J Periodontal Implant Sci. 2022 Jun;52(3):242-257
https://doi.org/10.5051/jpis.2006080304
pISSN 2093-2278·eISSN 2093-2286

JPIS
Journal of Periodontal & Implant Science

Research Article
Implant Science

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Bio-hybrid dental implants prepared using stem cells with β-TCP-coated titanium and zirconia

Ihab Nabeel Safi [1], **Basima Mohammed Ali Hussein** [2,*], **Ahmed Majeed Al-Shammari** [3]

[1]Prosthetics Department, Collage of Dentistry, University of Baghdad, Baghdad, Iraq
[2]Institute of Laser for Postgraduate Studies, University of Baghdad, Baghdad, Iraq
[3]Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq

OPEN ACCESS

Received: Aug 28, 2020
Revised: Sep 7, 2021
Accepted: Oct 20, 2021
Published online: Jan 3, 2022

***Correspondence:**
Basima Mohammed Ali Hussein
Institute of Laser for Postgraduate Studies, University of Baghdad, Al Jadiria, Baghdad, Iraq.
Email: yasbasima@yahoo.com
Tel: +9647707998929

ORCID iDs
Ihab Nabeel Safi
https://orcid.org/0000-0002-1455-0439
Basima Mohammed Ali Hussein
https://orcid.org/0000-0001-7614-0710
Ahmed Majeed Al-Shammari
https://orcid.org/0000-0002-2699-1514

Author Contributions
Conceptualization: Ihab Nabeel Safi, Basima Mohammed Ali Hussein, Ahmed Majeed al-Shammari; Formal analysis: Ihab Nabeel Safi, Basima Mohammed Ali Hussein, Ahmed Majeed al-Shammari; Investigation: Ihab Nabeel Safi, Basima Mohammed Ali Hussein, Ahmed Majeed al-Shammari; Methodology: Ihab Nabeel Safi, Basima Mohammed Ali Hussein, Ahmed Majeed al-Shammari; Project adminidtration: Ihab Nabeel Safi, Basima

ABSTRACT

Purpose: This study investigated periodontal ligament (PDL) restoration in osseointegrated implants using stem cells.
Methods: Commercial pure titanium and zirconium oxide (zirconia) were coated with beta-tricalcium phosphate (β-TCP) using a long-pulse Nd:YAG laser (1,064 nm). Isolated bone marrow mesenchymal cells (BMMSCs) from rabbit tibia and femur, isolated PDL stem cells (PDLSCs) from the lower right incisor, and co-cultured BMMSCs and PDLSCs were tested for periostin markers using an immunofluorescent assay. Implants with 3D-engineered tissue were implanted into the lower right central incisors after extraction from rabbits. Forty implants (Ti or zirconia) were subdivided according to the duration of implantation (healing period: 45 or 90 days). Each subgroup (20 implants) was subdivided into 4 groups (without cells, PDLSC sheets, BMMSC sheets, and co-culture cell sheets). All groups underwent histological testing involving haematoxylin and eosin staining and immunohistochemistry, stereoscopic analysis to measure the PDL width, and field emission scanning electron microscopy (FESEM). The natural lower central incisors were used as controls.
Results: The BMMSCs co-cultured with PDLSCs generated a well-formed PDL tissue that exhibited positive periostin expression. Histological analysis showed that the implantation of coated (Ti and zirconia) dental implants without a cell sheet resulted in a well-osseointegrated implant at both healing intervals, which was confirmed with FESEM analysis and negative periostin expression. The mesenchymal tissue structured from PDLSCs only or co-cultured (BMMSCs and PDLSCs) could form a natural periodontal tissue with no significant difference between Ti and zirconia implants, consequently forming a biohybrid dental implant. Green fluorescence for periostin was clearly detected around the biohybrid implants after 45 and 90 days. FESEM showed the invasion of PDL-like fibres perpendicular to the cementum of the bio-hybrid implants.
Conclusions: β-TCP-coated (Ti and zirconia) implants generated periodontal tissue and formed biohybrid implants when mesenchymal-tissue-layered cell sheets were isolated from PDLSCs alone or co-cultured BMMSCs and PDLSCs.

Keywords: Dental implant; Guided tissue regeneration; Mesenchymal stem cell transplantation; Periodontal ligament; Tissue scaffolding; Titanium; Zirconium oxide (Zirconia)

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Mohammed Ali Hussein, Ahmed Majeed al-Shammari; Writing – original draft: Ihab Nabeel Safi, Basima Mohammed Ali Hussein, Ahmed Majeed al-Shammari; Writing – review& editing: Ihab Nabeel Safi, Basima Mohammed Ali Hussein, Ahmed Majeed al-Shammari.

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

INTRODUCTION

Dental implants are used to replace teeth that are extracted or have been lost for various reasons. Many materials have been used to construct dental implants. Titanium has been widely used because of its biocompatibility, lack of cell toxicity, and weak inflammatory response in peri-implant tissues [1]. Among the disadvantages of titanium are its dark greyish colour, cellular sensitisation, and the galvanic side effect that is evident upon its contact with saliva or fluoride [2]. Yttria-stabilised zirconia, an oxide ceramic, became known as a biomaterial only a few decades ago. Yttria-stabilised zirconia has excellent mechanical properties and exhibits a good aesthetic quality. Zirconia appliances are highly suitable for constructing dental implant substrates. However, zirconia is a chemically inert material that cannot bind to bones [3].

Laser coating is a procedure wherein a laser beam is used to apply Ca/P powder upon a substrate. This type of modification is usually used to improve the performance of the material. Unlike conventional deposition techniques, laser melting processes can produce coatings with excellent mechanical properties and bonding. Laser coating is advantageous because it is used to manage and control the shape and size of the coat layer or particles and helps reduce the pre-surface preparation of the substrate and the requirements essential in other coating techniques [4].

The periodontal ligament (PDL) connects natural teeth to the surrounding tissues for efficient biological function [5]. Dental implants are fixed via osseointegration, closely resemble natural teeth, and have a high success rate [6]. However, some fundamental vulnerabilities are associated with osseointegration because of the mismatch between the living structure and the implant fixture in mechanical and physical properties, such as elastic modulus, resiliency, and response to functional loading. Furthermore, due to the absence of connective tissue via the PDL and the cementum, significant differences exist in the adaptation of the implant to occlusal forces as a result of biomechanical problems, affecting the healing mechanism between the implant fixture and the bone [7]. These issues are attributed to the absence of periodontal sensory mechanisms in the dental implant, including pain perception and proprioception, and undermining bone resorption. The focus of implant dentistry has changed from merely achieving osseointegration to the preservation and prevention of peri-implant hard- and soft-tissue losses. Consequently, implants with PDLs have been conceptualised and developed.

