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Original Article

Isolation and characterization of human periodontal ligament stem cells under the terms of use in clinical application: A pilot study

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ABSTRACT

Background: The aim of the present study is to determine the possibility of isolation and characterization of the human periodontal ligament stem cells (hPDLSCs) using limited harvested periodontal ligament (PDL) tissue of only one patient's wisdom teeth (2–4 teeth) under the more compatible terms of use in clinical application without using the fetal bovine serum (FBS).

Materials and Methods: In this pilot study, hPDLSCs were isolated from the impacted third molar, and tissue was scraped from the roots of the impacted third molar of 10 volunteers to enzymatically digest using collagenase. The cells were sub-cultured. The samples of the first seven patients and half of the eighth patient's sample were cultured in alpha modified of Eagle's medium (α-MEM) (−FBS) medium and the other part of the eighth patient's sample was cultured with prior medium supplemented with +FBS 15% as a control of the cultivation protocol. While for the past two patients (9th and 10th the α-MEM medium was supplemented with L-Glutamine, anti/anti 2X, and 20% knock-out serum replacement (KSR). Two more nutritious supplements (N2 and B27) were added to the medium of the tenth sample. Flow-cytometric analysis for the mesenchymal stem cell surface markers CD105, CD45, CD90, and CD73 was performed. Subsequent polymerase chain reaction was undertaken on three samples cultured with two growth media.

Results: Cultivation failed in some of the samples because of the lack of cell adhesion to the culturing dish bottom (floating cells), but it was successful for the 9th and 10th patients, which were cultured in the α-MEM serum supplemented with KSR 20%. Flow cytometry analysis was positive for CD105, CD90, and CD73 and negative for CD45. The PDL stem cells (PDLSCs) expressed CD105, CD45, and CD90 but were poor for CD73.

Conclusion: According to the limited number of sample tests in this study, isolation and characterization of PDLSCs from collected PDL tissue of one patient's wisdom teeth (2–4) may be possible by the proper setup in synthetic FBS-free serum.

Key Words: Adult stem cell, cell isolation, periodontal ligament

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INTRODUCTION

Taking advantage of stem cells, different strategies have been established to promote periodontal regeneration.[1] In general, stem cells are defined as undifferentiated cells that possess interesting

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properties such as self-renewal, high proliferation rate, and differentiation potential to different cell types.[2,3] Mesenchymal stem cells (MSCs) have been identified from multiple dental-related tissues, including periodontal ligament (PDL), dental pulp, apical papilla, and dental follicles.[4] PDL tissue contains some population of stem cells, which are responsible for periodontal tissue regeneration, generally called PDL stem cells (PDLSCs).[5,6] Several lines of studies have indicated that the transplantation of stem cells can be an effective approach for the treatment of periodontal defects.[2,7-9] In an inflamed periodontal environment, appropriate tissue regeneration does not occur because of the lack of enough stem cells for migration and regeneration.

Therefore, transplantation of exogenous stem cells is required to replenish the defect area and facilitate tissue regeneration.[10] As a consequence, current research has been directed toward developing cell-based techniques for periodontal regeneration. In this regard, several publications have directly compared the regenerative capacity of MSCs from different origins. Particularly, PDLSCs appear to have a greater capacity to regenerate dental-associated tissues, rather than other MSCs, which makes them highly amenable for use in periodontal regeneration.[11] Recently, some clinical studies have been reported using stem cells for the regeneration of periodontal tissues.[4] In the first preclinical studies, PDLSCs were harvested from pooling the PDL tissues of several individuals to increase the number of available stem cells in the samples and fetal bovine serum (FBS) was used whole during the isolation process to support the cellular survival and attachment. It is worth noting that pooling PDL tissue from different people and using FBS can increase the host's autoimmune response.[12-17] Recently, the number of studies on the clinical implementation of dental stem cells are increasing.[18-28] Clinical application of dental stem cells demands a comprehensive consideration of different concerns such as ethical factors, obtaining high-quality stem cells, minimal harvesting tissue morbidity, good access ability, and safety of the cultivation process.[29,30] Each mentioned concerning factor is important to be investigated by a large number of studies; however, a standard protocol for harvesting and *in vitro* expansion of PDLSCs under clinical condition, is not available so far, which highlight the necessity of optimized isolation, characterization, and expansion method.[31] The aim of the current study is to determine the possibility of isolation and characterization of the human PDL stem cells (hPDLSCs) using an individual PDL tissue under the more compatible terms of use in the clinical application without using the FBS as the culturing supplement.

