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Translational implications of a novel combination of iPRF and collagen scaffold for proliferation of gingival mesenchymal stem cells

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"Tissue engineering," is a concept that involves the use of scaffolds, cells, and growth factors/signaling molecules in the field of regenerative medicine. The present study was carried out to evaluate the attachment and depth of penetration of the Gingival Mesenchymal Stem Cells (GMSCs) onto the collagen scaffold preconditioned with Fetal Bovine Serum (FBS), Injectable Platelet Rich Fibrin (i-PRF) and a combination of FBS with i-PRF using histological analysis and environmental scanning electron microscopy (ESEM) respectively. In the present study, commercially available collagen membranes were used as scaffolds and divided into 3 groups where Group I was preconditioned with FBS, Group II was preconditioned with i-PRF, and Group III with a combination of FBS and i-PRF. Scaffolds were then seeded with GMSCs and incubated in a CO_2 incubator at 37 ^{0}C for 7 days. Both histological and ESEM analysis showed proliferation, attachment, and depth of penetration along with the combination of cell morphological changes in all the three groups. In group I cells showed mild depth of penetration along with a round morphological appearance. In group II the cells showed moderate depth of penetration and rounded morphology. In group III maximum depth of penetration was seen along with the round and spheroidal morphology of cells. The combination of the growth media showed the best effect on cell attachment, depth of penetration, and cell morphology. This is the first report demonstrating the effect of the combination of collagen and i-PRF supports the proliferation of GMSCs. Since FBS alone and i-PRF alone exhibited similar cell proliferation it is recommended that i-PRF could be used in clinical scenarios making it xenofree.

Keywords Tissue engineering, Gingival mesenchymal stem cells, Collagen Scaffold, I-PRF, Growth factors, Fetal bovine serum

Abbreviations

GMSCs	Gingival Mesenchymal Stem Cells
FBS	Fetal Bovine Serum
i-PRF	Injectable Platelet Rich Fibrin
ESEM	Environmental Scanning Electron Microscopy
CO_2	Carbon dioxide
PDGF-BB	Platelet Derived Growth Factor
IGF-1	Insulin like Growth Factor
BMP	Bone morphogenetic proteins
TGF-bs	Transforming growth factor-β
Bfgf	Basic fibroblast growth factors
PRP	Platelet-rich Plasma
PRF	Platelet-Rich Fibrin

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"Tissue engineering," a concept that involves the use of scaffolds, cells, and growth factors/signaling molecules which are broadly accepted in the field of regenerative medicine including periodontology to generate the lost tissues[1,2]. Biomaterials used in tissue engineering can be categorized in several ways, including their origin (natural or synthetic), physical properties (such as mechanical strength or biodegradability), and their intended applications (such as scaffolds, hydrogels, or microspheres)[3–5].

Scaffolds or matrices are specific compounds that transport cells or signaling molecules to the appropriate anatomic spot and provide mechanical support to growing tissue[6]. The scaffold's physical and chemical properties influence cell proliferation, cell penetration, and tissue in-growth. In tissue engineering, cells with scaffolds play an important role. Scaffolds that will hold cells together until they can establish contact with each other as well as produce their own matrix. The successful outcome of a tissue-engineered scaffold depends on the cell number, cell attachment, and cell penetration into the matrix.

Some of the signaling molecules used in the tissue-engineered scaffold are Platelet-derived growth factor (PDGF-BB) and Insulin-like Growth Factor IGF-1, Bone morphogenetic proteins (BMPs), Transforming growth factor -β (TGF-bs) and Basic fibroblast growth factors (Bfgf known as FGF) which were seen in Platelet-rich Plasma (PRP)[7]. Choukroun et al. (2001)[8]introduced second-generation platelet-rich fibrin (PRF). PRF is considered to be the richest source of the growth factors mentioned above which act as a signaling molecule and they also provide a natural fibrin architecture for cell adhesion and proliferation[9]. In a study conducted the efficacy of autologous platelet rich fibrin in bone augmentation and bone regeneration procedures in regard of soft and hard tissue healing in oral surgery treatment. PRF seems to help both soft tissues (gums) and bone heal better after surgery. PRF is a bonus because it's made from a patient's own blood and doesn't require any additional materials, making it potentially cost-effective[10,11]. PRF is also used for the treatment of furcation defects[12].

