistical difference between the study groups. (p= 0.36 at 24 hours, p= 0.41 at 48 hours). [Graph 2]

The results obtained can be explained because of the A-PRF matrix as it is reported to have a porous structure with an even distribution of immune cells thereby providing a continuous release of bioactive agents for a defined period of time as compared to other platelet concentrates [26,41]. However, with more time needed to attain specific amount of growth factors from A-PRF, this can be attributed to the difference between groups in the present study which was not significant at 24 and 48hrs.

The effectiveness of different types of PRF, L-PRF and A-PRF was evaluated on human gingival fibroblasts. At 24hrs, proliferation of gingival fibroblasts was increased by 87.5% for L-PRF and 52% for A-PRF as compared to NC (with 1% FBS; which was set as 100). However, at 48 hours, A-PRF showed 40% increase compared to L-PRF which showed 32.3% increase in the proliferation activity. The result reported was similar to the findings of the present study [25].

In a study where the proliferation of PDL fibroblasts was evaluated using L-PRF, A-PRF and FGF at 24hrs reported similar results. A-PRF showed increased proliferation by 69.2%, L-PRF by 49.6% and FGF by 26.7% compared to the control media. The intra-group comparison was also similar to the present study where, the two PRF groups and FGF group showed no statistical difference ($p>0.05$) [26].

Effect of optimised PRF with a low-speed concept was evaluated on gingival fibroblasts. L-PRF (control), A-PRF (14 minutes at 1300rpm) and A-PRF+ (8 minutes at 1300rpm) were compared and the results demonstrated that all the PRF experimental groups showed significant increase in cell proliferation at day 3 and day 5. However, they found that A-PRF and A-PRF+ groups showed slightly better results than L-PRF group at day 5. The marked difference in the centrifugation forces in A-

PRF lead to the formation of an A-PRF clot with even distribution of platelets, leukocytes and growth factors release over a definite period of time [19].

The proliferation of human periosteal cells was examined using A-PRF, CGF, PRGF and PRP. The in-vitro study reported that the order of growth factor release, the highest was from A-PRF and CGF which were almost similar followed by PRP and PRGF being the lowest amongst all. The proliferation of human periosteal cells was more in A-PRF and CGF group thus the presence of A-PRF enhanced the activity of periosteal cells leading to increased number of osteoblasts and promoting bone regeneration [34]. Present study reported similar results as to the above-mentioned experimental groups eliciting the positive effect of A-PRF in proliferation of PDL fibroblast [19,25,26,34].

Cell Migration of PDL fibroblasts:

Present study evaluated the migration of human PDL fibroblasts using the in-vitro scratch assay and the distance of wound closure was measured in μm using the ImageJ software [36] at 0hr, 24hrs and 48hrs [Figure 15]. The mean distance of wound measure at 0hr for Group1 and Group2 was 94333.33±1604.16 and 64100.00±4784.35 respectively [Table 8]. This difference between the groups at the start of the experiment could be attributed to the disparity caused by the difference in the width of the scratched wound.

Intra-group comparison for Group 1 and Group 2 was statistically significant. [Table 9] Intergroup comparison of Group1 and Group2 did not show statistical significance difference. [Table10]

At 24hrs, a significant wound closure was observed in Group 1 which was 11333.33±1761.63 (p=0.00) and at 48 hours, complete wound closure was seen for Group 1 i.e. 0.00±0.00 [Figure 15]. Whereas, for Group 2, a significant (p=0.00) and complete wound closure i.e. 0.00±0.00 was observed at 24 hours which was maintained at 48 hours. [Table 11, Graph 3]

In an in-vitro study cell migration of human gingival fibroblasts was evaluated using the same in-vitro scratch method for L-PRF and A-PRF. This study found that, at 24hrs, L-PRF significantly promoted the cell migration (p>0.05) as compared to control group (1%FBS). Whereas at 48hrs, the cell migration was significant in A-PRF group (p>0.05) compared to control group. Comparing the difference at 24 and 48 hours, only A-PRF group showed statistically significant increase in cell migration (p>0.001). This finding is similar in the present study where A-PRF showed better migration at both 24 hours and 48 hours [(25)].

