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Evaluation of biocompatibility of human dental pulp stem cells with plasma rich fibrin, two dimensional and three-dimensional fibrin glue scaffolds in rregenerative eendodontics- An in-vitro study

[1]Needa Ansari, Assistant Professor, Department of Conservative dentistry and Endodontics, Government Dental College and Hospital, Jamnagar, Gujarat, India.

[2]Abhishek Parmar, Assistant Professor, Department of Conservative dentistry and Endodontics, Government Dental College and Hospital, Ahmedabad, Gujarat, India.

[3]Girish Parmar, Professor and Dean, Department of Conservative dentistry and Endodontics, Government Dental College and Hospital, Ahmedabad, Gujarat, India.

[4]Shikha Kanodia, Associate Professor, Department of Conservative dentistry and Endodontics, Government Dental College and Hospital, Ahmedabad, Gujarat, India.

[5]Geeta Asthana, Professor and Head, Department of Conservative dentistry and Endodontics, Government Dental College and Hospital, Ahmedabad, Gujarat, India.

[6]Amee Krishnakumar, Assistant Professor, Department of Biotechnology, Institute of Science, Nirma University, Ahmedabad, Gujarat, India.

Corresponding Author: Needa Ansari, Assistant Professor, Department of Conservative dentistry and Endodontics, Government Dental College and Hospital, Jamnagar, Gujarat, India.

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Abstract

Objective(s): Regenerative endodontics has opened gateway for new era of revitalizing immature pulpless teeth. For regenerative endodontics, use of human dental pulp stem cells with appropriate scaffolds and growth factors is imperative. The aim of the study was to evaluate the biocompatibility of Plasma Rich Fibrin and Fibrin Glue scaffolds with Human Dental Pulp Stem Cells (HDPSC) used in regenerative endodontics.

Materials and Methods: Plasma rich fibrin is one of the natural scaffolds proven to be used in regeneration. Human dental pulp stem cells were seeded with three concentrations of PRF. Fibrin glue is one of commercial scaffold newly established in the field of tissue engineering as a scaffold. 2D and 3D scaffolds of fibrin glue were made. Also in 3D scaffold, reconstituted

fibrinogen was diluted to three different concentrations to modify gelation and network architecture of fibrin glue. Morphology of HDPSC was studied using inverted phase contrast microscope and Proliferation of cells with different concentration of both experimental scaffolds was assessed using MTT assay. Collected data underwent a two-way ANOVA test. The P-value of the study was kept 0.05 according to the sample size.

Results: Statistically, significant difference was present among PRF and Fibrin glue groups on day 1 and day 3.

Conclusion(s): The study concludes that Fibrin Glue shows more biocompatibility than PRF scaffold with Human Dental Pulp Stem Cells.

Keywords: Cell Proliferation; Dental Pulp; Fibrin Tissue Adhesives; Plasma Rich Fibrin; Regeneration; Stem Cells

Introduction

With each passing day, due to advancement of science and technology, the treatment concepts that were once perceived to be imaginative are today considered achievable. The knowledge generated through basic science research, in the fields of stem cell biology, biomaterials (scaffolds), and morphogenetic signaling molecules, coupled with the recent advances in clinical research has resulted into an era, where tissue engineering-based therapies can be applied in Endodontics. [1]

Currently, the two approaches to pulp tissue regeneration in regenerative endodontics are cell-free and cell-based. Clinical revascularization can be considered a cell-free approach. The cell-free approach is technically simpler than the cell-based approach because the former does not have to be concerned about stem cell source, and isolation. However, the unpredictable clinical outcomes associated with Revascularization have largely been ascribed to individual variations in intra-canal blood clot formation due to variable sizes of the apical foramen and inconsistencies in the amount of blood influx into the root canal space. The cell-based approach requires isolation and ex vivo expansion of Human Dental Pulp Stem Cells seeded in the scaffold and then transplanted into the canal space. The cell-based approach has been shown to be capable of differentiating into odontoblast-like cells and produce dentine-like mineralized tissue. Thus, this approach has more promise to result in true regeneration and is more predictable. [2]