Miron RJ et al[13]. conducted a study in which they compared PRP to i-PRF for fibroblast biocompatibility and migration. It was found that the i-PRF showed better and sustained release of growth factors when compared to PRP which was seen for over a period of 10 days[14,15]. The human periodontal ligament cells when placed in growth media had the potential to differentiate into osteoblast-like cells on a collagen scaffold[16–20].

GMSCs have the potential to differentiate into osteogenic, chondrogenic, and adipocyte cells. Recent advancements in research have led to the isolation and characterization of gingiva-derived mesenchymal stem cells (GMSCs), showcasing notable potential for regenerative and immunomodulatory applications[21–24].

To the best of our knowledge, there is no previously reported study available where the influence of signaling agents/growth factors derived from i-PRF on the attachment and depth of penetration of Gingival Mesenchymal stem cells (GMSCs) into a collagen scaffold is studied. Hence, the present study aimed to evaluate the attachment and depth of penetration of the GMSCs into the collagen scaffold preconditioned with different growth media like the Fetal Bovine serum (FBS), injectable platelet-rich fibrin (i-PRF) and a combination of FBS with i-PRF using histological analysis and environmental scanning electron microscopy (ESEM) respectively.

Materials and methods

The study was approved by Institutional Ethics Committee Dr. D Y Patil Dental College and Hospital Pimpri Pune (DPU/484/2/20221). All the methods performed in the study are in accordance with the relevant guidelines and regulations. Informed consent was obtained from all subjects before tissue sample collection. This study was conducted at the Regenerative Medicine Laboratory (RML) at Dr. D.Y Patil Dental College and Hospital Pimpri Pune, Dr. D. Y Patil Vidyapeeth Pimpri, Pune, Maharashtra India. In this study commercially available type I collagen scaffold, previously cryopreserved GMSCs and different growth factors like FBS, i-PRF were used.

Collagen scaffold

Commercially available collagen membranes (COLOGIDE)[25] were used as scaffolds in the present study based on the previously unpublished data. The COLOGIDE has a fibrous structure which creates porosity. It is obtained from bovine origin from a highly purified type I collagen (especially from controlled and certified animals to avoid any antigenicity).

Procurement of GMSCs

At RML the GMSCs were isolated from the excised gingival tissue obtained after the crown lengthening or gingivectomy procedures by the explant culturing method which were checked for their stemness. Confluent GMSCs were harvested and washed with phosphate-buffered saline (PBS) and characterized for stem cell surface markers such as CD34, CD45, HLADR, CD90, CD73, CD105 by using flow cytometry.

Procurement of the growth media/factors (FBS, i-PRF)

Fetal bovine serum (FBS (cat No. Himedia) was used for cell culture. FBS is the liquid fraction of clotted blood from foetal calves that is devoid of cells, fibrin, and clotting factors but includes a substantial amount of nutritional and macromolecular components necessary for cell proliferation. FBS's main component is bovine serum albumin. FBS growth factors are necessary for the maintenance and development of cultured cells. FBS also includes a range of tiny molecules such as amino acids, carbohydrates, lipids, and hormones[26].

The Injectable platelet rich fibrin (i-PRF)[13] preparation: 20 ml blood withdrawn from a healthy adult volunteer by venepuncture of the antecubital vein. The blood was collected in two tubes (10 ml each) that did not have any anti-coagulant present in the tube. The test tubes were then centrifuged at 700 revolutions per minute (RPM) for 3 min at room temperature in a Choukroun's Duo Quattro which resulted in the separation of three basic fractions because of differential densities: a yellow part, a buffy coat in the middle, and a red blood cell containing lower part. The i-PRF (1 ml from the top layer) was collected using an automated pipette. Because even little alterations in the fractionation process might alter the physical and biological features of the final

products, special care was taken to collect just the superficial yellow coat (above the leukocyte-rich buffy coat). The procedure was completed as promptly as possible.

A total of 18 collagen scaffolds measuring about 1 cm x 1 cm were selected and included in the study and divided into 3 groups (6 in each group). **Group I** = Collagen Scaffolds preconditioned with FBS. **Group II** = Collagen Scaffolds preconditioned with i-PRF. **Group III** = Collagen Scaffolds preconditioned with FBS and i_PRF.