In an in-vitro study it was reported that A-PRF + stimulated cultures secreted higher levels of eotaxins, CCL5, PDGF, and VEGF by 3-, 1.6-, 3-, and 1.2- folds respectively. Eotaxins and CCL5, pro-inflammatory chemokines are chemotactic for T cells and leukocytes. These agents are overexpressed during initial phase of tissue injury. VEGF and PDGF and promote chemotaxis of macrophages thereby favours migration of fibroblasts which can be anticipated in the results of the present study [(42)].

Cell migration of PDL fibroblast was evaluated using L-PRF, A-PRF and FGF. The results reported A-PRF and L-PRF to show significant cell migration of PDL fibroblasts (p>0.01) as compared to FGF. When speed of wound closure was assessed, A-PRF group showed higher speed of cell migration when compared to other groups which was statistically significant ($p>0.01$). The results suggest A-PRF+ contains a different and/or more pronounced cocktail of growth factors and chemokines promoting migration, and with less number of inhibitory components [26].

The effect of different forms of A-PRF was evaluated by optimising the centrifugation force. The effect of optimised PRF i.e. A-PRF (14 minutes at 1300rpm) and A-PRF+ (8 minutes at 1300rpm) was reported on cell migration of gingival fibroblasts. All the PRF experimental groups showed significant enhanced migration of human gingival fibroblasts where L-PRF showed 200%, A-PRF and A-PRF+ showed 300% increase in cell migration. No significant difference was seen in the cell migration with A-PRF and A-PRF+. The authors concluded that reducing the centrifugation force will lead to even distribution of leukocytes throughout the A-PRF clot in contrary to PRF clot where the leukocytes were located at the bottom of the clot [19].

The findings of the discussed studies along with results of the current study supports the role of A-PRF in promoting wound closure gap through migration of PDL fibroblast cells.

Attachment of PDL fibroblasts:

The evidence available in the literature indicates that root conditioning either with root planning alone [43,44] or root biomodification using various agents like tetracycline [45], EMD [46,47] facilitates attachment of PDL fibroblasts.

The attachment assay in the present study was performed according to the protocol given by Alleyn et al [37]. Briefly, 44 dentin root specimens were placed in 96-well plates. 22 tooth specimens were exposed to aliquot of DMEM+FBS and 22 were exposed to aliquot of DMEM+A-PRF for 24 hours. Following this, the live cells were calculated using 0.4% tryphan blue staining and Neuber's hemocytometer under a microscope.

The mean value of number of viable cells in Group 1 was 92,213±5107.6 and in Group 2 was 1,25,276±17695 respectively. A statistically significant result was noted when compared between the two groups ($p<0.001$). [Table 12]. The attachment of fibroblast cells seen in A-PRF group was 135% more than the control without A-PRF. [Graph 4] To the best of our knowledge there are no studies on the role of A-PRF for attachment assay of PDL fibroblasts and other different cell lines.

However, in an in-vitro study different concentrations of PRP were used to evaluate attachment of PDL fibroblasts on dentin slabs. In this study, 10% PRP significantly increased the fibroblast attachment as compared to 5% PRP and the control group. The release of growth factors from PRP, the presence of VWF, fibrinogen and attachment factors like fibronectin present in platelets would have influenced the attachment of cells to the dentin structure [(32)]. The concept of growth factor release is similar for PRP and A-PRF though they defer in the quantity of released growth factors [(26)], we can assume that the mechanism for attachment of the cells might be the reason for increased activity noted with A-PRF

In another study the attachment of osteoblasts was evaluated using PRF with WST-1 colorimetric assay from Roche. It was found that as compared to the control group (without PRF), PRF group showed significant increase in cellular attachment in a time dependent manner which was 35%, 55%, 91%, 98% and 100% at 0.5,1, 2 ,3 and 4 hours respectively and was statistically significant when compared to the control group ($p<0.05$). It was concluded that, PRF greatly enhanced cell attachment and accelerates wound healing [(48)].