An ideal scaffold for a cell-based approach should be biocompatible, with adequate, controllable porosity, with optimum mechanical strength, and should have biodegradability such that mature cells may completely replace the scaffold. [3] Fibrin glue, which is available commercially has been proposed to be a revolutionary tissue engineering scaffold. It has been widely used as a bio-adhesive in surgeries for hemostasis, wound closure, and a sealant. [4],[5],[6],[7]. Contrary to other scaffolds, fibrin gel presents many advantages, such as a controllable degradation rate similar to that of tissue regeneration, nontoxic degradation products, and outstanding biocompatibility. Furthermore, mechanical properties of fibrin glue can be adjusted by controlling the precursor concentration. [8] Its proposed use in DPSC tissue engineering is yet to be researched. Also, Fibrin Glue can serve as two-dimensional (2D) as well as 3D scaffold. [9]

Plasma rich fibrin, a platelet derivative has also been a very popular scaffold for regeneration. Platelets have been known to store growth factors such as PDGF and TGF-beta, which stimulate cellular proliferation and differentiation. Platelets release these growth factors when activated. Thus, blood-derived scaffolds containing platelets potentially stimulate tissue regeneration. [10]

Although various studies have been done to compare various natural and synthetic scaffolds for their use in

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Regenerative Endodontics but till present date, no study has been done to assess the Biocompatibility of organic Plasma Rich Fibrin and Fibrin glue. So, the aim of the study was to evaluate cell proliferation and morphology to assess biocompatibility of Human Dental Pulp Stem Cell in Fibrin Glue scaffold as well as Plasma Rich Fibrin Scaffold.

Materials and method

All the work was done in a laboratory designated for use Human origin material Biosafety Level II (BSL II) and Laminar Air Flow at Institute of Science, Nirma University, Ahmedabad.

For preparation of human dental pulp stem cells:

1. Initial Handling of cells

Human Dental Pulp stem cells were received from Hi-media Company in a T-25 flask. Cells were seen under inverted light microscope. The cells which were received in travel medium were changed to complete medium. The flask was capped and was shaken gently to ensure proper mixing and uniform distribution of cells. The flask was then incubated in CO_2 incubator at 37°C and 5% CO_2. Once the cells had reached 70-80% confluency, they were ready to be sub-cultured. The cells were monitored every day. The medium was changed after 2-3 hours. The cells were sub-cultured after they reach 70-80 % confluency. [Figure 1]

After human dental pulp stem cells were ready, scaffolds were prepared.

1) For preparation of scaffolds

(1) Preparing Plasma Rich Fibrin Scaffold

One hundred and forty mill-meter of blood was collected. The blood samples were centrifuged in centrifugation machine at 2700 rpm for 12 minutes. A white PRF clot was formed between the a-cellular plasma and RBCs. The PRF clot was seized by sterile forceps and separated from RBCs by scissor. The clot was positioned on the grid of endo-box and compressed by endo-box cover. After a minute, PRF clot was converted into membrane and the exudate was collected at the bottom of endobox below the grid. PRF exudate was centrifuged at 1800 rpm for 5 minutes to acquire exudate only without precipitated RBCs. It was filtered by 0.22 µm sterile syringe filters and stored in Eppendorf tubes at −20ºC immediately after preparation. PRF exudate was then diluted to 3 concentrations by mixing with growth medium viz 5%, 10% and 20%.

(2) Preparing Fibrin Glue Scaffold

The Tisseel Lyo Fibrin Glue kit contains four bottles of Fibrinogen, Thrombin, Aprotinin and Calcium Chloride. Aprotinin is aspirated in a syringe and injected into Fibrinogen till no un-dissolved particles are visible. Calcium Chloride is aspirated and injected into Thrombin till no un-dissolved particles are visible. Reconstituted Fibrinogen solution was subsequently diluted to 3 concentrations by mixing with Phosphate buffered Saline (PBS) which is 12.5%, 25%, 50%.

