Journal of Taibah University Medical Sciences (2020) 15(5), 363–373

Taibah University

Journal of Taibah University Medical Sciences

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Original Article

Preparing polycaprolactone scaffolds using electrospinning technique for construction of artificial periodontal ligament tissue

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Received 30 April 2020; revised 16 July 2020; accepted 20 July 2020; Available online 17 August 2020

الملخص

أهداف البحث: سمحت هندسة الأنسجة بتطوير علاجات قائمة على الخلايا الحية لإصلاح الأنسجة المفقودة أو التالفة، بما في ذلك الرباط حول اللثة، وبناء الغرسة الهجينة الحيوية. يهدف هذا العمل إلى عزل الخلايا الجذعية للرباط اللثوي البشري وزرعها على سقالة من مادة البولي كبرولاكتون المصنعة لتجديد أنسجة الرباط اللثوي الطبيعي.

طرق البحث: تم جمع الخلايا الجذعية لأنسجة الرباط اللثوي البشري من الضواحك البشرية المستخرجة ثم زراعتها وتوسيعها للحصول على خلايا الرباط اللثوي الطبيعي. تم الكشف عن علامة محددة للرباط اللثوي الطبيعي (بيريوستين) باستخدام المقايسة الكهربائية المناعية. تم تطبيق التثبيت الكهربائي لتصنيع البولي كبرولاكتون بتركيزات ١٣٪ و١٦٪ و٢٠٪ من الوزن/الحجم في شكلين، وتم فحصها بعد ذلك بواسطة الفحص المجهري الإلكتروني للانبعاثات المجهرية. كما تم زرع الخلايا الجذعية للرباط اللثوي البشري المعزولة على البولي كبرولاكتون المصنع. بعد ٢١ يوما، تم إجراء الفحص المجهري الإلكتروني للانبعاثات المجهرية لتقييم السقالات المزروعة، وتم إجراء اختبار سمية خلوية من ميثيل تترازوليوم لتوصيف الاستجابة البيولوجية لسقالة البولي كبرولاكتون عند فترات تعرض خلايا مختلفة لمدة ٢٤ و٤٨ و٧٢ ساعة.

النتائج: تم التعبير عن البيريوستين في خلايا الرباط اللثوي الطبيعي الموسعة، والتي أظهرت أن سقالة البولي كبرولاكتون المصنعة للوزن/الحجم بنسبة ٢٠٪ بحجم المسام أكثر من ١٠ ميكرومتر كانت الخيار الأنسب. كانت معدلات نمو الخلايا الجذعية للرباط اللثوي البشري عالية. وأظهر اختبار السمية الخلوية لسقالة البولي كبرولاكتون المصنعة عدم وجود تغيير كبير في قابلية بقاء الخلية مقارنة مع التحكم السلبي وبدون آثار متدهورة أو مثبطة للنمو بعد فترات مختلفة.

الاستنتاجات: نجحت هذه الدراسة في تطوير نسيج خلوي باستخدام البولي كبرولاكتون المصنعة كسقالة لتغطية غرسات الأسنان وتعزيز ارتباط خلية الرباط اللثوي الطبيعي وانتشارها ونموها لبناء غرسة هجينة حيوية.

الكلمات المفتاحية: الخلايا الجذعية للرباط اللثوي البشري؛ بيريوستين؛ التثبيت الكهربائي؛ سقالة بولي كابرولاكتون

Abstract

Objectives: The strategies of tissue-engineering led to the development of living cell-based therapies to repair lost or damaged tissues, including periodontal ligament and to construct biohybrid implant. This work aimed to isolate human periodontal ligament stem cells (hPDLSCs) and implant them on fabricated polycaprolactone (PCL) for the regeneration of natural periodontal ligament (PDL) tissues.

Methods: hPDLSCs were harvested from extracted human premolars, cultured, and expanded to obtain PDL cells. A PDL-specific marker (periostin) was detected using an immunofluorescent assay. Electrospinning was applied to fabricate PCL at three concentrations (13%, 16%, and 20% weight/volume) in two forms, which were examined through field emission scanning electron microscopy (FESEM). The isolated hPDLSCs were implanted on the fabricated PCL. After 21 days, FESEM was conducted to evaluate the implanted scaffolds, and

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Peer review under responsibility of Taibah University.

Production and hosting by Elsevier

 https://doi.org/10.1016/j.jtumed.2020.07.007

an MTT assay was performed to characterize the biological response of the PCL scaffold at different cell exposure durations (24, 48, and 72 h).

Results: Periostin was expressed in the expanded PDL cells, and this result revealed that 20% weight/volume PCL scaffold with a pore size of more than 10 μm was the best. The growth rates of PDLSCs were high. Cytotoxicity test of fabricated PCL scaffold demonstrated no significant change in the cell viability when compared with the negative control and no deteriorating or inhibitory effect on growth after different durations.

Conclusions: A cell sheet was successfully formed by using PCL as a scaffold to cover dental implants and promote PDL cell attachment, proliferation, and growth for biohybrid implant construction.

