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Dentin-Pulp Regeneration using Autologous Fibrin Matrix in Human Dental Pulp Stem Cells

사람치아 치수줄기세포에서 자가 피브린 매트릭스를 이용한 상아질-펄프 재생

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Abstract

Dentin-Pulp Regeneration using Autologous Fibrin Matrix in Human Dental Pulp Stem Cells

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Background and Purpose of the Study

Xenogeneic serum is widely used as a growth supplement for cell culture medium; however, animal-borne pathogens increase the risk of transmitting infectious agents. Human autologous supplements, including platelet derivatives such as platelet-rich fibrin (PRF), have been assessed in clinical studies as a possible replacement for fetal

bovine serum. However, concentrated leukocytes may affect catabolic gene expression in tendons and ligaments, and concentrated red blood cells (RBCs) cause inflammation due to numerous pro-inflammatory interleukins. It was hypothesized that minimizing the number of leukocytes would contribute to the differentiation activity of human dental pulp stem cells (hDPSCs). This study aimed to explore the ability of autologous fibrin (AF) serum prepared after leukocyte removal to induce the differentiation of hDPSCs *in vitro* and *in vivo.*

Materials and Methods

Human DPSCs were obtained from extracted third molars. To characterize the immunophenotype of the hDPSCs, the expression of mesenchymal stem cell-associated surface markers at passage 5 was analyzed by flow cytometry. Next, AF was prepared by high-speed centrifugation and most of the leukocytes were removed. The AF serum was then subjected to three freeze-thaw cycles and used *in vi tro* for the cell culture. hDPSCs were treated with AF serum, and then odontogenic-associated markers were investigated by real-time polymerase chain reaction (PCR).

For the *in vivo* experiments, autologous fibrin matrix (AFM) was

fabricated on a plate and hDPSCs cultured on the AFM formed a cell-fibrin complex. Then, the potency of the differentiation induction of the hDPSCs in the cell-fibrin complex was evaluated via real-time PCR and Western blot. The hDPSCs mixed with a cell-fibrin complex were subcutaneously transplanted into nude mice and allowed to grow for 8 weeks. Then, the cell-fibrin complex was examined how induced odontoblastic differentiation and dentin formation of the hDPSCs in the mice by immunohistochemical (IHC) analysis.

Results

Flow cytometric analysis showed that approximately 90% or more of the hPDLSCs expressed CD13, CD90, and CD146, and 1.58% expressed CD34. The multi-lineage differentiation capacity of hDPSCs *in vitro* in osteogenic, chondrogenic, and adipogenic medium was observed. Scanning electron microscopy (SEM) images verified that leukocytes were almost absent from the fibrin matrix after high-speed centrifugation. A cytokine array showed that AF serum released sufficient cytokines to induce the differentiation of hDPSCs. AF serum accelerated mineral nodule formation *in vitro* and increased the expression of odontoblast-associated genes, such as alkaline phosphatase *(ALP)*, bone sialoprotein *(BSP),* dentin matrix protein 1

(DMP1), dentin sialophosphoprotein *(DSPP)*, runt-related transcription factor 2 *(Runx2)*, and vascular endothelial growth factor *(VEGF)*. In addition, the cell-fibrin complex also was observed that it significantly up-regulated the levels of the proteins DMP1, osteopontin (OPN), and DSPP during odontogenic differentiation. Furthermore, IHC staining revealed proteins were expressed after the transplantation of the hDPSCs and the cell-fibrin complex induced the hDPSCs to differentiate into odontoblasts-like cells and to regenerate dentin-pulp tissue *in vivo*.

Conclusions

The present study investigated the potential safe methods to expand autologous cells in a risk-free environment, and the cell-fibrin complex scaffold could be generated without the introduction of foreign scaffold materials. The results of experiments confirmed that

1. AF serum prepared after removal of leukocytes to could be used for the proliferation and promotion of hDPSCs odontogenic differentiation under animal serum-free conditions.

2. Human DPSCs cultured on the AFM formed a cell-fibrin complex which became stable and secreted their own matrix to form an

autogenous 3D structure.

3. AFM can be effectively used as a carrier for successful regeneration of dentin-pulp complexes, which has been demonstrated by the *in vivo* subcutaneous transplantation in nude mice.

4. As a clinical implication, AFM possesses good bio-compatibility and cell infiltration and is suitable for dental tissue regeneration in hDPSCs.

Keywords : autologous fibrin, human dental pulp stem cells (hDPSCs), platelet-rich fibrin (PRF), leukocyte, dentin, regeneration

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I. INTRODUCTION

Complete or partial loss of tooth structures can be caused by mechanical trauma, periodontal diseases, and endodontic complications. Therapeutic approaches include restorations, fixed or removable prostheses, caries, and implants. Regeneration of the lost structures of the tooth has attracted the attention of biologists in recent years, given that it protects the integrity of the natural tooth structure and prevents tooth loss [1]. Cell transplantation became the principal strategy of choice in regenerative medicine. Stem cell technology can induce the differentiation of mesenchymal stem cells into various cells *in vitro*; it can also be used to treat several diseases such as leukemia, dementia, and Parkinson's disease [2].

Human dental mesenchymal stem cells (hDMSCs), which exhibit multi-lineage differentiation abilities, can be obtained from various types of dental tissues, such as pulp, periodontal ligaments, apical papilla, and periapical follicles, and attempts have been made to regenerate these tissues from stem cells [3-6]. Human dental pulp stem cells (hDPSCs) are able to differentiate to form both dentin and pulp tissues, and therefore a combination of these cells with various

scaffold materials is currently being investigated for use in endodontic treatments [7].

When culturing human stem cells, the culture medium is usually supplemented with xenogeneic additives such as bovine thrombin or fetal bovine serum (FBS) [8]. Subsequently, autologous platelet-rich plasma (PRP) was developed, and it can be used as a replacement for FBS in cell culture [9-11]. However, PRP treated with anticoagulants has only limited effects on bone regeneration [12,13]. To overcome this disadvantage, platelet-rich fibrin (PRF) was developed, which does not contain any anticoagulants [14,15]. PRF is a fibrin clot with a solid structure, including platelets, leukocytes, and concentrated red blood cells (RBCs), and these cells are mostly distributed in the buffy coat [14].

The low-speed centrifugation concept (LSCC) was later proposed to separate a new type of liquid PRF without any anticoagulant [16,17]. Liquid PRF obtained with a low-speed relative centrifugal force (RCF) is mainly used as an autologous carrier system because it contains higher concentrations of leukocytes, lymphocytes, and neutrophils compared to high RCF [18,19]. However, concentrated leukocytes may affect the PRF matrix and induce catabolic gene

expression in tendons and ligaments [20]. The inclusion of leukocytes in PRF products is always controversial.

Because of the limitations of leukocytes, the development of a leukocyte-free product is important in tissue regeneration for the *in vitro* expansion of a sufficient number of cells. The involvement of human-derived serum is necessary for the culture and differentiation of hDPSCs owing to the immunogenic responses to xenogeneic FBS. In addition, the optimal concentration of liquid PRF is important for the differentiation of hDPSCs. At present, the biggest challenge is to mitigate the immune response triggered by residual white blood cells in PRF.

Implementing a foundation of tissue engineering might allow for better management of the cells and tissues of interest and contribute to more reliable results. Therefore, these cell-based experiments adhere to the standard tissue engineering model that includes the transfer of stem cells and recombinant growth factors in a scaffold. Various scaffold materials used to carry the stem cells are placed into tooth-derived models in order to demonstrate differentiation into odontoblasts, pulp-like tissue, vascularization, and the formation of tubular dentin [21,22]. Nevertheless, the cells may closely resemble

intrinsic tissue and create a more favorable environment when they regenerate a 3D tissue without the use of extrinsic materials to form the scaffold.

Overall, it was hypothesized that minimizing the number of leukocytes and reducing the activity of residual immunogens in human autogenic serum would contribute to preserving the differentiation activity of hDPSCs. It would also allow the serum to be suitable for the differentiation of hDPSCs. Thus, the primary objective of this study was to explore the ability of autologous fibrin (AF) serum prepared after removal of leukocytes to induce the differentiation of hDPSCs *in vitro*. The secondary objectives were to determine the optimal concentration of AF serum required to culture hDPSCs in comparison to 10% FBS by assessing the cell survival rates and gene expression profiles. Then, the effects of autologous fibrin matrix (AFM) as a cell-fibrin complex on the odontogenic differentiation of hDPSCs was evaluated *in vivo* by mixing the cells with AFM and transplanting them into mice.

II. MATERIALS AND METHODS

1. Samples

Human third molars and blood samples were collected from three healthy women aged 18-25 years. The use of anonymous tooth and blood samples for research purposes was approved by the local ethics committee, and informed consent was acquired from all donors. The study was approved by the Institutional Review Board (IRB) of Seoul National University Dental Hospital, Seoul, Korea (IRB no. 05004). The animal studies were performed after receiving the approval of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (No. SNU-190426-13).

2. Fabrication of AF and AF serum

The collected blood samples were placed in a sterile tube without anticoagulants and immediately centrifuged at 950 g using a Union 32R Plus centrifuge (Hanil Science Industrial Co., Ltd., Incheon, Rep. of Korea) for 16 min at room temperature (Fig. 1). The blood was then stratified into the following three layers: a yellow liquid serum (top), a red portion full of RBCs (bottom), and a middle layer

comprising the leukocyte pellet. The liquid serum (top layer) was carefully collected into a new polypropylene tube, avoiding RBC contamination, and after letting it stand for a few minutes, it separated into AF and supernatant serum (SS).

To break the platelet membranes and release the growth factors, the AF was passed through at least three freeze-thaw cycles. Then, the AF was divided into a fibrin membrane and a liquid fraction [AF extraction (AFe) + SS]. The AF serum (AFe + SS) was stored at -70℃ for 24 h and then immersed in a 50℃ water bath for 30 min. Finally, the obtained AF serum was sterilized using a 0.2 μm GVS filter (Filter Technology, Inc., Chicago, IL, USA) for cell culture. After centrifugation at 400 g for 12 min, conventional PRF separates into three layers and is left at room temperature for a while. When it clots into a gel-like state, the upper portion is usually called PRF (Fig. 2A) [23,24].

3. Histological analysis of AF and PRF

The AF and PRF were histologically examined to determine their microstructure. PRF was prepared as described previously [24]. Briefly, the specimens were fixed in 3.7% paraformaldehyde (PFA) at

4℃ for 1 day. Samples were embedded in paraffin, and 4-mm-thick serial sections were transversely cut from the center of the exposed part. Then, the sections were dehydrated through an alcohol series of increasing concentrations (70%, 80%, 90%, 95%, and 100%) every 2 min and processed for hematoxylin and eosin (H&E) staining.

4. Scanning electron microscopy (SEM) analysis

To observe the cell bodies trapped in the AF and the overall architecture of the fibrin network, samples were washed with 0.1 M of phosphate-buffered saline (PBS) and fixed in modified Karnovsky's fixative buffer at 4℃ for 2 h. The AF was washed three times using 0.1 M of PBS at room temperature for 15 min and then fixed in 0.1 M of cacodylate buffer (pH: 7.4) containing 1% osmium tetroxide for 1 h. After rapid dehydration through an ethanol gradient (70%, 80%, 90%, 95%, and 100% v/v), the samples were coated with platinum before observation with a field emission S-4700 scanning electron microscope (Hitachi, Tokyo, Japan) using an acceleration voltage of 15 kV.