MATERIALS AND METHODS

This pilot study was approved by the Ethics Committee of the Isfahan University of Medical Sciences (Ethical Code IR.MUI.REC.1394.3.124). PDL tissues were obtained from 32 impacted third molars of 10 volunteers (3 men and 7 women) aged 20–44 years following the International Review Board guidelines. The study design was based on the extraction of PDL tissue from impacted wisdom teeth to minimize the potential destructive effect of the inflammatory reactions on the PDL stem cells.[3,10] Patients who met the following inclusion criteria were selected based on panoramic radiographs [Figure 1] and clinical examination.

1. No systemic problem or behavior that can affect their oral tissues
2. At least two impacted wisdom teeth
3. Wisdom teeth which were placed in jaw positions that there was no need to be cut to remove (to minimize damage possibility of the PDL tissue)
4. Subjects who had the ability to cooperate during the surgery process
5. Teeth with complete development of root apex (to avoid catching the pulp tissue during PDL tissue harvesting from the root)
6. The extracted samples were sent to the laboratory in <1 h.

To reduce the number of oral microorganisms, Phase I of perio dontal treatment was performed and the patient was advised to brush their teeth and tongue using separated toothbrushes before surgery.

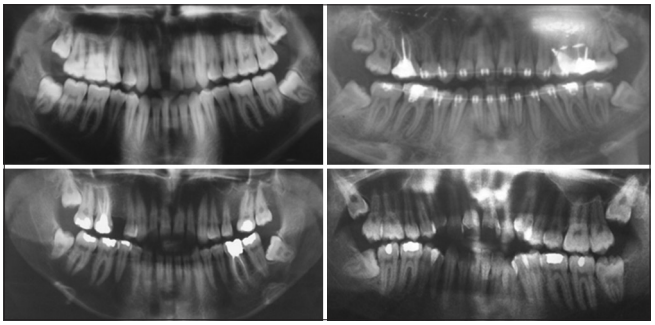

Figure 1: Examples of selected patients' panoramic radiographs.

After obtaining written informed consent from all the volunteers, 50 mL of blood was taken from each patient for human serum extraction, povidone-iodine 1% was used for skin antisepsis, local anesthesia (lidocaine 2%, adrenaline concentration 1/80,000, Loghman, Iran) was injected, and patients rinsed their mouths by mouth wash (Chlorhexidine [CHX] 0.02%, Darupakhsh, Iran) for 30 s and patients' mouth was washed with diluted CHX 0.02% (1/5 CHX 0.02% and 4/5 normal saline). All the surgeries were done atraumatic and under sterile conditions. During extraction, the teeth were held by the crown and avoid trauma to the roots. Any number of wisdom teeth that the patient agreed were extracted by doing a full-thickness flap and then removing tooth without sectioning. The PDL tissue was taken gently from the middle part of the roots using a surgical blade [Figure 2].

Each patient's extracted PDL tissue along with his/her blood sample transferred to the laboratory (first and second patients in PBS+ medium (phosphate-buffered salt contains Pes/str, both from Gibco, Paisley, UK) and about the rest seven patients in an alpha modified of Eagle's medium (α-MEM Sigma, Munich, Germany) which all put in separated sample ice dishes and each tissue sample was transferred to the cellular laboratory (Department of Cellular Biotechnology at Cell Science Research Center Royan Institute for Biotechnology Isfahan, Iran) individually by one of researchers in <1 h. Suturing was done, and postsurgery medicine (Ibuprofen 400 mg q6 h Najo, Iran, mouth rinse CHX 0.02% Bd, Najo, Iran Darupakhsh, Iran) and instructions were prescribed.

