Development of a Mouse Model for Dental Pulp Disease and Biodistribution of Human Dental Pulp Stem Cells transplanted Intra-pulpally in the Mouse

Sunil Kim

The Graduate School

Yonsei University

Department of Dentistry

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Directed by Professor Euiseong Kim

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This certifies that the Doctoral Dissertation

of Sunil Kim is approved.

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Thesis Supervisor : Euiseong Kim

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Chan-Young Lee

_____

Han-Sung Jung

_____

Su-Jung Shin

_____

Sun-Young Kim

The Graduate School

Yonsei University

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먼저 논문의 시작부터 끝까지 저를 이끌어 주시고 격려해 주신 김의성 교수님께 진심으로 감사드립니다. 2014년 초여름에 논문 실험 주제를 정했을 때, 과연 내가 할 수 있을까? 라는 의문이 있었으나 2년의 기간 동안 교수님의 소중한 가르침으로 의문이 확신으로 바뀔 수 있었고, 이렇게 박사 논문을 마무리할 수 있게 되었습니다. 그리고 수련 기간과 강사로 근무하는 동안 관심과 격려를 아끼지 않으셨고, 이번 논문이 완성되는 데 큰 도움을 주신 이찬영 교수님과 힘들 때마다 진심 어린 조언을 해 주신 신수정 교수님께 감사의 말씀을 드립니다. 조직학 분야에 대한 정확한 감수를 도와주신 정한성 교수님과 논문 구성을 꼼꼼히 살펴 주신 김선영 교수님께도 깊은 감사를 드립니다. 또한, 존경하는 이승종 교수님, 석사 과정 때부터 많은 가르침을 주신 노병덕 교수님, 언제나 격려해 주시는 박성호 교수님, 따뜻한 조언을 아끼지 않으시는 정일영 교수님, 늘 모범이 되어 주시는 박정원 교수님, 곁에서 도움 주신 신유석 교수님께도 감사드립니다.

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김 선 일

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Abstract

Development of a Mouse Model for Dental Pulp Disease and Biodistribution of Human Dental Pulp Stem Cells transplanted Intra-pulpally in the Mouse

Sunil Kim

Department of Dentistry

The Graduate School, Yonsei University

(Directed by Professor Euiseong Kim)

The aims of this study were (1) to establish the mouse model for pulp-dentin complex regeneration research and (2) to test *in vivo* fate of intra-pulpal transplanted dental pulp stem cells in comparison with those of intravenously trasnplanted DPSCs using the developed the mouse model.

In order to establish the mouse model, a total of 45 mice had their pulp exposed, and five mice each were sacrificed at 1, 2, 4, 7, 9, 12, and 14 days after pulp exposure. As a control group to check normal pulp status, unprepared teeth were used. In order to verify the pulp necrosis and formation of apical lesions, five mice were sacrificed 28 and 35

days after pulp exposure. Histologic changes over time were observed by light microscope. In order to test *in vivo* biodistribution of intra-pulpal and intravenously transplanted human DPSCs, normal impacted third molars were collected from volunteer and hDPSCs were isolated and cultured using the method, as described by Gronthos et al. Under deep anesthesia, 1 x 10^5 hDPSCs were transplanted a mouse through the tail vein for intravenous transplantation or pulp chamber for intra-pulpal transplantation. After transplantation of hDPSCs, mice were sacrificed at various time periods, and the number of hDPSCs in the organs were analyzed quantitatively. Also, qualitative analysis was performed to detect intra-pulpally transplanted hDPSCs in the pulp chamber.

Up to seven days after exposure of the dental pulp in mice, inflammation and necrosis was limited to the area superior to the orifice level. By 14 days after pulp exposure, necrosis had progressed as far as the root apices, and all the cells and tissues in the root canals had undergone necrosis. Intravenously injected hDPSCs were mostly distributed to the lungs. The number of hDPSCs in the lungs continued to decrease at 15 minutes, 30 minutes, and up to 12 hour after hDPSCs transplantation. hDPSCs were rarely detected in other organs except the lungs throughout all time periods. The hDPSCs transplanted into the pulp chamber rarely migrate to other organs across all time periods. Even after 12 hours had passed, the viable cells were found within the pulp chamber.