Tissue engineering (stem cells, scaffold, and growth factors) aims to reconstruct natural tissues. Therefore, a new approach to the replacement of tooth loss has been introduced (i.e., tissue engineering of the PDL). In this approach, tissue-engineered PDL cells are formed on the dental implants, thereby acting as a natural tooth. This new dimension in the field of implant dentistry is known as ligaplants [8]. The PDL contains undifferentiated mesenchymal cells (multipotent stem cells) that can differentiate into many mesenchymal lineages [9]. Mesenchymal stem cells (MSCs) isolated from the PDL are named PDL stem cells (PDLSCs): these cells were found to generate specific attachments of the tooth PDL-like complex in mice [10]. A plethora of technical problems have been identified in the isolation of PDLSCs, including the risk of contamination, tumorigenesis, and the vulnerability of stem cells' condition [11]. In addition, primary cultures of PDLSCs are insufficient to generate a cell sheet for PDL cells [12]. Allogenic PDLSC transplantation is the key to overcoming the limitations of autologous PDLSC transplantation [13]. Bone marrow mesenchymal cells

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(BMMSCs) can differentiate into many cell types. These stem cells have been successfully utilised for *in vitro* and *in vivo* studies, driving their use in clinical trials and theraputical associated studies [14]. Complete periodontal regeneration is performed by combining stem cells and scaffolding (tissue engineering) [15,16]. The determination of a suitable material for a bio-hybrid PDL-implant is an important topic for future research because titanium may not always be the best choice [17].

This study used laser coating to fabricate a beta-tricalcium phosphate (β-TCP) layer (bioactive ceramic) to construct a biohybrid dental implant from commercially pure titanium grade 1 or zirconia (bioinert ceramic) in a novel structure composed primarily of BMSCs and PDLSCs. We also compared these 2 materials in terms of their ability to stimulate the formation of PDL components and their interactions with the surrounding living jawbone of a rabbit. The biohybrid implants (titanium and zirconia) were evaluated with the structure of a natural tooth and its supporting tissues.

MATERIALS AND METHODS

This study was approved by the College of Dentistry of the University of Baghdad. Healthy New Zealand White rabbits between 10 and 12 months old and 2–2.5 kg of weight, with no clinically evident diseases, were selected for the experiments. The rabbits were housed under standard conditions, with a controlled temperature (23°C–25°C), a 12-hour light/12-hour dark cycle under artificial illumination, and food and drink (pellets with protein, jet and carrot) provision. All the animals were weighed to calculate the dose of the anaesthesia and antibiotics to be administered during surgery.

Selection of implant size

Cone-beam computed tomography (CBCT)

The facial anatomy and amount of bone jaws was evaluated using CBCT (ProMax, 3D Implant Planning Module, Planmeca, Helsinki, Finland). Under general anaesthesia, two 3-dimensional (3D) images were taken: one before and the other after the extraction of the lower right central incisor. The rabbit was tied in an upright position to the tray of the device, which was set at 90 KW, 12.5 mA, and 12 s, as shown in **Figure 1A**.

Implant preparation

A cylindrical form (measuring 2.5 mm in diameter and 6 mm in length) was prepared from commercially pure Ti grade 1 rods of 99.9% purity (Baoji Jinsheng Metal Material, Baoji, China). The size was set according to the CBCT radiography results. With silica carbide paper, all specimens were ground and polished and then cleaned ultrasonically in ethanol and dried for half an hour. To remove the oxide film, a 100 mL etch solution (containing 6 mL of nitric acid, 2 mL of hydrofluoric acid, and 92 mL of distilled water) was used for 2 minutes. Then, the specimens were rinsed with acetone before coating [18]. The same measurements were used to prepare zirconia specimens using partially sintered zirconia (VITA YZ, Zahinfabrik, Spitalgasse 3, D-79713 Bad Sackingen, Germany). The Exocad dental CAD software was used to design the zirconia substrates, which were prepared using a dental digital 3-axis milling system (CAD/CAM, CORiTEC 250i; imes-icore, Eiterfeld, Germany). The zirconia substrates were finally sintered in a sintering furnace at a temperature up to 1,650°C (VITA, ZYRCOMAT 6000 imes-icore, Eiterfeld, Germany) according to the manufacturer's instructions. Before coating, the zirconia substrates were cleaned ultrasonically in absolute ethanol for 30

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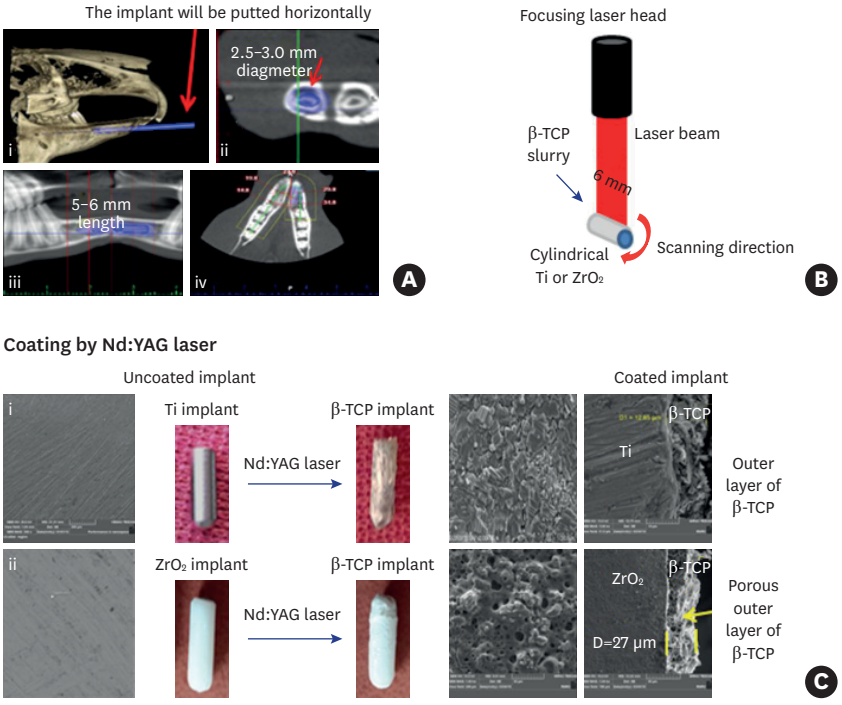

Figure 1. (A) Cone-beam computed tomographic images assessing the required size of the implant when it will be placed horizontally, i) 3-dimensional X-ray lateral view, ii) cross-sectional view, iii) lateral view, iv) occlusal view (B) Schematic drawing of the laser coating process for cylindrical implants, (C) FESEM images of the topography and cross-section substrate i) at the Ti-β-TCP interface area, ii) at the ZrO_2-β-TCP interface.
β-TCP: beta-tricalcium phosphate.

minutes and then air-dried. A pulsed Nd:YAG laser of 1064 nm wavelength (Han's Laser, Shenzhen, China) was used to coat the substrates with β-TCP (≥98% β-phase basis, 49963; Sigma Aldrich, St. Louis, MO, USA). The laser coating parameters and the procedure used for coating the titanium substrates have been described elsewhere [19], as have the laser parameters for coating the zirconia substrates [20]. Cylindrical dental (titanium and zirconia) substrates were moved in a rotary motion during the coating process through a especially manufactured device to fix the samples during laser application, as shown in **Figure 1B**. The Mach3Mill CNC controller software was used to control the scanning speed. The titanium and zirconia implants with β-TCP coating were examined by field emission scanning electron microscopy (FESEM) (MIRA3 TESCAN, Brno, Czech Republic), as shown in **Figure 1C**.

