Original Article

BIOACTIVATED MARINE COLLAGEN MATRIX WITH INJECTABLE PLATELET RICH FIBRIN IN SOFT TISSUE AUGMENTATION - AN IN-VITRO WOUND HEALING STUDY

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Abstract

Objectives: The study intended to assess the efficacy of collagen matrix of marine origin impregnated with injectable plasma rich fibrin (I-PRF) on fibroblast migration and viability in comparison with plain collagen matrix.

Methodology: Standard I-PRF obtained by centrifugation of 10ml of whole blood at 700 rpm (60G force) for 3 minutes was impregnated in collagen scaffold, was checked for fibroblast biocompatibility at 24 hours (MTT assay & fluorescent microscopy); for fibroblast migration at 24, 48, 72 hours, and results were compared with plain collagen matrix.

Result: Cell viability assay showed the viability of 98.8% for collagen and 98.1% for collagen with I-PRF. Cell migration at 24, 48 and 72 hours were 4.2% and 12.5%, 47.9% and 50%, 77% and 98.75% for collagen and collagen with I-PRF respectively.
Cell viability assay showed no cytotoxicity with collagen and collagen impregnated with I-PRF and cell migration was found to be better in collagen impregnated with I-PRF.

Conclusion: Marine Collagen and marine Collagen with I-PRF showed the potential for tissue regeneration via induction of fibroblast behavior. Bioactivation of collagen matrix with I-PRF might enhance soft tissue healing and tissue regeneration.

Keywords: Collagen, Fibroblast, Platelet rich fibrin, Tissue engineering.

Introduction

Regeneration is the restoration of lost tissues where the architecture and function could entirely be reestablished. Though, regeneration is a naturally occurring phenomenon in several primitive species, in humans, healing mostly occurs by tissue repair rather than tissue regeneration. With the advent of tissue engineering concept, regeneration of any lost tissue could now be a possible reality. The constituents of tissue engineering triad are 'the cells'- the building block of tissues, 'the signaling molecules'-that enhances or initiates a series of responses that would decide the activity of the cell, and "the scaffold"- that supports or facilitates the repopulation of cells for regeneration forming the triad that continuously interacts with each other.[1]

Collagen is an eminent protein of the extracellular matrix that constitutes about 1/3rd of the total protein in the body. Over 20 different types of collagen have been identified of which type I & III contribute significantly in

wound healing events. [2] Collagen matrix acts as a biological scaffold allows adherence of platelets, [3] which on activation releases thromboxane A2 that facilitates the formation of the blood clot, while the 3D matrix by itself helps in strengthening of the clot. Collagen attracts monocytes that debride the wound. [4] It acts as a scaffold for growth of blood vessels and tissues through directed migration of cells, [5] like endothelial cells, fibroblasts [6] and, keratinocytes [7] all of which facilitates deposition of oriented and organized fibres that increase the integrity of the tissue. [8] It can also induce the release of growth factors and cytokines involved with tissue regeneration like cellular attraction, multiplication and differentiation, neo vascularization, interstitial matrix production that result in transition from inflammatory to the remodeling phase expediting the process of regeneration. The scaffold used in this study is type 1 collagen of marine origin that has been used widely and successfully in various fields of medicine (diabetic foot ulcers, chronic ulcers, burns, post-amputation surgeries, etc.) The advantages of collagen of marine origin are that there is no threat of zoonoses such as BSE (bovine spongiform encephalitis), TSE (transmissible spongiform encephalitis), and FMD (foot mouth disease). [9] It presents with higher content of collagen and greater absorption owing to its low molecular weight. It elicits a low inflammatory response and is metabolically compatible and has minor regulatory and quality control problems.