The results suggest that there seems to be no safety problem in cell based approach which transplants hDPSCs into pulp chamber for pulp-dentin complex regeneration.

Keywords: mouse model, biodistribution, dental pulp stem cell, dental pulp, regeneration

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I. Introduction

Since Kakehashi reported that bacteria caused pulpitis in his mouse experiment in 1965, endodontic treatment has developed with the goal of the complete removal of bacteria from the root canal (Kakehashi et al., 1965). It was believed that once exposed to bacteria, the dental pulp would fall into a state of irreversible pulpitis that could never be restored to the normal state and that the progression of inflammation would result in necrosis. The dental pulp tissues that are damaged due to bacterial invasion or dental trauma were considered should be removed. Therefore, the conventional root canal treatment focuses on three-dimensional mechanical preparation, disinfection, and the tight sealing of the

root canal space to completely eliminate the dental pulp and bacteria in the root canal and prevent re-infection (Schilder, 1974). However, this treatment does not regeneration of the pulp-dentin complex but repair. Therefore, many researchers have been trying for regeneration of the pulp-dentin complex through tissue engineering, using stem cells.

Mesenchymal stem cells (MSCs) were first discovered by Friedenstein et al. (Friedenstein et al., 1976) from bone marrow. MSCs have the potential to differentiate into a variety of cell types forming bone, cartilage, muscle, tendon, ligament and fat (Gao et al., 2001). Since Friedenstein's discovery of these cells, the interest in adult MSCs has been progressively growing. In recent years, various stem cells have been isolated from the oral cavity, including dental pulp stem cells, stem cells from exfoliated deciduous teeth, periodontal ligament stem cells, dental follicle progenitor cells, alveolar bonederived MSCs, stem cells from apical papilla, tooth germ progenitor cells, and gingival MSCs (Liu et al., 2015).

In 2000, Gronthos and Shi isolated dental pulp stem cells (DPSCs) from human third molars for the first time, and these DPSCs were characterized as highly proliferative cells with self-renewal multi-differentiation properties *in vitro* (Gronthos et al., 2002; Gronthos et al., 2000). They also reported that when DPSCs were transplanted into immunocompromised mice, they generated a dentin-like structure lined with human odontoblast-like cells that surrounded a pulp-like interstitial tissue (Gronthos et al., 2000). DPSCs differentiate not only as osteoblast and odontoblast, but also adipocytes, neurons, chondrocytes (Gronthos et al., 2002). The dental pulp is vascularized and characterized

by innervated loose connective tissue that contains heterogeneous cell populations (Trowbridge, 2003). Therefore, the strong angiogenic and neurogenic potentials of DPSCs which can be easily isolated from teeth have attracted much attention in the study of pulp-dentin complex regeneration (Huang, 2009; Nakashima et al., 2009).

There have been many studies attempting to use DPSCs to achieve pulp-dentin complex regeneration at an *in vitro* level. Unlike *in vitro* environment that can be easily control several factors, transplanting a mixture of DPSCs, growth factors and scaffolds into animal teeth should require particularly advanced skills and techniques. Because of these difficulties, *in vivo* studies on pulp-dentin complex regeneration up to now have usually involved ectopic transplantation of the candidate substance into the subcutaneous tissue or the renal capsule, rather than orthotopic transplantation directly into the teeth (Kim et al., 2015). However, before applying these candidate cells in clinical trials, their treatment efficacies should be evaluated *in vivo* orthotopic transplantation using animal teeth.Therefore, several researches on the orthotopic transplantation of DPSCs have been performed. These studies have been performed in large animals, such as dogs, pigs, ferrets and monkeys (Iohara et al., 2014; Iohara et al., 2013; Iohara et al., 2004; Iohara et al., 2009; Kodonas et al., 2012; Torabinejad et al., 2011; Zhu et al., 2012), because mice or rats teeth of which diameters of only 2-5 mm are too small to transplant a mixture of DPSCs, growth factors and scaffolds. However, experiments using large animals are restricted by the breeding and costs involved in securing a sufficient number of experimental animals. Whereas mice are relatively inexpensive, reproduce quickly, and

can be easily manipulated genetically. Therefore, mice have been a species of choice to study stem cell biology in mammals (Harding et al., 2013).