5. Human cytokine antibody array

Focused protein array analysis was performed using the human cytokine antibody array panel Proteome Profiler Array (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Human cytokine array panels were used on the AF serum, and they detected angiogenin, brain-derived neurotrophic factor (BDNF), B lymphocyte chemoattractant (BLC), bone morphogenetic protein (BMP)-4, BMP-6, casein kinase β8-1 (CK β8-1), ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), eotaxin, eotaxin-2, eotaxin-3, fibroblast growth factor (FGF)-6, FGF-7, Fit-3 ligand, fractalkine, granulocyte chemotactic protein 2 (GCP-2), glial cell-derived neurotrophic factor (GDNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-C motif) ligand 1 (I-309), interferon gamma (IFN-ɣ), insulin-like growth factor-binding protein (IGFBP)-1, IGFBP-2, IGFBP-4, IGF-I, interleukin (IL)-10, IL-13, IL-15, IL-16, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, Leptin, LIGHT, monocyte chemoattractant protein 1 (MCP)-1, MCP-2, MCP-3, MCP-4, M-CSF, macrophage-derived chemokine (MDC), monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1δ, MIP-3α,

neutrophil-activating peptide-2 (NAP-2), neurotrophin-3 (NT-3), pulmonary and activation-regulated chemokine (PARC), platelet-derived growth factor-BB (PDGF-BB), regulated upon activation normal T-cell expressed and secreted (RANTES), stem cell factor (SCF), stromal cell-derived factor 1 (SDF-1), thymus and activation regulated chemokine (TARC), transforming growth factor (TGF)-β1, TGF-β3, tumor necrosis factor (TNF)-α, TNF-β, growth related oncogene (GRO), and hemoinfiltrate C-C motif chemokine 4 (HCC-4).

6. Primary cell culture

Dental pulp was separated from the root canal of extracted human third molars to obtain the hDPSCs. Separated tissues were digested in a solution containing 3 mg/mL of collagenase type I (Worthington Biochem, Freehold, NJ, USA) and 4 mg/mL of dispase (Boehringer Ingelheim, Ingelheim am Rhein, Germany) at 37℃ for 1 h. At this point, single-cell suspensions were obtained by passing the cells through a 40-mm strainer (BD Labware, Franklin Lakes, NJ, USA) and then they were cultured in an alpha modification of minimum essential medium (Alpha MEM)(WELGENE, GS, Rep. of Korea)

supplemented with AF serum 100× antibiotic (GIBCO USA) and incubated at 37℃ in 5% CO2 [25]. All primary cells used in this study were collected from passages 2-5.

7. Proliferation assay

To study the effects of AF serum on cell proliferation, hDPSCs were plated in 96-well plates at a density of 5×10^3 cells per well. AF serum was added to FBS-free α-MEM at different final concentrations of 1%, 2%, 5%, 8%, and 10%. For comparison, cells cultured in 10% FBS complete medium were prepared separately. At 1, 4, 7, and 14 days of culture, the cell proliferation was measured using a WST-8 assay Viability Assay Kit (MediFab Co., Ltd., Rolleston, New Zealand) and a plate reader at an optical density of 450 nm.

8. Flow cytometry analysis

To characterize the immunophenotype of the hDPSCs, the expression of MSCs-associated surface markers were analyzed by flow cytometry as described previously (Becton Dickinson

Immunocytometry Systems, San Jose, CA, USA) [25]. Briefly, cells (1×10^6 cells) were collected and fixed in 4% PFA for 10 min and then resuspended in PBS containing 1% bovine serum albumin (BSA) for 30 min for blocking. The cells were incubated with specific antibodies against CD13, CD34, CD90, and CD146 (BD Biosciences, San Jose, CA, USA) at 4°C for 1 h, followed by incubation with fluorescent secondary antibodies at room temperature for 1 h. The percentage of CD13-, CD90-, and CD146-positive and CD34-negative cells was measured with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and the results were analyzed using CellQuest Pro software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

9. Multi-differentiation of hDPSCs

For osteogenic differentiation, hDPSCs were seeded into 24-well plates at a density of 4×10^4 cells per well with α-MEM containing 1%, 2%, 5%, 8% and 10% AF serum, whose effects were compared those of 10% FBS supplemented with 10 mM of β-glycerophosphate, 50 mg/mL of ascorbic acid, and 1 mM of dexamethasone. After osteogenic differentiation induction, calcium deposition was assessed

using Alizarin Red S (Sigma Aldrich Corp., St. Louis, MO, USA) staining. For induction of chondrogenic or adipogenic differentiation of the hDPSCs, the cells (4×10^4 cells) were seeded into 24-well plates and induced with Stempro Chondrogenic or Stempro Adipogenic induction media kits (Lonza, Walkersville, MD, USA) supplemented with the appropriate supplements (Lonza, Walkersville, MD, USA). The medium was changed after the first 24 h and then every 3 days. After chondrogenic and adipogenic induction, the cells were washed with PBS and fixed in 3.7% paraformaldehyde for 10 min. The cells were stained with 1% Alcian Blue (Sigma Aldrich Corp.) and 0.3% Oil Red O dye (Sigma Aldrich Corp.) for the detection of proteoglycans and fat vacuoles as indicators of chondrogenic and adipogenic differentiation. The cells were observed and photographed with an Olympus U-SPT microscope (Olympus, Tokyo, Japan).

10. Gene expression of the hDPSCs

To evaluate their gene expression, hDPSCs (5.0×10^5 cells) were seeded in 6-well plates and cultured for 7, 14, and 18 days under differentiation-inducing conditions. Total RNA was extracted from the samples using TRIzol reagent (Life Technologies, NY, USA), and

complementary DNA (cDNA) was synthesized from 2 μg of total RNA using a reverse-transcriptase Superscript II Preamplification System (Invitrogen, Carlsbad, CA, USA). Real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR Master Mix in a Step One Plus real-time PCR detection system (Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicate and normalized to the reference gene glyceraldehyde 3 phosphate dehydrogenase *(GAPDH)*. The specific primer sets used for this analysis are listed in Table 1.

11. Fabrication of autologous fibrin matrix (AFM) and dental pulp stem cells culture on the AFM (cell-fibrin complex)

After incubation of the serum for 2 hours at room temperature, the serum was divided into 100 μL, 200 μL, 300 μL, 400 μL, 500 μL and 1000 μL in a 24-well culture plate and left to clot. Thus, AFM was fabricated on the plate and 1×10^4 hDPSCs were seeded into each well onto the AFM sheets and incubated for 1, 3, 5, and 7 days. A WST-8 assay (Viability Assay Kit, MediFab Co., Ltd.) was used to

measure the proliferation ability of the cells on the AFM and the results were read at a wavelength of 450 nm with a plate reader.

12. The invasion of the hDPSCs into the AFM

To assess the cell infiltration and morphology of the hDPSCs cultured on AFM, 1×10^4 hDPSCs were seeded on the surface of the AFM (300 μL) for a period of 3 days, so it became a cell-fibrin complex. The cell-fibrin complex was fixed with modified Karnovsky's solution for 2 hours and processed as described for the SEM methods. The samples were washed 3 times with PBS buffer for 15 min and fixed with 1% osmium tetroxide (EMS). Later, the samples were washed with distilled water and dehydrated with graded concentrations (70, 80, 90, 95, and 100% v/v) of ethanol and then treated with hexamethyldisilazane (HMDS) for 20 min. Subsequently, the samples were coated with platinum before observation with a field emission S-4700 scanning electron microscope (Hitachi, Tokyo, Japan) with an acceleration voltage of 15 kV at three different magnifications: ×10000, ×5000, and ×1000.

13. Immunofluorescence analysis of cell adhesion

Cell-fibrin complexes were fixed with 4% paraformaldehyde in PBS for 15 min. Then, the cells were rinsed three times with PBS, then incubated in PBS containing 0.5% Triton X 100 for 15 minutes at room temperature. Samples were blocked for 1 h, and then the permeabilized cells were incubated with actin green 488 fluorescent dye for 30 min at room temperature. Finally, as a nuclear counterstain, 4′, 6 diamidino 2 phenylindole (DAPI) with the mounting solution was added before imaging. Cells were visualized using fluorescence microscopy (AX70, Olympus Optical Co, Tokyo, Japan).

14. Evaluation of the differentiation of hDPSCs on the AFM by quantitative real-time PCR

To evaluate gene expression levels, hDPSCs (5.0×10^4 cells) were seeded on the AFM in a 24-well plate (3 wells/sample) and cultured for 7 days under induction conditions in DMEM (Welgene, Fresh Media, Korea) containing 1× antibiotics, 10 mM glycerol 2-phosphate, 50 μg/mL ascorbic acid and 1 mM dexamethasone or with 10% FBS.

Total RNA was harvested from the samples using TRIzol reagent (Life Technologies, NY, USA) and cDNA was synthesized from 2 μg of total RNA using reverse transcriptase (Superscript II Preamplification System; Invitrogen). The expression of the selected osteogenic genes alkaline phosphatase *(ALP)*, dentin matrix protein 1 *(DMP1),* dentin sialophosphoprotein *(DSPP),* and osteopontin *(OPN)* were quantified using SYBR green gene expression assays on a StepOnePlus system (Applied Biosystems) according to the manufacturer's instructions. All reactions were run in triplicate and were normalized to the reference gene *GAPDH*. The specific primer sets used for this analysis are listed in Table 1.

15. Western blot analysis

Human DPSCs (5 x 10^5 cells/dish) were seeded in 48 mm dishes and cultured for the indicated times. Cell lysate protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein (20 mg/lane) were loaded on SDS-PAGE gels and afterward transferred to polyvinylidene difluoride membranes (GE Healthcare). Primary antibodies against DMP1, DSPP, and OPN were used to study the mechanism of action. Blots were

developed using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and visualized using an enhanced chemiluminescence kit (GE Healthcare).

16. Transfection of AFM into a rat calvarial defect

To explore whether it works well as a scaffold in vivo, the AFM was transplanted into rat calvarial defects (Hsd:RH-Foxn1Rnu, 3 males, 6 weeks old, Envigo, New Jersey, USA). The rat was anesthetized with a combination of anesthetics intraperitoneally. An 8 mm diameter round defect was made with a trephine bur in the unilateral non-suture-associated parietal bone under constant irrigation. An 8 mm defect is thought to be critical in the rat calvaria. Extreme care was taken to avoid injury to the underlying dura mater, and each defect was rinsed with saline to remove bone debris. In the control group, PRF was implanted into the defect area, and in the negative control group, nothing was implanted into the defective area, while in the experimental groups, fresh 8 mm diameter AFM or PRF pieces were implanted into the defect. Subsequently, the

skin was sutured, and the animal was kept under surveillance. After 6 weeks, the rat was sacrificed, and the composites were harvested.

17. Micro-computed tomography imaging analysis

Samples were harvested 6 weeks after surgical implantation and then fixed in 3.7% paraformaldehyde for 48 h at 4℃. Representative skull samples were trimmed to the proper size for the analysis chamber of the micro-CT device (SkyScan1172 high-resolution micro-CT) and mounted on a turntable that could be shifted automatically in the axial direction. Six hundred projections were taken over 180 object rotations (0.3 degrees). The X-ray shadow projections were digitized as 1024 × 1024 pixels with 4096 brightness gradations (12 bit) for the cooled camera or 256 gradations (8 bit). The spatial resolution obtained was 15 mm. The samples were then trimmed for decalcification in 10% EDTA for 3 weeks and embedded in paraffin. Then, 4 μm serial buccolingual sections were stained with H&E and examined under a light microscope (Olympus U-SPT).

18. Preparation of human dentin matrix

The dental pulp tissue and the periodontal tissues were completely removed using a surgical bur, with grinding along with the tooth root profile for removing the pre-dentin (inside) and the cementum (outside). The final size of the dentin was 5 mm in length, 5 mm in diameter, with a thickness up to 1.0 mm. The dentin was immersed in deionized water and mechanically cleaned using an ultrasonic cleaner for 20 min. The dentin was washed 3 times with ethylene diamine tetra acetic acid (EDTA) (Sigma Aldrich Co., St. Louis, MO, USA) before it was used as human dentin matrix.

The human dentin matrix was stored in sterile PBS supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (Biofluids, Rockville, MD, USA) for 72 h, washed in sterile deionized water for 10 min in an ultrasonic cleaner, and finally stored in α-MEM at 4℃ [26].

19. Transplantation

To assess the effects of the AFM on the regeneration of dentin-pulp tissue, hDPSCs (1.0×10^6 cells) were mixed with the gelled scaffolds (group 1: hDPSCs were mixed with 0.5% fibrin gel as a control; group 2: hDPSCs were mixed with AFM as experiment

1; group 3: hDPSCs were mixed with the cell-fibrin complex as experiment 2) and were placed into the canal space of the human dentin matrix, and then the outside was wrapped with the gelled scaffolds. The entire system was transplanted subcutaneously into immunocompromised mice (BALB/c-nu, 3 males, 6 weeks old, Orient, Seongnam, Rep. of Korea).