After preconditioning for 24 h with 3 different preconditioned medias all the collagen scaffolds were then seeded with cryopreserved 2 million GMSCs in 10% α MEM (Gibco) and incubated in a CO_2 incubator (THERMO FISSURE) at 37 ^{0} C for 7 days. The 10% α MEM was changed every 2–3 days.

For the evaluation of attachment and depth of penetration of the GMSCs all the 18 pieces of collagen scaffold of 1 cm x 1 cm each seeded with 2 million cryopreserved GMSCs in the 3 groups were further divided (cut) into 2 parts (total of 36). They were fixed in 4% formalin and carried in an Eppendorf tubes. 18 parts were sent for the histopathological analysis at the Department of Oral Pathology, Dr. D Y Patil Dental College and Hospital Pimpri Pune. Other 18 parts were sent for ESEM analysis at Kiran Icon Analytics, Sanpada, Navi Mumbai.

Histological analysis

18 samples were fixed in 4% formalin and carried in Eppendorf tubes. Pre-conditioned Collagen scaffold seeded with GMSCs were re-fixed with 10% formalin for 24 h. Dehydration was carried out using 60%, 70%, 80%, 90% alcohol (1/2 hour each) and with two changes of absolute alcohol (1 h each). Clearing with xylene (with two changes, 1 ½ hours each.). After clearing the scaffold were embedded in paraffin wax (with two changes of 1 ½ hours each.) Blocks were made with the embedding machine. Then thin section about 3–5 microns were made followed by H and E stain protocol. The slide was warmed for 3–4 min with a slide warmer to remove the excess of paraffin wax. After which it was kept in xylene for 3 min (with two changes each) followed by Alcohol for 3 min (with two changes each) and in distilled water for 10 min. After that haematoxylin staining was done for 4–5 min and was washed with running tap water for 1–2 min till bluing of the slide occurred. Eosin staining was done for 1 min followed by washing it under the running tap water. Lastly, it was kept in the alcohol dip and xylene dip followed by mounting with DPX. Microscopic analysis for the number of cells attached/ penetrated into the collagen scaffolds was performed using a microscope (40x) in 5 different areas using the phase contrast microscope (OLYMPUS).

Environmental Scanning Electron Microscope (ESEM) analysis

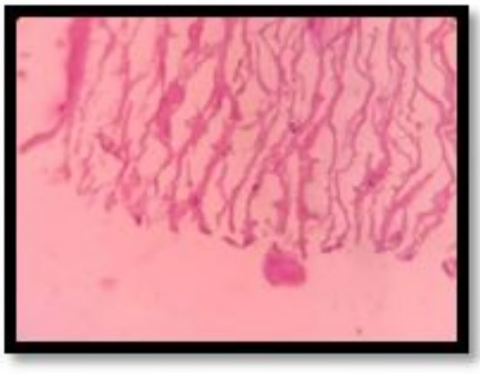
Eighteen samples were fixed in 4% formalin and carried in Eppendorf tubes to the canter (Kiran Icon Analytics). The samples were analysed in Qaunta 200 model make FEI manufactured in the Netherlands at Kiran Icon Analytics Sanpada, Navi Mumbai. The samples were dried using a blower and lint-free paper. Once the samples were dried they were loaded and fixed on to the aluminium stubs using double-sided carbon tapes. The stubs were then placed into the chamber and were examined under low vacuum under 15kv and 20kv at room temperature using different magnifications (1000x, 2000x, 4000x, 8000x).

Results

The present study was carried out to evaluate the influence of growth factors derived from Injectable Platelet rich fibrin (i-PRF) on the attachment and depth of penetration of human gingival mesenchymal stem cells. A total of 18 collagen scaffolds of 1 cm x 1 cm each were divided into 3 groups (Group I -FBS Group II -i-PRF, Group III- FBS and i-PRF). The cell attachment, depth of penetration and cell morphology were evaluated.

Histological analysis

In group I it was observed that the cells proliferated on the collagen scaffold surface with minimum attachment and mild depth of penetration into the collagen scaffold. In group II it was observed that the cells proliferated on the collagen scaffold surface with moderate attachment of cells and moderate depth of penetration into the collagen scaffold. In group III it was observed that the cells proliferated on the collagen scaffold surface with maximum attachment and maximum depth of penetration into the collagen scaffold.