2) Seeding of well plates:

(1) For PRF Extract Scaffold

Cells were seeded at a density of 3000 cells/ well in micro-titer plate. Human dental pulp stem cells with 10% FBS served as positive control. [Figure1] Cells in serum free media served as negative control. Duplicate samples were seeded with each concentration of PRF exudates; 5%, 10% and 20%. Cell morphology was examined at various time intervals under an inverted microscope [Figure 2]

(2) For Fibrin Glue Scaffold

For 2D scaffolds, two sets of experiments were carried out. In one set, cells were pre-seeded. After that, 25 micro liter of fibrinogen and 25 micro liter of thrombin were supplemented above it to formulate fibrin glue. In another fibrin-glue pre-coated set, after fibrin glue was made by

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mixing fibrinogen and thrombin, cells were added with growth medium.

For formulating 3D scaffolds, 25 micro liter of thrombin was added, and then, cells along with culture medium were added and gently mixed. After that, different concentration of reconstituted fibrinogen solution was supplemented for the glue to form with cells trapped in the fibrin scaffold. Cell morphology was examined at various time intervals under an inverted microscope [Figure 3].

3) MTT Assay:

MTT Assay is done to check proliferation of cells under various conditions. It is a three-day protocol. First of all, human dental pulp stem cells were suspended in culture medium at seeding density of 3000 cells per well in a micro-titre plate. Cells were incubated in the well plate for 24 hours so that cells form half- confluent mono-layer. Cells were then examined under inverted light microscope to make sure that cells were evenly distributed in the well plate. After 24 hours incubation, the culture medium was aspirated. Now in each well, treatment media was added and cells were incubated for 24 hours at 37ºC and 5% CO_2. After 24 hours incubation on day 3, the entire treatment medium was aspirated. Per each well, 4 micro-litres of MTT solution was added in each test well. The plates were incubated for 5 hours at 37ºC and 5% CO_2. After that, MTT solution was removed and 100 microlitre dimethyl sulphoxide (DMSO) was added. The plate was then transferred to plate reader and absorbance was read at 570 nm (Reference was 650 nm). Viability of cells was calculated using following formula:

$$\text{Viability}(\%) = \frac{100 \times OD(570)a}{OD(570)b}$$

Where OD (570) a = Measured optical density of test matter

OD (570) b = Measured optical density of blank without test matter

The readings of MTT assay for PRF extract scaffold and Fibrin Glue was taken at Day 1 and Day 3.

4) **Statistical Analysis**

SPSS 20.0 was used for statistical analysis. The results of the absorbance measured using multi-plate reader were analyzed using two-way analysis of variance (ANOVA).

Results

On day 1 and 3, statistically significant difference was present among various concentrations of PRF Group. Cell viability was highest in 5% PRF group. In 3D scaffolds group, Cells seeded in thrombin with 25% fibrinogen showed maximum cell viability on day 1 and 3. Statistically, significant difference was present among PRF and Fibrin glue groups on day 1 and 3. (Table 1, Graph 1)

Fibrin Glue shows more cell viability than PRF scaffold ($p < 0.05$)

Table 1: % Cell Viability of HDPSC in experimental groups at various time interval.

Groups	Sub Groups	Concentrations	% Cell Viability on Day 1		% Cell Viability on Day 3	
			Mean Reading	SD	Mean Reading	SD
PRF	-	5% PRF supplemented with Serum free media	179.00	9.89	60.00	12.72
	-	10% PRF supplemented with Serum free media	109.00	19.79	46.00	15.55
	-	20 % PRF supplemented with Serum free media	96.50	4.95	30.50	2.12