Whether the transplanted hDPSCs are limited to the pulp chamber-the target site-is also important for preventing side effects due to the multipotency of MSCs. MSCs have been shown to possess the capability to differentiate into a variety of cell type. Also, recent studies indicate that stem cells and tumor cells share many common master regulatory genes (Ben-Porath et al., 2008; Chiou et al., 2008; Sperger et al., 2003). The interwoven nature of multipotency and tumorigenicity programs is revealed by the molecular machinery shared by them, and it has become a major challenge to untangle the determinants of multipotency from those responsible for tumorigenicity. Furthermore, the ability of intravenously administered MSC to migrate to the sites of microscopic tumor lesions was confirmed by micro-PET imaging (Hung et al., 2005). Human immortalized MSC injected in the tail vein of mice with preestablished colon cancer cells localized in capillary-like structures and within the inner layer of small arterioles and in connective tissue of the tumor, and enhanced tumor growth (Hung et al., 2005). Similarly, human fetal BM-MSC injected into tail vein of SCID mice with human malignant melanoma incorporated into tumor vessels (Sun et al., 2005). These findings indicate that hMSC may contribute to the development of vascular and connective tissue component of tumor stroma.

Because of these characteristcs of MSCs, there is a risk of malignancies if hDPSCs transplanted into the pulp chamber travel to other organs in the blood. Thus, it is crucial

to not only assess the efficacy of pulp-dentin complex regeneration by DPSC transplantation through animal studies, but also to verify the *in vivo* fates of DPSCs transplanted into the body. Previous studies showed that most of MSCs were entrapped in the lung immediately after intravenous transplantation, with some MSCs undergoing apoptosis (Bagi and Kaley, 2009). After 10 min, these trapped MSCs gradually returned to the blood circulation and redistributed to other organs (Lee et al., 2009). The mechanisms of these phenomena are still unclear (Wei et al., 2013). In addition to intravenous transplantation, there have been studies analyzing the systemetic distribution and retention of stem cells in the target organ after direct transplantation into a target organ (Shim et al., 2015; Wang et al., 2016; Wood et al., 2012). While *in vivo* fate studies using stem cells are being actively performed in other areas of medicine, current understanding of the *in vivo* fate of systemically and locally administrated DPSCs is limited in dentistry.

In the field of pulp-dentin complex regeneration, the lack of research on the fate of transplanted stem cells and treatment efficacies might be due to the absence of a small animal model. The efficacy of pulp regeneration should be assessed qualitatively and quantitatively via the use of animal model. Thus, the development of a reproducible transplantation animal model is essential for obtaining precise and accurate data *in vivo* (Obokata et al., 2011).

Therefore, the purposes of this study were (1) to establish the mouse model for pulp-dentin complex regeneration research and (2) to test *in vivo* fate of intra-pulpal

transplanted DPSCs in comparison with those of intravenously trasnplanted DPSCs using the developed the mouse model.

II. Material and Methods

1. Animal Model for Dental Pulp Disease

1.1 Experimental Group Design

In order to verify the appearance of inflammation progression over time, a total of 45 mice had their pulp exposed, and five mice each were sacrificed at 1, 2, 4, 7, 9, 12, and 14 days after pulp exposure. As a control group to check normal pulp status, unprepared teeth were used. In order to verify the pulp necrosis and formation of apical lesions, five mice were sacrificed 28 and 35 days after pulp exposure.