Isolation of stem cells

BMMSCs were harvested from rabbit tibia and femur, and the PDLSC culture was collected from the freshly extracted lower incisor. The cells were co-cultured for 21 days to induce BMMSC differentiation in the PDL cells, and the methods of isolation followed those described elsewhere Minimum essential medium was used, containing 20% foetal bovine serum, FBS 100 μg/mL ascorbic acid (US Biological, Salem, MA, USA) [22,23], 12.5 μg/mL fibroblast growth factor (US Biological), 100 μg/mL streptomycin, 100 U/mL ampicillin, and 100 μg/mL amphotericin B (Capricorn Scientific, Ebsdorfergrund, Germany) [21,23].

Cell sheet engineering

Temperature-responsive culture dishes (Nunc UpCell surface; Sigma-Aldrich, Z688851-30EA Japan) were used with the supplied membrane and collagen graft (CSD2030, GENOSS,

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Suwon, Korea) to harvest cell monolayers to generate 3D tissue models. The methods of forming the constructs, including collagen graft and 3-layered cell sheets, are described in a previous study [21]. A histological assessment of the sheets (the cross-section of the construct and the adhesion) was performed. The topography was assessed using FESEM. Then, the 3 layers with collagen were cut (approximately 9 mm wide × 7 mm long) and transplanted onto the coated (Ti and zirconia) implants to cover the implant instantly before implantation. A collagen graft was used to wrap the implant so that it came in direct contact with the alveolar bone at the outer side and with the co-culture layered-cell sheet that faced the implant. The same technique was used to prepare both BMMSC- and PDLSC-layered cell sheets, as shown in **Figure 2A and B**.

Transplantation

Sample grouping

According to the type of implant materials, 80 specimens were divided into 2 groups (Ti and zirconia). Each group was subdivided into 2 subgroups according to the healing period (either 45 or 90 days). Afterwards, each subgroup was subdivided into 4 groups according to the types of layered cell sheet stem cells. All these groups were evaluated using histological tests. The natural lower central incisors were used as controls.

Tooth extraction

Male New Zealand White healthy rabbits weighing 2–2.5 kg were used. Six weeks before the implant procedure, their right lower central incisors were extracted as described elsewhere [21]. The left lower central incisors were maintained for mastication [13]. The implant procedure was completed when the alveolar bone healed.

Implant procedure

A combination of ketamine at 10% (0.5 mL/kg; Kepro, Deventer, The Netherlands) and xylazine at 20% (0.5 mL/kg; Kepro) was used as a general anaesthetic solution. Lidocaine (at 2%, 1/80,000 adrenaline; Septodont, Saint-Maur-des-Fossés, France) was used for local anaesthesia. In the lower rabbit jaw, at the lower central incisor area, the socket was prepared using sequentially larger burs from 2 mm until the desired size and depth of 3 mm × 6 mm (diameter and length) were achieved. The drilling procedure was performed under continuous irrigation using a special kit (Mini Implants; Easy Implant, Chavanod, France) In the biohybrid implant, either Ti or zirconia was transplanted into the prepared site using small mosquito forceps. Over-pushing should be avoided because of the relative brittleness of the rabbit mandible and the location of the implant near the midline. The soft tissue was sutured using threaded absorbable synthetic polyglycolic 4/0 suture (Dtec, Kesteren, The Netherlands). After completing the surgical procedure, the animals were injected with 0.5 mL/kg of penicillin as an antibiotic (Kepro) for 3 days, and a dental care solution (chlorhexidine digluconate Trixie, Tarp, Germany) was added to the drinking water one day before extraction and five days after extraction. The animals were placed on soft diet containing vitamins and proteins. CBCT was used for post-implant assessment, as shown in **Figure 2C**.

Histological evaluation

Stereoscopic analysis

After 45 and 90 days, bone blocks containing the implant and the adjacent tooth were cut off using a disc and prosthetic engine at low speed with continuous cooling. The bone blocks were horizontally placed in a mould, filled with acrylic resin, and then mounted and sliced with MecatomeT210 (Presi, Eybens, France). The bone blocks were cooled and lubricated

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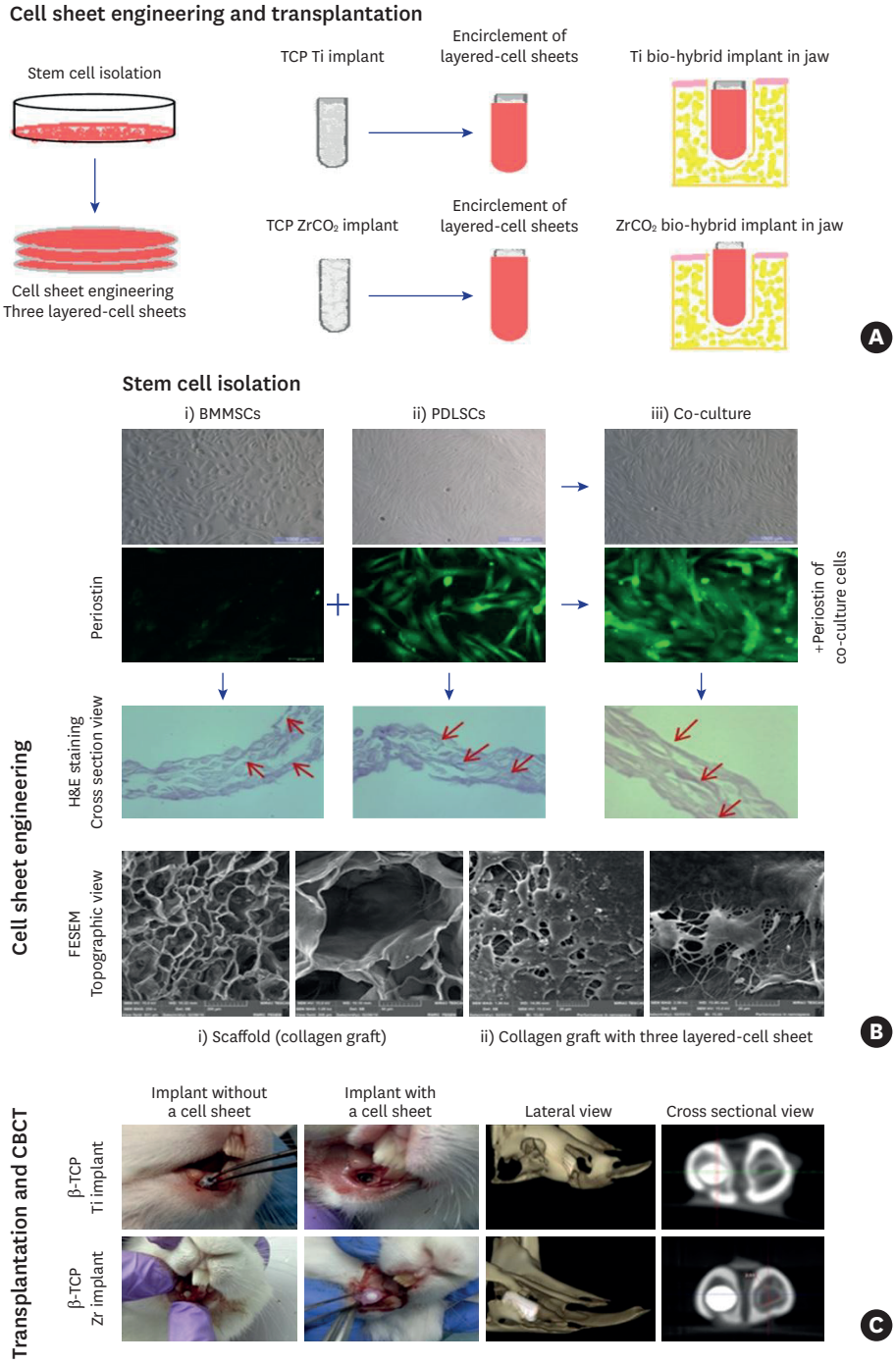

