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Evaluation of The Effect of Surface Modification of 3D-Printed Poly L-lactic Acid Scaffolds by Freeze-Dried Platelet-Rich Fibrin on Migration and Attachment of Dental Pulp Stem Cells

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ORIGINAL STUDY

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Abstract

Purpose: The goal of that research is to appraise the surface modification effect of three-dimensional (3D)-printed poly L-lactic acid (PLLA) by freeze-dried platelet-rich fibrin (PRF) on some of the properties of stem cells from dental pulp. **Patients and methods:** Stem cells extracted from dental pulp (DPSCs) were isolated and cultured from teeth indicated for extraction from donors with ages ranging from 19 to 25. Seeding of the 6th passage of the isolated cells was done on 3D-printed PLLA scaffolds sterilized by gama radiation. The Scaffold surface was modified using PRF by a freeze-thaw cycle. Scaffolds before and after modification were examined for characterization under a scanning electron microscope. Assessment of the characteristics of seeded DPSCs represented in cell attachment and migration on modified and unmodified scaffolds was done. Cell attachment was assessed using an electron microscope while evaluation of cell migration was done using a transwell migration assay. **Results:** Scaffolds modified by surface coating using freeze-dried PRF provided a better attachment substrate for DPSCs. The migration ability of DPSCs was significantly increased toward modified scaffolds. **Conclusion:** Modifying PLLA 3D-printed scaffolds' surface with freeze-dried platelets rich with fibrin could serve as a better scaffold choice for several applications of tissue engineering.

Keywords: Platelet-rich fibrin, Poly L-lactic acid, Scaffolds, Stem cells

1. Introduction

Bone loss due to trauma, cancer, genetic disorders, or even aging is a critical problem that should be managed. The goal of tissue engineering is to cause the damaged tissues to be repaired by regeneration by developing biological substitutes. The triad of tissue engineering includes stem cells, a highly porous scaffold, and suitable growth factors [1].

Scaffold used in tissue engineering must have certain characteristics related to its biological, physical, and chemical properties [2].

A scaffold's biological properties affect scaffold interaction with tissues and organs. This includes biocompatibility, biodegradability, cellular biocompatibility, and nontoxicity. The physical properties of a scaffold define its ability to withstand the *in vivo* exerted forces to offer support to its structure until new tissue is formed. Moreover, the engineered scaffold must be of controlled porosity to allow proper vascularization and mass transport. Also, the chemical properties of a scaffold include its ability to be like the native extracellular matrix (ECM)'s structure and biology [3].

Traditional methods of creating scaffolds cannot produce polymeric scaffolds that can satisfy the requirements of the material as these methods have many limitations on the scaffold's architecture and pore interconnectivity. Researchers mainly focus on

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providing a three-dimensional (3-D) environment, or scaffold, for the enhancement of cell attachment and growth to mimic the *in vivo* environment. Poly L-Lactic acid (PLLA) is the preferred polymer to be used for its tunable properties mechanically and biologically such as biocompatibility and biodegradability [4]. Therefore, it was important to undergo modification to the surface properties to boost cell-material interaction [5].

Platelet-rich plasma (PRP) which is affluent in growth factors is best to be utilized in tissue engineering for both soft tissues and bone [6]. A lot of studies showed the importance of contributing to the interaction between cells and the extracellular matrix during the process of regeneration [7]. However, some limitations have been raised regarding anti-coagulant usage for preparing PRP [8]. Platelet-rich fibrin (PRF) is known to be the platelet concentrate second generation without the addition of any anticoagulants [9]. Recently, a liquid form of PRF termed injectable PRF was investigated and showed significantly high levels of long-term production of growth factors. The biological properties of stem cells were found to be improved after they were seeded on a three-dimensional printed PCL scaffold with freeze-dried PRP coating [10].

Therefore, this work assessed the surface modification's effect of 3D-printed PLLA by freeze-dried PRF on some stem cells extracted from dental pulp (DPSCs) properties.

2. Patients and methods

2.1. Scaffold preparation and surface modification

The PLLA Scaffolds were prepared by Rubik 3D; Egypt. Using Rubik I3 fused deposition modeling (FDM) 3D Bio-printer, alkaline modified, sterilized by gama radiation.

The scaffolds were divided into three groups:

(a) Group (A): unmodified/unsterilized PLLA scaffolds, as the control group.
(b) Group (B): modified/sterilized PLLA scaffolds.
(c) Group (C): unmodified/sterilized PLLA scaffolds.