Keywords: Electrospinning; Human periodontal ligament stem cells; Periostin; Polycaprolactone scaffold

Introduction

Tissue engineering is a new therapeutic strategy that combines the use of a scaffold and cells with bioactive agents (e.g. growth factors) to regenerate damaged and diseased tissues.[1,2] For example, it can be used to repair damaged tissues in periodontitis, an inflammatory oral disease that is the main cause of tooth loss.[3] While dentists can control periodontal inflammation with conventional treatments, they cannot restore the destroyed periodontal tissue. For tissue repair, scaffold biomaterials should be able to support cell adhesion.[1,4] In engineering tissues, two methods are used[1]: inserting cells and a scaffold precursor into a patient's body and[2] culturing the scaffold in vitro and inserting the resultant engineering tissue into a patient's body.[4] A scaffold material should be biodegradable, and its degradation byproducts should be biocompatible with stem cells. Scaffolds should have pores to enhance cell penetration, attachment, and proliferation.[5,6] Biocompatible materials, such as metals, ceramics, and polymers, have been used extensively[7,8]; however, metals and ceramics are not biodegradable, thereby limiting their use in tissue engineering. Polymers have been widely considered for tissue engineering applications because of their biodegradability.[6] Various techniques have been developed to improve scaffold designs by controlling pore characteristics (size and interconnectivity) and porosity to be suitable for cell attachment.[9] Nanofibrous scaffolds made from biodegradable polymer nanofibers can be manufactured using various techniques, including electrospinning, self-assembly, and phase separation.[10] Among these, electrospinning is the fastest and simplest technique used to create nanofibrous scaffolds. Electrospinning is an interesting and flexible technology for the fabrication of ultrafine fibres whereby the nano-structural orientation of the resultant fibrous materials is altered to a significantly reduced dimensional size, to produce outstanding mechanical responses and perform the essential biomedical activities.[11]

Periodontal ligament stem cells (PDLSCs) play an important role in the regeneration of periodontal tissues in human and animal models,[12] and the periodontal ligament (PDL) can be used as a source of stem cells[13]. In different models of periodontal defect, a cell sheet technique has been applied to induce periodontal regeneration, and the efficacy of PDL cell sheets has been assessed in preclinical studies. In a periodontal defect, a calcium phosphate–polycaprolactone scaffold induces bone formation.[14] Fibroblast periodontal cell sheets are delivered utilising a melt electrospun membrane.[15]

Because of its tailorability, Polycaprolactone (PCL) scaffold material can be used in engineering many types of soft tissues by modifying its properties and decreasing its molecular weight.[16] This study aimed to isolate human PDLSCs and to use them to fabricate sheets by utilising a PCL scaffold made via an electrospinning technique. PDLSCs also enhanced cell adhesion, proliferation, and support to create cell sheets for PDL tissue regeneration.

Materials and Methods

All the experimental procedures are listed in Figure 1.

Isolation of hPDLSCs

Forty sound and permanent premolars were collected from young adults (13–26 years) undergoing routine orthodontic extraction at the Oral and Maxillofacial Unit of the Education Dental Hospital/College of Dentistry at the University of Baghdad. This study was approved by the Human Ethics Committee of the College of Dentistry at Baghdad University. Written consents were obtained from

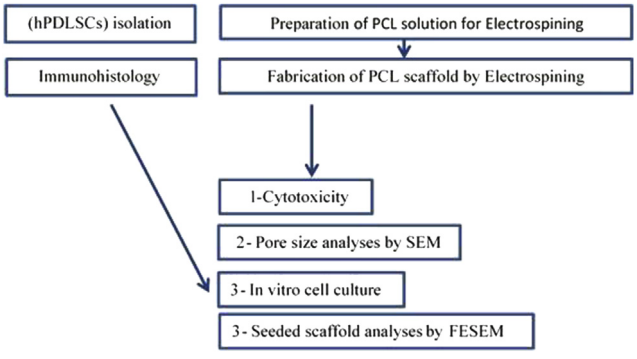

Figure 1: Schematic drawing of the steps of the experimental design.

all donors. Patients with periodontitis, gingivitis, and teeth with caries and periapical lesions were excluded from the study. Dental extraction was performed under local anaesthesia. The extracted teeth were placed immediately in transport phosphate buffered saline (PBS) media (Chemical Point, Germany) or in free media with out fetal bovine serum (FBS) at 4 °C. Under a sterilised hood in a tissue culture laboratory, the extracted teeth were placed in a sterile Petri dish containing free minimal essential medium (MEM) (mediaCapricorn, USA). Premolar teeth were rinsed five times with PBS containing 100 µg/ml amphotericin, 100 µg/ml streptomycin, and 100 units penicillin (Capricorn-Scientific, Germany) for 5 min. For PDL collection, a surgical scalpel was used to scrape the roots (Figure 2). The collected PDL tissue was dispersed with 0.75 µg/ml collagenase/dispase (Sigma–Aldrich, Germany) at 37 °C with shaking for 7 h to obtain a single cell suspension. The test tube containing the cell suspension was placed inside a centrifuge (Hettich, Germany) and centrifuged at 377 *xg* rpm and 17 °C for 3 min. The supernatant was discarded, and 8–10 ml of the MEM culture was added to the test tube. The suspension was cultured and incubated in an incubator containing 5% CO_2 (Memmert, Germany) at 37 °C. After 7 days, the medium was replaced with MEM containing 20% FBS (Capricorn-Scientific, Germany), 80 µg/ml ascorbic acid (US-Biological, USA),[17] and 12.5 ng/ml fibroblast growth factor (FGF2, US-Biological, USA), which was freshly added with each change. The medium was changed every 3 days. On day 21, the cells reached approximately 80% confluence.

Immunofluorescence assay

Human PDLSCs were seeded at a density of 5×10^5 cells in tissue culture chambers with MEM containing 20% FBS.[18] The medium was aspirated from culture slides that were subsequently washed with PBS. Cold acetone at −20°C (Chemical Point, Germany) was added for 3 min to fix the cells after which the cells were allowed to dry. A Papen (Daido Sangyo, Japan) was used to draw a circle on the slides. The slides were then washed with PBS for 5 min, incubated with a blocking reagent (Santa Cruz, Biotechnology, USA) for 1 h, and washed again with PBS for 5 min. Afterwards, the cells were stained with 1:50 periostin antibody (Santa Cruz, Biotechnology, USA) at 4 °C overnight and washed with PBS for 5 min. Coverslips were fixed on microscopic slides, and the slides were examined in the dark using a fluorescence microscope (Leica, Germany).[19]

Scaffold preparation

A scaffold was prepared from PCL (Sigma–Aldrich, UK) via electrospinning in an electrospray system (Nano NC, ESB-200, South Korea; Figure 3).