Isolation

Since in this study, we aimed to harvest PDLSCs from individuals, PDLs from each patient were processed separately. After three times rinsing with PBS-solution, each PDL tissue was segmented to 1–3 mm size pieces and digested in a solution of 3 mg/mL collagenase Type I (Gibco, Paisley, UK) and 4 mg/mL Disphase (Gibco, Paisley, UK) for 1 h at 37°C. Enzyme solution was diluted with culture medium contains (fetal calf serum, Gibco, Paisley, UK) and single-cell suspensions were passed through a 40 μm strainer. Next, cell precipitation was down in 1800 rpm and cultured in α-MEM media supplemented with 15% FBS (Gibco, Paisley, UK), 1% L-Glu (Gibco, Paisley, UK), 1% penicillin/streptomycin (Gibco, Paisley, UK). After 24 h medium was exchanged following washing cells with PBS+ (contains Ca^{2+} and Mg^{2+}) (Gibco, Paisley, UK) the samples of the first seventh patients and half of the eighth Patient's sample were cultured in α-MEM (−FBS) medium supplemented with L-Glutamine, pen/strep 2X and HS20% and the other part of the eighth patient's sample cultured with prior medium (supplemented α-MEM with L-Glutamine, anti/anti 2X + FBS 15%) as a control of the cultivation protocol while for the last two patients 9th and 10th, the α-MEM medium was supplemented with L-Glutamine, anti/anti 2X, and 20% Knock Out Serum Replacement (KSR) (Gibco, Paisley, UK) and two more nutritious supplements (N2 and B27) were added to the medium of the tenth sample too. Cells were then cultured for 10 days in 5% carbon dioxide at 37°C to reach to 70%–80% confluency (suitable for passage). The medium was changed every 3–4 days for each cell groups and cells were used for further assays at the third-seventh passage.

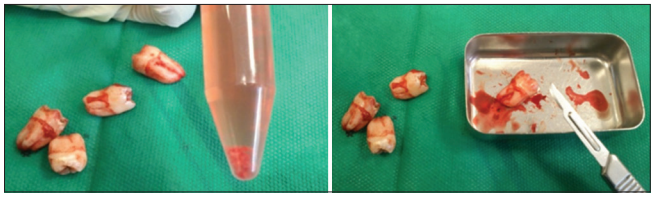

Figure 2: Extracting the periodontal ligament tissue.

Characterization

Expression of surface markers using flow cytometry

To verify the identity of the resulting cells, expression of the surface markers CD90, CD105, and CD73 (all from Millipore), which are known as minimal criteria of MSCs and CD45 as an indicating marker of hematopoietic cells were analyzed by flow cytometry. After fixing the cells with 4% paraformaldehyde, samples were treated with the primary antibody for one night and then for 2 h with a secondary antibody (Sigma, Munich, Germany). The percentage of cells expressing each marker was analyzed by FACS Caliber cytometer (Becton Dickinson, San Jose, CA, USA) by collecting 10,000 events per sample at a flow rate of around 100 events per second.

The data analysis was performed using the open-source software WinMDI 2.9.

Reverse transcription-polymerase chain reaction

RNA extraction was performed using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA was used for reverse transcription with Revert Aid TM H Minus First

Strand cDNA Synthesis Kit (Fermentas). Subsequent polymerase chain reaction (PCR) was undertaken on three samples cultured with two growth media. The primer sequences and the size of the amplification product are indicated. PCR products were then separated by electrophoresis on 1% agarose gel and photographed by an ultraviolet transilluminator.