Figure 2. (A) Schematic drawing of the cell sheet engineering and transplantation process. (B) Inverted microscope images i) BMMSCs, ii) PDLSCs, iii) and co-culture with their fluorescence microscopic images at ×20, showing positive periostin expression in the PDLSCs and co-cultured cells (expression show in green), Photomicrographs of H&E-stained cross-sections, three layered-cell sheets at ×40, and FESEM images: i) topographic view of the scaffold (collagen graft) at ×250 and ×1,000, respectively; ii) collagen graft with 3-layered cell sheets in a magnified view showed stable adhesion between the cells and the sheets; (C) transplantation and CBCT images after implants insertion; i) titanium with and without a cell-layered sheet in position, ii) zirconia with and without a cell-layered sheet in position.
BMMSCs: bone marrow mesenchymal cells, PDLSCs: periodontal ligament stem cells, H&E: haematoxylin and eosin, FESEM: field emission scanning electron microscopy, CBCT: cone-beam computed tomography.

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during the cutting process by the self-contained internal system. The samples were examined using a sterioscopic microscope (Olympus, Tokyo, Japan). For each implant, the average of 4 readings was taken. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The equality of the means was tested using the t-test and analysis of variance.

Haematoxylin and eosin staining

Some blocks were prepared for haematoxylin and eosin (H&E) staining. Tissue sections were fixed immediately in 10% freshly prepared formalin and decalcified. After complete decalcification, the implants were withdrawn gently from the bone blocks. Then, the blocks of tissue were washed with tap water for 30 minutes. The bone blocks were then gradually dehydrated in a series of alcohol concentrations (40%, 60%, 80%, 95%, and absolute alcohol). Finally, the bone blocks were processed in 2 jars of xylene for 30 minutes. The specimens were moulded in paraffin (Bemis, Neenah, WI, USA). The blocks were sectioned into 4-μm-thick semi-serial cross-horizontal sections using a microtome and mounted on glass slides for routine H&E staining. The slides were examined under a light microscope for histological study [24].

Immunohistochemistry (immunofluorescence)

The blocks were sectioned (4 μm) and mounted on positively charged microscope slides (Leica, Wetzlar, Germany) for immunohistochemistry. The slides were placed in xylene, and three changes of 5 minutes each were conducted. Gradually, the sections were hydrated using graded alcohol solutions. The sections were washed in ethanol twice for 15 minutes each and 90% ethanol twice for 15 minutes each time. The slides were then washed in deionised water for 1 min with stirring. The slides were placed in a sodium citrate buffer at a pH of 6 and heated for 15 minutes at 95°C. Then, the slides were allowed to cool in the buffer for 20 minutes. The slides were washed 3 times with phosphate-buffered saline (PBS) (Chemical Point, Oberhaching, Germany) for 3 minutes to eliminate the remaining antigen retrieval solution and neutralise the acidity. Excess liquid was aspirated from the slides at this step using a pap pen (Daido Sangyo, Kawasaki, Japan), and a circle was drawn on the slide around the sections. The slides were incubated with a blocking reagent for 1 hour and then washed 3 times with PBS for 5 minutes. Subsequently, the cells were stained with periostin antibodies (1:30, Santa Cruz Antibodies, Dallas, TX, USA) and incubated for 2 hours at room temperature in a dark chamber. The cells were washed 3 times with PBS for 5 minutes.

The coverslips were fixed on the microscope slide using 50% PBS with 50% glycerine. Then, the slide was examined in the darkroom using a fluorescence microscope (Leica).

FESEM

FESEM was used to evaluate the PDL connection between the bone and the implant surface. After the specimens were horizontally cross-sectioned as described in section "Stereoscopic analysis," they were prepared via a special method for examination and fixed with PBS with 4% glutaraldehyde at 4°C for 24 hours. The specimens were washed with PBS 3 times for 10 minutes at room temperature. The specimens were postfixed with 2% osmium tetroxide solution (OSO_4) for 2 hours. Again, the specimens were washed 3 times with PBS at room temperature for 10 minutes. Then, the specimens were dehydrated in absolute ethanol with different concentrations (50%, 70%, 90% and 100%) for 10 minutes [25].

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RESULTS

Cell sheet engineering

The photomicrographs of the H&E-stained cross-sections showed the results of the engineered cell sheet with 3 layers, constituting a 3D construct (cell sheets), and a stable adhesion between them (**Figure 2**). At high magnifications, FESEM of the cells showed stable adhesion to one another with good adhesion between the sheets (**Figure 2B**).

Histological H&E staining

The transplantation of coated (Ti or zirconia) dental implants without a cell sheet stimulated the formation of a new bone, as shown in **Figure 3B**.

The mesenchymal-tissue-layered cell sheets, whether structured from PDLSCs alone or co-cultured BMMSCs and PDLSCs, formed natural periodontal-like tissues at different intervals (45 and 90 days), as shown in **Figure 3C**.

The mesenchymal-tissue-layered cell sheet isolated from the BMMSCs failed to form complete PDL-like fibres with the bone, as shown in **Figure 3E**.