PCL solutions with three different PCL concentrations (13%, 16%, and 20% W/V) were prepared by dissolving PCL powder in glacial acetic acid. The resulting solutions were mixed using a magnetic stirrer at room temperature (25–30°C) for 6 h to obtain homogeneity. The solution was pumped at a flow rate of 1 ml/h through a small metallic needle (diameter = 0.7 mm) and a 20 kV voltage source on a flat collector to prepare a flat disc scaffold and on rod (drum) collectors with diameters of 3 mm to make a cylindrical scaffold. This procedure was conducted at a 300-rpm rotary speed to shape the scaffolds. The capillary-collected distance was 20 cm (Figure 3). The resultant scaffolds were examined through field emission scanning electron microscopy (FESEM) and a cytotoxicity test.[20] All the scaffolds were sterilised by exposing them to UV light for 24 h.

Cytotoxicity test

The PCL scaffold was evaluated in terms of its cytotoxicity and biocompatibility in vitro in accordance with ISO 10993-Part 5. hPDLSC cultures were maintained in a flask and incubated in a humidified incubator with 5% CO_2 at 37 °C. The medium was changed every three days, and the growth factor was added freshly with each change. When the cells reached 80%–90% confluence, they were harvested through trypsinisation.

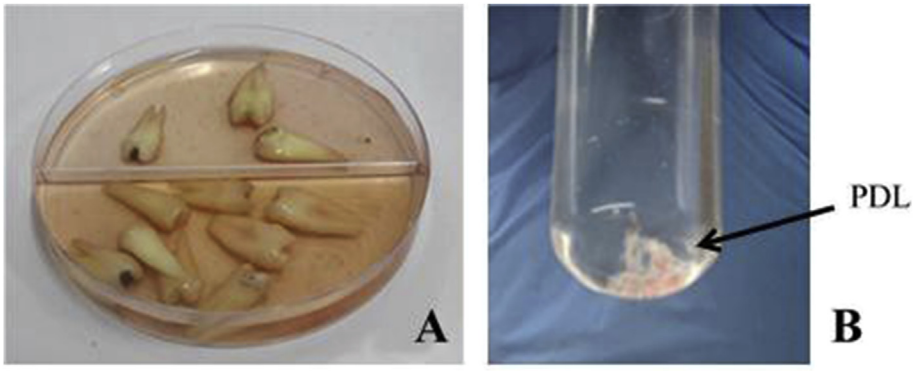

Figure 2: hPDLSC isolation: A) Premolar extracted teeth, B) PDL.

PCL scaffold discs (diameter = 12 mm) were prepared and placed in 96-well flat-bottom labelled plates containing 100 µl of MEM supplemented with 20% FBS. PDL cells were seeded at a cell density of 10,000 cells per well. The cells incubated in standard MEM were used as a negative control, while those incubated in media containing a cytotoxic product (10% ethanol diluted in media) were set as a positive control. The containers of the plates were sealed and incubated under standard culture conditions for 24, 48, and 72 h to achieve cell confluence. At the end of the incubation periods, the cell culture viability was estimated using a methyltetrazolium (MTT; Santa Cruz, USA) solution cytotoxicity assay, and the culture medium was removed from each well. In a dark room, 100 µl of 5 mg/ml fresh MTT solution was added to each well. The plates were then incubated for 4 h. The mitochondrial dehydrogenase of living PDLSCs converted MTT to formazan crystals. The supernatant was removed; 50 µl of dimethyl sulfonic oxide (DMSO; Santa Cruz, USA) was added to each well, and the plates were incubated for 30 min. The dimethyl sulfonic oxide (DMSO) solution was transferred to new 96-well flat-bottom plates, and optical density was measured at 570 nm using a fully automated microplate-based multidetection reader (FLOUstar OPTIMA, microplate BMG LABTECH, Germany).[21–23] Each experiment was repeated three times.

Scanning electron microscopy of cells cultured on PCL scaffolds

PDLSCs were cultured on PCL scaffolds with 13%, 16%, and 20% W/V PCL in 24-well cell culture plates containing 500 µl of MEM supplemented with 20% FBS and PDL cells seeded at a cell density of 100,000 cells per well. After 21 days, FESEM was used to evaluate the implanted scaffolds.[24,25] The implanted scaffolds were fixed with 4% glutaraldehyde at 4°C for 24 h, washed with PBS at room temperature for 10 min, fixed with 2% osmium tetroxide solution for 2 h, washed again with PBS for 10 min at room temperature, and dehydrated in absolute ethanol at different concentrations (50%, 70%, 90%, and 100%), 10 min for each concentration.[25]

Results

hPDLSC culture

On day 7 of culturing, PDLSCs appeared as small colonies of adherent cells (Figure 4). The medium was changed to remove cell debris. On days 10–20, the cells began to proliferate and migrate, and appeared as spindle-like fibroblasts. The size of the colonies ranged from 0.5 mm to 3 mm in diameter, and the cells reached more than 80% confluency on day 21.

Immunofluorescence

More than 90% of the PDLSCs were found to be positively stained for periostin (Figure 5).

Scaffold structure

Microparticles were formed with 13% W/V PCL (Figure 6A and B). In comparison, spindle-shaped beads and fibres were observed with 16% W/V PCL (Figure 6C and D). Smooth fibres with a pore size of more than 10 µm were obtained with 20% W/V PCL (Figure 6E and F).