1.2 Animal Experiment

All animal experiments were performed in accordance with the Guideline for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Yonsei University, and were approved by Committee (experimental protocol number 2015-0029). Dental pulp inflammation animal model experiments were performed using 7-week-old male ICR mice supplied by Orient Bio, Inc. (Seungnam, Kyonggi-Do, Republic of Korea). All procedures were performed under the surgical operating microscope (Global Surgical, St. Louis, MO, USA), except for the anesthesia. Following anesthesia by intra-peritoneal injection of 0.018 cc of Zoletil 50® (Virbac, Carros, France) and 0.012 cc of Rompun®

(Bayer, Leverkusen, Germany) mixture (Fig. 1A), a cavity was prepared with a carbide bur (diameter 0.5mm) (H1.FG.005; Komet, Gebr Brasseler GnbH & Co KG, Lemgo, Germany) on the occlusal aspect of the maxillary first right molar, in the center of the tooth according to the mesio-distal plane until the pulp was exposed. After pin point pulp exposure, access opening was subsequently enlarged mechanically using an endodontic hand file of #15 and #20 (K-file®; Dentsply Maillefer, Ballaigues, Switzerland) (Fig. 1B);

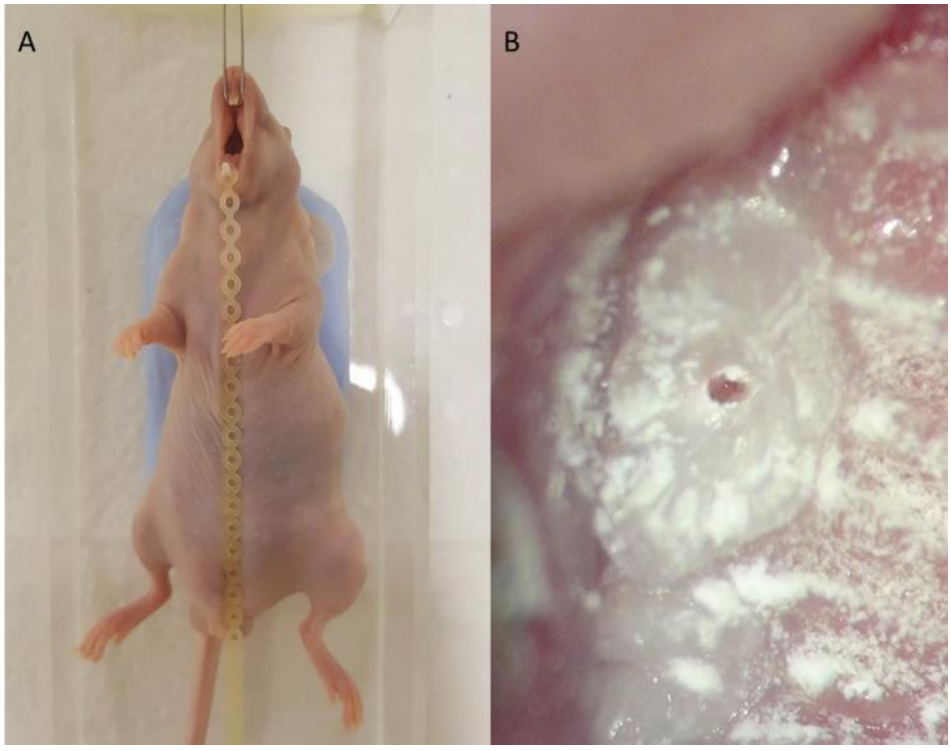

Fig. 1. Clinical procedure of pulp exposure. (A) After anesthesia by intra-peritoneal injection of Zoletil 50® and Rompun®, mouse was fixed with wire and elastic. (B) After preparing cavity with a bur, the pulp was exposed using an endodontic hand file of #15 and #20.

this approach enabled control of pulp exposure size to approximately 200 μm (size of the tip of the K-file). The cavity was not sealed to maintain the bacterial invasion into the dental pulp.

Animals were sacrificed at various time periods following the experimental design. In order to obtain a better pulp tissue fixation, prior to the tooth and the alveolar bone collection, animals were sacrificed by intra-cardiac perfusion with 4% paraformaldehyde (PFA) (Sigma Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) 0.1 mol L^{-1} through the left ventricle. Following removal of most of the soft tissues, maxilla of mice were immersed 4% PFA for 24 hour at 4 ℃.