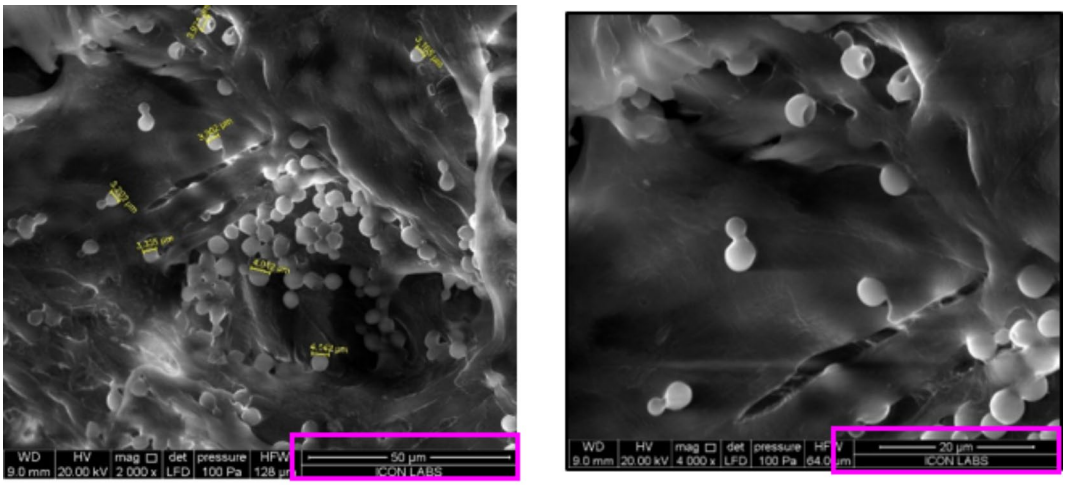


Fig. 1. Group I Cells showing round morphological appearance.

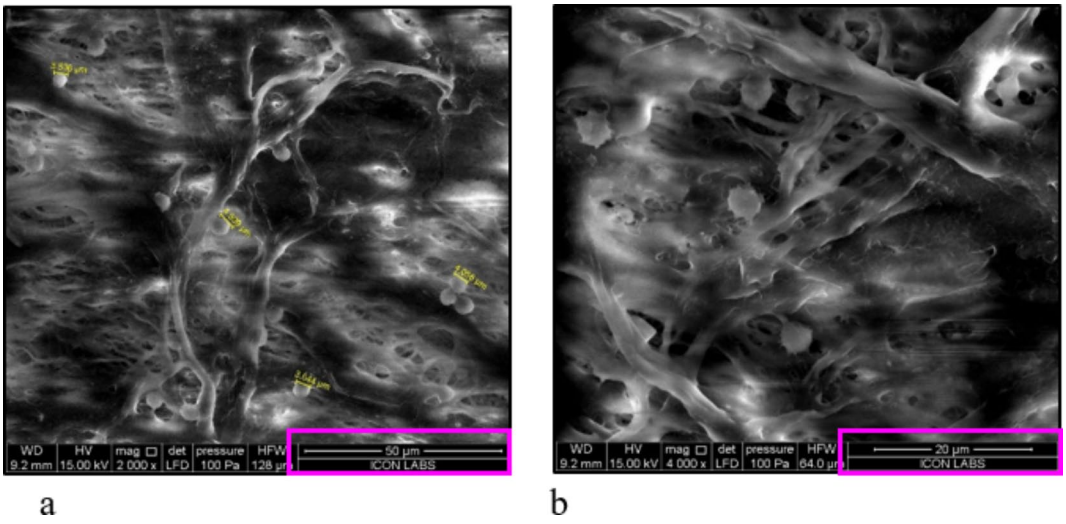


Fig. 2. Group II cells showing cells appearing elongated with spikes/filaments.

In the intergroup comparison, it was observed that Group III had maximum cell attachment and depth of penetration in the collagen scaffold compared to group II and group I which showed moderate and mild attachment and depth of penetration on the collagen scaffold respectively. The cells in Group I had a folded morphology when compared to Group II and Group III that showed rounded morphology with cytoplasmic extensions.

Environmental Scanning Electron Microscope (ESEM) analysis

In group I mild depth of penetration along with clusters of cells and proliferation of cells into the collagen scaffolds was observed under the given field. The morphology appeared round measuring around 3.1 –4.1 µm. (Fig. 1a, b)

In group II moderate depth of penetration along with the proliferation of cells into the collagen scaffolds was observed under the given field. The morphology appeared slightly elongated measuring around 3.5 –4.5 µm and they had spike/filament present on the surface (Fig. 2a, b).