2.2. Scaffold characterization

For scaffold characterization, scaffolds in all groups were dried. Then they were glued on the holder, then gold coating was done under a vacuum with an ion coater and examined under the scanning electron microscope to investigate the surface morphology of scaffolds before and after sterilization.

2.3. Surface modification of scaffolds with freeze-dried platelet-rich fibrin (freeze-thaw cycle)

After obtaining the informed consent, 20 ml of fresh venous blood was taken from each donor and put equally in two glass Vacutainer test tubes each with 10 ml volume, with no addition of anticoagulants, and immediate centrifugation was done at 3000 rpm for 20 min. The product formed consisted of three layers: the upper first layer, the acellular PRP; the layer in the middle, the nontransparent PRF gel and the third final layer the RBCs. PRF gel was carried out using scissors for usage in the next step. PLLA scaffolds in group B were immersed in PRF at room temperature and then placed at −80 °C without coverage to allow complete evaporation of any excess fluid, then coated scaffolds were subjected to cyclic thawing and freezing three times. All groups were assessed under the scanning electron microscope to compare the surface morphology of scaffolds before and after surface modification. Steps of modification of scaffold surfaces are shown in Fig. 1.

2.4. Isolation and culture of human dental pulp stem cells

The approval of this study was by the Research Ethics Committee (REC) of the Faculty of Dental Medicine for Girls, Al-Azhar University (REC-MA-23-01). Three human third molar teeth to be extracted were taken under sterile conditions from

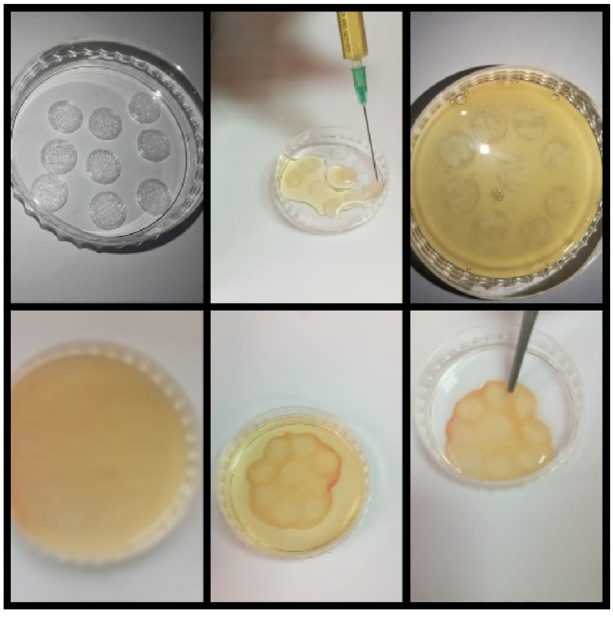

Fig. 1. Steps of coating scaffold surfaces with platelet-rich fibrin.

donors with ages ranging from 19 to 25 and were kept at 4 °C in Dulbecco modified Eagle's media (DMEM, Biowhittaker, GIBCO, Sigma) supplemented with penicillin/streptomycin (Invitrogen Co, USA) and 15% fetal Bovine Serum (FBS, Life Science, UK). The pulp extracted was cut into fragmented parts and then digestion was done using collagenase type two with a percentage of 0.2 for about 1 h long at 37 °C. Cells that were isolated were put in culture in DMEM supplemented with penicillin/streptomycin and 15% FBS and then were inserted in the incubator at 37 °C and 5% CO_2. When cells adhered to the dish and showed to have 70–80% confluency, cell passage was made. Sixth passage cells were handled in the next experiments.

2.5. Cell seeding and cell attachment evaluation

DPSCs cells at the 6th passage were detached and each scaffold in groups B and C was seeded with 1×10^5 cells. After 24 h, getting rid of the culture medium was done and scaffold fixation and dehydration were done in glutaraldehyde and in an alcohol series. Scaffolds were examined under the electron microscope as previously described.