1.3 Histology

The maxilla of 45 experimental animals and control animals were dissected, rinsed in PBS for 60 min, and decalcified for 4 weeks in a 5% ethylenediamine tetraacetic acid (EDTA) with 4% sucrose in PBS with pH 7.4 and agitation at room temperature. The solution was renewed every week. After decalcification, samples were dehydrated through increasing grades of isopropyl alcohol (Duksan, Seoul, Republic of Korea). The dehydration process consisted of a series of isopropyl alcohol rinses starting from 70% solution for one hour and followed by 80%, 90%, 95% and 100% solution for one hour. After dehydration, samples were embedded in paraffin block according to standard procedures. After embedded in paraffin, the samples were cut with a microtome (Leica,

Berlin, Germany) in sections of 6 μm, and then stained with haematoxylin and eosin (H&E). Slides were observed under a high magnification of 40x to 400x by light microscope (Nikon, Tokyo, Japan).

2. Biodistribution of Intravenous and Intra-pulpal Transplanted Human Dental Pulp Stem Cells in Mouse

2.1 Isolation, Culture, and Characterization of hDPSCs

hDPSCs were isolated and cultured using the method, as described by Gronthos et al (Gronthos et al., 2000). Volunteers were recruited from patients who were scheduled to extract third molar in the Department of Conservative Dentistry or Oral and Maxillofacial Surgery, Yonsei University Dental Hospital. Informed consent was obtained from each subject and the research protocol was approved by the Institutional Review Board of Yonsei University Dental Hospital, Seoul, Republic of Korea (IRB number: 2-2015-0055). Normal impacted third molars were collected from adults (19-29 years of ages). The teeth were extracted gently paying attention not to be fractured. After extraction, tooth surfaces were cleaned and cut around the cementum-enamel junction by using sterilized dental carbide burs to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root and then digested in a solution of 4 mg/ml collagenase type I (Gibco, Carlsbad, CA, USA) and 4 mg/ml dispase (Gibco) for 1 h at 37℃. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (Falcon). Single-

cell suspensions (0.01 to 1 x 10^5 / well) of dental pulp were seeded into 100 mm culture dish (Falcon) with alpha modification of Eagle's medium (Gibco) supplemented with 15% FBS (Gibco) / 100 μM L-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka, Japan) / 2 mML-glutaminey100 units/ml penicillin / 100 μg/ml streptomycin (Gibco), and then incubated at 37℃ in 5% CO_2.

hDPSCs were characterized with respect to cell surface marker expression and differentiation potential. Cell surface marker expression was examined by flow cytometry (FACSverse; BD Biosciences, San Jose, CA, USA) using specific antibodies against CD45, CD73, CD90 and CD105 (BD Biosciences). Flow cytometry data were analyzed using FlowJo (v. 10.0, FlowJo, Ashland, OR, USA).

Osteogenic, adipogenic and chondrogenic differentiation was assessed by von Kossa, Oil red O and Alcian Blue staining, respectively. Bone marrow mesenchymal stem cells (Stem cell therapy center, Yonsei university health system, Seoul, Republic of Korea) were used as a control. For osteogenic differentiation, hDPSCs were plated at 5 x 10^3 cells/cm^2 in 12-well plates and incubated in α-MEM supplemented with 10% fetal bovine serum (FBS) for 7 days and then the cultures were incubated in α-MEM supplemented with 10% FBS based osteogenic differentiation medium that was supplemented with 50μg/ml ascorbate 2-phosphate (Sigma Aldrich), 10^{-8} M dexamethasone (Sigma Aldrich), and 10 mM β-glycerophosphate (Sigma Aldrich). The culture was incubated at 37℃ with 5% CO_2, and the medium was changed every 3 days for 4 weeks. The cells were fixed with 4% formaldehyde for 10 min at room temperature and stained with von Kossa.