RESULTS

The conducted cultivation of all samples which were cultured in the α-MEM serum supplemented with FBS 15% was successful; however, cultivation of the PDL tissue in the α-MEM, which was supplemented with 20% HS in the first five patients failed because of the bacterial infection, it failed in the 6th patient because of the lack of vital cells and it failed in the 7th and half of the 8th patient's sample because of the lack of cell adhesion to the culturing dish bottom (floating cells); however, the cultivation process was successful for the second part of the eighth patient's sample which was cultured in an FBS + supplemented medium as the control of the cultivation protocol. PDL tissue cells of the 9th patient, which were cultured in the α-MEM serum supplemented with KSR 20% were attached to the dish successfully [Table 1] and the same result happened with the cultivation of the second group sample of the 10th patient which was cultured in the α-MEM supplemented with KSR 20%, N2, and B27 that using of these supplements promoted the amount of cell proliferation too. According to the last two patients' samples (9th and 10th), it was observed that the morphological characteristics of cells that were cultured in serum-free medium (KSR 20%) at the checking times was similar to the FBS 15% containing medium [Figure 3]. On the 10th day in both media, Fibroblast-like cells reached 90% confluence and cells cultured in serum-free medium preserved normal features of stem cells in passage 6.

Flow cytometry analysis

After passage five cultured cells in both mediums were positive for CD105 and CD90 (common MSC markers) and negative for CD45 (leukocytic factor), but CD73 had adequate expression just in serum-free medium and we found it very low in normal culture condition [Figure 4].

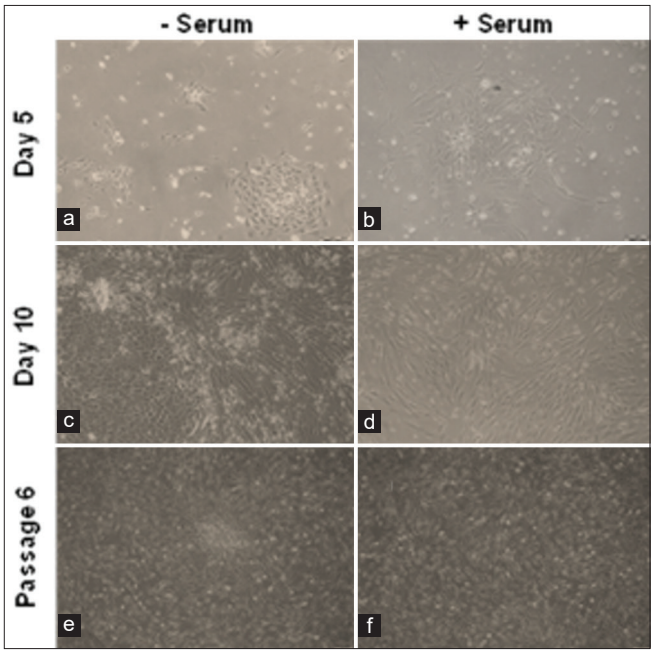

Figure 3: Phase-contrast morphological features of periodontal ligament stem cells (PDLSCs) cultured under fetal bovine serum (FBS)-free and FBS-containing conditions. Periodontal ligament-derived cells showed nodule-like morphology in primary culture (P0) after 5 days in both culture conditions (a and b). In the approximately same manner in both media, flattened fibroblast–like cells reached to 90% confluency during 10 days (c and d). PDLSCs preserved their normal feature in designed FBS-free condition (e) as well as in the usual FBS-containing medium (f).

Table 1: Cultivation methods and results of them in 10 cases

Case number	Number of used teeth	Cultivation medium	result
1st	4	α-MEM + L-Glutamine + HS20%	Failed
2nd	4	α-MEM + L-Glutamine + HS20%	Failed
3rd	1	α-MEM + L-Glutamine + HS20%	Failed
4th	4	α-MEM + L-Glutamine + HS20%	Failed
5th	2	α-MEM + L-Glutamine + HS20%	Failed
6th	2	α-MEM + L-Glutamine + HS20%	Failed
7th	4	α-MEM + L-Glutamine + HS20%	Failed
8th	3	1/2α-MEM + L-Glutamine + HS20% and1/2α-MEM + L-Glutamine + FBS15%	½ failed and ½ successful
9th	4	α-MEM + L-Glutamine + KSR	Successful
10th	4	α-MEM + L-Glutamine + KSR + N2 + B27	Successful