In group III severe depth of penetration along with the proliferation of cells into the collagen scaffolds was observed under the given field. The size of the cells varied from 3.4 to 4.4 µm. They showed a mixed morphology of cells spheroidal as well as elongated cells were present (Fig. 3a, b).

In the intergroup comparison, it was observed that Group III had severe depth of penetration in the collagen scaffold compared to Group II and Group I which showed moderate and mild depth of penetration into the collagen scaffold respectively. Morphologically the cells in Group I appeared rounded and in Group II appeared

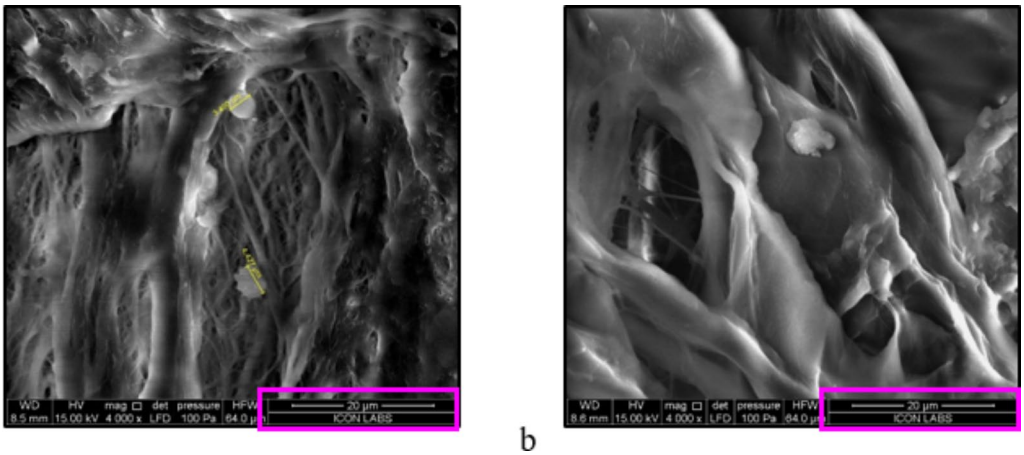


Fig. 3. Group III showing a mixed morphology of cells.

elongated with spikes/filaments attached to it. Group III showed a mixed morphology of cells both rounded and elongated with spikes/filaments.

Both histological and ESEM analysis showed maximum attachment and severe depth of penetration in Group III along with a combination of cell morphology i.e. both spheroidal and filamentous.

Discussion

The aim of the present study was to evaluate the attachment and depth of penetration of the GMSCs into the collagen scaffold preconditioned with different growth media like the Fetal Bovine serum (FBS), injectable platelet-rich fibrin (i-PRF) and a combination of FBS and i-PRF. According to the available literature[16–19] the proliferation and migration of human periodontal ligament cells and osteoblast-like cells on the collagen scaffold have been studied, where they have evaluated the attachment and proliferation of the cells.

GMSCs have the potential to differentiate into osteogenic, chondrogenic, and adipocyte cells.[20] However, this is a first study reported where the attachment and depth of penetration of GMSCs on the collagen scaffold are assessed using the histopathological analysis and correlated with the environmental scanning electron microscope analysis (ESEM). The ESEM analysis overcomes the washout phenomenon of cells which is a drawback of the histological analysis.

Using the principles of tissue engineering proposed by Langer and Vacanti[1] this invitro study was carried out to examine the influence of growth factors derived from Fetal bovine serum (FBS)and Injectable Platelet-rich fibrin (i-PRF) on the attachment and depth of penetration of human gingival mesenchymal stem cells on the commercially available collagen scaffold. Here in this study, we used the cryopreserved GMSCs, commercially available collagen membrane (COLOGIDE) as scaffolds, and FBS, i-PRF as signaling agents/molecules The cryopreserved GMSCs were seeded on the pre-conditioned collagen scaffold using different signaling molecules as mentioned above and evaluated through histological and ESEM analysis for the cell attachment and depth of penetration.