Platelet concentrates are obtained from a patient's blood where activated platelets get meshed within fibrin matrix and liberate cytokines and growth factors that ameliorate host response, and play an effective role in processes of tissue regeneration. It has been successfully utilized for various treatment modalities in both medicine and dentistry for over two decades. PRF is a second-generation platelet concentrate. It was developed by Choukroun et al in 2001 and is known to accelerate wound healing, as well as hard and soft tissue remodeling with added advantages like easy chair side preparation with no additives, inexpensive and ready for immediate use. [10]

A 'low-speed centrifugation concept' postulated that by reducing the G-force the number of inflammatory cells & growth factors would increase, heightening the healing and regenerative properties.[11] A liquid form of PRF was developed by Mourao et al in 2015 termed as injectable PRF released larger concentrations of different growth factors and increased fibroblast migration and enhanced expression of PDGF, TGF-β, and collagen1,[12] with antibacterial activity against periodontal pathogens like P.gingivalis, A.actinomycetum comitans,[13] enhanced angiogenic activity with up-regulation of wound healing[14] and increased osteoblast migration, adhesion, proliferation, differentiation.[15]

In this study we have used commercially available fibroblast cell lines as "cells", the growth factors released by the alpha granules of activated platelets as "signaling molecules", and type 1 collagen matrix of marine origin as the "scaffold" and checked for viability and migration of 'fibroblasts'- the cells that take part in the extracellular matrix and collagen synthesis. The study was an in-vitro analysis done prior to clinical trial comparing the efficacy of plain marine collagen matrix and marine collagen matrix impregnated with I-PRF in treatment of Miller's class I and II gingival recession defects The study aims to evaluate the non toxicity and improved wound healing characteristics of the combination.(marine collagen+I-PRF)

Materials and methods

The study got its approval from the institutional review board (IRB NO: SRMDC/IRB/2018/MDS/NO.501).

Patient selection

3 systemically healthy subjects undergoing routine blood investigations were chosen for the study. 2 females and 1 male patient aged between 23 to 40 years who had normal platelet counts and no history of any systemic diseases or conditions, not pregnant, nonsmokers were included in the study after obtaining informed consent.

Cell culture

Murine fibroblast cell line (3T3 cell line) was purchased from National centre for cell sciences (NCCS, Pune, India). EDTA-trypsin 0.25% was used to detach the cells from tissue culture plastic. The cells were allowed to reach confluence. Cells from 4^{th} to 6^{th} passage were utilized for the study. Cell culture was done in a humidified

atmosphere at 37ºC in a medium consisting of DMEM (Gibco), FBS 10% (Gibco), and antibiotics 1% (Gibco). The media was changed twice per week.

I-PRF Protocol

10ml of whole blood was drawn from the subjects from the anti cubital vein and was collected in manufacturer-specified I-PRF tubes without any additives. It was centrifuged at a pre-programmed spin for I-PRF (700 rpm for 3 minutes with 60 g force) at room temperature in a pre programmed centrifuge*. After centrifugation was complete, the blood split into a yellow-orange upper phase and a red lower phase. The upper phase which was the liquid PRF was then retrieved using a sterile syringe. [Figure1a-d] I-PRF remains in its liquid state for up to 10-15 minutes.

Sample preparation

The type I collagen matrix of marine origin# was cut into small squares of length 2mm. (plain collagen matrix) 0.1ml of I-PRF was infused into the collagen. (Collagen matrix impregnated with I-PRF) [Figure1e]

The fibroblasts obtained from cell culture were treated with test samples of plain marine collagen matrix (T) and marine collagen matrix impregnated with I-PRF(C) for cell viability and migration studies.

*Dentifuge LD C-10® Labtech Disposables, Ahmedabad-380015, Gujarat, India

Biofil sponge® Eucare PharmaceuticalsThirumudivakkam, Chennai-600044 Tamil Nadu, India

Cytotoxicity effect on cells– MTT assay

The cytotoxicity on murine fibroblasts (3T3) to collagen matrix (C) and collagen matrix impregnated with I-PRF (T) was assessed by MTT assay. [16]

The principle behind the MTT Assay was that when tetrazolium dye was added to cells it reacts with NADPH dependant oxidoreductase enzyme produced by mitochondria of metabolically active cells and produces insoluble formazan crystals which were purple. It was then dissolved in appropriate solvent and intensity of the color was read spectrometrically. The intensity of the purple color was compared with the standard control and then the cell viability was calculated.