At 8 weeks after the transplantation, the nude mice were euthanized and the complexes were removed for analysis. The samples fixed with 4% phosphate-buffered paraformaldehyde and decalcified in 12% EDTA (pH 7.4) solution at 4℃. All samples were embedded in paraffin, sectioned, and stained with H&E for histological analysis. Some sectioned samples were also used for immunohistochemistry (IHC).

20. Histological analysis

Samples were fixed in 3.7% paraformaldehyde solution (prepared from a 95% paraformaldehyde powder (Sigma-Aldrich) that was diluted in PBS (3.7 g/100 mL)) for 48 h at 4℃ before decalcification with 12% EDTA (pH 7.4) at 4℃. The decalcified samples were embedded in paraffin and were cut serially. Semi serial 5 μm sections

were prepared for hematoxylin and eosin (H&E) staining. The stained sections were observed under a light microscope (Olympus U-SPT).

21. IHC analysis

Some sections of the samples were further processed as described previously [27]. The deparaffinized sections were immersed in 0.6% H_2O_2/methanol for 20 min and pre-incubated with 1% bovine serum albumin in PBS for 30 min. Then, these sections were incubated overnight at 4℃ with rabbit polyclonal antibodies against dentin matrix protein 1 (DMP1; 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), dentin sialoprotein (DSP; 1:200, Santa Cruz Biotechnology), and von Willebrand factor (vWF; 1:200, EMD Millipore Co., USA). The sections were incubated for 1 h at room temperature with the appropriate secondary antibodies (1:1000) and then reacted with avidin-biotin-peroxidase complexes (VECTASTAIN ABC Systems; Vector Laboratories, Inc., Burlingame, CA, USA) in PBS for 30 min. After color development with 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (DAB Peroxidase Substrate; Vector Laboratories), the stained sections were counterstained with hematoxylin.

22. Statistical analysis

Data were analyzed using IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA). A non-parametric Mann-Whitney U-test was used to evaluate the level of significance in the differences among samples. The level of significance was set at $p < 0.05$.

III. RESULTS

1. Characterization of AF

H&E staining results showed a large number of dark-purple concentrated leukocytes, pink cross-linking mesh-like fibers, and a moderate number of pink RBCs in the PRF (Fig. 2B, C). The total number of blood cells (i.e., leukocytes and RBCs) in the AF were significantly decreased compared with those of the PRF (Fig. 2E, F). In addition, the matrix of the AF exhibited a primary fibrin network structure (Fig. 2F).

SEM analysis revealed a large-size cell complex (~4-8 μm in diameter) containing platelet aggregates, RBCs, and leukocytes (Fig. 3B, C) in the middle layer (leukocyte pellet) at a low magnification, and single leukocytes exhibited spherical structures with irregular surfaces (Fig. 3D).

Centrifugation of the abovementioned serum was conducted again for 15 min at 1,880 g before fibrin coagulation (Fig. 3E), where residual cells were enmeshed into the fibrin network by matrix shrinkage (Fig. 3F). At a low magnification, platelets were observed in the supernatant, and the fibrin was found to be denser with a

solid, thick mesh due to extensive aggregation and clotting (Fig. 3G). The typical size of the cells became smaller (~1-2 μm in diameter) (Fig. 3H). The size and morphology of the platelets were different from those of RBCs or large leukocytes.

This was centrifuged at a higher rotational force and for a longer time to identify residual cells in the yellow liquid serum of the upper layer. The results showed that the middle layer that we discarded contained a large number of platelets, and the upper liquid layer also contained some platelets. After the AF was subjected to three freeze-thaw cycles, the fibrin was condensed and compacted as the fibrin membrane shrunk to a compact size (Fig. 3J, K), and almost no platelets or leukocytes were observed in the fibrin membrane (Fig. 3L).

2. Detection of cytokines in the AF serum

The cytokine levels of the AF serum were detected by a human cytokine antibody array, and 12 cytokines (angiogenin, EGF, FGF-6, I-309, IGFBP2, leptin, MIP-1δ, NAP-2, PDGF-BB, RANTES, GRO, and HCC-4) were detected in the AF serum (Fig. 4). These results

indicated that the AF serum still released enough cytokines to induce the differentiation of hDPSCs.

3. Characterization of hDPSCs in the AF serum

At passage 5, the hDPSCs were cultured with the AF serum, and the expression of DMSC-related markers was confirmed by fluorescence-activated cell sorting analysis. The expression of CD13, CD90, and CD146 was strongly positive in hDPSCs at >90%. CD34, a hematopoietic stem cell marker, was not expressed, present at <2% (Fig. 5). These results suggested that the isolated cells were mainly mesenchymal cells and they preserved the stemness of the hDPSCs up to passage 5. Assessing the hDPSCs′ colony-forming capacity showed the formation of a shuttle-like morphology and proliferation into colonies within 7 days (Fig. 6).

To investigate the multi-differentiation potential of the hDPSCs after mineral induction, the hDPSCs were found to form an extensive amount of Alizarin Red S-positive mineral deposits throughout adherent layers. Then, after treatment with adipogenic-inductive supplements for 3 weeks, the hDPSCs demonstrated the ability to differentiate into adipogenic cells. In addition, the hDPSCs appeared

as Alcian Blue-positive nodules after three weeks of chondrogenic induction (Fig. 6). hDPSCs treated with AF serum formed several mineral nodules of Alizarin Red S-positive throughout adherent layers after 14 and 18 days (Fig. 7). According to the characteristics of the differentiation of the hDPSCs, 18 days was selected as the time point of differentiation of the hDPSCs for subsequent experiments.

4. Effects of the AF serum on the hDPSCs in vitro

The optimized concentration of the AF serum for the proliferation of the hDPSCs was 5%. The effects of AF serum on proliferation were inferior to 10% FBS (Fig. 8, 9). Real-time PCR after 7, 14, and 18 days of culture in AF serum showed a significant increase in the expression of the odontogenic-related genes *ALP*, bone sialoprotein (*BSP*), *DSPP, DMP1*, and runt-related transcription factor 2 (*RUNX2*) in the hDPSCs compared with culture in FBS. hDPSCs cultured in 1% AFC serum significantly expressed these genes at 7 days. After 18 days, *DSPP* and *DMP1* expression decreased with increasing AFC serum concentration compared with FBS. Interestingly, 1% AFC serum significantly increased *RUNX2*

expression as an index of early differentiation at 7 days but slightly down-regulated *RUNX2* expression at 18 days. In contrast, 1% AFC serum decreased vascular endothelial growth factor (*VEGF)* expression at 7 days but up-regulated *VEGF* expression at 18 days (Fig. 10).

5. Optimal thickness of the AFM for the hDPSCs culture

To investigate the optimal AFM thickness for the hDPSCs cultured on AFM, the results first showed the proliferation of cells on the AFM did not show any significant difference over time, and on the 5th day, the proliferation was already sufficient. On Day 5, there were no significant differences on thicknesses of 100 μL, 200 μL, or 300 μL, and there was a tendency for proliferation to decrease from 400 μL. The AFM thickness tended to decrease gradually with time. The thicker the AFM, the less cell proliferation. However, since cell proliferation tended to decrease from 400 μL, it can be confirmed that 300 μL is the optimal thickness of AFM for the culture of hDPSCs in 24-well plates (Fig. 11, 12).

6. Infiltration and morphological observation of the hDPSCs on AFM

Next, the structural appearance of the hDPSCs grown in three-dimensional (3D) AFM was determined. As demonstrated by SEM (Fig. 13), the hDPSCs extended out of the matrix surface on day 1, and on the second day, the cells clumped together. On the third day, the cells infiltrated into the fibrin. As a result, on the third day, the cells adhered closely to the AFM and entered a stable phase.

The morphological appearance of the hDPSCs culture on the 3D AFM was compared with 2D culture on a bare plate. IF analyses indicated that the hDPSCs spread over the sheet and still kept their cell morphology after being seeded on AFM for 3 days (Fig. 14). The hDPSCs cultured on AFM formed significantly better cell-to-cell connections than on a bare plate. Also, the expression data indicated that the cells on AFM maintained their cell viability, biocompatibility, and invasive ability.

7. The odontogenic influences of hDPSCs on the AFM

The expression levels of odontogenic-related genes, including *ALP, DMP1, DSPP,* and *OPN,* were significantly increased in hDPSCs grown in AFM compared to FBS at 7 days (Fig.15). Next, the protein levels of DMP1, DSPP, and OPN in hDPSCs differentiated in an odontogenic medium for 7 days by western blot analysis were evaluated (Fig. 16). Intrinsic expression of the DSPP and OPN proteins was higher on the hDPSCs in AFM than in FBS. DMP1 protein expression in AFM increased dramatically relative to the FBS. These data indicate that DSPP, DMP1, and OPN protein expression in hDPSCs is significantly up-regulated when cells are cultured in AFM compared with FBS during odontogenic differentiation.

8. Effects of AFM on the rat calvarial defect model

The results of micro-computed tomography (micro-CT) and histological analysis showed that the defect size of the negative control group (no transplantation group) remained larger than the size of the experimental group (AFM transplantation group) at 6 weeks after implantation (Fig. 17), which showed no severe inflammatory reaction. Meanwhile, the experimental group showed new bone

formation in most of the defects more so than in the control group (the PRF transplantation group) by micro-CT. The H&E staining further showed that bone-like tissues were generated in the cavity of the calvarial defects in the experimental group (Fig. 17). However, in the control group, no obvious clearance was present, and bone-like tissue formation was observed, which demonstrated that AFM was able to realize osseointegration.

9. Histological analysis of human dentin matrix surface in vivo

An extracted human tooth was used to fabricate human dentin matrix. SEM images indicated that the insides of the dentinal tubules were sufficiently exposed (Fig. 18D), which means the successful fabrication of human dentin matrix. The composite of hDPSCs + cell-fibrin complex placed inside the dentin matrix for tooth root regeneration was transplanted subcutaneously into nude mice for 8 weeks. No severe inflammatory reaction was observed (Fig. 19). H&E staining showed that cellular dentin-like tissues were generated on the inside of the human dentin matrix (Fig. 20). However, in the control group, no obvious dentin-like tissue was observed, which

demonstrated that AFM promoted dental pulp-like tissue regeneration.

Inside the pulp cavity, dental pulp-like cells were uniformly distributed over the dental pulp-like tissues in the experimental groups (Fig. 20B, C). In contrast, a greater number of cells were concentrated on the inner boundary of the human dentin matrix, similar to that observed in the odontoblastic layer (Fig. 20E, F). What's more, pre-dentin matrix-like tissues were observed on the interface between the dental pulp-like tissues and the dentin matrix in the experimental group (cell-fibrin complex scaffold group) (Fig. 20C, F). In addition, blood vessels were observed in the dental pulp-like tissues (Fig. 20C, F arrowhead), which served as transporters of nutrients. In the control group, no abundance of cells and only sparse collagen fibers were observed in the chamber of the dentin matrix. Then, IHC analysis was used to further identify the characteristics of the regenerated tissues. The results showed that the dental pulp-like tissues were positive for DMP1, DSP, and vWF (Figs. 21, 22). In the control, all experimental tissues were negative for all tested markers.

IV. DISCUSSION

With the aim of developing a xenofree cell culture medium to improve the quality and safety of the expanded hDPSCs for clinical applications, the present study explored the feasibility of AF serum as an alternative to FBS. The results revealed the potential of AF serum to maintain the characteristics of hDMSCs and to promote the proliferation and differentiation of hDPSCs.

Research on MSC, which is more popular now than ever, is helpful in identifying the characteristics of stem cells and treating various diseases [28,29]. In dentistry, hDMSCs have been primarily focused on because of their potential ability to differentiate into dental tissues that were considered irreparable. Riecke et al. have reported that a sufficient number of hDMSCs plays a critical role in the thickness of the floor of the maxillary sinus [30].

As FBS contains various infectious agents, such as animal-borne pathogens, research attention has been focused on human serum supplements, which contain platelet derivatives such as PRF and can be used to culture hDMSCs. Because of its autologous origin and the absence of anticoagulants, PRF can accelerate wound healing and tissue regeneration [29]. However, the standard PRF matrix contains

an extremely large number of host immune cells (platelets, leukocytes, and T-lymphocytes), leading to different unwanted effects on MSC [31,32]. Leukocytes can affect the mechanical properties of their fibrin scaffolds and are directly related to cell inflammation and proliferation [33]. The functions of leukocytes in PRF have always been debatable.