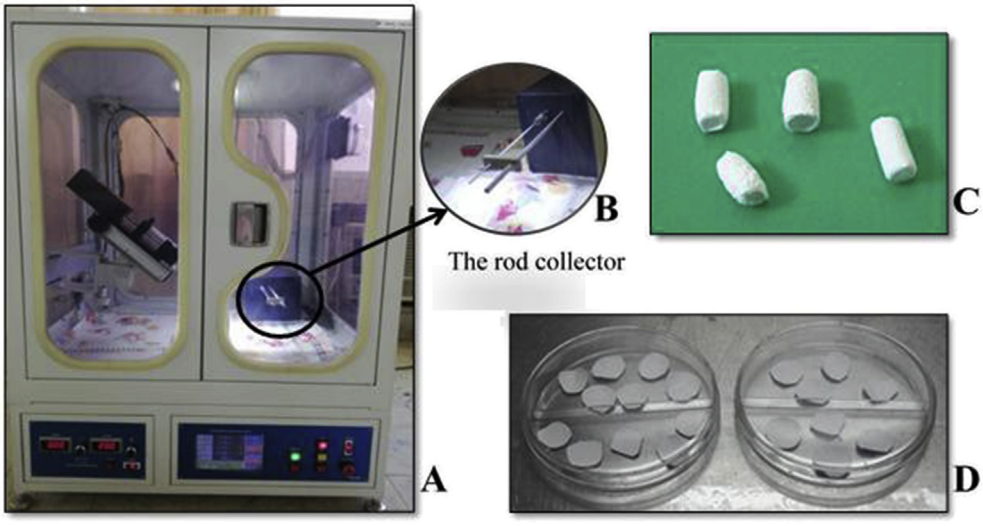

Figure 3: A) Bio-Electrospinning/Electrospray system, B) Rod collector for scaffold shaping, C) Cylindrical PCL scaffold, D) Discs PCL scaffold.

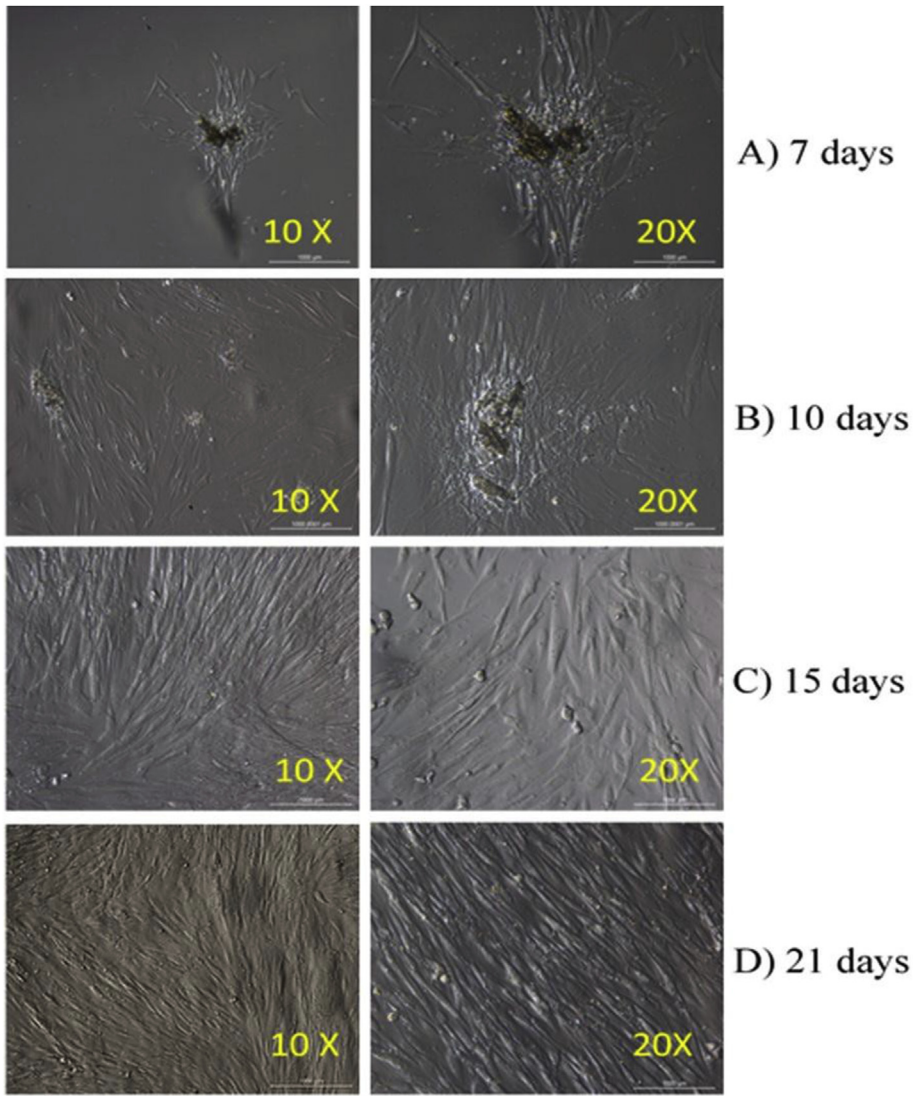

Figure 4: Inverted microscope images for hPDLSCs; A) 5 days, B) 10 days, C) 15 days, D) 21 days.

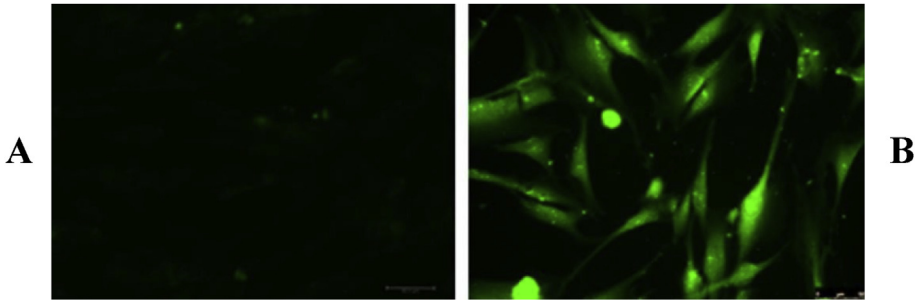

Figure 5: Fluorescence microscopic images at 20X, A) control hPDLSCs (negative expression), B) + periostin of hPDLSCs (positive green expression).