In our study, the cells in Group I where the collagen scaffolds were preconditioned with FBS showed proliferation, attachment, and depth of penetration on the collagen scaffold. The cells appeared spheroidal with maximum concentration in the centre of the scaffold. The number of cells evaluated in the histological analysis showed mild attachment and depth of penetration into the collagen scaffold. ESEM analysis showed a number of cells on the collagen scaffold after 7 days of culture and these cells were seen as well spread on the collagen scaffold. This was in corroboration with a study where the proliferation of Human Mesenchymal Stem Cells isolated from bone marrow onto the collagen scaffold was done and evaluated at different time intervals[17]. Fetal bovine serum (FBS) constitutes the supernatant derived from the coagulation of blood obtained from fetal calves. It is distinguished by its lack of cellular elements, fibrin, and clotting factors while boasting a significant presence of nutritional constituents and macromolecular entities crucial for facilitating cell proliferation. Predominantly, bovine serum albumin stands out as the principal constituent within FBS. The indispensable growth factors inherent to FBS play pivotal roles in the sustenance and advancement of cultured cells. Furthermore, FBS encompasses an array of minute molecules, encompassing amino acids, carbohydrates, lipids, and hormones, all of which contribute to its biological functionality.

i-PRF is rich in growth factors that can control regenerative procedures with improved biological characteristics compared to other platelet concentrations. To study the impact of platelet concentrate on cell proliferation, GMSCs were seeded on the collagen scaffold and cultured in experimental i-PRF-based culture conditions. In our study, the cells in Group II where the collagen scaffolds were preconditioned i-PRF resulted in the proliferation of GMSCs along with morphologic changes that were seen on the collagen of the gingival mesenchymal stem cells. The cells appeared to be rounded with projections on the surface of the cells which looked like filaments. These filaments helped the cell to attach to the collagen scaffold and penetrate deep into the individual collagen scaffold. These filaments on the cell surface could be attributed to being formed due to the effect of growth factors like the PDGF-AA, PDGF-AB, EGF, and IGD-1 present in the i-PRF.

In our study, the cells in Group III where the collagen scaffolds were preconditioned FBS with i-PRF showed maximum proliferation attachment and depth of penetration on the collagen scaffolds.

The present study showed successful results with respect to cell attachment, proliferation and depth of penetration of the GMSCs into the collagen scaffold in all three groups. Group I where GMSCs were seeded onto the collagen scaffold with FBS alone showed cell adhesion, proliferation, and depth of penetration into the collagen scaffold due to the bovine serum albumin which is the major growth factor in FBS. Group II where GMSCs were seeded onto the collagen scaffold along with the i-PRF having growth factors like PDGF-AA, PDGF-AB, EGF and IGD-1 showed better cell adhesion, proliferation, and depth of penetration into the collagen scaffold compared to Group I. Group III where a combination of signaling molecules, like the FBS with i-PRF was used showed the best result when compared to Group I and II in relation to cell adhesion proliferation and depth of penetration. The growth factors influenced the cell attachment, depth of penetration, and cell morphology.

Conclusion

In the present study, cryopreserved GMSCs were seeded on a collagen scaffold (COLOGIDE) which was preconditioned with signalling agents FBS and i-PRF incubated for 7 days. A total of 18 collagen scaffolds measuring about in 1 cm x 1 cm were selected and included in the study and divided into 3 groups and incubated for 1 week at 37^0 C in CO_2 incubator, the 10% α MEM solution was changed every 2–3 days for 7 days. After 7 days of culture maximum cell attachment, proliferation and depth of penetration was seen in the Group III where a combination of the signalling molecules was used. The growth factors had an effect on the cell attachment, depth of penetration and cell morphology. In clinical scenarios it is not feasible to always send the patient blood to the lab for the culture of GMSCs, so further studies are required to evaluate the effect of commercially available growth factors to check for their cell attachment, proliferation, and depth of penetration into the collagen scaffold.

The limitation of the present study was that the entire membrane could not be evaluated in the ESEM and Histological analysis. Commercially available platelet-derived growth factor were not used. In future further studies are required to evaluate the influence of growth factors from commercially available platelet-derived growth factors compared to autologous platelet derived growth factors to assess the cell attachment, proliferation, and depth of penetration into the collagen scaffold and also the influence of incubation at different time intervals.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions
The basic structure of the study was proposed by K.G. S.K, S.Sample collection and processing in stem cell lab was done by K.G and A.K S.K performed all the histological evaluation. K.G & S.K.S. wrote the main manuscript text. K.G, S.K.S, A.K. and A.K. analysis of the results R.B. and D.G revised the manuscript.All authors have read and approved the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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