The use of autogenic serum is especially important for tissue regeneration because of the need for *in vitro* expansion of a sufficient number of cells. A reduced number of leukocytes could result in a decreased anti-immune reaction and less inflammation for autogenic applications. Cell-specific properties such as weight, size, and density can be controlled by changing the centrifugal force and time [23]. Different PRFs were investigated by applying different centrifugal forces and time durations and comparing the structure and composition of the resulting PRF matrices. The results showed that leukocytes were collected in the middle layer and changed shape into a pellet, thus making it easier to remove the upper layer and eliminating the leukocytes before coagulation of the serum. Moreover, the histological staining of the AF convincingly showed the absence of leukocytes compared with that observed in PRF (Fig. 2C, F). In addition, the fibrin mass structure of the AF was more uniform

compared with that of PRF. These observations were in accordance with the results obtained by Wend et al., [18] who reported that high-speed RCF significantly decreased the levels of multiple growth factors.

Another method of reducing the potential risk of alloimmunization is freeze-thawing, which can reduce the presence of platelet antigens against major histocompatibility complex class I [11]. SEM images showed that most leukocytes and a portion of the platelets were arrested in the middle pellet layer in high-speed centrifuged RCF (Fig. 3A-D). The residual platelets released various growth factors such as angiogenin, EGF, FGF-6, IGFBP-2, and PDGF-BB, which are sufficient to maintain hDMSCs growth (Fig. 4A, B). These findings supported the findings of previous reports that platelets release various growth factors such as IGF-1, FGF, TGF-β, VEGF, PDGF, and EGF, which promote cell growth [34].

Bieback et al. proposed that human platelet lysate-containing media could be used as a substitute for FBS-containing media to expand MSCs [10]. Some studies stated that autogenic serum could be an alternative to FBS in supporting *in vitro* cell expansion [35-37]; however, none of these studies used the latest high-speed autogenic

liquid PRF to compare and investigate the hDPSCs that are the most significant to dentists. In this study, hDPSCs treated with AF serum were found to be mainly mesenchymal cells. The MSC properties of the hDMSCs were characterized by their cell surface markers and by evaluating their multi-differentiation potential under AF serum conditions through passage 5.

In proliferation assays, the hDPSCs were cultured in AF serum, and then their cellular proliferation was evaluated. The results showed that no significant effect was found on cell proliferation by treatment with a low concentration of AF serum (<5%), but a concentration of AF serum between 5%-10% promoted cell proliferation; however, cell viability was reduced by treatment with AF serum at a high concentration (>10%). Previous studies on other types of stem cells also recommended a concentration of 10% PRF [38]. The optimal concentration of AF serum for the proliferation of hDPSCs is 5%. These results showed that the effects of AF serum on cell proliferation are different depending on the stem cell type. However, the effects of AF serum on cell proliferation were less than those of FBS, and this finding is consistent with the results reported by Arpornmaeklong et al. who stated that human serum is inferior to FBS in its ability to support in vitro MSC expansion [35]. The

mechanism underlying cell proliferation induced by AF is unclear, and the response varies in different types of stem cells, which warrants further research.

The potential of differentiation is closely associated with hDPSCs-based root regeneration, and the present study showed that the optimum concentration exhibited a positive effect on the odontogenic differentiation of hDPSCs *in vitro*. Simultaneously, osteogenic differentiation of hDPSCs was inhibited by AF serum, which is thought to exhibit a conflicting relationship with odontogenic differentiation. These results are the same as those of some previous studies, which reported that autogenic serum could promote the differentiation of some MSCs [39]. The appropriate concentration of AF serum is important for the differentiation of various cell types, suggesting that exploring the optimum concentration of AF serum is essential for its application in tissue engineering. The present study findings provide guidance for the application of AF in hDPSCs-based root regeneration.

Dentin is comprised of mineralized tissues that share some common characteristics with bone in terms of composition and mineralization processes. The expression of *ALP, BSP, DMP1, DSPP,* and *Runx2*

were all increased and facilitated *VEGF* expression at a later stage by the hDPSCs. All of these genes are expressed in common by osteoblasts and odontoblasts, and they play important roles in hard tissue mineralization. As suggested by the above results, with time, the hDPSCs were induced to display odontogenic differentiation under AF serum conditions. AF serum secretes growth factors and is beneficial for odontogenic differentiation of hDPSCs. However, the mechanism of the interactions between the molecules has yet to be elucidated and should be studied further.

Successful tissue engineering allows scaffold biomaterials to stimulate the differentiation of stem cells in the desired direction by providing a special microenvironment in which cells are captured and proliferate [40]. The scaffold also plays a key role in tissue regeneration by regulating the survival, self-renewal, and differentiation of stem cells by simulating the extracellular matrix (ECM) microenvironment [41]. The tooth root is a complex apparatus consisting of different hard (dentin and cementum) and soft (pulp and gingiva) tissues. In this study, the tooth root structure was created by combining a cell-fibrin complex (soft scaffold) and a human dentin matrix (hard scaffold) to mimic the ECM microenvironment and to confirm its practical value in future dental clinical applications.

Therefore, to establish a clinically useful method, and autologous fibrin matrix for fabricating hDPSCs constructs that could be transplanted into the tooth root canal was developed.

Traditionally, cells are combined with exogenous materials before being transplanted into the body in order to facilitate the formation of a 3D structure, to help localize the cells to the region of regeneration, or to direct cell behavior. Scaffolds for dental pulp engineering have been designed mainly from polymeric materials [42] or from natural materials such as collagen [43] or peptides [44]. However, exogenous materials are far from being able to recapitulate the multifaceted extracellular matrix scaffold that the cells generate for themselves. Recent work evaluated natural materials for their suitability for dental pulp tissue engineering; fibrin-based materials revealed excellent cytocompatibility and supported pulp-like tissue formation in vivo [45]. Because the compounds released by platelets in autologous PRF can stimulate cell migration and proliferation, it acts as a chemoattractant for cells from local tissues and promotes cell homing.

This autologous ECM scaffold formed from biodegradable polymers serves as a temporary supporting structure for growing cells and tissues before its degradation. However, the *in vivo* implanted AFM

without cell seeding in rat calvaria presented remodeling without necrosis in the center. Neither skin necrosis nor a significant inflammatory response was observed at the implantation site of AFM in the rat, indicating that the manufactured AFM possessed good biocompatibility for regeneration. Furthermore, the micro-CT and histological results indicated that AFM was a superior scaffold in subcutaneous implants relative to PRF due to the extensive penetration and invasion by surrounding cells. Although PRF includes more leukocytes and growth factors than AFM, the interactions between the many factors in PRF leads to the inhibition of bone regeneration. The fibrin architecture in AFM ensured that the infiltrated cells received enough oxygen and nutrients, which benefited the preserved vessel architectures and portions such as the laminin. These distinct structural properties will play important roles in inducing mitosis and cell migration and recruitment during dental pulp regeneration. In addition, the cells on the surface of the AFM could continue proliferating and even migrate inside the materials, suggesting that AFM possesses good biocompatibility and cell infiltration, and is suitable for tissue engineering of dental pulp.

To utilize fabricated cell constructs as a transplanted tissue, cells in the constructs must be able to survive [46]. In the hDPSCs

constructs fabricated in this study, almost all hDPSCs were alive. hDPSC constructs may also initially contain abundant fibrous ECM. A 3D cell system with AFM provides an easy method to control the shape and size of the cell spheroids. hDPSCs constructs were obtained by shaping sheet-like aggregates of hDPSCs seeded on sheets of AFM. In the self-assembled system, the cells arranged themselves in their own preferred 3D structure, therefore fully controlling their environment and behavior. Taken together, these results indicate that the self-organizing ability of the 3D hDPSCs construct was exerted within the pulpless root canal *in vivo*.

After tooth transplantation, the key requirement for successful pulp-dentin regeneration is that sufficient pulp cells occupy the tooth surface rather than the surrounding host cells. This indicates that scaffold materials have to meet higher demands to be suitable for cell homing than for cell transplantation. As mentioned above, in this study, wrapping the dentin matrix root model in a cell-fibrin complex was required to prevent the host tissue from readily entering and filling the dentin matrix canal. This autologous cell-fibrin complex scaffold formed from biodegradable polymers served as a temporary supporting structure for the growing cells and tissues before degradation.

The *in vivo* implantation in nude mice for 8 weeks showed that dentin-pulp-like tissue complexes were formed and expressed root-related antibodies. The IHC results demonstrated that the dental pulp-like tissues were positive for DMP1 and DSP throughout the hDPSCs under cell-fibrin complex induced conditions. DMP1, as an extracellular matrix protein, is essential for the mineralization of dentin [47]. At the same time, DSP is always expressed by new odontoblasts and is related to the secretion of pre-dentin matrix [48]. Angiogenesis is thought to be a key factor in pulp regeneration, because only the blood vessels are generated in the canal space, leading to the long-term stability of the newly formed tissues [22]. The positive expression of vWF in pulp suggested that the pulp-like tissue with blood supply was successfully regenerated. The expression of DMP1, DSP, and vWF showed that the dental cavity of the dentin matrix was successfully formed inside a dentin-pulp complex. The above *in vivo* results suggested that complete tooth root tissues had been successfully constructed , including dental dentin-pulp complexes, after subcutaneous transplantation in nude mice using hDPSCs and a cell-fibrin complex as a scaffold.

Researchers have speculated that an optimal combination of a hard scaffold and bio-scaffold cues in consideration of a target cell type

should be achieved for desirable bio-root tissue regeneration. Additional studies may include investigations of signaling pathways involved in bio-scaffold cues that induce cell differentiation for periodontium regeneration.

The present study proved that high-speed centrifugation removes many host immune cells, but a small amount of platelets remain in the supernatant serum. In addition, when the hDPSCs were exposed to autogenic serum conditions, they maintained their characteristic stemness. However, the hDPSCs required different concentrations for proliferation and different directions for promoting odontogenic differentiation. The hDPSCs were transplanted into the root canal of human teeth via cell-fibrin complex engineered scaffold tissues. hDPSCs cultured on the AFM formed a cell-fibrin complex that became stable and was easy to place in contact with the implanted suspended cells that secreted their own matrix to form an autogenous 3D structure that could then be delivered to the root canal.

V. CONCLUSIONS

Autologous fibrin (AF) serum is useful in root regeneration with the application of human dental pulp stem cells (hDPSCs). These conditions are suitable for cultivating hDPSCs under animal serum-free conditions in human-derived medium additives only. They provide a method of cultivating stem cells for tooth root regeneration without FBS. This study showed the potential of methods to expand autologous cells in a risk-free manner, and a cell-fibrin complex scaffold of hDPSCs to regenerate tissues could be generated autologously without the introduction of foreign scaffold materials.

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FIGURES AND TABLE

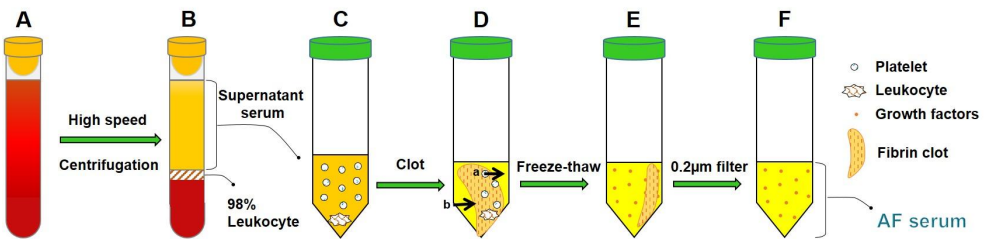

Figure 1. Fabrication of an autologous fibrin (AF) and autologous fibrin serum (AF serum).

(A) A sample of 10 mL of blood; (B) Separated into 3 layers by high-speed centrifugation; (C) Liquid serum; (D) AF (a: supernatant serum, b: AF); (E) After three freeze-thaw cycles, the AF was more compacted and formed a solid fibrin membrane; (F) AF serum after filtering.