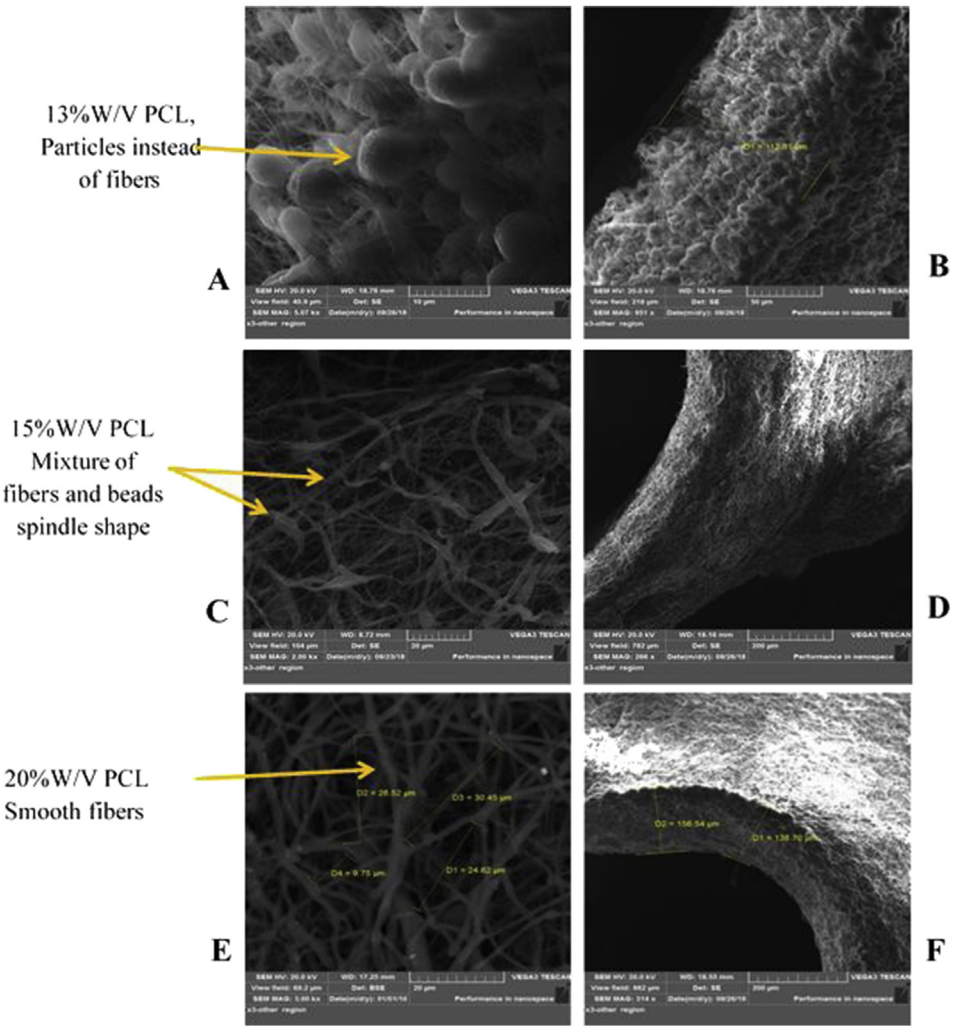

Figure 6: SEM topographic and cross section images of scaffold: A,B) images of 13%W/V PCL; C,D) images of 16% W/V PCL; E,F) images of 20% W/V PCL.

Cytotoxicity test

The tested scaffold showed no significant change in cell viability (Figure 7A) and did not cause any deteriorating or inhibitory effect on cell growth after 24, 48, and 72 h of exposure of cells to the scaffold materials (Figure 7B).

FESEM of the seeded scaffold

FESEM analysis revealed that the 13% W/V PCL scaffold had weak growth rates (Figure 8A), the 16% W/V PCL scaffold had low growth rates with nonuniform proliferation (Figure 8B), and the 20% W/V PCL scaffold had high growth rates (Figure 8C). The average pore size was more than 10 µm (Figure 9). Furthermore, the 20% W/V PCL scaffold with PDLSCs at different durations favoured the formation of a typical confluent monolayer (Figure 10).

Discussion

In regenerative medicine, stem cells of various origins are utilised to treat different conditions, such as neurological and endocrine deficits, cartilage abnormalities, and dental conditions.[26–32] In this study, the cultured PDL cells began to proliferate and migrate between 10 and 20 days of culture; they also manifested a spindle-like fibroblasts. Colonies and confluency of periodental ligament stem cells (PDLSCs) have also been described in another study.[19] The enzymatic dissociation method was used to obtain PDLSCs with greater proliferation rates, better colony-forming efficiency, and stronger differentiation capacity in culture than those obtained using the outgrowth method. Furthermore, the success rate of primary culture was higher with type I collagenase than with trypsin, which is in agreement with previously reported results.[33]