96-well microplates were used to seed the fibroblasts. (1 x 10^6cells/well) Incubation was done for 24 hours using 5% CO_2 incubator with temperature maintained at 37°C until cells reached 80% confluence. After replacement of the medium, [DMEM (Gibco), FBS 10% (Gibco)] the cells were exposed to the samples (plain collagen and collagen impregnated with I-PRF) and incubated for another 24 hours. Cells were washed with phosphate buffer saline (pH7.4). 20µL of MTT solution (5 mg/mL in PBS) was added in to each well. It was left undisturbed for 1-3 hours in the dark at a temperature of 37ºC. Formazan crystals were dissolved in 100 µL DMSO (dimethyl sulfoxide). The absorbance rate was studied spectrometrically at 570nm. The changes in unexposed cells (standard tissue culture plastic - control) and exposed cells (collagen matrix (C) and collagen matrix impregnated with I-PRF (T) - test samples) were examined after 24 hours under a bright field microscope and photographed. [Figure2 a-d]

Scratch wound healing assay

The standardized protocol given by Liang et al., in 2007 [17] was utilized to evaluate fibroblast migration between plain collagen (C) and I-PRF impregnated collagen (T). 6-well plates were used to seed the 3T3 murine fibroblasts (8×10^5 cells/well) and were allowed to reach a confluence of 90%. A scratch with a P10 pipette tip was made in

the centre of monolayer of cells which simulated an injury. A fresh medium [DMEM (Gibco), FBS 10% (Gibco)] was used to eliminate the debris. After exposure to the samples, [collagen matrix (C) and collagen matrix impregnated with I-PRF (T)] incubation was done in 5% CO_2 incubator at a temperature of 37°C. Under an inverted microscope wound closure examination was done. 4 digital images at 0hr (t0), 24hrs (t1), 48hrs (t2), and 76 hrs (t3) were taken. [Figure3a-h] The scratch closure rate [18] was assessed using the ImageJ software.

Statistical analysis

Every step was done in triplicate. Statistical analysis was done using SPSS software version 17. Mean and standard errors were calculated. Staistical significance was calculated using one way ANOVA and comparative pairwise analysis was done using Post Hoc test for cell viability assay. Staistical significance was calculated using Independent T test for scratch wound healing assay. P value of <0.05 was considered statistically significant

Results

Cell viability assay

Cell viability of murine fibroblasts (3T3 Cells) with plain collagen, and collagen impregnated with I-PRF at 24 hours showed good compatability with high number of living cells. [Figure2 a-d] One way ANOVA analysis reveal that there were significant difference in cell viability among study groups with p value 0.001. Pairwise comparison using Post Hoc assay suggested that significant differences were noted between control and plain collagen, control and I-PRF impregnated collagen in terms of cell viability. There was no significant difference between plain collagen and I-PRF impregnated collagen. Graph was prepared with Y-axis representing cell viability (%) and X-axis representing the samples. [Graph1]

Scratch wound healing assay

Cell migrations at 24, 48 and 72 hours were recorded for collagen, and collagen with I-PRF (Table2,Figure3 a-h) T test analysis showed that there was statistically significant differences in wound closure rate between test and control groups at all time points. ($p<0.001$) Graph was prepared with scratch closure rate (%) at Y-axis and time on X-axis. [Graph2]

Discussion

Healing is a multi-staged process that involves platelets, leukocytes & growth factors. Platelets being the first cells to appear during trauma execute a crucial role in hemostasis & wound healing. PRF is an autologous fibrin-based, living biomaterial, used for regenerative procedures in fields like endodontics, periodontal and implant surgeries, oral and maxillofacial surgeries, with propitious outcomes. I-PRF one of the second-generation platelet concentrates could have an extended and or alternative use by exploitation of the regenerative properties of PRF in the liquid (injectable) form. I-PRF protocol follows the LSCC where limiting the relative centrifugal force (RCF) leads to an increased number of regenerative cells with elevated levels of growth factors in comparison with other formulations of PRF. [12]

Various in-vitro studies demonstrate that I-PRF has increased leukocyte numbers, can direct and recruit various cell types, and can influence the activity of gingival fibroblasts. [19] I-PRF also showed increased osteoblast migration, adhesion, proliferation, differentiation, via increased expression of genes runx2, alkaline phosphatase, collagen1 and osteocalcin. [15] The higher levels of PDGF, TGF-β, IL-10, fibronectin, [19] the anti-inflammatory effect by influencing macrophage and dendritic cells [20] signifies its potential to enhance wound healing and facilitate hard and soft tissue regeneration