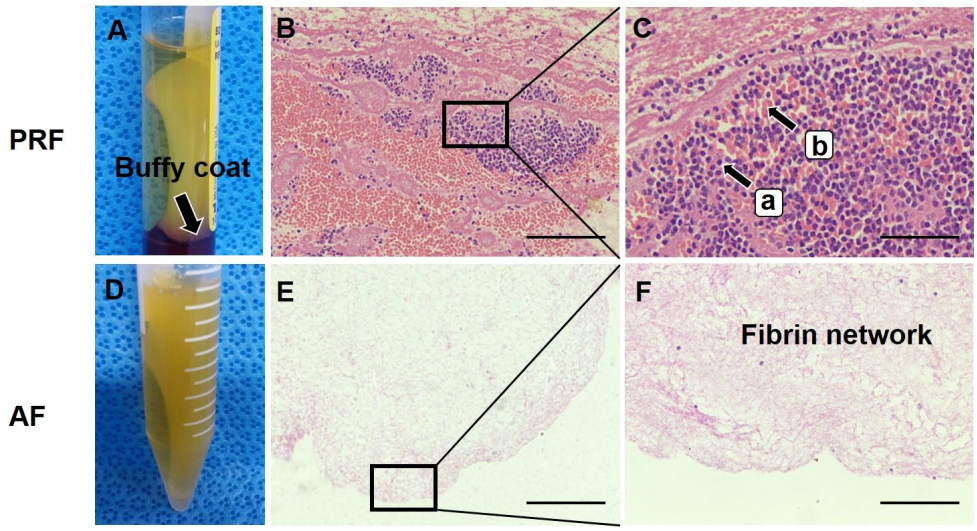

Figure 2. Autologous fibrin (AF) compared with platelet-rich fibrin (PRF).

AF and PRF were histologically examined to determine their microstructure. The total numbers of blood cells (i.e., leukocytes and RBCs) in the AF were significantly decreased compared with those of the PRF. (A) Buffy coat (arrows); (B) Low magnification of the buffy coat; (C) High magnification of the buffy coat (a: leukocytes, b: RBCs); (D) AF; (E) Low magnification of the AF; (F) High magnification of the AF (fibrin network structure). Scale bars: 100 μm, 50 μm. RBCs, red blood cells.

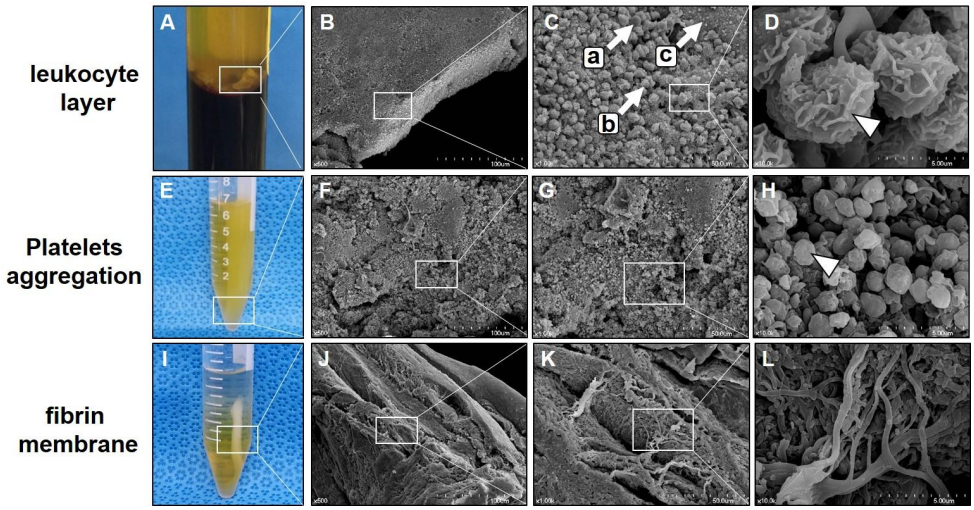

Figure 3. SEM evaluation of the autologous fibrin (AF).

Cell bodies trapped in the AF and the overall architecture of the fibrin network by SEM analysis. High-speed centrifugation traps most of the leukocytes and platelets in the middle pellet layer. Some platelets remain in the AF. After the AF was subjected to three freeze-thaw cycles, almost no platelets or leukocytes were observed in the fibrin matrix. (A) Leukocyte pellet (middle layer); (B) Low magnification of the leukocytes layer (C) (a: RBCs, b: leukocytes, c: platelet); (D) Leukocyte with irregular surface (arrow head); (E) Fibrin coagulation after the liquid serum was centrifuged again at 15 minutes at 1880× g; (F and G) Platelets aggregation (H) Platelet (arrow head); (I) Fibrin membrane after three cycles of freeze-thaw; (J and K) Fibrin membrane shrinks more compactly; (L) No platelets

can be observed in the fibrin membrane. Three different magnifications: ×500, ×1,000, and ×10,000; Scale bars: 100 μm, 50 μm, and 5 μm. SEM, scanning electron microscopy; RBCs, red blood cells.

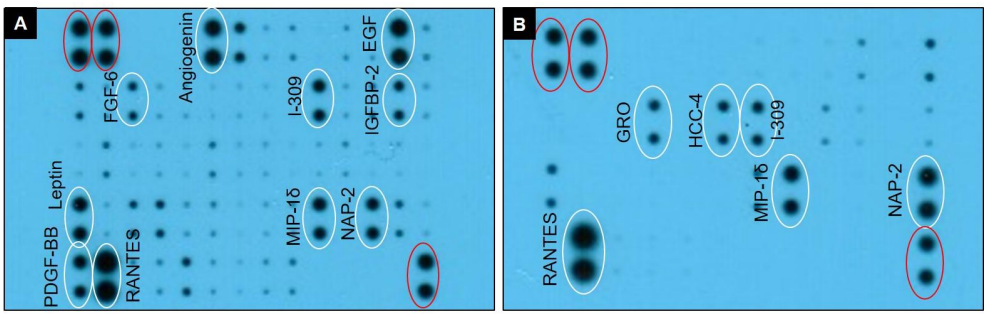

Figure 4. Evaluation of a human cytokine/chemokine array for autologous fibrin serum (AF serum).

Three positive controls (red circle) were designated in the two corners. The cytokines in the AF serum were detected. EGF, epidermal growth factor; FGF6, fibroblast growth factor 6; IGFBP2, insulin-like growth factor-binding protein 2; MIP-1δ, macrophage inflammatory protein-1δ; NAP-2, neutrophil-activating peptide-2; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated upon activation, normal T-cell expressed, and secreted; GRO, growth related oncogene; HCC-4, hemoinfiltrate C-C motif chemokine 4.

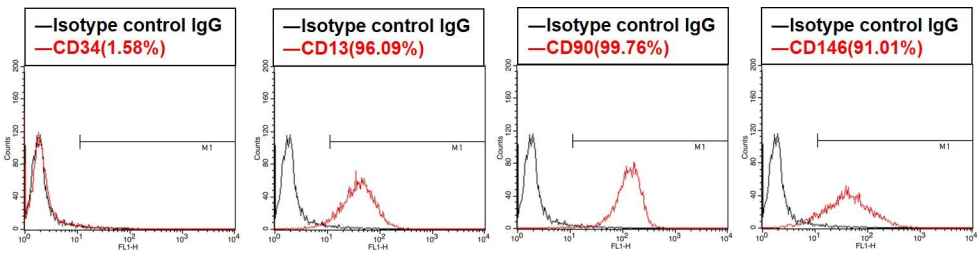

Figure 5. Characterization of human dental pulp stem cells (hDPSCs) at passage 5 by flow cytometry assay.

The hDPSCs were negative for hematopoietic cell surface markers (CD34) and showed high expression levels of multiple mesenchymal stem cell surface markers (CD13, CD90, and CD146) on their cell surface in fluorescence-activated cell sorting analysis. The percentages of the cells to the right of the M1 gate were measured (n = 3).

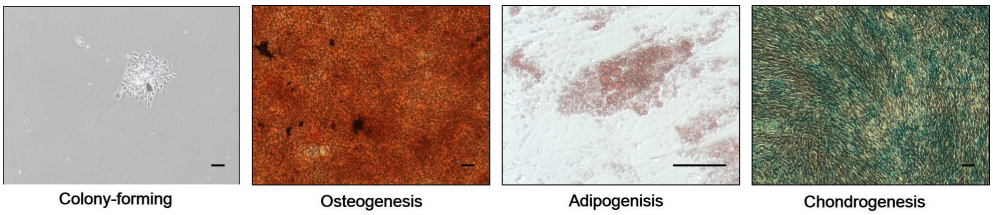

Figure 6. Characterization of human dental pulp stem cells (hDPSCs).

hDPSCs were isolated from extracted human third molars and their colony-formation was confirmed via microscopic views. To investigate the multi-lineage differentiation potential of the hDPSCs, the cells were cultured with osteogenic, chondrogenic, and adipogenic induction medium for 18 days, 21 days, and 21days, respectively. After osteogenic, chondrogenic, and adipogenic induction, hDPSCs formed extensive Alizarin Red S-positive mineral deposits, Oil Red O-positive lipid droplets throughout the adherent layers, and Alcian Blue-positive nodules, respectively. scale bar: 50 μm.

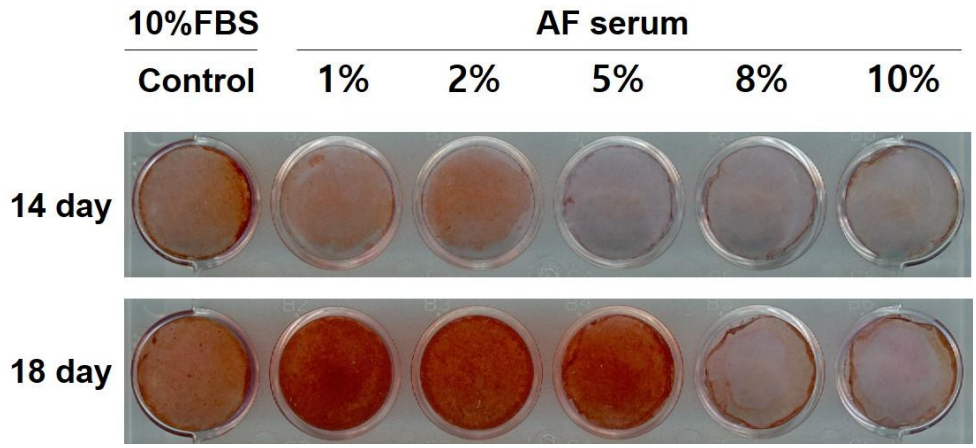

Figure 7. Osteogenic differentiation of human dental pulp stem cells (hDPSCs) *in vitro*.

hDPSCs were cultured in osteogenic differentiation medium supplemented with autologous fibrin (AF) serum compared with 10% FBS for 14 or 18 days and then stained with Alizarin Red S. Day 18 was selected as the time point of differentiation of the hDPSCs for subsequent experiments. FBS, fetal bovine serum.

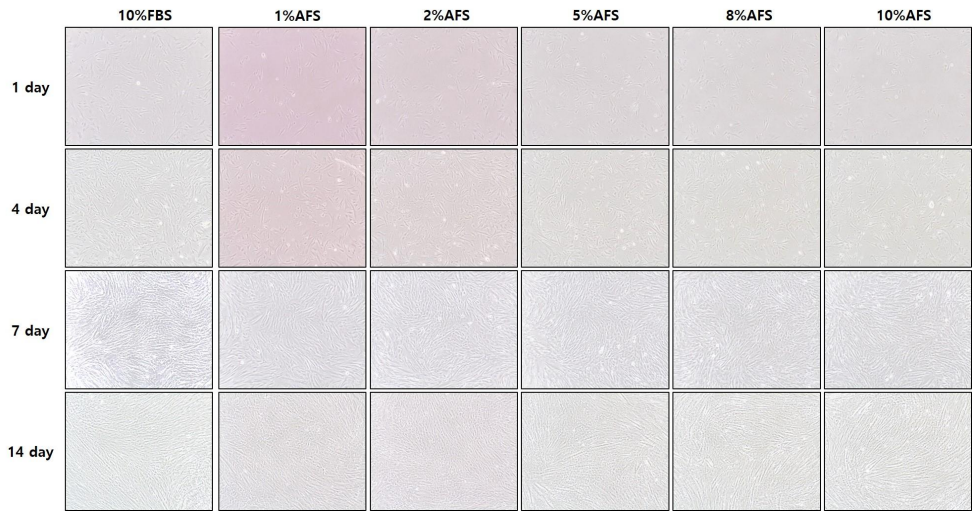

Figure 8. Morphology of human dental pulp stem cells (hDPSCs) cultured in autologous fibrin serum compared with FBS.