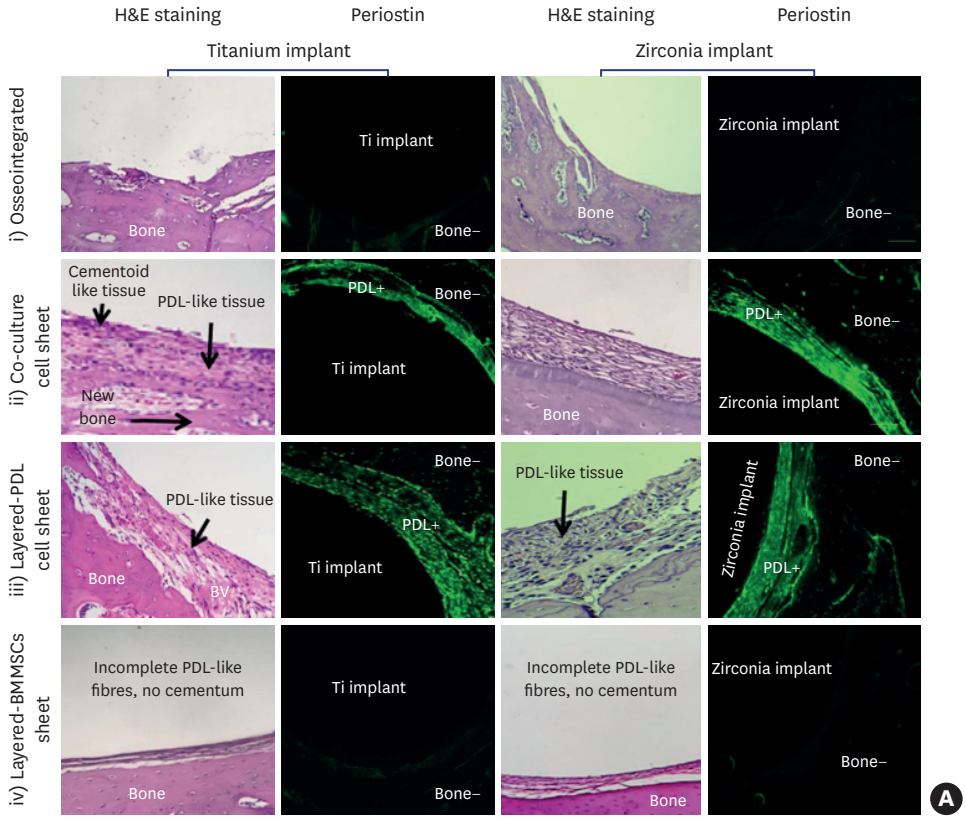

Figure 3. (A) H&E staining and immunohistological analysis of periostin in section of implants with different types of layered-cell sheet stem cells that coated the implant before transplantation. (i) (Ti, ZrO_2) implants without layered cell sheets, (ii) biohybrid (Ti and ZrO_2) implants encircled with co-cultured cell sheets, (iii) biohybrid (Ti and ZrO_2) implants with layered PDLSC sheets, (iv) Ti and ZrO_2 implants encircled with layered BMMSC sheets. At 45 days post-transplantation. (B) H&E staining and immunohistological analysis of periostin in sections of implants with different types of layered-cell sheet stem cells that coated the implant before transplantation in comparison to the natural tooth. (i) Natural tooth, (ii) osseointegrated (Ti, ZrO_2) implants without layered cell sheets, (iii) biohybrid (Ti and ZrO_2) implants encircled with co-cultured cell sheets containing BV, (iv) biohybrid (Ti and ZrO_2) implants with layered PDLSC sheets containing BV, (v) Ti and ZrO_2 implants encircled with layered BM-MSCs sheets. At 90 days post-transplantation.
H&E: haematoxylin and eosin, BMMSCs: bone marrow mesenchymal cells, PDLSCs: periodontal ligament stem cells, BV: blood vessel.

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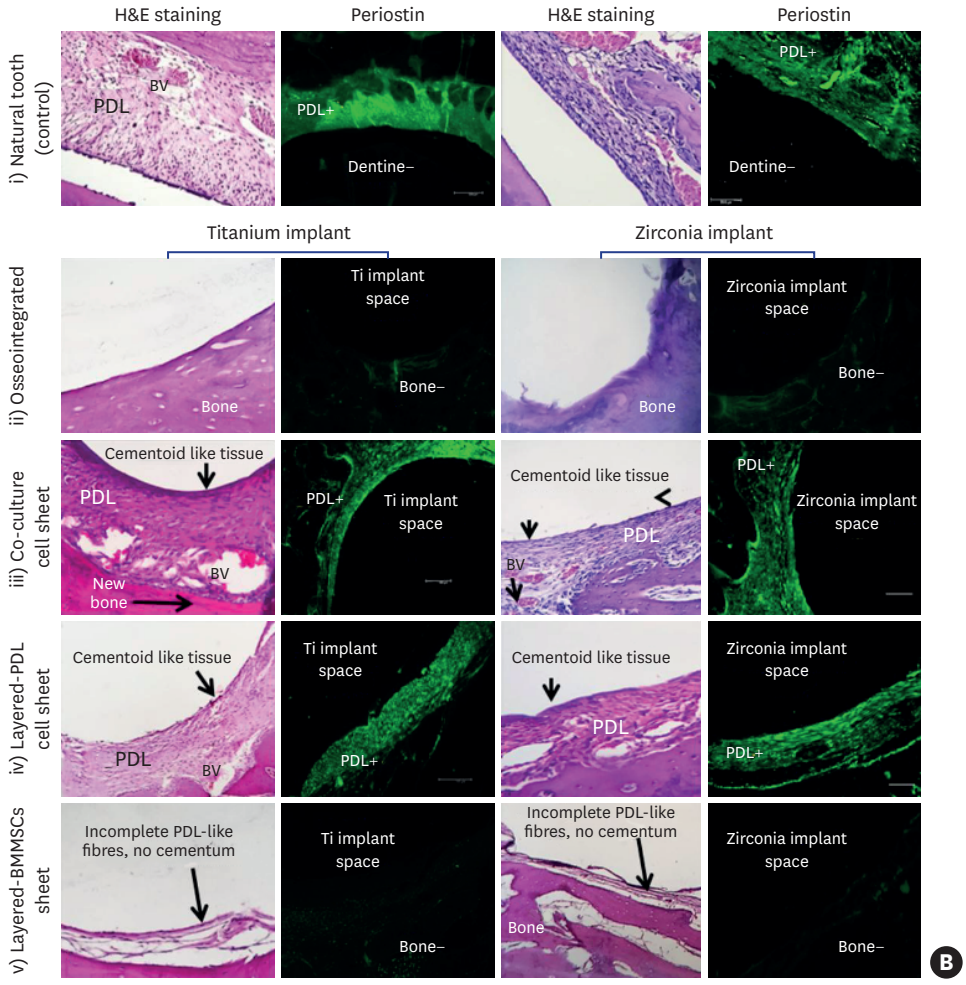

Figure 3. (Continued) (A) H&E staining and immunohistological analysis of periostin in section of implants with different types of layered-cell sheet stem cells that coated the implant before transplantation. (i) (Ti, ZrO_2) implants without layered cell sheets, (ii) biohybrid (Ti and ZrO_2) implants encircled with co-cultured cell sheets, (iii) biohybrid (Ti and ZrO_2) implants with layered PDLSC sheets, (iv) Ti and ZrO_2 implants encircled with layered BMMSC sheets. At 45 days post-transplantation. (B) H&E staining and immunohistological analysis of periostin in sections of implants with different types of layered-cell sheet stem cells that coated the implant before transplantation in comparison to the natural tooth. (i) Natural tooth, (ii) osseointegrated (Ti, ZrO_2) implants without layered cell sheets, (iii) biohybrid (Ti and ZrO_2) implants encircled with co-cultured cell sheets containing BV, (iv) biohybrid (Ti and ZrO_2) implants with layered PDLSC sheets containing BV, (v) Ti and ZrO_2 implants encircled with layered BM-MSCs sheets. At 90 days post-transplantation.
H&E: haematoxylin and eosin, BMMSCs: bone marrow mesenchymal cells, PDLSCs: periodontal ligament stem cells, BV: blood vessel.