2.6. Transwell migration assay

Assessment of cell migration was done by utilizing a transwell system with two chambers (8 mm pore size and 6.5 mm diameter, Greiner bio-one, Switzerland) modified (group B), and unmodified (group C). PLLA Scaffolds were inserted in the lower wells of a 24-well plate and submerged in DMEM supplemented with FBS 15%. DMEM supplemented with 15% FBS without any scaffolds was used as a control. Migration Chambers were inserted in place and 0.5×10^5 cells' suspension in 100 µl DMEM supplemented with 15% FBS was done, and then seeded on the migration chambers. The upper chambers were moved to the lower wells and incubation at 37 °C was made. After 24 h, the migration chambers were removed and the migrated cells were inserted in 4% formaldehyde for fixation 2 min long, and then staining with Giemsa stain was done for 15 min. The cells which did not undergo migration were rubbed out using a cotton swab from the chamber. Observation of chambers was then done under an inverted microscope (Leica DMi1 Inverted Phase Contrast Digital Microscope).

Pictures were taken under the microscope to analyze cell migration using Image J software. Triplicate independent experiments were performed [11–13].

2.7. Statistical analysis

Data of cell migration were reported as mean ± standard error. Statistical analysis was done utilizing Microsoft (MS) Office Excel Software. One-way analysis of variance was utilized for the evaluation of the groups 'differences and calculation of *P* values was done with an unpaired Student's *t*-test using IBM SPSS version 21. *P* values that were less than 0.05 were considered with statistically significant differences.

3. Results

3.1. Scaffold characterization

Observation of the surface of the scaffolds was done by SEM. As all scaffolds were alkaline modified, group A surfaces showed rough surfaces with micro-size pores enough for cell attachment initially as shown in Fig. 2. Scaffold surfaces in group B showed randomly distributed PRF around the scaffold surfaces which caused rougher surfaces than group A as shown in Fig. 3. Group C showed no observed difference from group A (Fig. 4).

3.2. Cell attachment

Images taken from the surfaces of scaffolds by using SEM showed that cells were more adhered to the deep pores of the modified scaffolds than to the surfaces of the pores of unmodified scaffolds (Figs. 5 and 6).

3.3. Isolation of stem cells from dental pulp stem cells

Isolation of stem cells was done successfully from three human third molar teeth pulp which were

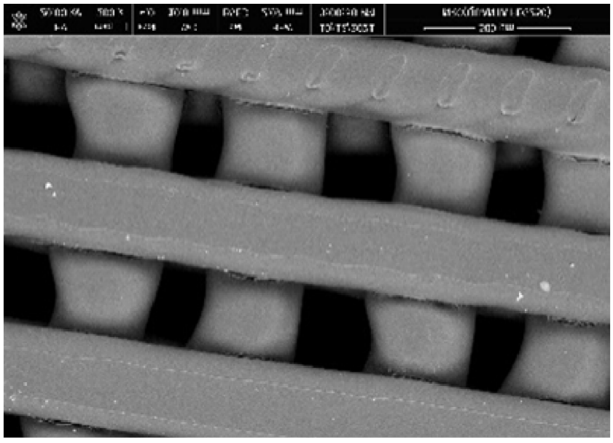

Fig. 2. SEM image showing the scaffold surface of group A.

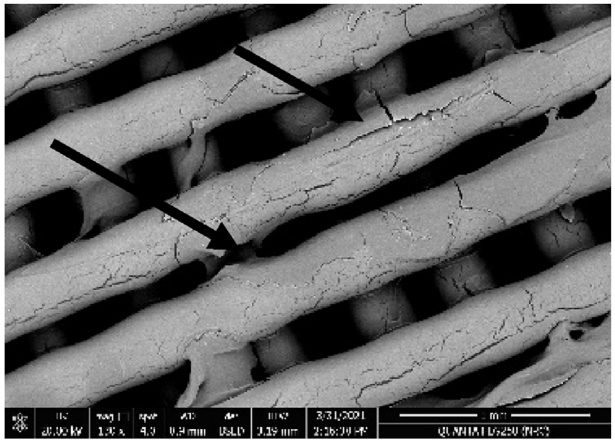

Fig. 3. SEM image showing the scaffold surface of group B.

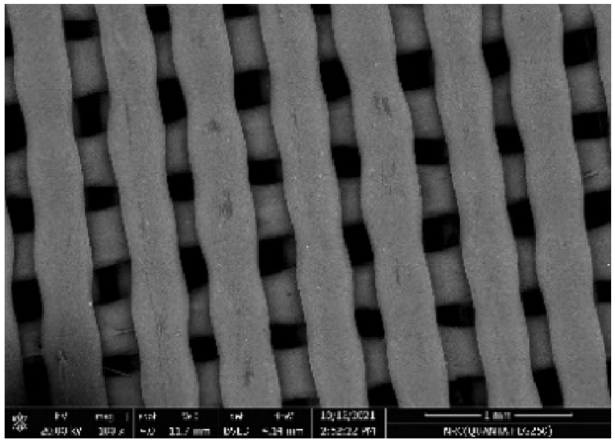

Fig. 4. SEM image showing scaffold surface of group C.