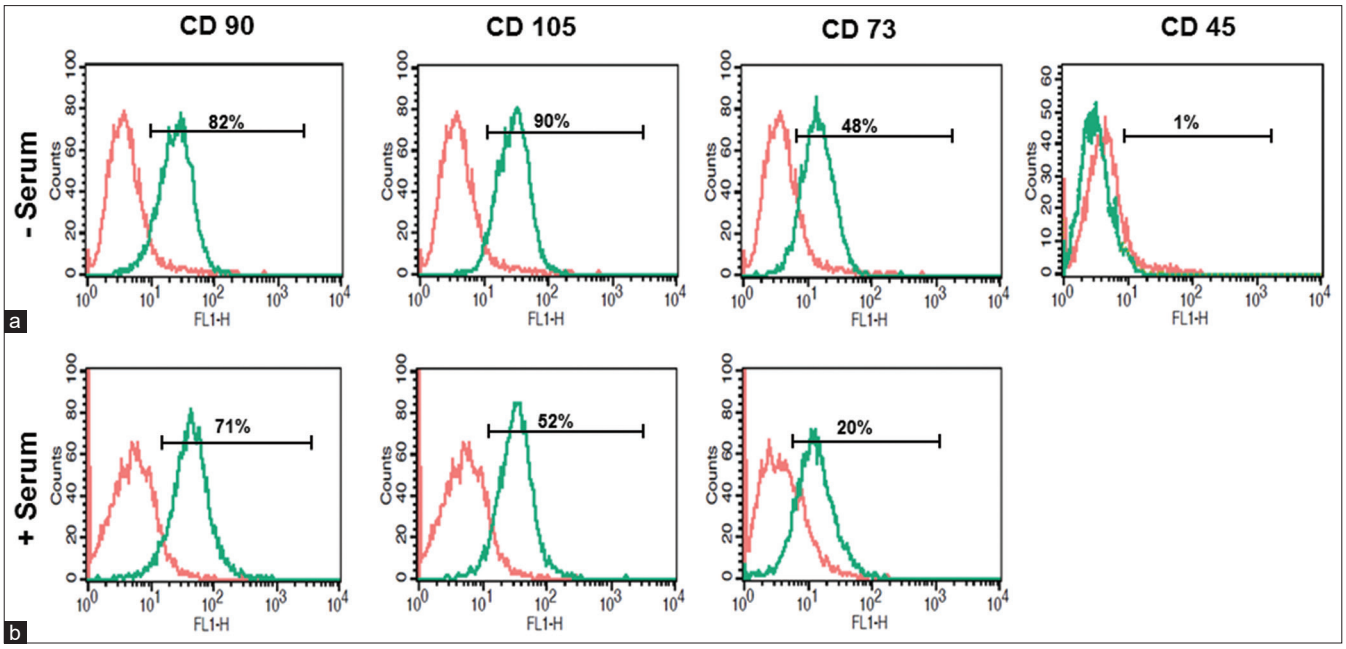

Figure 4: Representative histograms for flow cytometry analysis of the expression of mesenchymal stem cell (MSC) markers in cultured periodontal ligament stem cells. After five passages cells were positive for common MSC markers such as CD105 and CD90 and negative for the leukocyte antigen CD45 in both serum-free (a) and serum-containing (b) media. While CD73 showed an adequate expression in the absence of fetal bovine serum. The red lines represent isotype control Immunoglobulin G expression and green lines depict the marker expression.

Reverse transcription- polymerase chain reaction results

The results of reverse transcription subsequent PCR, compared the gene expression of the markers of cultured PDL cells, showed that the gene expression of the CD105, CD45, and CD90 markers was normal in both environments, but gene expression of CD73 marker was poor in cells grown in medium containing bovine serum [Figure 5]. β-actin was used as a housekeeping gene. PCR primer sequences are mentioned in Table 2.