Immunohistochemistry

In the natural teeth, positive findings for periostin expression (green fluorescence) were observed in the PDL of the natural lower incisor. In addition, negative expression was found in the dentin, enamel, dental pulp, alveolar bone and cementum, as shown in **Figure 3A**.

In the implants, negative findings for periostin expression were observed for the bone in the osseointegrated implants (**Figure 3B**), whereas using a layered co-culture cell sheet produced positive expression, as shown in **Figure 3C**. Furthermore, a layered-PDL cell sheet produced positive expression around the Ti and zirconia biohybrid implants at 45 and 90 days after transplantation, as shown in **Figure 3D**. However, the mesenchymal-tissue-layered cell sheets isolated from the BMMSCs produced negative results, as shown in **Figure 3E**.

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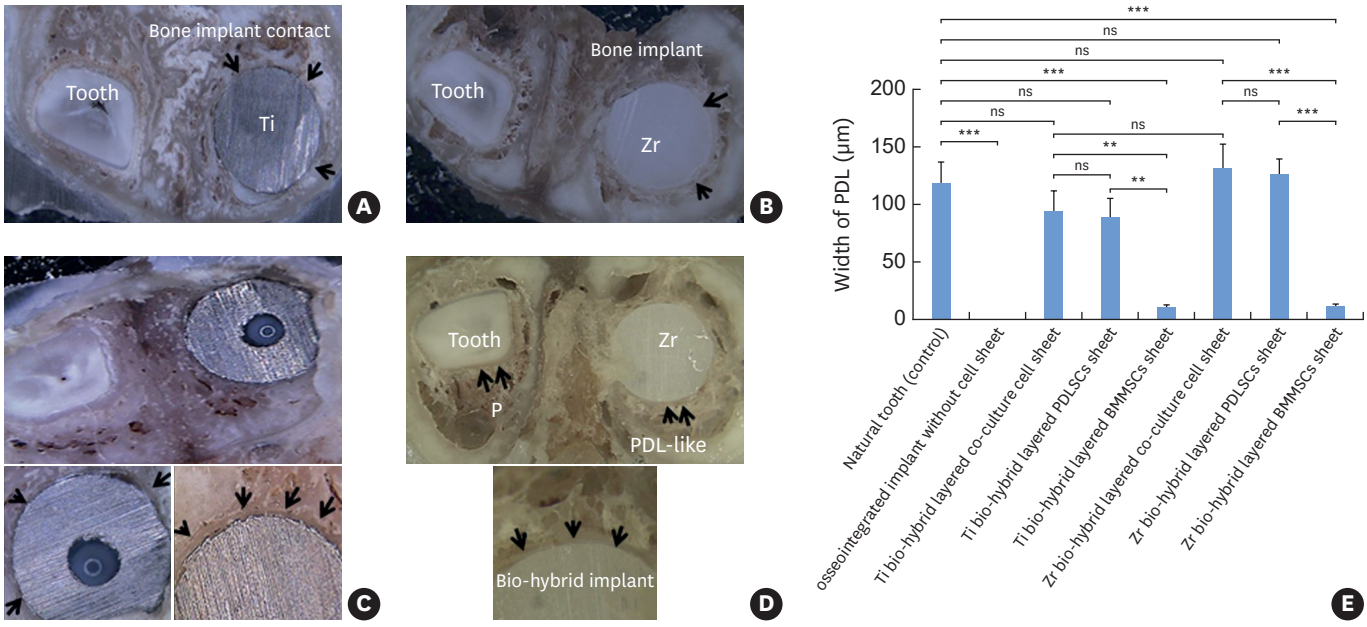

Figure 4. Histological analysis: stereoscopic images, cross-sections of the lower rabbit jaw (×10). (A) Section of the osseointegrated Ti implant at 90 days post-transplantation (×60). (B) Section of the osseointegrated coated zirconia implant at 90 days post-transplantation (×25, ×60). (C) Section of the bio-hybrid Ti implant at 90 days post-transplantation (×10, ×25, ×60). (D) Section of the bio-hybrid zirconia implant at 90 days post-transplantation (×10, ×60). (E) Chart comparing different groups in term of measurements of the width of the periodontal area (GraphPad Prism 7), using 5 samples from each group.
ns: not significant.
**Highly significant, ***Very highly significant.

Stereoscopic analysis to measure the width of the PDL

The PDL width of the Ti biohybrid implants was slightly lower than that the natural teeth, with a statistically non-significant difference, whereas the PDL width of the zirconia biohybrid implants was slightly higher than that of the natural teeth, again with a statistically non-significant difference. A statistically non-significant difference in PDL width existed between the Ti and zirconia biohybrid implants (**Figure 4**).

FESEM

FESEM analysis revealed that the osseointegrated (Ti and zirconia) implants were connected to the surrounding bone. The energy- dispersive X-ray spectroscopy (EDX) compositional line scan data of the implant–bone interface at different areas showed the element composition of the implant and the bone, as shown in **Figures 5A, B**, and **6**.

The FESEM data revealed that PDL-like fibres tightly filled the space between the implant socket and the implant. The PDL-like fibres were connected to the implant surface when the layered co-culture cell sheet was used, as shown in **Figures 5C, D**, and **6**.

DISCUSSION

Bioactive coatings (CaP) were used to promote cementum deposition around implants and to improve the stability of dental implants [7]. Sintered β-TCP powder was used in this study to produce a bioactive coat that could withstand the laser effect without changes in the Ca:P ratio (1.5), which is close to that in the natural bone (approximately 1.5–1.65) depending on its location. The same finding has been described in other studies [26,27] β-TCP has more