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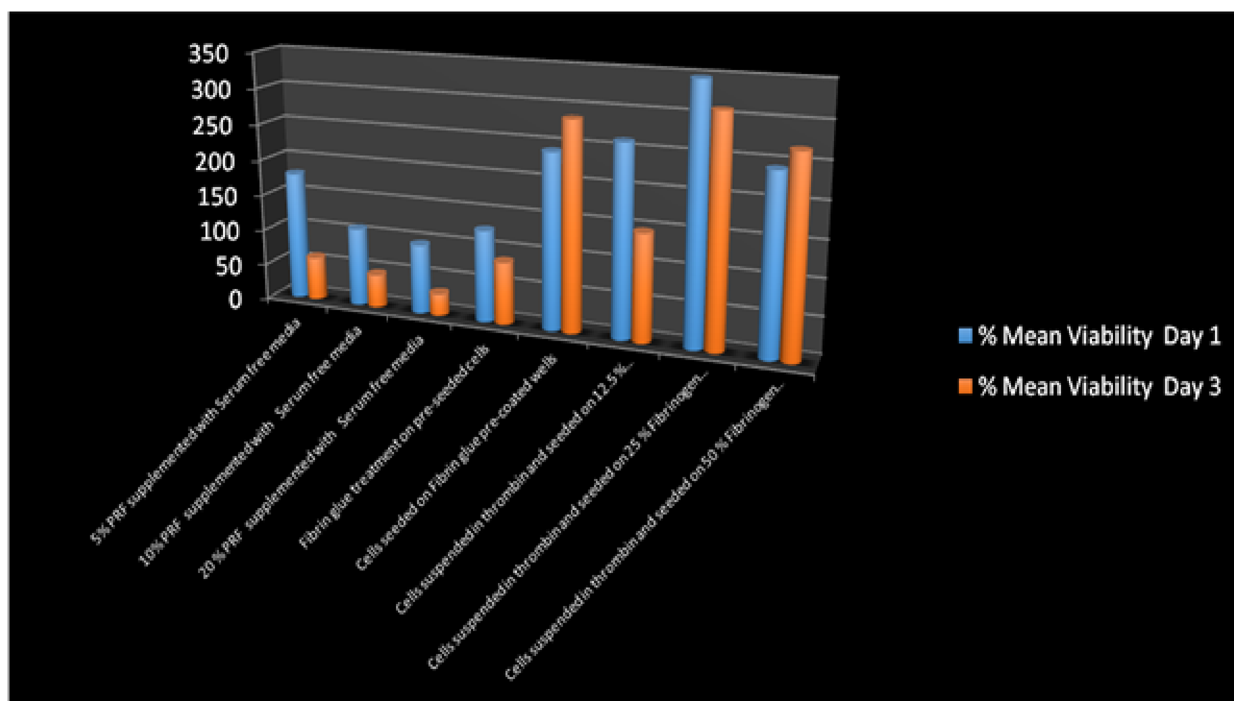
Fibrin Glue	2D	Fibrin glue treatment on pre-seeded cells	127.00	19.79	86.24	10.94
		Cells seeded on Fibrin glue pre-coated wells	241.50	14.84	285.75	6.01
	3D	Cells suspended in thrombin and seeded on 12.5 % Fibrinogen treated wells	262.00	73.53	147.25	8.83
		Cells suspended in thrombin and seeded on 25 % Fibrinogen treated wells	347.00	72.12	310.25	9.54
		Cells suspended in thrombin and seeded on 50 % Fibrinogen treated wells	243.00	42.42	268.50	26.16

Graph 1: % Cell Viability of HDPSC in experimental groups at various time interval.



Discussion

When dental caries reaches the dental pulp, the treatment of choice is generally pulpectomy. [11] Following pulpectomy and root canal filling, chances of postoperative pain [12], apical periodontal lesions caused by micro-leakage from the tooth crown [13], and vertical root fracture [14] are there. Most recent advances have aided stem cell therapy to regenerate the pulp/dentin complex for conservation and complete structural and functional restoration of the tooth by the triad of tissue engineering: 1) Human Dental Pulp Stem Cells (HDPSC) 2) Growth factors and 3) Scaffolds. [15]

HDPSCs have the property to differentiate into odontoblasts; these cells can be used directly for dental therapy for regeneration of dentin-pulp complex.[16] It is obligatory that the scaffold used for clinical application should have the capacity to adhere the cells to the surface of this scaffold and proliferate as well as undergo differentiation. It is also necessary to have good mobility of these cells on this scaffold. Also, the scaffold should be replaced by regenerated tissue after its implantation.