The specific spindle shape was confirmed via microscopic views. The hDPSCs morphology was elongated and spindle shaped. The hDPSCs morphology after treatment with AF serum gave results largely consistent with culturing in 10% FBS. FBS, fetal bovine serum.

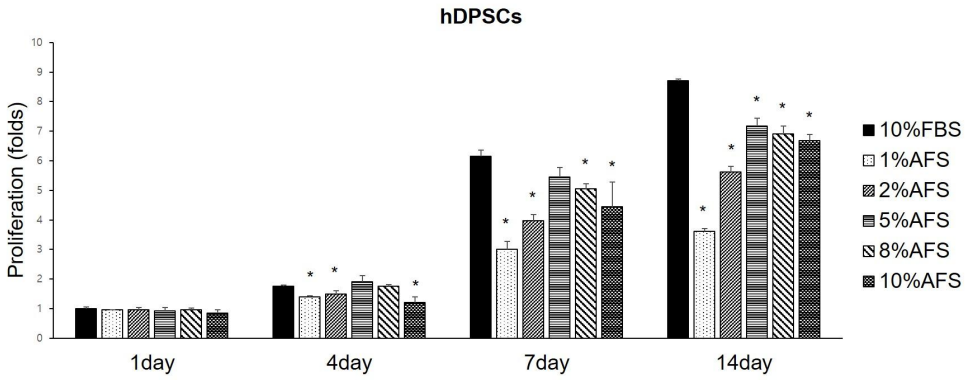

Figure 9. Effects of autologous fibrin (AF) serum on the proliferation of human dental pulp stem cells (hDPSCs) compared with 10% FBS *in vitro*.

To determine the effects of AF serum on hDPSCs proliferation *in vitro,* the cells were cultured in 1%, 2%, 5%, 8%, and 10% of AF serum compared with 10% FBS for 1, 4, 7, and 14 days. The optimized concentration of AF serum for the proliferation of hDPSCs was 5%; meanwhile, the effects of AF serum on their proliferation was inferior to 10% FBS. Significant differences versus the 10% FBS (n = 4; $*p < 0.05$).

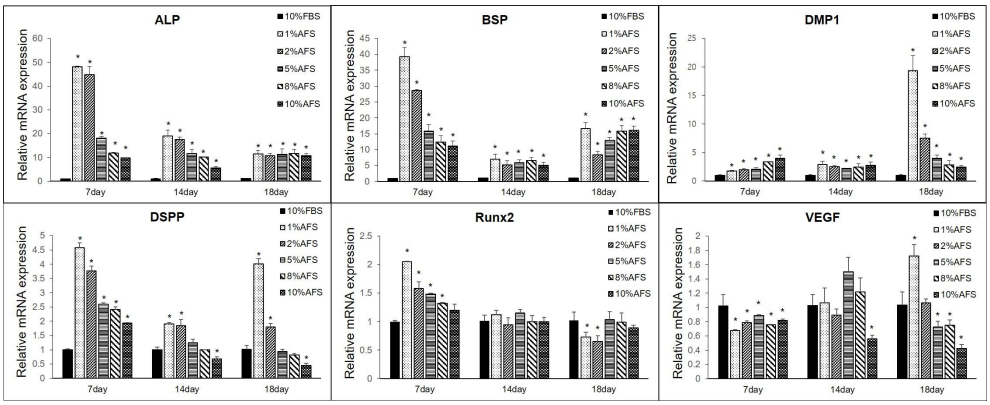

Figure 10. Effects of autologous fibrin (AF) serum on the odontogenic influence of human dental pulp stem cells (hDPSCs) *in vitro.*

To investigate the effects of AF serum on differentiation, hDPSCs were cultured in 1%, 2%, 5%, 8% and 10% AF serum compared with 10% FBS. The mRNA levels of odontogenic-related genes *ALP, BSP, DMP1, DSPP, RUNx2,* and *VEGF* on days 7, 14, and 18. Significant differences versus the 10% FBS (n = 3; $*p < 0.05$).

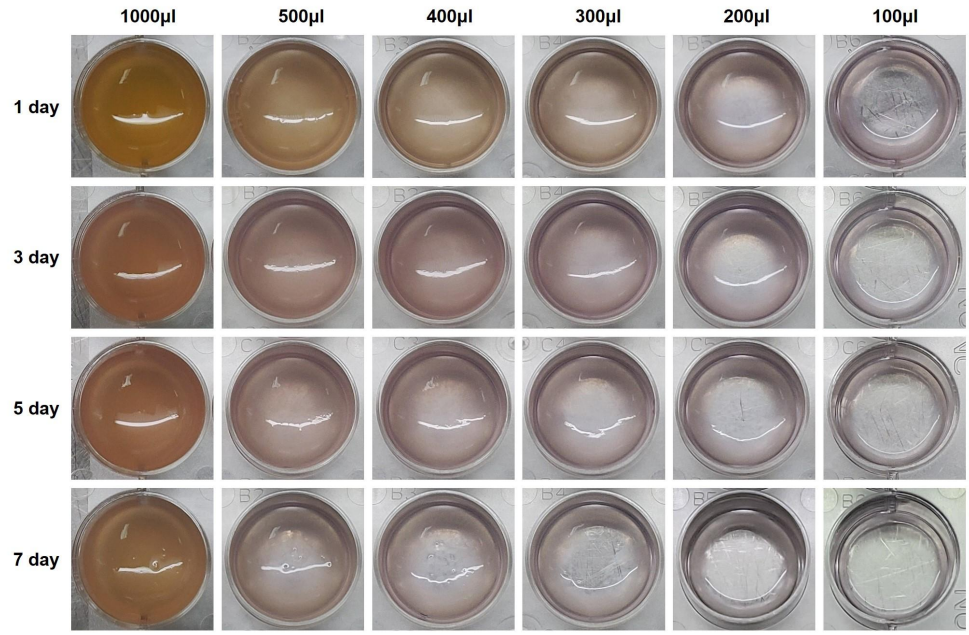

Figure 11. Images of human dental pulp stem cells (hDPSCs) cultured on the autologous fibrin matrix (cell-fibrin complex).

Autologous fibrin matrices (AFM) (100 μL, 200 μL, 300 μL, 400 μL, 500 μL, and 1000 μL) were fabricated on the 24-well plates and hDPSCs (1×10^4) were seeded onto the AFM and cultured for 1, 3, 5, and 7 days. The AFM thickness tended to decrease gradually with time.

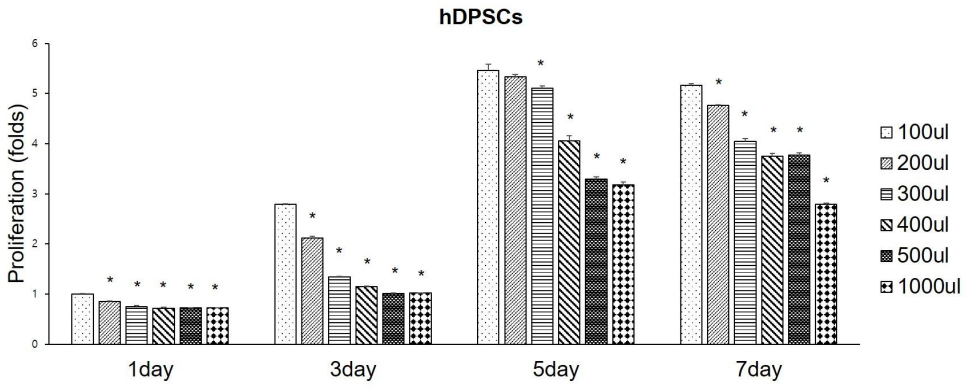

Figure 12. Optimal autologous fibrin matrix (AFM) thickness for human dental pulp stem cells (hDPSCs) culture.

The optimal AFM thickness for hDPSCs (1×10^4) cultured on AFM for 1, 3, 5, and 7 days. The thicker the AFM, the less the cell proliferation. Significant differences versus 100 μL of AFM (n = 3; $*p < 0.05$).

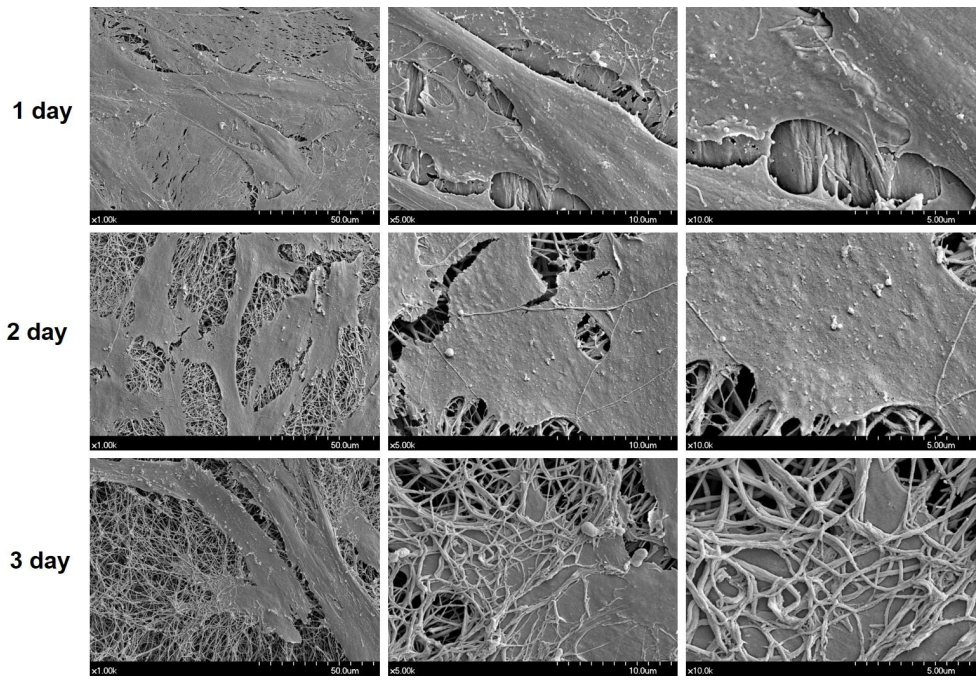

Figure 13. Infiltration of human dental pulp stem cells (hDPSCs) on autologous fibrin matrix (AFM).

The structural appearance of the hDPSCs in the three-dimensions (3D) of AFM for 3 days. As demonstrated by SEM, the hDPSCs extended out of the matrix surface on day 1, and on the second day, the cells clumped together. On the third day, the cells infiltrated into the fibrin. Three different magnifications: ×1000, ×5000, ×10000; Scale bars: 50 μm, 10 μm, and 5 μm. SEM, scanning electron microscopy.

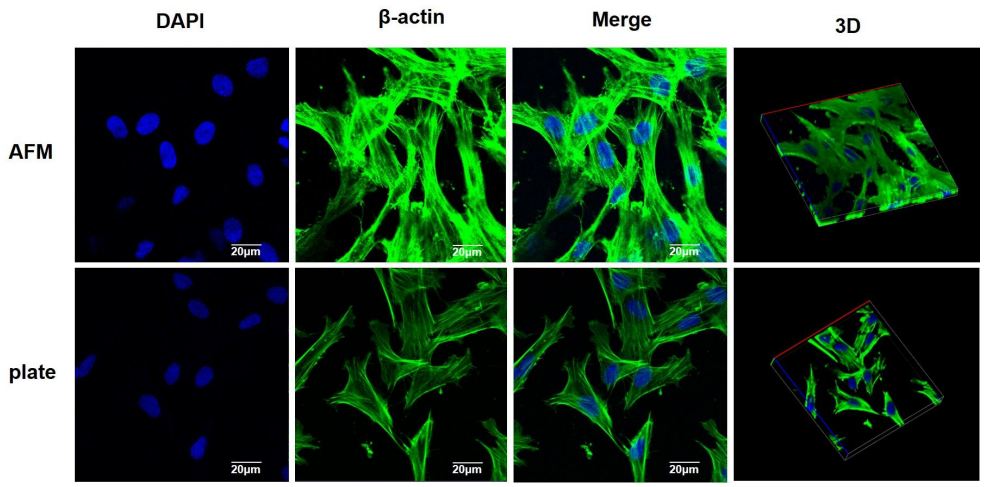

Figure 14. Morphological observation of human dental pulp stem cells (hDPSCs) on autologous fibrin matrix (AFM).