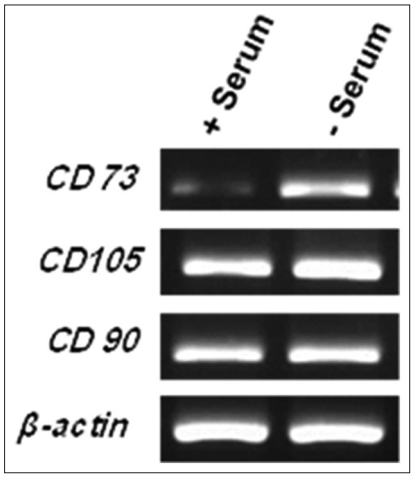

Figure 5: Gene expression in cultured periodontal ligament stem cells. Reverse transcription-polymerase chain reaction was applied to compare the expression levels of CD73, CD105, CD90, and CD45 between two culture conditions. Data revealed a normal expression of mesenchymal stem cell-specific genes by cultured periodontal ligament stem cells in serum-free medium. Although the expression of CD73 was week in + fetal bovine serum culture condition.

DISCUSSION

The PDLSCs are adult tissue-harvested MSCs and can only differentiate to a limited number of tissues. Methods to confirm the safety of these types of stem cells have been established by Washio *et al*. and Lalu *et al*., who have investigated the tumorigenicity of these cells and their guideline for the clinical use of dental stem cells has mentioned as having low cytotoxicity.[31,32] Moreover, articles which have compared the regeneration capacity of PDLSCs to the other MSC populations have shown remarkable properties compare to other MSCs such as greater regeneration capacity and incidence of fibroblastic colony-forming units than the reported for bone marrow and adult pulp stem cells[8,11,33,34] as in the present study the fibroblast-like cell reached 90% confluency in 10 days both in FBS-containing and FBS-free conditions which confirms the high quality of these stem cells. Access ability, high growth, and multipotential capacity of the hPDLSCs

Table 2: Polymerase chain reaction primer sequences

Gene	Accession number	Primer sequence
THY-1 (CD90)	NM_006288.4	F: 5`-CACCTCACCCTCTCCGCACA-3` R: 5`-CTGGCTTCCCTCTTCACGAAC-3`
Endoglin (CD105)	NM_000118.3	F: 5`-AATGAGGCGGTGGTCAATATCC-3` R: 5`-GGAAGTGTGGGCTGAGGTAG-3`
NT5E (CD73)	NM_002526	F: 5`-TCAAAGGACACGAGAGAAAGGAA-3` R: 5`-AAGAAAGAGGACAGAGGCAGAG-3`
B-actin	NM_001101.3	F: 5`-CGTGACATTAAGGAGAAGCTGTGC-3` R: 5`-CTCAGGAGGAGCAATGATCTTGAT-3`

have made them a demanded source for utilization in cell therapy. The first clinical dental stem cell studies pooled the harvested PDL or pulp tissue of several donors to be able to get enough stem cells for isolation,[12-17] while the mentioned potential characteristics and tissue morbidity decreasing reasons have motivated investigators to work on obtaining and rising stem cells from as much as a smaller number of teeth that is possible as Iwata *et al*. obtained autologous PDL stem cells same as our study[18] or Park *et al*. got appliable stem cells even from the inflamed PDL tissue hoping that the required tissue be harvested during periodontal treatment.[5]

One of the main aims of this work was the isolation and characterization of the PDLSCs from the limited PDL collected from one patient's wisdom teeth (2–4 teeth). Clinical setting to get enough stem cells from this limited resource can be more challenging, considering the bacterial concentration in the oral cavity that is a usual concern in PDLSCs harvesting, which threatens the samples and cell delivery strategies[9] as we lose the samples of the first–fifth patients because of it. Despite the vibration, increasing the volume of the transferring buffer and concentration of the antibiotics according to the primer culturing setup, under the microscopic observations report, the most bacterial contamination was cocci, which are considered the normal flora of saliva; therefore in a constant effort to decrease unwanted bacterial load a diluted CHX rinse (1/4 CHX 0.02% and — 3/4 sterile serum) was used immediately before and after the surgical incision and luxation of each tooth. All the teeth were held by crown all the time during tooth extraction. The surgeon changed his sterile gloves immediately before tooth extraction (due to possible contamination with saliva during the injection or surgery) and the extracted PDL tissue was transferred to the laboratory in an Alfa modification of Eagles medium. The sixth case (two teeth) was dropped because the cultivation dish was empty of any vital cells this time. Thus, the concentration of the CHX solution declined (1/5 CHX 0.02% and 4/5 sterile serum) and rinsing was performed just before the surgical incision and did not continue during the flap elevation or luxation of teeth to prevent the surface of the roots from being soaked with the CHX solution, so for the rest of the cases the bacterial contamination was reduced to a controllable level.