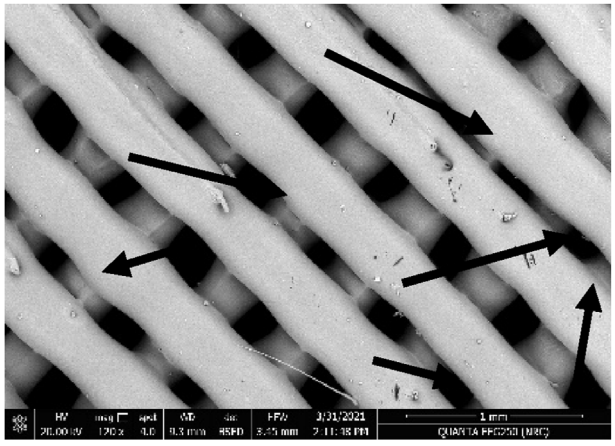

Fig. 5. SEM image of modified scaffold surface after cell attachment.

indicated for extraction. After steps of the isolation and culturing, the cells started attaching to the dishes' bottom. Different morphologies of cells appeared, some were spindle-like, others satellite-shaped, and fibroblastic appearance was also shown in others revealing the difference in pulp cells morphology (Fig. 7). Proliferation and propagation of cells continued until 70–80% coverage of the dish area with cells was observed by day 5, showing high proliferation of cell population in the dental pulp as shown in Fig. 8.

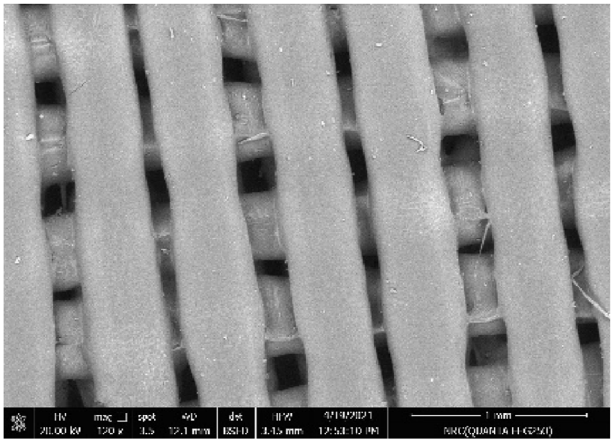

Fig. 6. SEM image of unmodified scaffold surface after cell attachment.

3.4. Cell migration

Image J analysis revealed no significant difference in the sum up of migrated cells towards non-modified scaffolds and the control group ($P > 0.05$). While the sum up of migrated cells toward modified scaffolds was significantly more than that of the control ($P < 0.00001$). Moreover, the sum up of migrated cells in the modified group was shown to be with significantly increased than in the non-modified group ($P < 0.001$) (Fig. 9).

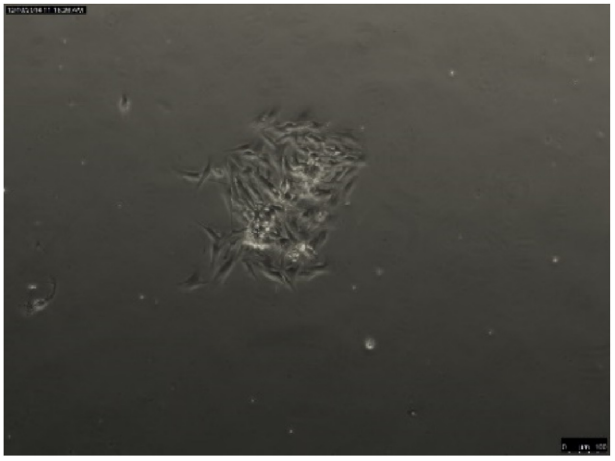

Fig. 7. Primary cultured dental pulp stem cells.