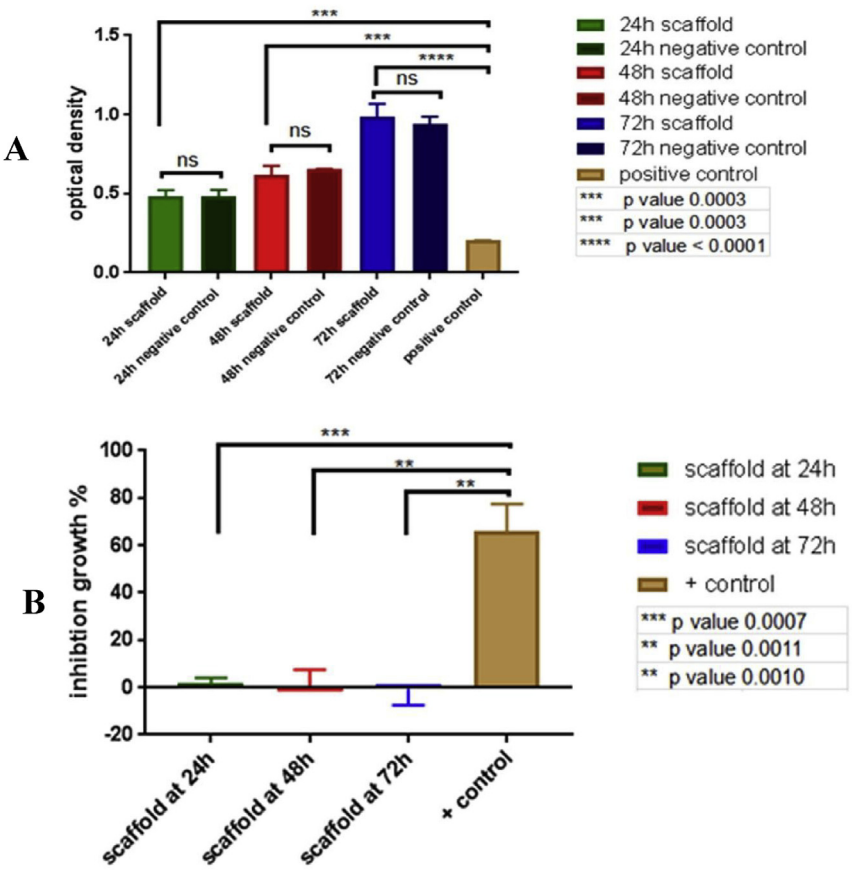

Figure 7: A) Cell viability at different exposure times (24h, 48h, and 72h) to the scaffold, B) Optical density inhibition growth % scaffold at (24, 48, and 72).

The immunophenotyping analysis of PDLSCs revealed that more than 90% of the cells were positive for periostin, which is a protein expressed mainly in PDL and associated with the functions of PDL.[13,34] In this study, electrospinning, which is a simple and efficient method, was performed to fabricate PCL. A triphasic scaffold (90% PCL/10% hydroxyapatite) was manufactured by fused deposition modelling. The three phases have the same chemical compositions, but each phase has a modified architecture.[35]

The resultant scaffold has a micro-scale fibrous structure. The viscosity of the polymer is the most important factor in determining the morphological characteristics of the resultant fibres; polymer concentration and viscosity are related to each other.[36] A pilot study was conducted to select suitable concentrations of the polymer to fabricate scaffolds with a structure (pore size and fibre diameter) suitable for PDL cell penetration and proliferation in order to initiate the 3D growth required for tissue regeneration. When the polymer concentration (viscosity of the polymer) was low (e.g., 13% W/V PCL), electrospray instead of electrospinning led to the formation of microparticles instead of microfibers (Figure 6A and B). When the polymer concentration increased to 16% W/V PCL, a mixture of spindle-shaped beads and fibres formed (Figure 6C and D). When the polymer concentration increased to 20% W/V PCL, smooth nanofibers were obtained with a pore size of more than 10 μm (Figure 6E and F). Therefore, a polymer concentration of 20% W/V was used in this study. Our results corroborate previous findings,[25] that is, the diameter of smooth nanofibers without microparticles is 1252 nm, resulting in the formation of a PCL scaffold with a concentration of more than 15% W/V PCL. The methyltetrazolium solution cytotoxicity (MTT) assay was used to study the biocompatibility of the PCL scaffolds with PDL stem cells. The results revealed that 20% W/V PCL had good biocompatibility and no noxious or cytotoxic effects on PDLSCs. This study revealed that no toxic leached ions were released from the PCL scaffold, and the scaffold was biocompatible with vital tissues.

The tested scaffold showed no significant change in cell viability with the negative control and exhibited a very significant change with the positive control. The scaffold did not cause any deteriorating or inhibitory effect on cell growth after the cells were exposed to it for different durations. FESEM analysis of the 13% W/V PCL scaffold showed weak growth rates. No cell growth (dead cells) was due to the architecture of the scaffold, and the average pore size was lesser than 1 μm. The presence of large rounded beads with fibres formed beads on a string structure, and this finding was related to the low viscosity of the PCL solution that led to the break-up of jets in solutions; the formed beads