Clinically I-PRF has been used in the management of diabetic and nonhealing ulcers, eardrum perforations, in cosmetic procedures like smoothening fine lines, wrinkles, accentuate nasolabial folds, lip augmentation, acne treatment, scar removal, in hair fall treatments, etc. In oral surgery for management of TMJ disorders, and in regenerative procedures, where a blend of I-PRF and bone grafts called 'steaky bone'- is used in guided bone regeneration.[21] In vertical ridge augmentation prior to implant surgery, [22] for treating gingival recession defects, [23] in treatment of intrabony defects, [24] in direct sinus lift procedures with simultaneous implant placement,[25] in PAOO,[26] in management of endo perio lesions [27] and in treatment of cleft palate.[28] I-PRF treated CTG demonstrated improved clinical outcomes in management of gingival recession defects.[29]

Collagen matrices are biocompatible, physiologically metabolized, are chemotactic for fibroblasts, acts as a barrier for migrating gingival epithelial cells, and acts as a scaffold allowing early vascular and tissue ingrowths, and also an ideal carrier vehicle for the release of cytokines, growth factors, and live-cell therapies. Marine collagen might be a valuable substitute to bovine collagens for its structural analogy and better physiologic and biochemical properties. [30] In dentistry collagen with MTA has been utilized for endodontic treatment of open apices in immature teeth, and [31] in socket preservation procedures.[32] A pilot study used the combination of porcine collagen and I-PRF in sinus augmentation with implant placement and demonstrated satisfactory defect fill radiographically at end of 6 months. [25]

Most of the previous studies have used bovine/porcine collagen matrix for gingival soft tissue augmentation. Our study uses collagen from marine source which has relatively less religious and ethical constraints along with other advantages like higher content of collagen, greater absorption without the threat of zoonoses. The synergistic effects of marine collagen with I-PRF on fibroblasts were studied before commencing the clinical trial.

The cell viability assay showed that the fibroblasts were compatible with plain collagen as well as I-PRF impregnated collagen. Plain collagen demonstrated better cell viability when compared to I-PRF impregnated collagen but with insignificant difference. The scratch wound healing assay showed that plain marine collagen showed appreciable levels of cell proliferation and migration whereas I-PRF impregnated marine collagen showed comparatively higher cell migration at all 3 time periods. The better cell proliferation and migration could be attributed to the healing and regenerative properties of I-PRF. With the above results we can infer that collagen impregnated I-PRF is biologically safe and has potential to hasten wound healing and impel regeneration. Miron et al. [12] studied cell vibility and migration of human gingival fibroblast(HGF) on exposure to platelet rich plasma (PRP) and I-PRF. Wang et al.did a similar study comparing viability and migration of HGF on titanium implant surfaces [33] Both studies concluded that I-PRF had excellent biocompatibility and positively influenced cell migration analogous with our study results. Marine collagen matrix could be used as a carrier to deliver growth factors, signaling molecules and pharmaceuticals while I-PRF could be agglomerated with different biomaterials for propitious regenerative outcomes. Fibroblast adhesion, proliferation and quatification of growth factors could have been studied together to further validate the potentiality of the combination of collagen with I-PRF in soft tissue healing.

Conclusion

The findings of our study demonstrated that marine collagen and marine collagen with I-PRF showed capability for tissue regeneration via induction of fibroblast behavior. This study is a first in an attempt to demonstrate in-vitro characteristics of fibroblast cell lineage when both marine collagen and I-PRF are used together. Research on a larger and more varied sample is necessary to determine binding and release kinetics of biologic factors to collagen as a carrier device & controlled clinical trials to validate the use of I-PRF impregnated marine collagen matrix as a biological substitute in stimulating tissue regeneration in various domains of regenerative surgery for extrapolating its potential clinical benefits.