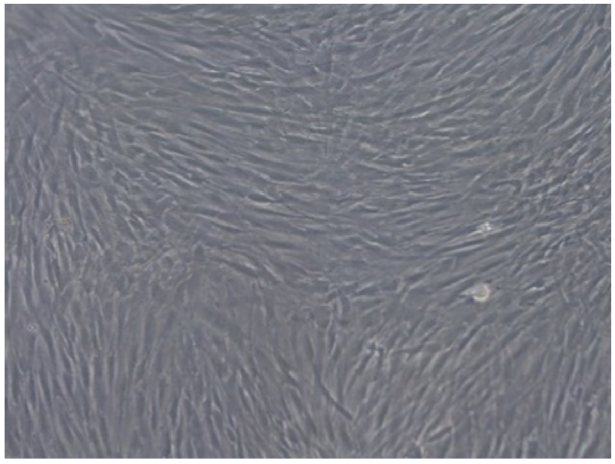

Fig. 8. Confluent cultured dental pulp stem cells.

4. Discussion

Scaffold fabrication done by 3D-printing technology allows controlling the design of microstructure and interconnected porous structure. Recently, there has been a rapid spread of 3D-printed scaffolds in all fields of tissue engineering [14]. PLLA is a polymer used recently for 3D printing [4]. PLLA was chosen in this study as it is a crystalline homopolymer of the PLA family, a thermoplastic aliphatic polyester [15–17]. PLLA has favorable mechanical properties, low toxicity, and biodegradability that allow for the different fabrication methods used to produce different micro and nanostructures of porous scaffolds [18–21].

Scaffolds that are synthesized from this material usually have high values of tensile strength (60–70 MPa) and modulus (2–4 GPa) but low elongation percentage (2–6%) when put in comparison with other synthetic polymers, such as PCL and PDLA [22]. One of the main advantages of PLLA is its degradation by-products mainly lactic acid which is one of the body components and excretion is done in the form of water and carbon dioxide [23–25]. PLLA degradation kinetics depend on its crystallinity and microstructure [26]. The rate of degradation is inversely proportional to the degree of crystallinity. A lower degree of crystallinity is accompanied by a faster degradation rate. PLLA is with slow degradation kinetics due to the extra methyl group causing hydrophobic characteristics and resistance to hydrolysis [22].

The selection of fabrication technology that is compatible with the biomaterial properties is very important. Scaffolds fabricated with conventional fabrication techniques have limitations like controlling scaffold pore size, geometry, and porosity. Also, these methods make it difficult to control scaffold shape and dimensions [14].

In this study, the scaffolds used were fabricated by FDM which is from additive manufacturing techniques. FDM technique uses heat in the synthesis of 3D scaffolds. The FDM technique was selected for its advantages like the absence of using solvent and providing flexibility in the processing and handling of the material. Preformed fibers are required for this technique with certain sizes and mechanical

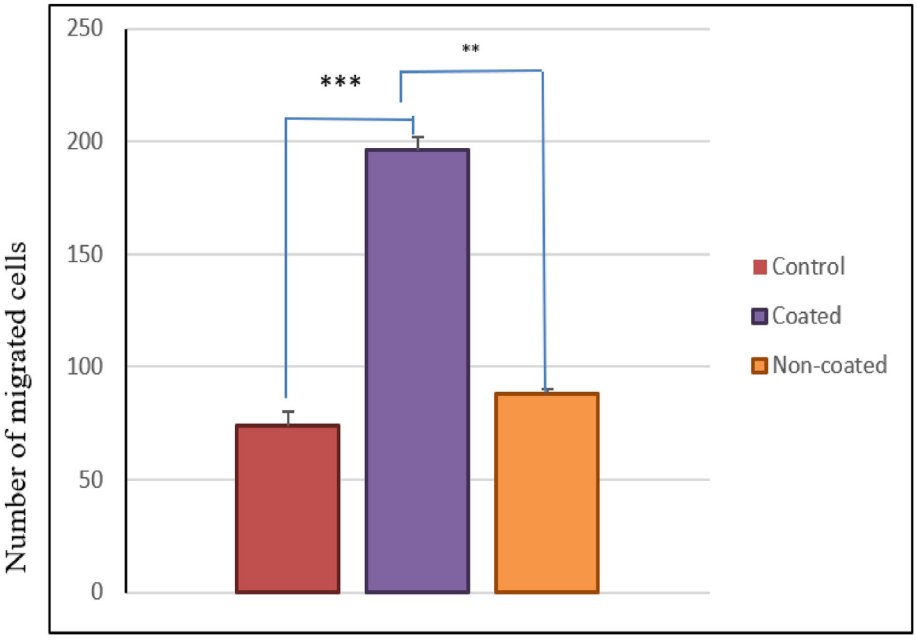

Fig. 9. Bar chart representing the mean numbers of migrated cells in all groups.

properties appropriate to be suitable for feeding through rollers and nozzle [27].