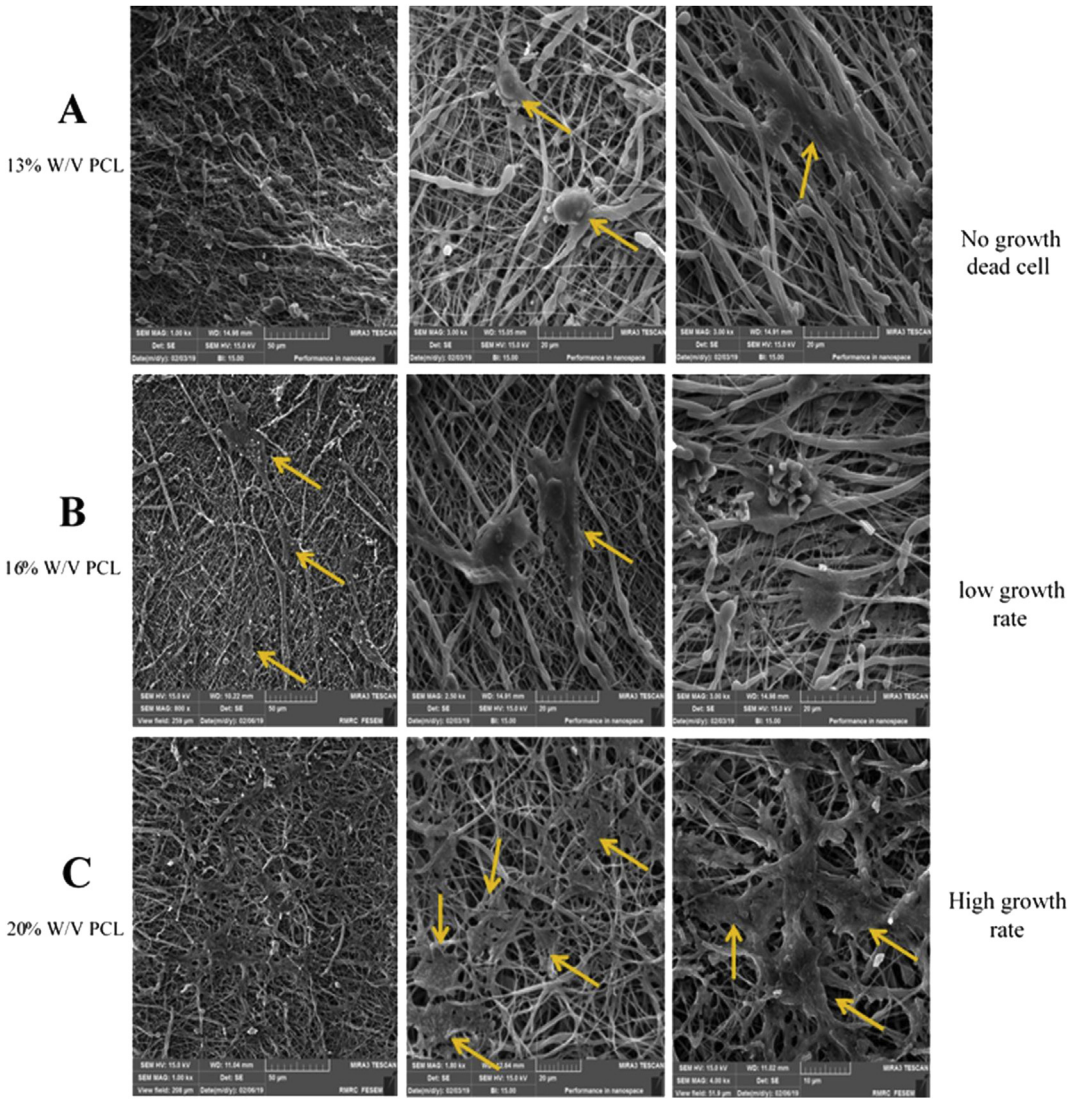

Figure 8: FESEM image of seeded PCL scaffold at day 10, A) No growth on the seeded scaffold (13% W/V PCL) with stem cells at 1000X, 2000X, and 3000X, B) Low growth rate on the seeded scaffold (16% W/V PCL) with stem cells at 1000X, 2000X, and 3000X, C) High growth rate on the seeded scaffold (20% W/V PCL) with stem cells at 1000X, 2000X, and 3000X.

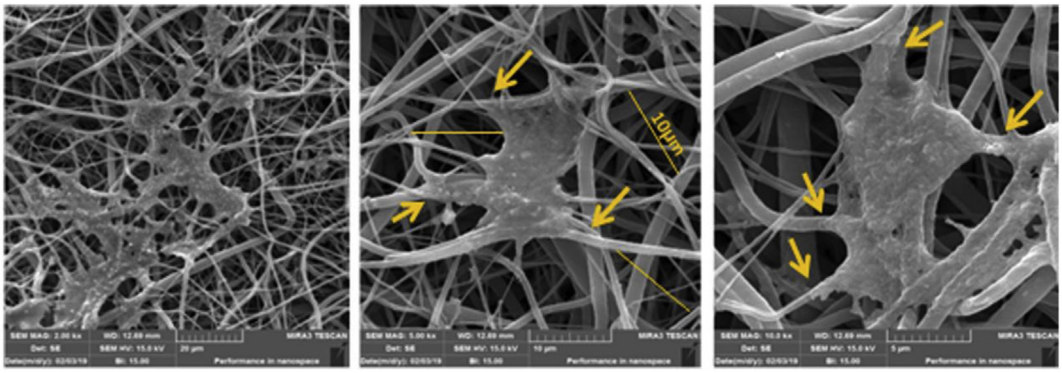

Figure 9: FESEM image of 20% W/V PCL scaffold with different magnification 2000×, 5000×, 10,000X. Arrows are pointing to cell attachment and growth following fibres of PCL.