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Legends to figures

Figure 1: I-PRF protocol: a.) Collection of blood from anti cubital vein in specified vacutainers **b.)** Centrifugation done at 700RPM for 3 minutes at 60G force with Dentifuge LD C-10 **c.)** I-PRF separated as upper liquid yellow phase **d.)** I-PRF retrieved with 2ml syringe **e.)** Collagen impregnated with I-PRF

Figure 2: Cell viablity assay: a & b) Plain collagen at 24 hours under light and immunofluorescent microscopy **c & d)** Collagen + I-PRF at 24 hours under light and immunofluorescent microscopy

Figure 3: Scratch wound healing assay: a) 3T3 cells after reaching confluence of 90% **b)** Scratch made at the centre of cell monolayer **c & d)** Migration at 24 hours for collagen (4.16%) & collagen + I-PRF (12.50%) **e & f)** Migration at 48 hours for collagen (47.92%) & collagen + I-PRF (50%) **g & h)** Migration at 72 hours for collagen (77.08%) & collagen + I-PRF (98.75%)

Table 1: cell viability assay

Table 2: scratch closure rate (%)

Graph 1 shows cell viability % for control, collagen & collagen + I-PRF

Graph 2 compares cell migration at 24, 48, 72 hours for plain collagen and collagen + I-PRF

Declarations

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Availability of data and material: Data transparency maintained

Code availability: Not applicable

Authors' contributions:

Conceptualization, Methodology, Data Curation, Writing - Original Draft, Investigation: Dr.Deepikha Krishnaraj, Dr.Harinath Parthasarathy

Supervision, Review & Editing: Dr. Anupama Tadepalli, Dr. Deepa Ponnaiyan

Ethical statement: This study was approved by the Institutional Ethical Committee and the Scientific Review Board with the approval number SRMDC/IRB/2018/MDS/No.501. The research protocol was in accordance with the guidelines put forth by the Helsinki Declaration of 1975.