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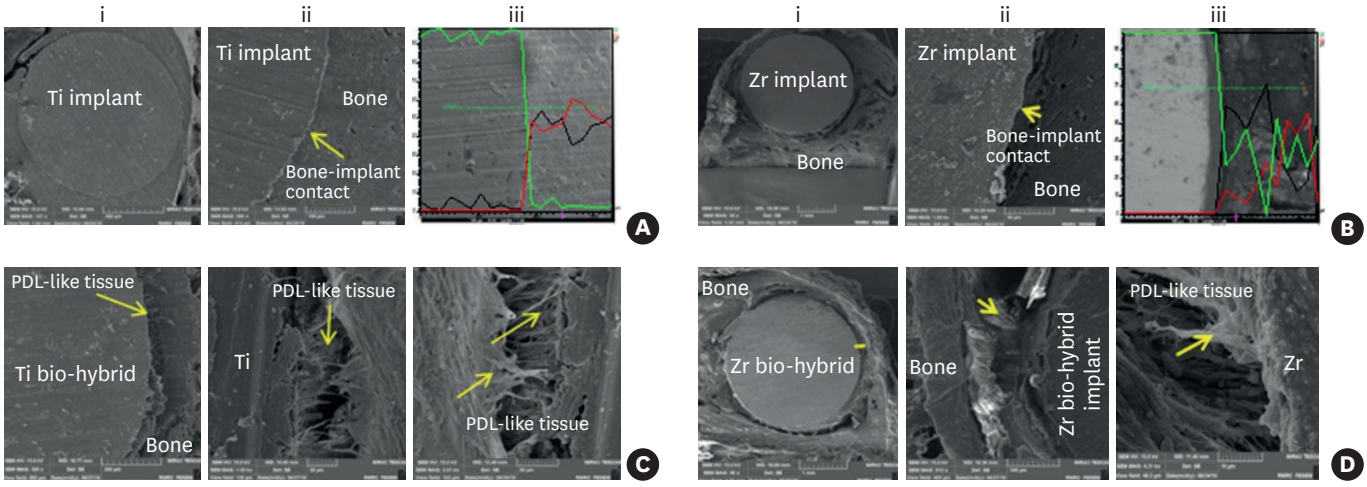

Figure 5. FESEM images. (A) (i, ii) Cross-sections of the lower rabbit jaw showing the osseointegrated Ti implant at 90 days post-transplantation (×100, ×500). (iii) EDX compositional line scan data of the implant-bone interface at different selected area interface. (B) (i, ii) Cross-sections of the lower rabbit jaw showing the osseointegrated zirconia implant at 90 days post-transplantation (×38, ×1,000), (iii) EDX compositional line scan data of the implant-bone interface at different selected area interfaces. (C) Cross-section of the lower rabbit jaw showing the Ti biohybrid implant at 90 days post-transplantation at different magnification, using a layered co-culture cell sheet. (D) Cross-section of the lower rabbit jaw showing the zirconia biohybrid implant at 90 days post-transplantation at different magnifications, using a layered co-culture cell sheet.
PDL: periodontal ligament, EDX: energy-dispersive X-ray spectroscopy.

advantages than α-TCP [28]. Laser coating makes it possible to control the process parameters, such as managing the size and shape of the coating layer and reducing the requirements for the substrate pre-surface preparation. This finding is consistent with those of previous studies [19,20,29], and this aspect of laser coating prevents unwanted problems within living organisms on the coat debris. The same findings have been described elsewhere [30,31].

A construct was fabricated successfully through temperature-responsive culture. This method results in superior transplantation efficiency and higher cell density than that of cells isolated through an enzymatic method. A similar finding is described by Iwata et al. [32]. FESEM was performed to examine the cross-section of the scaffold (collagen graft), indicating a porous (collagen graft) scaffold that supports cell proliferation, penetration, and attachment for the tissue-like construct. The scaffold architecture can be used as a guide for perpendicular insertion into the newly formed bone. The engineered tissues were composed of 3-layer cell sheets.

The transplantation of coated (Ti or zirconia) dental implants without a cell sheet stimulated new bone formation (well-developed bone) around them. Active osteoblasts indicated osseointegrated implants both healing intervals (45 and 90 days), as shown in **Figure 3**. This finding can be attributed to the chemical bonding of β-TCP to the bone through the exchange of ions that stimulated the differentiation of osteogenic cells and led to the formation of new bone *in vivo*. These results are consistent with the results of other researchers [33-35].

Mesenchymal-tissue-layered cell sheets, whether structured from PDLSCs alone or co-cultured BMMSCs and PDLSCs, could form natural periodontal-like tissue, including PDL, cementum, and alveolar bone, on the Ti and zirconia-coated dental implants (biohybrid implants) at different intervals (45 and 90 days), as shown in **Figure 3C**. This phenomenon occurred because the scaffold (collagen graft) with PDLSCs in the co-culture around the implant in the site acted as a specialised region or microenvironment (niche) with special

https://doi.org/10.5051/jpis.2006080304

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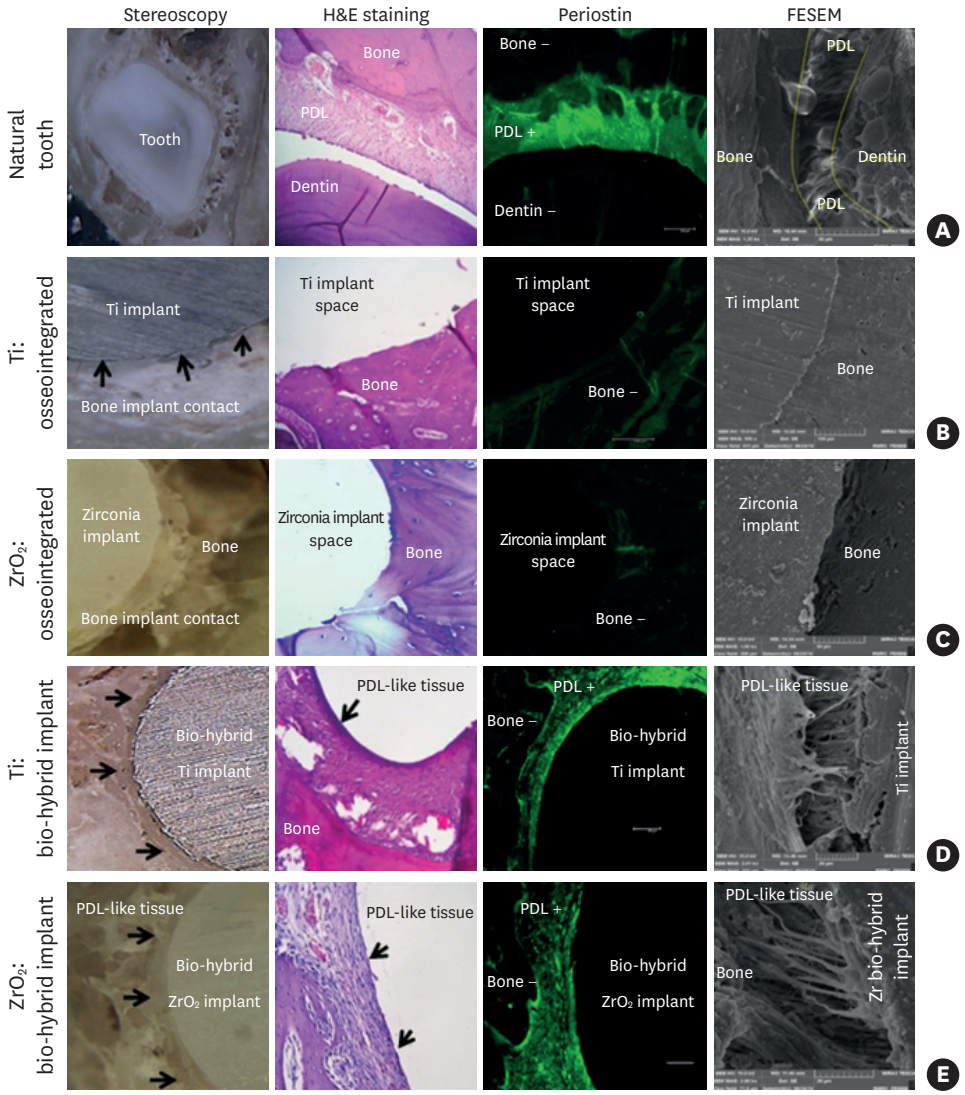