Overall, A-PRF has shown to promote the cellular behaviour of PDL fibroblasts by enhancing its proliferation, migration and attachment to dentin surfaces. The use of A-PRF proves to be advantageous when compared to other platelet preparations in relation to the number of growth factor release as reported by Kobayashi E et al [22]. The mechanical strength of A-PRF membrane is proven to be superior than other platelet concentrates thereby can be a reservoir of growth factors and serve as a scaffold for tissue regeneration [34,41].

The shortcomings of the present study include the variation caused by the difference in the width of the scratches obtained during artificial wound creation for cell migration assay which could be minimised by use of special migration plates. Secondly, the A-PRF preparation which involved the use of different donor blood samples for each cell assay could have been obtained from one individual. As the literature mentions that the PRF membranes obtained from younger age group individuals have more number of platelets, improved fibrin matrix architecture and enhanced antimicrobial property, this directly affects the growth factor release pattern from the PRF membrane [49]. So, the present study could have focused on collecting blood from one individual for preparing A-PRF.

SUMMARY AND CONCLUSION

The aim of the current study was to assess the effect of advanced platelet rich fibrin (A-PRF) on proliferation, migration and attachment of PDL fibroblasts. Study was conducted in the Department of Periodontics, KLE VK Institute of dental sciences, Belagavi and the laboratory procedures were carried out in the KLE's Dr. Prabhakar Kore Basic Scientific Research Centre, Belagavi.

PDL fibroblasts were harvested from healthy individuals who were planned for orthodontic extraction. Middle third portion of the root was scrapped to collect PDL fibroblasts. The cells from 3-8 passages were taken for the experiment. The cells were equally divided in two groups, in Group1 cells were exposed to DMEM+FBS and in Group2 cells were exposed to DMEM+A-PRF for 24hrs.

The Cell proliferation of PDL fibroblasts was evaluated at 24hrs and 48hrs using the MTT assay and the groups were compared to negative control (only DMEM). At 24hrs, A-PRF group did not show a statistical difference in proliferation rate when compared to Group 1 (p= 0.36) and negative control group (p= 0.09). However, at 48hrs, A-PRF group showed statistical difference in proliferation rate when compared to negative control (p= 0.018). Compared to Group1, difference was not statistically significant (p= 0.41).

Cell migration was done using in-vitro scratch wound assay which measured the distance covered by the PDL fibroblasts when exposed to Group1 and Group2 at 0, 24 and 48hrs using the ImageJ software. At 24hrs, complete wound closure was attained in Group 2 which was not noted in Group 1 with statistical significant difference (p=

0.00). At 48 hours, Group1 showed complete wound closure, which was also maintained for Group2 as seen at 24hrs.

The literature lacks in providing evidence to show the attachment of PDL fibroblasts when exposed to A-PRF. In the present study, the attachment of PDL fibroblasts reported a highly significant difference between two groups (p= 0.00) where, Group 2 showed 135.8% increased rate of attachment when compared to Group1.

Within the limitations of the present study, following conclusions can be made:

1. At 48hrs A-PRF showed increased proliferation by 136% as compared to Group 1. However, the activity of A-PRF at 24 hours was not significant as compared to Group1.
2. At 24 hours A-PRF showed complete wound closure and the difference between the groups was significant. Whereas, Group 1 showed complete wound closure at 48 hours which was maintained for A-PRF at 48 hours.
3. The attachment of PDL fibroblasts was significant in A-PRF group which was highly significant and increased by 135.8% more than Group 1.

Hence to conclude, A-PRF showed a positive impact on cellular behaviour of PDL fibroblasts which includes cell proliferation, migration and attachment. However, in-vitro and in-vivo studies are required which will provide an insight to validate the utility of A-PRF in periodontal wound healing and regenerative therapy.