Consent to participate: Required informed consent has been obtained

Consent for publication: Required consent has been obtained

Figures Tables & graphs

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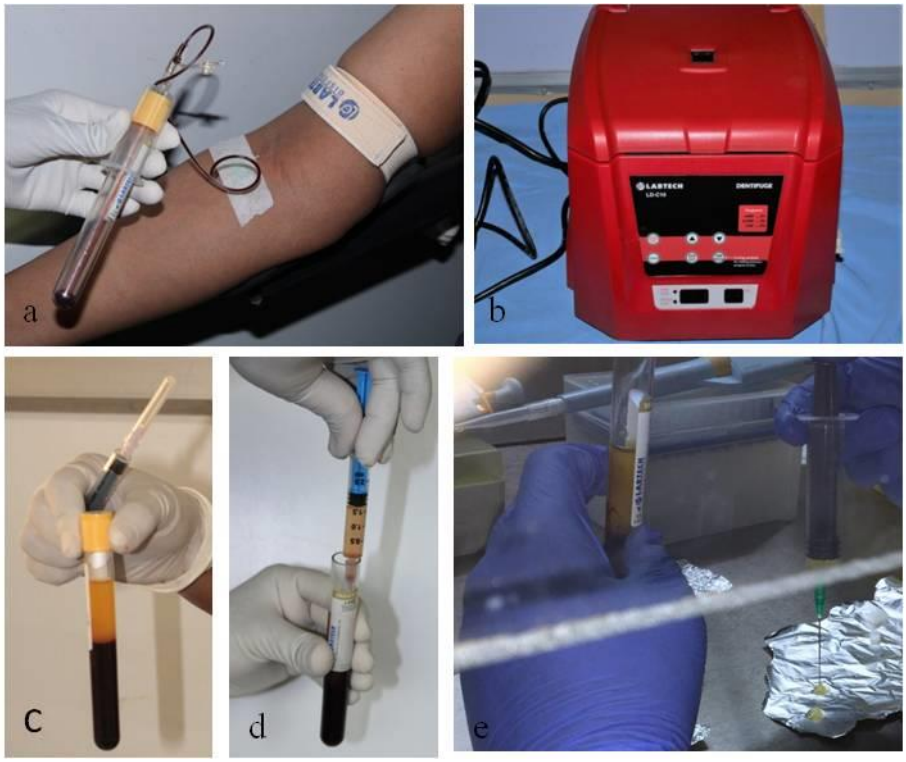
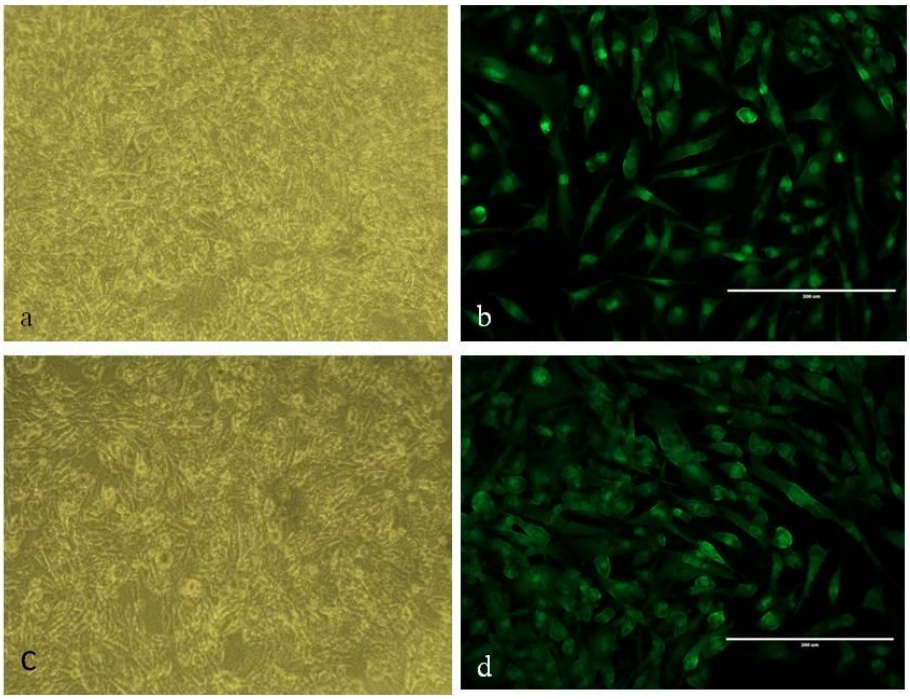


Figure 2: Cell viablity assay: a & b) Plain collagen at 24 hours under light and immunofluorescent microscopy c & d) Collagen + I-PRF at 24 hours under light and immunofluorescent microscopy



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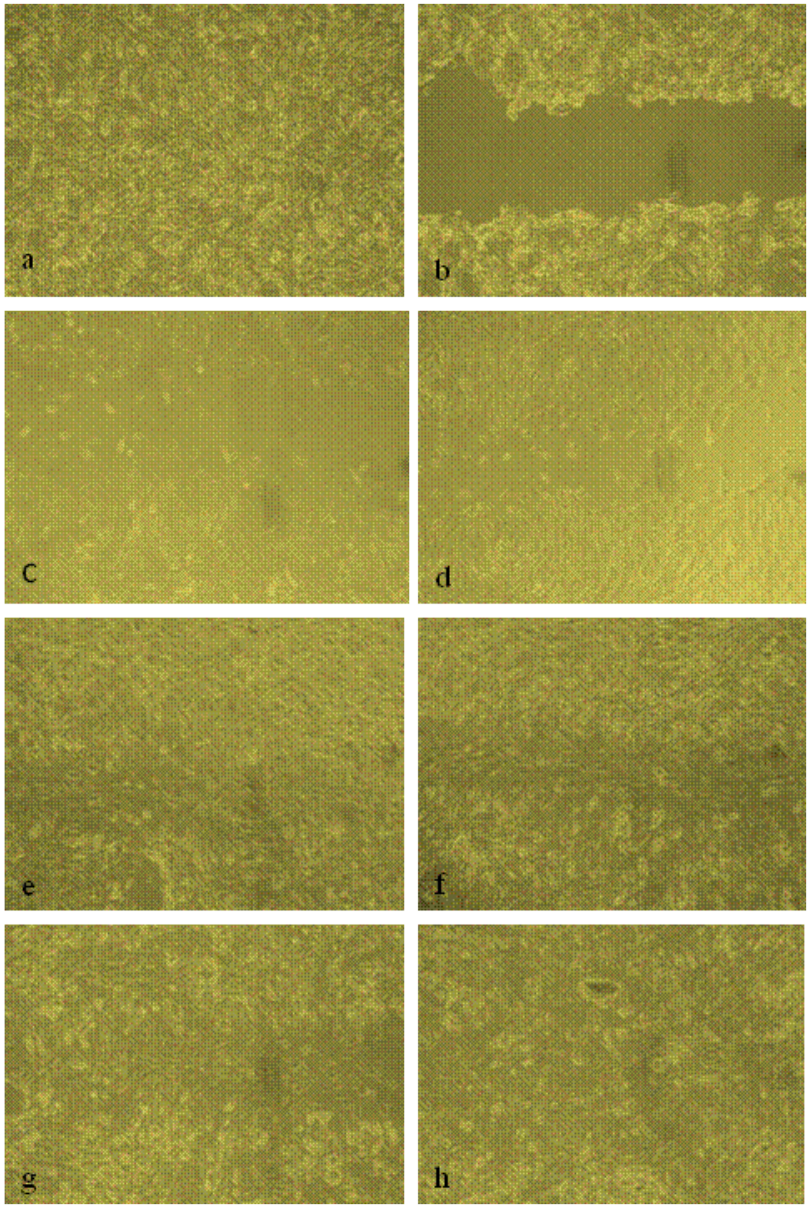