Figure 6. Stereoscopic analysis, H&E staining, immunohistological analysis for periostin, and FESEM images for cross-sections of implants that were enveloped with layered co-cultured cell sheets at 90 days post-transplantation in comparison to natural tooth. (A) Natural tooth, (B) osseointegrated Ti implants without layered cell sheets, (C) osseointegrated zirconia implants without layered cell sheets, (D) biohybrid Ti implants with layered co-cultured cell sheets, (E) biohybrid zirconia implants with layered co-cultured cell sheets.
H&E: haematoxylin and eosin, FESEM: field emission scanning electron microscopy.

properties that regulated the BMMSCs' quiescence, survival, and differentiation into the PDL cells. PDLSCs secrete soluble factors in niches, which serve as signals for BMMSC differentiation. Moreover, the extracellular matrix proteins of PDLSCs (periostin) provide a scaffold for BMMSCs to attach, and the blood vessels provide nutrition. The relationships between the niches and the stem cells are dynamic. Any alteration in the niche results in the stimulation of stem cell proliferation, mobilisation, or retention, resulting in PDL-like fibres of biohybrid implants composed of longitudinal and transverse fibres that are equivalent to the natural incisor teeth. The magnified images show a cementoid-like tissue that developed on the Ti and zirconia implants and the PDL space, which was filled with periodontal-tissue-like fibres and blood vessels between the implant and the newly formed bone. A similar result was described by Kaoru et al. [36].

https://doi.org/10.5051/jpis.2006080304

The mesenchymal-tissue-layered cell sheet isolated from the BMMSCs failed to form complete PDL-like fibres with the bone, and no cementum was found around the β-TCP-coated (Ti and zirconia) dental implants after transplantation, as shown in **Figure 3E**. This result might be attributed to the random differentiation of the BMMSCs to osteoblasts any other cell types on the borders with available ideal microenvironment for osteoblast differentiation, and the loss of guidance (microenvironment) for differentiation to PDL-like fibres [37]. This result is the opposite of that obtained by other researchers [38], who found that the undifferentiated MSCs of goat BMMSCs could differentiate into PDLs.

Histopathological analysis showed that the PDL-like tissue showed an increase in proliferation and cellularity at 90 days compared with the growth at 45 days. In this study, a histological analysis was conducted after 3 months to observe the PDL-like tissue's proliferation. The developed PDL remained present without any loss, deflation, or teratoma.

To confirm whether the tissues between the alveolar bone and the implant were PDLs that originated from the mesenchymal-tissue-layered cell sheets, periostin protein detection was performed. Periostin is secreted from the PDLs. For the natural tooth, periostin showed positive expression (green fluorescence) in the PDL of the natural lower incisor tooth that was used as a control. The result shows that periostin was clearly expressed in the PDLs rather than in other tissues. This finding is consistent with that of Yamada et al. [39]. In addition, no positive reaction (negative expression) was observed in the dentin, enamel, dental pulp, alveolar bone, or cementum. This result is consistent with that reported by Afanador et al. [40], as shown in **Figure 3A**. For the implants, periostin produced negative expression for the bone in the osseointegrated implant (**Figure 3B**), whereas green periostin fluorescence was detected clearly around the Ti and zirconia biohybrid implants at 45 and 90 days after transplantation using a layered co-culture cell sheet, as shown in **Figure 3C**. Furthermore, green periostin fluorescence was detected clearly around the Ti and zirconia biohybrid implants at 45 and 90 days after transplantation using a layered PDL cell sheet, as shown in **Figure 3D**. This finding agrees with that obtained by other researchers [41], who provided evidence that periostin plays an important role in the regeneration of the PDL after periodontal surgery.

β-TCP-coated (Ti and zirconia) implants could generate periodontal tissue and form biohybrid implants with the development of artificial PDL when using mesenchymal-tissue-layered cell sheets isolated from PDLSCs alone or co-cultured BMMSCs and PDLSCs. This result is in agreement with those of previous studies [39,41]. The mesenchymal-tissue-layered cell sheets isolated from BMMSCs produced negative results, as shown in **Figure 3E**.

The width of the PDL that formed around the bio-hybrid implants was comparable to that of a natural tooth (the control), as determined using a stereoscope. A non-significant difference was found when the width of the PDL around the biohybrid implants, both Ti and zirconia, was compared with the natural tooth. The result indicates that this tissue persisted as a PDL without any loss, deflation, teratoma for 3 months after transplantation. This finding is consistent with that of a previous study [5]. In the construction of the biohybrid implant, both Ti and zirconia produced encouraging results that support PDL regeneration with the help of the tissue engineering technique and stem cells. The finding of PDL-like tissue formation after transplantation is a promising result that may help overcome many problems or shortages related to osseointegrated dental implants.

https://doi.org/10.5051/jpis.2006080304

FESEM analysis revealed that the osseointegrated (Ti and zirconia) implants were connected to the surrounding bone. Soft tissue was not observed between the implant and the healed alveolar bone. The EDX compositional line scan data of the implant–bone interface at different areas showed the element composition of the implant and the bone, as presented in **Figures 5A, B**, and **6**.

The FESEM data showed that PDL-like fibres tightly filled the space between the implant socket and the implant. The correct formation of cementum on the surface of the (Ti and zirconia) biohybrid implants and the invasion of PDL-like fibres perpendicular to the cementum of the biohybrid implant could be observed. PDL-like fibres were connected to the implant surface when a layered co-culture cell sheet was used, as shown in **Figures 5C, D**, and **6**. The perpendicular direction of the fibres improved their function and contributed to the load distribution and the bone load tolerance. These findings are in agreement with the results of previous research [7].

In conclusion, stem cells were isolated from PDL and bone marrow (adult tissue sources; not sourced from embryonic tissues to avoid ethical issues) and used to produce the PDL surrounding artificial implants to generate a biohybrid dental implant. Co-culture is the key to mitigating the problems regarding the number of PDLSCs. Three-layered cell sheets were successfully constructed. Temperature-responsive tissue culture dishes and collagen graft were used as a scaffold to envelop the implants. Mesenchymal-tissue-layered cell sheets were isolated from PDLSCs alone and co-cultured BMMSCs and PDLSCs and used to generate periodontal tissue. The mesenchymal-tissue-layered cell sheets isolated from BMMSCs alone could not generate periodontal tissue. This report presents next-generation biohybrid implants for the replacement of lost tooth and future biohybrid artificial organ replacement therapy. However, as a limitation, this study did not test the regenerated PDL in the dental implant for impact force in comparison with natural teeth and the osseointegrated implant using the stress analysis method (with a strain gauge and finite element analysis).

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