The morphological appearance of hDPSCs cultured on the three-dimensional (3D) of the AFM compared with the 2D of the plate. The hDPSCs were seeded on the surface of the AFM at 300 μl for 3 days. Immunofluorescence analyses indicated that the hDPSCs cultured on the AFM formed significantly better cell-to-cell connections than on the plates. Scale bar : 20 μm.

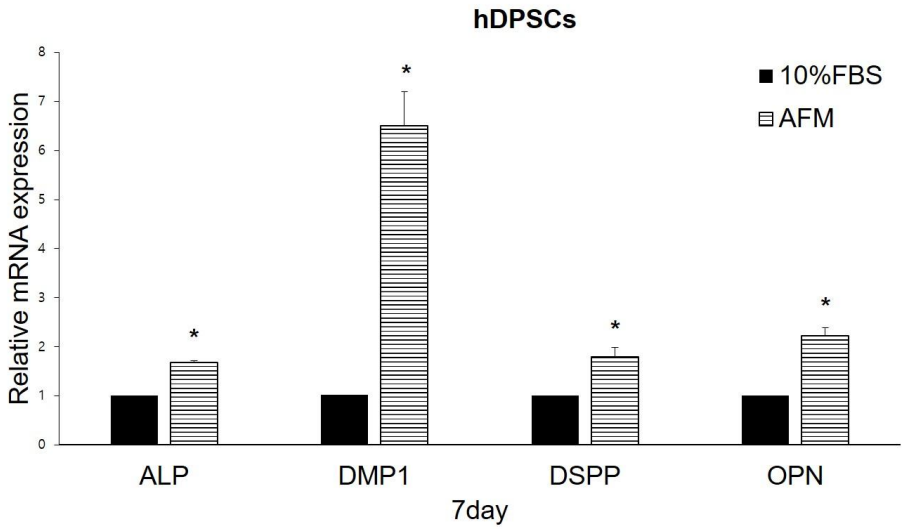

Figure 15. Evaluation of the odontogenic differentiation of human dental pulp stem cells (hDPSCs) cultured on the autologous fibrin matrix (AFM).

The expression of odontogenic-related genes in hDPSCs cultured on AFM analyzed using quantitative real-time polymerase chain reaction. The expression of *ALP, DMP1, DSPP*, and *OPN* were significantly increased in hDPSCs on AFM compared with FBS at 7 days. Significant differences versus the 10% FBS (n = 3; $*p < 0.05$).

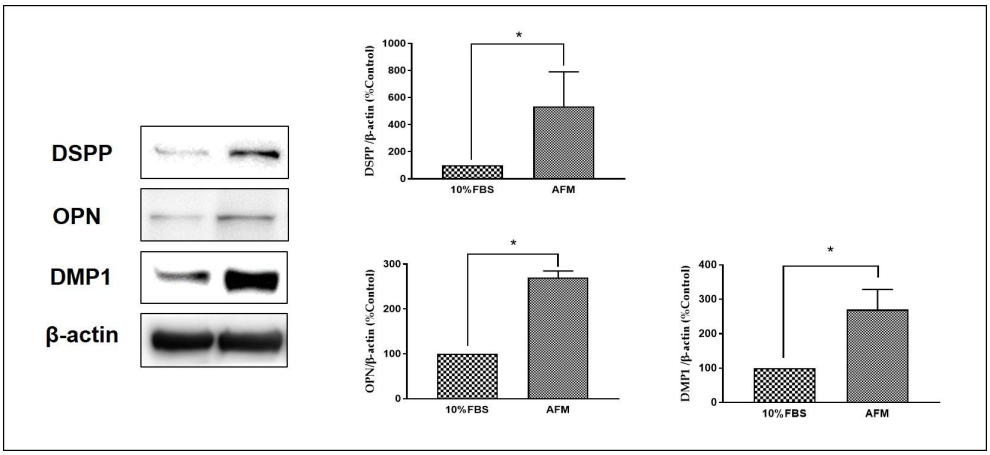

Figure 16. Investigation of odontogenic-specific protein expression by Western blot assay.

Human dental pulp stem cells (hDPSCs) were cultured on autologous fibrin matrix and compared with those cultured on a plate. The protein levels of DSPP, OPN, DMP1 in hDPSCs differentiated in odontogenic medium for 7 days by Western blot analysis where β-actin served as an internal control (left). The bands were measured by densitometric analysis of autoradiograph films (right). Significant differences versus the 10% FBS (n = 3; $*p < 0.05$).

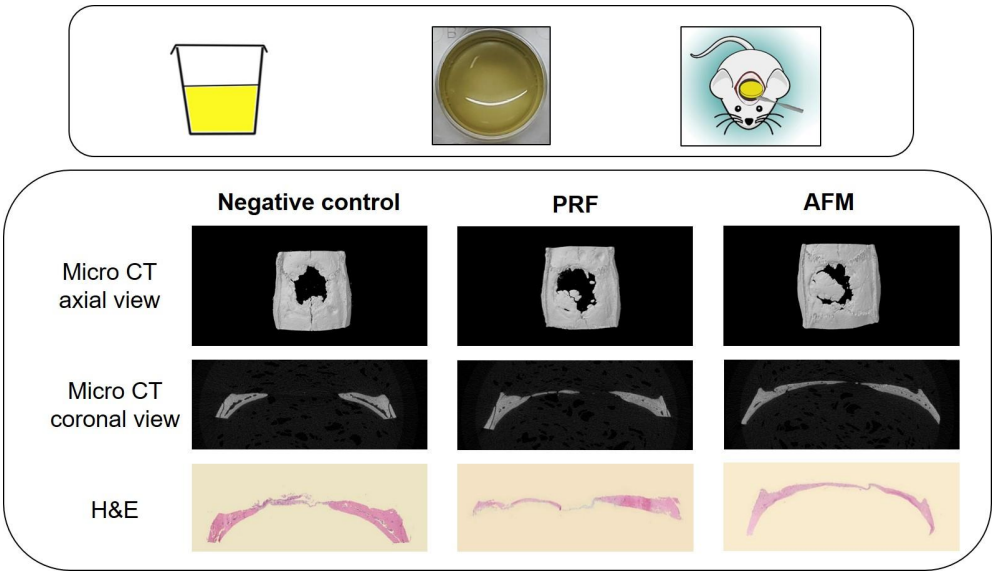

Figure 17. Effects of autologous fibrin matrix (AFM) on the rat calvarial defect model.

Three-dimension (3D) reconstructed images and healing of calvarial defects represented as a fraction of the total defect area after 6 weeks by micro-CT and H&E. Negative control group (8 mm diameter defect only); Control group (PRF transplantation); Experimental group (AFM transplantation). Micro-CT, micro-computed tomography; H&E, hematoxylin and eosin; PRF, platelet rich fibrin.

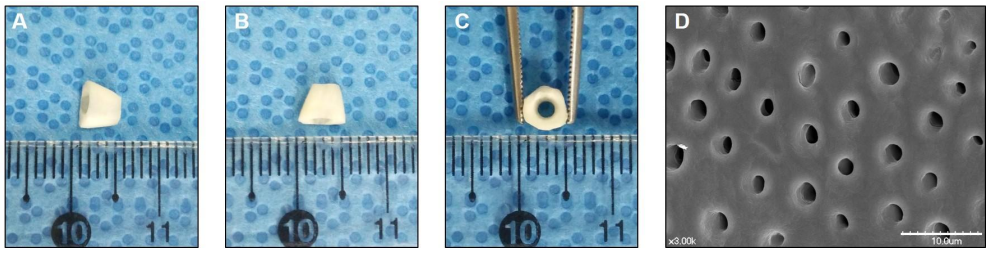

Figure 18. Morphology of the human dentin matrix.

(A–C) The final size of the dentin was 5 mm length, 5 mm diameter, with a thickness up to 1.0 mm; (D) SEM image indicates that the dentinal tubules were sufficiently exposed. SEM, scanning electron microscopy.

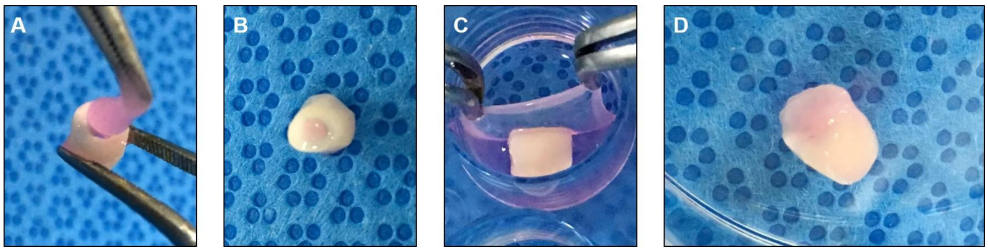

Figure 19. Image of individual components of the transplantation composite and the final construct.

Human dental pulp stem cells (hDPSCs) (1.0×10^7 cells) were mixed with the gelled autologous fibrin matrix (AFM) scaffolds and were placed into the canal space of the human dentin matrix. Then, it was wrapped in some more gelled scaffold and the entire system was transplanted subcutaneously into immunocompromised mice (BALB/c-nu) for 8 weeks. Group 1 (Control): hDPSCs + 0.5% fibrin gel; Group 2 (Experiment 1): hDPSCs + AFM; Group 3 (Experiment 2): hDPSCs + Cell-fibrin complex.

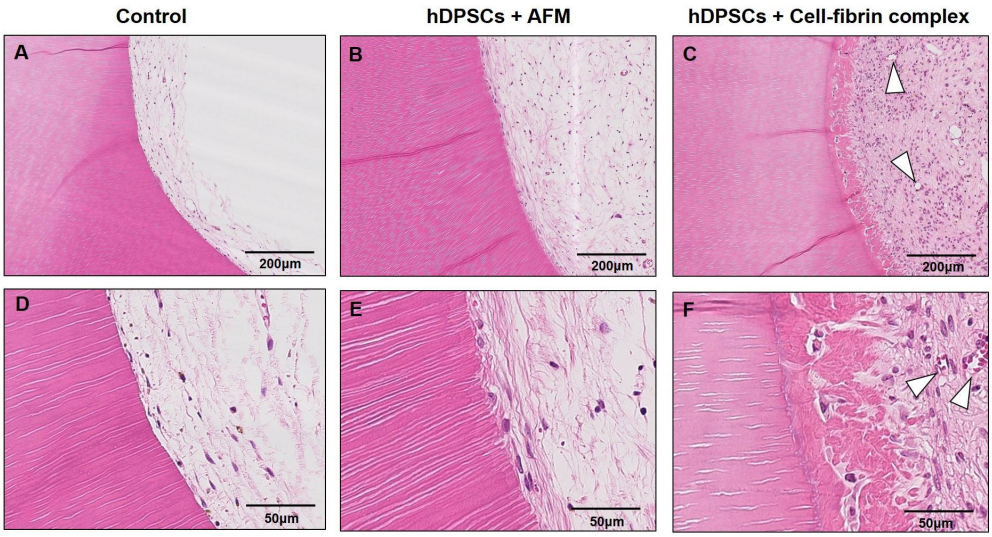

Figure 20. Effects on the human dentin matrix (AFM) surface *in vivo*.

(A and D) In the control group, no obvious dental-like tissue and sparse collagen fibers; (B and C) In the experimental groups, dental pulp-like cells are uniformly distributed over the dental pulp like-tissues; (E and F) A greater number of cells were concentrated on the inner boundary of the human dentin matrix, similar to that observed in the odontoblastic layer; (C and F) In the experimental group (cell-fibrin complex scaffold group), pre-dentin matrix-like tissues were observed on the interface between the dental pulp-like tissues and dentin matrix, along with blood vessels (arrowhead). Scale bars: 200 μm, 50 μm.