One of the major difficulties facing the clinical implementation of dental stem cells is the safety and high quality of the *ex vivo* cultured stem cells that use the autologous MSC sources to manage this issue to a large extent.

The first studies on the clinical use of dental stem cells manipulated the tissue of several patients or used autologous tissue but cultured them in the FBS serum, such as what Ji *et al*., d'Aquino *et al*., Behnia *et al*., Banavar SR *et al*., Sheth *et al*., Gan *et al*., Chen *et al*., Jhaveri *et al*., Piva *et al*. and Pini Prato *et al*. did between 2000 and 2017[12-17,21-25] while utilizing the stem cells in this way, puts regaining the periodontal structures at the risk of facing local allogenic and xenogeneic immune reactions during cell transplantation or has potential risk of infection from animals or other people. Using autologous stem cell resources and cultivation in the autologous human serum or synthetic FBS-free serum going to be preferred methods in more recent studies, and several countries have begun to establish setups for preventing allergic and foreign body reactions as Nakashima *et al*. used autologous cell and serum.[19] Xuan *et al*. used autologous cells too[20] and Piva *et al*.[24] used human serum; however, they pooled the tissue of several patients to get enough stem cells and all of these clinical studies are on pulp stem cells. Hernández-Monjaraz *et al*. used the FBS-free medium however they utilized allogenic pulp stem cells,[26] but in line with this goal, Iwata *et al*. performed a clinical study on autologous PDLSCs in autologous serum.[18] Trubiani *et al*.[27] have introduced a novel xenofree culture method and Tarle *et al*.[28] have reported a chemically defined serum-free medium same as our work which can provide the basis for useful manufacturing procedure of autologous PDLSC to facilitate the clinical using of stem cells. The seventh case failed because of the floating cells (available cells did not attach to the bottom of the cultivation dish). To check the efficacy of the study's protocol on the cultivation

of a small volume of PDLSCs, the eighth patient's sample (3 teeth) was divided into two parts. One part cultured in an FBS 15% contained medium and the other part in the same human serum as in prior cases. The cells cultured in the FBS-supplemented medium showed successful adhesion to the dish, but a lack of cell adhesion happened for the other part cells again. The probable reason can be that the FBS is more nutritious than the human serum, which results in better nutrition and proliferation of the limited number of PDLSCs used in this study. The processing procedure applied for the isolation of the ninth and tenth cases was the same as the previous ones although the FBS was replaced with KSR to enrich the medium and promote cell proliferation in clinical conditions. Indeed, N2 and B27 supplements were used to provide some essential factors required for MSCs' growth and expansion, such as Transferrin, Insulin, Progesterone, Sodium Selenite, Putrescine, Albumin, triiodo-I-thyronine (T3), Vitamin A, and Biotin.[34,35] All antibacterial/antimitotic components were also removed to eliminate any probable interfering effect of them on isolated cells. The successful cell adhesion occurred after 24 h, which was confirmed by the characterization, (flow cytometry analysis and PCR), established that this paper's protocol can be applicable for the isolation and characterization of hPDLSCs from the limited amount of tissue of each patient's wisdom teeth. Continued investigations are still needed to design new and more effective clinical strategies for stem cell-based therapies.

CONCLUSION

According to the limited number of sample tests in this pilot study, isolation and characterization of PDLSCs from the limited PDL tissue collected from one patient's wisdom teeth (2–4) may be possible by proper setup for preventing allergic and foreign-body reaction in a chemically defined serum-free medium which can provide the basis for useful manufacturing procedure of autologous PDLSCs to facilitate clinical application of stem cell.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial, in this article.

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