Earlier studies have reported the potential of using platelet concentrates as scaffolds in tissue regeneration. Platelet concentrates are autologous, simple to prepare in a dental setting, and contain high concentrations of

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growth factors including transforming growth factor-beta (TGF-beta), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF). [17] Platelet-rich plasma (PRP) is a 1st generation platelet derivative. Platelet-rich fibrin (PRF) which is a second-generation platelet concentrate has many advantages over PRP. To start with, the preparation of PRF does not require exogenous agents like thrombin. Also, PRF forms an organized fibrin network in which platelets and leukocytes are trapped. These entrapped cells serve the purpose of a reservoir of various growth factors for long-term release. The mechanical properties of PRF may also facilitate the condensation of overlying MTA. Thus, it is rational to expect PRF to be an optimal scaffold for pulpal regeneration. [18]

Fibrin gel is able to function as both two-dimensional as well as three-dimensional scaffold. Although the traditional two-dimensional scaffold provides an understanding as to how cells interact with the fibrin glue, it cannot mimic the physiological environment of cells in vivo. Three-dimensional scaffolds became well-accepted because of their ability to be a model of tissue physiology and provide a better understanding on the interaction of cell and matrix, as well as their function.[19]

PRF entails prospective research as a potential scaffold for regenerative endodontic therapy. PRF exudate was diluted to 3 concentrations by mixing with growth medium viz. 5%, 10% and 20%. Cells with 10% FBS were taken as a positive control. In the previous studies, the optimum concentration of PRP varied from 50% to 10% [20, 21] to less than 1%. [22] Soffer et al. considered 0.5-1% PRP as the optimum concentration for cellular proliferation and mineralization rates. [22] However, Ferreira et al. found that 50% PRP was the optimum concentration for osteoblast proliferation. [20] On the other hand, 10% PRP was sufficient to induce marked cell proliferation of MSC, derived from adipose tissue. [23] Hence, the experiments were conducted on three concentrations of PRF exudates (5%, 10%, and 20%) to assess HDPSC viability.

The proliferation and morphology of the cells were checked on day 1 and day 3. This is because studies have reported that PRF increased proliferation of the cells on day 1 but started inhibitory growth patterns on the 3rd day. [24]

The morphology of human dental pulp stem cells was studied. The normal morphology of human dented pulp stem cell is spindle-shaped, fibroblast-like morphology which adheres to the culture vessel. [25] This morphology was retained maximum in the 5% PRF group. Even though debris which is a metabolic product of the cells was seen on day 1 also, it increased on day 3. The least morphology was retained in the 20% PRF group.

Cell Viability was highest in the 5% PRF group on Day 1 which decreased on Day 3 but still was highest in the 5% PRF group. These results indicate that 5% PRF supplemented with serum-free media is the most preferable and recommended concentration for human dental pulp stem cell viability.

In another similar study conducted by Saeed et al, 10% PRF exudate showed maximum proliferation of human dental stem cells. This may be because of the difference in the amount of growth factors which is variable in the blood of individuals. [24] In our study, 10% showed neither proliferative nor inhibitory action on day 1 but showed marked inhibitory action on day 3. While 20% PRF exudates had no proliferative effect on day 1 as well as on day 3 which may be because earlier reported studies stated that too much excessive promotive stimulus present because of growth factors present in PRF could lead to loss of maturation leading to inhibitory action resulting in reduced proliferation. [24]

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Cell proliferation decreased on the 3rd day which is because of a decrease in the release of growth factors with time as reported in the studies. [26] In 20% PRF, the release of TGF- β, and CGF-e would be responsible for the decreased proliferation because even though the concentration would be decreased with time but would be higher than that of the lower concentrations. [27] A study by Ling et al stated that Levels of TGF-β at days 21 and 28 were close to that of day 1 and the rest of the growth factors like PDGF-AB and IGF decreased significantly on days 21 and 28. [28] These studies prove that the stimulative growth factors decrease with the passage of time and inhibitory factors stay constant which may lead to a decrease in cell proliferation in 20% PRF exudates.

On 1st and 3rd day, statistically significant difference was present between both pre-seeded and pre-coated 2D Fibrin glue group. Pre-coated group with seeded cells showed more cell viability. Pre-seeded cells showed decreased viability because the growth medium had to pass through fibrin glue to reach pre-seeded cells. Though, fibrin glue is porous, its viscosity might have reduced permeability of nutrients, respiratory gases as well as growth factors to reach the cells adequately.