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ANNEXURES:

1. **Ethical clearance certificate**

Research and Ethics Committee

KLE V K INSTITUTE OF DENTAL SCIENCES

KLE University

KLE UNIVERSITY EMPOWERING PROFESSIONALS

Accredited 'A' Grade by NAAC   Placed in Category 'A' by MHRD (GoI)

Nehru Nagar, Belagavi - 590 010, Karnataka State

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Sl. No. : 1314

CERTIFICATE

This is to Certify that the synopsis titled ASSESSMENT OF THE EFFECT OF ADVANCED PLATLET RICH FIBRIN (A-PRF) ON PROLIFERATION, MIGRATION AND ATTACHMENT OF HUMAN PERIODONTAL LIGAMENT FIBROBLASTS - IN VITRO STUDY. *Submitted by Dr.* NIKITHA. SHETTY *P. G. Student / Staff, Guided by* DR. MENAKA. K.B. *from Department of* PERIODONTICS *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
KLBVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belgaum

2. Consent Form

DEPARTMENT OF PERIODONTICS

KLE V.K. INSTITUTE OF DENTAL SCIENCES

BELAGAVI.

ASSESSMENT OF THE EFFECT OF ADVANCED PLATELET RICH FIBRIN (A-PRF) ON PROLIFERATION, MIGRATION AND ATTACHMENT OF CULTURED HUMAN PERIODONTAL LIGAMENT FIBROBLASTS – AN IN-VITRO STUDY.

Principal Investigator: **IK0219005**

I ________________________________, aged ________ years have been informed about my involvement in the study.
I agree to give my personal details like Name, Age, Gender, Residential Address, past and Present dental history and any other details if required for the study to the best of my knowledge.
I will co-operate with the dentist.
I will follow the instructions given by the dentist during study.
I permit the dentist to utilize the information given by me and the results obtained from this study for presentation and publication without disclosing my identity.
I have been informed that the blood sample drawn from me will be used to prepare a newer agent which will be used for the study, and I permit the dentist to perform the same.
If by chance any complications arise during the above said procedure, I permit the dentist to take necessary actions to prevent the same.
In my full consciousness and presence of mind, after understanding all the procedures and related complications if any, in my vernacular language, I am willing and give my consent to participate in this study.

Date:
Name of the Patient:

Signature:

Address & Ph. No:

Name of witness/guardian:

Signature:

3. Optical densities of three groups at 24 and 48 hours

TIME	DISTANCE OF WOUND CLOSURE MEASURED IN µm		
	GROUP 1	**GROUP 2**	**Negative control**
24HOURS	0.679 0.506 0.481	0.506 0.487 0.487	0.455 0.339 0.31
48 HOURS	0.872 0.461 0.874	0.62 1.021 0.974	0.455 0.339 0.31

4. Distance of wound closure measured using IMAGEJ software

TIME	DISTANCE OF WOUND CLOSURE MEASURED IN µm	
	GROUP 1	**GROUP 2**
0 HOUR	96000 94200 92800	68100 65400 58800
24HOURS	13200 11100 9700	**0**
48 HOURS	**0**	**0**

5. Attachment of PDL fibroblasts in two groups at 24 hours.

GROUP 1	Number of cells attached	GROUP 2	Number of cells attached
1	97,350	1	1,21000
2	91,675	2	146000
3	98251	3	1,32000
4	92,666	4	1,40000
5	90,123	5	1,49000
6	82,970	6	1,41200
7	87,561	7	1,19870
8	85,341	8	98,000
9	89,900	9	1,27890
10	91,000	10	1,10000
11	99,950	11	99,175
12	95,712	12	1,57210
13	93,119	13	1,32000
14	81,980	14	1,30900
15	88,999	15	1,19000
16	90,560	16	97,289
17	94,390	17	94,461
18	96,671	18	1,42980
19	88,381	19	1,33000
20	95,340	20	1,21000
21	97,100	21	1,16,000
22	99,653	22	1,28100