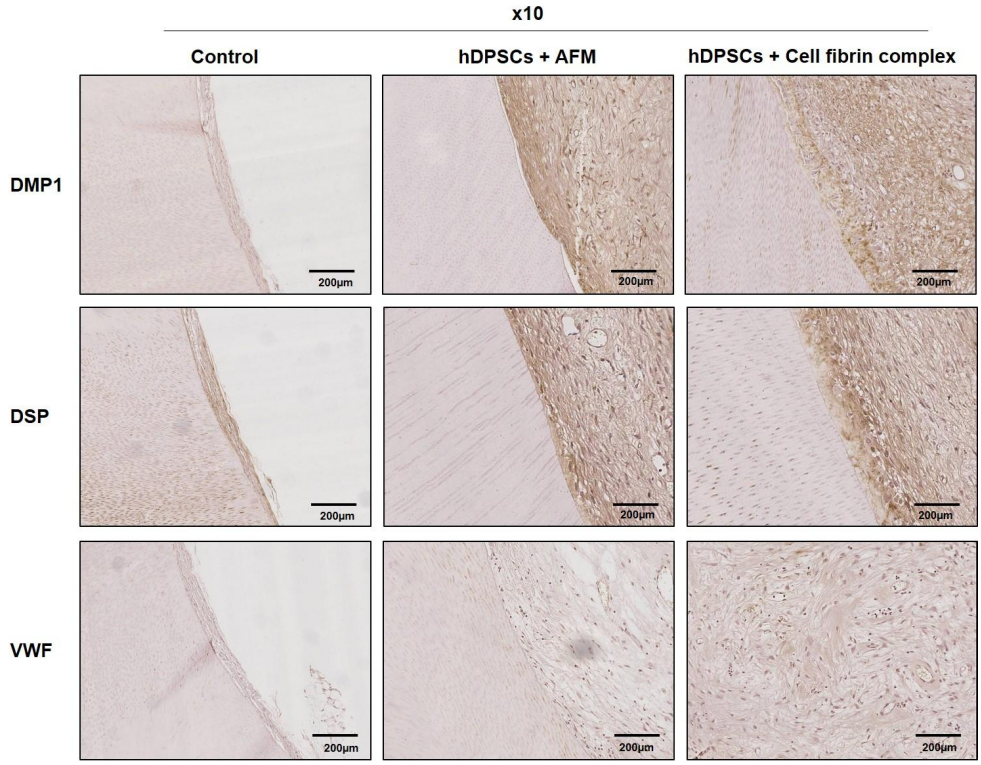

Figure 21. Immunohistochemical analysis showing the effect of the human dental pulp stem cells (hDPSCs) cultured on the autologous fibrin matrix (AFM) *in vivo* (low magnification).

Experimental groups were all positive for DMP1, DSP, and vWF antibody staining in the human dentin matrix cavity. In the control, all experimental tissues were negative for all tested markers. Scale bar: 200 μm.

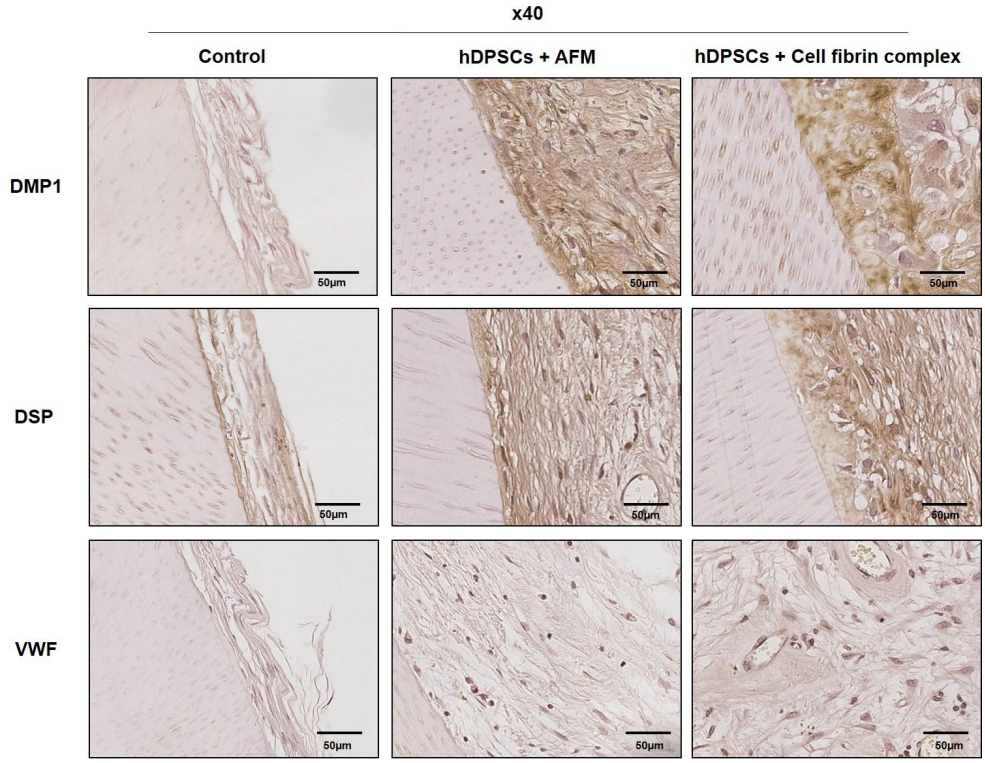

Figure 22. Immunohistochemical analysis showing the effect of the human dental pulp stem cells (hDPSCs) cultured on the autologous fibrin matrix (AFM) *in vivo* (high magnification).

Experimental groups were all positive for DMP1, DSP, and vWF antibody staining in the human dentin matrix cavity. In the control, all experimental tissues were negative for all tested markers. Scale bar: 50 μm.

Table 1. Primer sequences for the real-time polymerase chain reactions

Target cDNA	*Forward primer sequence (5'-3')*	*Reverse Primer sequence (5'-3')*
ALP	TAAGGACATCGCCTACCAGCTC	TCTTCCAGGTGTCAACGAGGT
BSP	GATTTCCAGTTCAGGGCAGTAG	CCCAGTGTTGTAGCAGAAAGTG
Runx2	CTTTACTTACACCCCGCCAGTC	AGAGATATGGAGTGCTGCTGGTC
DMP-1	CTCGCACACACTCTCCCACTCAAA	TGGCTTTCCTCGCTCTGACTCTCT
DSPP	GCAGCAATAGCAGTGAGAGCAGTGA	GCTGCTGTCACTATCGCTGCTGTTA
VEGF	NAGGGAAGAGGAGGAGATGAG	GCTGGGTTTGTCGGTGTT
OPN	CAGCCATGAATTTCACAGCC	GGGAGTTTCCATGAAGCCAC
GAPDH	CTTTGGTATCGTGGAAGGACTC	GTAGAGGCAGGGATGATGTTCT

ALP, alkaline phosphatase; *BSP*, bone sialoprotein; *Runx2,* runt-related transcription factor 2; *DMP-1*, dentin matrix protein 1; *DSPP,* dentin sialophosphoprotein; *VEGF,* vascular endothelial growth factor; *OPN,* osteopontin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase

국문 초록

사람치아 치수줄기세포에서 자가 피브린 매트릭스를 이용한 상아질-펄프 재생

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1. 연구목적

사람치아줄기세포를 배양 시 이종첨가제를 주로 사용하여 왔는데 이는 이종 기원이기 때문에 동물성 병원체 등의 감염원을 지닐 가능성이 있다. 최근 fetal bovine serum (FBS)를 대체하기 위한 임상 연구에서 혈소판 풍부 피브린 (Platelet rich fibrin; PRF)과 같은 혈소판 유도체를

포함한 인간 자가 보충제가 많이 연구되었다. 그러나 PRF는 농축된 백혈구와 적혈구를 함유하고 있으며, 그중 백혈구는 건(tendon)과 인대(ligament)에서 유전자 발현에 영향을 줄 수 있고, 적혈구는 염증반응을 촉진하는 인터루킨을 다량 함유하기 때문에 의도치 않은 염증이 유발될 수 있다고 보고되어왔다. 하여 백혈구 수를 최소화하면 인간치아 치수줄기세포 (human dental pulp stem cells; hDPSCs)의 분화에 기여할 것이라고 가설을 세웠다. 이에 본 연구를 통해 백혈구를 제거한 자가 피브린으로 체외 및 생체 내에서 사람치아 치수줄기세포 의 분화를 유도하는 능력에 대해 알아보고자 한다.

2. 연구방법

발거된 제3대구치의 치수조직에서 사람 치수줄기세포를 추출하였으며, 이를 대상으로 줄기세포 특성을 조사하였다. 사람 혈액을 고속 원심 분리하여 자가 피브린을 만들었고 그 피브린 추출물로 *in vitro* 실험을 위한 세포 배양에 사용되었으며, 또한 자가 피브린 매트릭스를 사용하여 *in vivo* 실험을 시행하였다. 우선, 혈액을 원심 분리하여 얻은 자가 피브린 매트릭스에서 백혈구가 제거되었는지 확인하기 위하여 조직학적 분석 및 주사전자현미경(scanning electron microscopy; SEM) 촬영을 통해 확인하였고, 사람 치수줄기세포에 자가 피브린 추출물을 처리한 뒤 qRT-PCR (quantitative real-time polymerase chain reaction)을 통해

치성 관련 유전자 발현을 조사하였다. 그리고 치수줄기세포를 자가 피브린 매트릭스 위에서 배양하여 줄기세포-피브린 매트릭스 혼합체를 얻어 그 효능을 Western blot을 통해 평가하였다. *in vivo* 환경에서의 영향을 확인하기 위해 누드마우스를 대상으로, 실험군으로 치수줄기세포와 자가 피브린 매트릭스 혼합체를 한 쪽 피하에 이식하였고, 대조군으로 자가 피브린 매트릭스를 실험군 반대편에 이식하였다. 8주 후 이식한 조직을 채취하여 상아질모세포(odontoblast) 분화와 상아질모세포에 의한 상아질 생성 및 특정된 단백질 발현을 immunohistochemistry로 비교 확인하였다.

3. 연구결과

유세포 분석 결과 치수줄기세포 표지인자인 CD13, CD90, CD146이 발현 되었으며, 분화를 통해 다중 분화 능력이 있는 줄기세포의 특징을 가지고 있음을 확인하였다. 조직학적 분석과 SEM으로 확인한 결과 자가 피브린 매트릭스에서 백혈구는 거의 확인되지 않았다. *In vitro*에서 자가 피브린 추출물은 경조직 분화 마커인 alkaline phosphatase *(ALP),* bone sialoprotein *(BSP),* dentin matrix protein 1 *(DMP1),* dentin sialophosphoprotein *(DSPP),* runt-related transcription factor 2 *(Runx2),* vascular endothelial growth factor *(VEGF)*의 발현이 증가한 것을 확인하였다. 또한 줄기세포-매트릭스 혼합체는 상아질모세포의 분

화과정에서 DMP1, osteopontin (OPN) 및 DSPP의 단백질 발현을 유의하게 촉진함을 확인할 수 있었다. 누드마우스의 피하에 이식한 매식체의 조직학적 분석결과 대조군에 비해 치수줄기세포와 자가 피브린 매트릭스 혼합체의 이식군에서 상아질 특정 단백질이 더욱 강하게 염색되었으며, 상아질-펄프 조직이 재생되어 있음을 확인하였다.

4. 결론

이 연구는 무위험 환경에서 자가 세포를 확장시키는 잠재적인 안전한 방법을 조사했으며, 세포 스캐폴드 복합체는 외인성 물질의 도입없이 치아 조직이 생성 될 수 있다.

1. 자가 피브린 추출물은 백혈구를 제거한후 제조되었으며 동물 무 혈청 조건하에 치아줄기세포의 증식과 분화에 유용하게 이용가능하다.

2. 자가 피브린 매트릭스에서 배양된 치아줄기세포는 세포-피브린 복합체를 형성하였으며, 이는 안정적이고 3차원적인 자가 매트릭스 구조를 형성하였다.

3. 자가 피브린 매트릭스는 생체 내 누드마우스 피하에 이식하여 상아질-펄프 복합체의 성공적인 재생에 우수한 운반체로서 유용하게 이용할 수 있음이 입증되었다.

4. 임상적 의미에서 자가 피브린 매트릭스는 우수한 생체 적합성 및 세포 침윤을 보유하며 치아줄기세포에서 치아조직 재생에 유용하다는 것을 알 수 있다.

주요어 : 자가 피브린, 사람치수 줄기세포, 혈소판 풍부한 피브린, 백혈구, 상아질, 재생

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