Table 1: cell viability assay

Samples	**Absorbance**			**Average**	**Cell viability (%)**
	I	**II**	**III**		
Control	0.933	0.938	0.934	0.935	100
C	0.923	0.928	0.921	0.924	98.823
T	0.92	0.915	0.917	0.9173	98.110

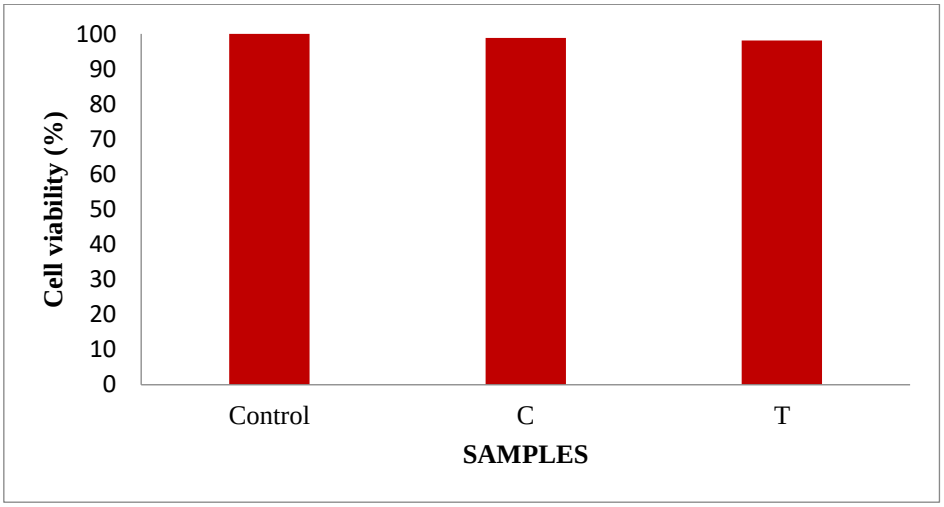
C – Collagen, T – Collagen + I-PRF

Table 2: scratch closure rate (%)

Time (hrs)	C (%)			T (%)		
	I	II	III	I	II	III
24 hrs	4.166	4.2421	4.4031	12.509	12.04	13.236
48 hrs	47.916	47.891	48.095	50.085	50.879	51.089
72 hrs	77.083	76.978	76.047	98.753	98.881	99.041

C – Collagen, T – Collagen + I-PRF

GRAPH 1 showing cell viability percentage for Control, Collagen (C) & Collagen + I-PRF (T)



GRAPH 2 comparing cell migration at 24, 48, 72 hours for plain collagen (C) & collagen + I-PRF (T)