In this study, fibrinogen solution was diluted to three concentrations by mixing with Phosphate Buffered Saline which is 12.5%, 25%, and 50%. This was done according to the study by Zhao et al. that by modifying thrombin and fibrinogen concentration, gelation and network architecture can be controlled. [29] In a previous study, Catelas et al. discarded the effect of thrombin concentration and explained the importance of concentration of fibrinogen on release of growth factors.[30] For this reason, in this study, the concentration of thrombin was fixed. Significant difference was not present statistically among various 3D fibrin glue groups on the first day, but it was significant on the third day proving that cell homing on is better with the time interval. 25% fibrinogen group had highest cell viability on the 1st as well as 3rd day. Hence, it is the most favourable concentration for HDPSC. A study by Parmar et al proved that 25% fibrinogen concentration had highest cell viability in contrast to other higher and lower concentrations.[31] Previous studies have indicated that the highest proliferation of cells occurs when 25% fibrinogen is used in contrast to other concentrations of fibrinogen. [31] Another publication [32] found that equine mesenchymal stem cell migration into fibrin hydrogels had higher cell viability in both autologous and commercial fibrin sealant with lower fibrinogen precipitate solution percentage (25% superior to 50% and 75%).[33] A study evaluated the long-term therapeutic protein expression of differentiated adipose cells in vitro using 3D fibrin sealant scaffolds and found that fibrinogen concentration was important factor.[34]

On next day, cell viability of the cells in 12.5% fibrinogen decreased because probably very low concentration of fibrin glue will not possibly provide optimum growth factors for cells to grow and survive as reported by previous studies.[29] Cell viability of the cells suspended in 50% fibrinogen was lower than that of 25%, probably because dense fibrin network would provide too compact environment for cells to thrive. Cell viability increased on the following day which also proves that because of too dense network, growth medium would require ample time to reach cells for them to proliferate. Overall cell viability decreased on 3rd day suggesting that cells after a phase of proliferation, enters differentiation stage depending on growth medium. There was no statistical difference between mean cell viability between two dimensional and three-dimensional scaffolds on both days. However, viability was greater in 3D scaffold with 25% fibrinogen (347 ± 72.12) than in 2D scaffold (285.75 ± 6.01).

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According to a study by Hakkinen et al., fibroblasts migrate at least 1.3 times faster in 3D culture compared with their corresponding 2D culture in fibrin.[35]

When Plasma Rich Fibrin scaffold (179 ± 9.89) was compared with Fibrin Glue Scaffold (347 ± 72.12), significant difference was there on 1st as well as on 2nd reading. This report is similar to study done by Vendamin et al. They questioned whether PRF could disrupt the phenomenon of inosculation and revascularization and stated that the higher the density of the fibrin mesh, the more likely the arrival of platelets and their growth factors, proving that excess fibrin is harmful.[36]

Altmeppan et al. performed a study in which they evaluated the characteristics and composition of PRP into glue and compared with commercially available glues. It proved that adhesion strength decreased as the platelet concentrations increased which may explain how high platelet concentration can block formation of fibrin network.[37]

But this study is one of its kinds because there has never been any comparative study between organic Plasma Rich Fibrin scaffold and commercial Fibrin glue scaffold on human dental pulp stem cells.

Both of them are fibrin scaffold but fibrin glue proved to be better because its excellent biocompatibility, optimum mechanical properties, controllable viscosity by changing the fibrinogen concentration and controllable degradation rate by changing aprotinin concentration, its adhesive property and its ability to support cell proliferation. Plasma rich fibrin scaffold, inspite of having pool of growth factors because of its weak mechanical properties and inability to chemically modify it has been proven to be inferior scaffold than Fibrin Glue. But fibrin glue also has limitations like chance of potential disease transmission; shrinkage of gel, antibody reaction to synthetic aprotinin still needs to be addressed for the wide adoption of fibrin glue in tissue engineering.

Degradation time of PRF in the body is after 28 days and that of Fibrin glue is 30 days.[40] This study evaluates the proliferation of human dental pulp stem cells with PRF and Fibrin glue only for 4 days. So long term evaluation of the scaffolds with dental pulp stem cells need is needed to support the results of the present study.

But, within the limitations of the study, Fibrin glue proved to be significantly better than Plasma Rich Fibrin Scaffold.