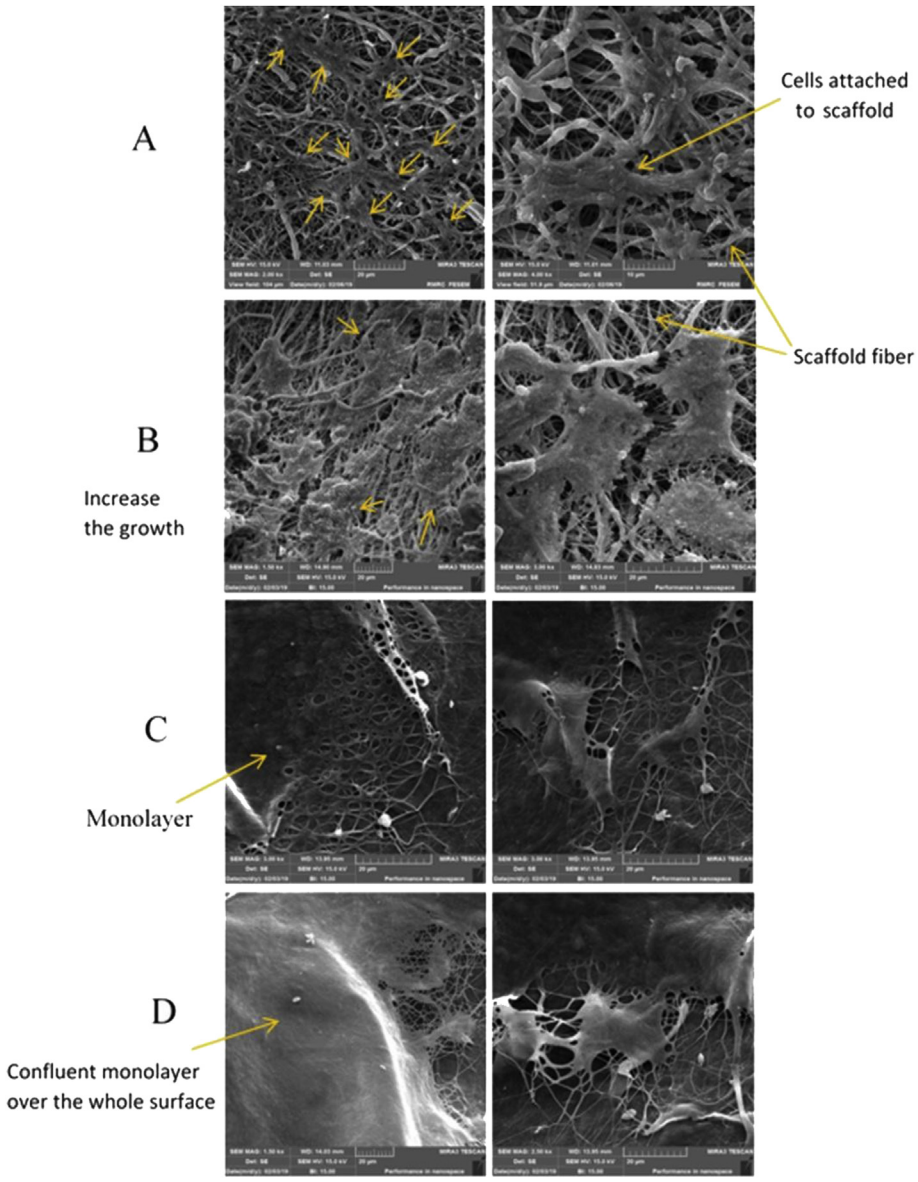

Figure 10: FESEM image of seeded PCL (20 V/W) scaffold with increase in cell growth at different time, A) 7 days, B) 14 days, C) 21 days, D) 30 days.

may also reduce the pore size, making it difficult for cells to penetrate the scaffold and thereby weakening cell proliferation.[37] FESEM analysis of the 16% W/V PCL scaffold showed low growth rates with nonuniform proliferation on the whole scaffold surface, and these characteristics should be further optimised to develop a scaffold structure with larger pore size. The average pore size was lower than 5 μm (Figure 8B). Moreover, the 20% W/V PCL scaffold showed high growth rates, and therefore, was a good scaffold structure that was suitable for the attachment and stability of cells (Figure 8C).

The average pore size of the 20% W/V PCL scaffold was more than 10 μm, which was suitable for PDL cell penetration and proliferation to initiate 3D cell growth required for tissue regeneration.[38] The scaffolds were examined every week, until the cultures became semiconfluent with adherent cells after four weeks. On day 28, approximately 90% confluence was observed, and on day 30, a cell monolayer was formed (Figure 10). FESEM analysis of the 20% W/V PCL scaffold seeded with PDLSCs at different durations revealed that a typical confluent monolayer was formed and the scaffold completely disappeared on the whole surface. The cell sheet engineering technique was used, since the single cell suspension injection technique in tissue reconstruction is accompanied with many disadvantages.[39] One of the limitations of this study is that a vital tooth was necessary. The tooth was important because it ensured preservation of stem cells (higher proliferation rate and higher gene expression in cell proliferation), which were isolated within 15 min after extraction. In this study, teeth were collected from young adults (13–26 years) undergoing routine orthodontic extraction. Multipotent PDLSCs can be isolated from human impacted third molars and from human exfoliated

deciduous teeth via modified procedures. There are many problems associated with the isolation of PDLSCs from retained teeth, such as the risk of contamination, periapical inflammation, and vulnerable condition of stem cells. The 3D artificial construct (cylindrical scaffold and seeded cells) has potential clinical applications in the covering of dental implants and production of biohybrid implants. Further vivo studies could aim to cover dental implants (titanium or zirconia) with sintered β-TCP[40,41] in order to simulate natural periodontal tissues.

Conclusion

The PDL of an adult human is an ideal and successful source for harvesting and expanding PDLSCs. The rates of cell growth on the prepared scaffold depended on the pore size of the scaffold, and we found that a pore size of 10 μm is ideal and leads to better cell invasion, penetration of the scaffold, and continuation of tissue-like structure formation. The study found that a 20% W/V PCL scaffold can support cell sheet formation and has potential clinical applications in covering dental implants and producing biohybrid implants that can promote PDL cell attachment, proliferation, and growth.

Source of funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

Ethical approval (number 085219, 24-2-2019) regarding the protocol of the study was granted by the Research Ethic Committee in College of Dentistry at University of Baghdad.

Authors contributions

AMA conceived and designed the study. INS conducted research and collected and organised the data. MAU provided research materials. BMA analysed and interpreted the data. All authors wrote the initial and final drafts of the article and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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How to cite this article: Safi IN, Al-Shammari AM, Ul-Jabbar MA, Hussein BMA. Preparing polycaprolactone scaffolds using electrospinning technique for construction of artificial periodontal ligament tissue. J Taibah Univ Med Sc 2020;15(5):363–373.