In a recent review discussing PLLA as a material used in tissue engineering, surface modifications of pure PLLA scaffolds were discussed which are used to improve surface bioactivity to aid in cell adhesion [28]. Different types of bioactive molecules can be deposited on the surface causing improvement in the biology of PLLA-based scaffolds, such as hydroxyapatite, chitosan, and collagen [16]. Many types of research showed that there was an improvement in cellular attachment, proliferation, and migration when coated with such molecules [16,28]. Moreover, oxygen plasma treatment on PLLA scaffolds caused a reduction in the contact angle of the polymer and a rise in the free surface energy making PLLA more hydrophilic [29].

Platelet concentrates were used as bioscaffolds for tissue engineering [30]. PRP plasma is considered to be the first generation of platelet concentrate found to improve wound healing [31,32]. It has some issues with its biosecurity and stability and is difficult to store in its liquid form [33,34]. Therefore, in this study, it was preferred to use the second generation of platelet concentrate which is PRF as it is simple to produce and needs no addition of any artificial additives, and can promote cell attachment, proliferation, and differentiation [35–37]. Freeze-thaw cycles were used to modify the surface of PLLA with PRF [37]. There are many other significant ways to modify the surface of scaffolds but the freeze-thaw cycle was preferred as it is a cheap and easy way to retain adhesion and proliferation properties while maintaining the same amount of high growth factors it has [38]. It solves the problem of storage as PRP is not easily stored in liquid form [33,34].

In a previous study, PRP components could promote endometrial mesenchymal stem cells to proliferate and migrate [39], which held the same point of view as the results of this study that showed that modifying scaffolds with PRF enhanced attachment and migration of stem cells. Another study comparing PRF and concentrated growth factor effect on properties of human stem cells of apical papilla which are proliferation, migration, and attachment showed significant differences between migratory cells in the PRF and those in the control group, in PRF were shown to be denser than in the control group [38]. This study's results agreed with the resultant outcomes of this work, the results of modified scaffolds with PRF revealed a significant difference in the way DPSC migrated in both control and unmodified groups because of the high level of growth factors PRF contains [38].

Stem cells derived from dental pulp were selected for use in this study as they are more simply obtained than stem cells derived from bone marrow and exhibit differentiation into other cells [40]. Also, a recent study comparing stem cells derived from and stem cells from adipose tissue used to regenerate bone revealed that stem cells from dental pulp showed an enhancement of the ability of colony formation, the ability of proliferation, the ability of migration, and expression of genes of angiogenesis. They also showed the secretion of more vascular endothelial growth factors in comparison to adipose tissue stem cells [41].

A previous study used coated PCL scaffolds with PRP and showed better results in cell attachment, migration, and bone regeneration in coated scaffolds than bare scaffolds which supports the results of this study that modification with freeze-dried PRF showed significant results in cell attachment and migration than unmodified and bare scaffolds [10]. This could be attributed to the randomly distributed PRF which caused surface roughness leading to better cell attachment to modified scaffold surfaces and the high content of growth factors promoting better migration and bone regeneration.

4.1. Conclusions

Under the limitations of the current research, it was concluded that:

Modifying 3D-printed PLLA scaffolds with freeze-dried PRF enhances DPSCs' attachment and migration to scaffold surfaces.

4.2. Recommendations

Further studies are recommended with other different surface modifications for 3D-printed PLLA scaffolds and compare them with modifications done in this study.

Ethics information

The Research Ethics Committee of the Faculty of Dental Medicine for Girls, Al-Azhar University is constituted and operates according to ICH GCP guidelines and applicable local and Institutional regulations and guidelines which govern IRB operation. The committee met on May 2023. Study Title: "Evaluation of The Effect of Surface Modification of 3D-Printed Poly L-lactic Acid Scaffolds by Freeze_-Dried Platelet-Rich Fibrin on Migration and Attachment of Dental Pulp Stem Cells" Protocol Code: P-MA- 19-12. Final Code: REC-MA-23-0.

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Conflicts of interest

There are no conflicts of interest.

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Though he is no longer here and we are all sorry for that, the great contribution of Dr, Ahmad Farouk Abdelazeem Researcher of Oro-Dental Genetics, Human Genetics and Genome Research Division, National Research Centre, should be mentioned and thanked.

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