Conclusions

This is the first ever study evaluating Biocompatibility of Plasma rich fibrin and Fibrin glue scaffold with Human Dental Pulp Stem Cells. Within the limitations of this study, it can be proved that Fibrin Glue scaffold has better mean cell viability of human dental pulp stem cells than Plasma Rich Fibrin scaffold ($p < 0.005$).

Figure 1: Human Dental Pulp Stem Cells (Blue Arrow Depicting Spindle shaped Stem Cells and Red Arrow depicting Cellular Matrix)

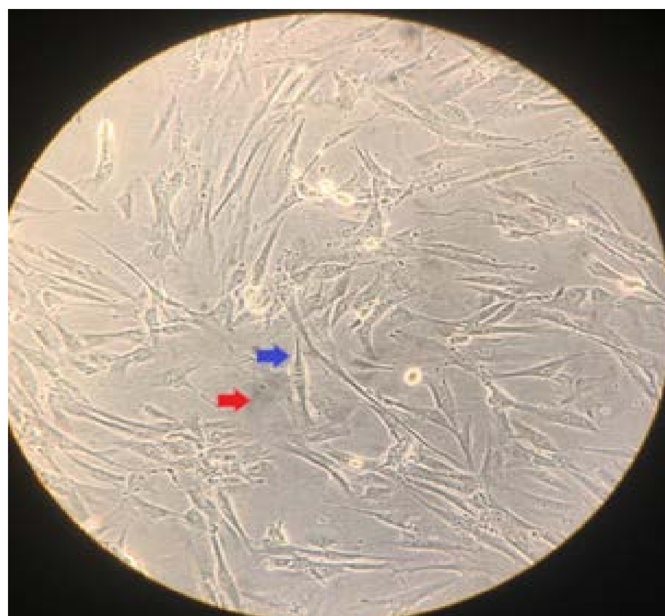

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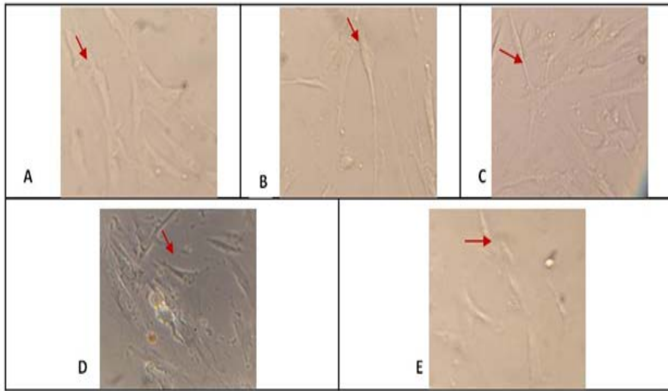
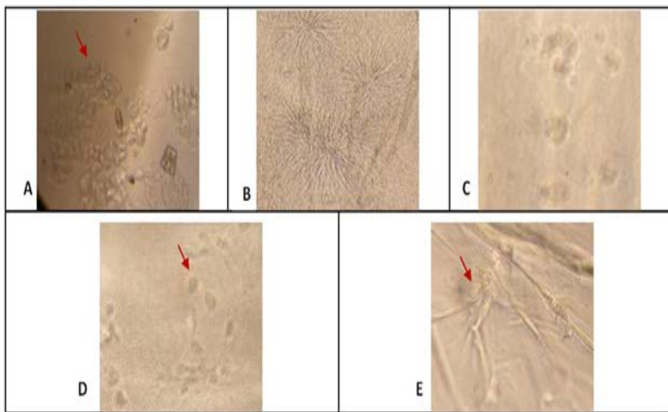
Figure 2: Hdpsc in Prf Scaffold.



A- HDPSC in Serum Free Media B- HDPSC in Plasma Rich Fibrin Positive Control C- HDPSC in 5% PRF D- HDPSC in 10% PRF E- HDPSC in 20% PRF Red Arrow depicting Human Dental Pulp Stem Cells in different scaffolds.

Figure 3: Hdpsc in Fibrin Glue Scaffold.



A- HDPSC in fibrin glue pre-coated cells B- Fibrin glue treatment on pre-seeded HDPSC

C- HDPSC with 12% fibrinogen 3D scaffold D- HDPSC with 25% fibrinogen 3D scaffold E- HDPSC with 50% fibrinogen 3D scaffold.

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