For adipogenic differentiation, hDPSCs were plated at 5 x 10^3 cells/cm^2 in 12-well plates and incubated in adipogenic differentiation medium (Gibco, StemPro® Adipogenesis Differentiation Kit). The culture was incubated at 37℃ with 5% CO_2, and the medium was changed every 3 days for 7 weeks. The cells were fixed with 4% formaldyhyde for 10 min, and stained with Oil Red O.

A pellet culture system was used for chondrogenic differentiation. Approximately 2.5 x 10^5 hDPSCs were placed in a 15-ml polypropylene tube (Falcon, Bedford, MA, USA), and centrifuged to a pellet. The pellet was cultured at 37℃ with 5% CO_2 in 500 μl α-MEM supplemented with 10% FBS based chondrogenic differentiation media (hMSC Differentiation BulletKit® –Chondrogenic, cat#PT-3003, Lonza, Cologne, Germany) supplemented with 10 ng/ml TGF β3 (Sigma Aldrich). The pellet culture medium was changed every 3 days for 4 weeks. For microscopy, the pellets were fixed with 4% formaldehyde for 10 min at room temperature and embedded in paraffin, the samples were cut with a microtome (Leica), and then stained with Alcian Blue.

2.2 Experimental Group Design

2.2.1. Intravenous Transplantation Group

After transplantation of hDPSCs into the tail vein, mice were sacrificed after 15 minutes, 30 minutes, 1 hour, 6 hours, and 12 hours, and the number of hDPSCs in the heart, liver,

spleen, kidneys, lungs, brain, and lymph nodes was analyzed quantitatively. Four mice were used for each group.

2.2.2. Intra-pulpal Transplantation Group

After transplantation of hDPSCs into the pulp chamber, mice were sacrificed after 15 minutes, 30 minutes, 1 hour, 6 hours, and 12 hours, and the number of hDPSCs in the heart, liver, spleen, kidneys, lungs, brain, and lymph nodes was analyzed quantitatively. Also, qualitative analysis was performed by immunohistochemical (IHC) staining to detect intra-pulp transplanted hDPSCs in the pulp chamber. Four mice were used for each group.

2.3 Animal Experiment - *in vivo* Transplantation of hDPSCs

In vivo experiments were performed using 7-week-old male Balb/c nude mice supplied by Orient Bio, Inc.

2.3.1. Intravenous Transplantation Group

Under deep anesthesia by intra-peritoneal injection of 0.018 cc of Zoletil 50® and 0.012 cc of Rompun® mixture, mice were trasnplanted with 1 x 10^5 hDPSCs through the tail vein for intravenous transplantation. hDPSCs at passage four were used for experiments.

To assess whether mouse tissues contain human DNA, animals were sacrificed at different times after hDPSCs transplantation and tissues were analyzed by real time polymerized chain reaction (RT-PCR). Under deep anesthesia with 0.018 cc of Zoletil 50® and 0.012 cc of Rompun® mixture, animals were sacrificed by 100% carbon dioxide inhalation. The heart, liver, spleen, kidney, lung, brain and lymph node were excised and collected for genomic DNA (gDNA) extraction.

2.3.2. Intra-pulpal Transplantation Group

All procedures were performed under the surgical operating microscope except for the anesthesia. Following anesthesia by intra-peritoneal injection of 0.018 cc of Zoletil 50® and 0.012 cc of Rompun® mixture, a cavity was prepared with a carbide bur (diameter 0.5mm) on the occlusal aspect of the maxillary first right molar, in the center of the tooth until the mesial orifice and distal orifice of the root canals were exposed. After preparation of access cavity, 1 x 10^5 hDPSCs pellet inserted into the pulp chamber. hDPSCs at passage four were used for experiments. Subsequently, the access cavity was sealed with self-adhesive resin cement (RelyX™ U200; 3M ESPE, St Paul, MN, USA).

Animals were sacrificed at various time periods according to the experimental design. Under deep anesthesia with 0.018 cc of Zoletil 50® and 0.012 cc of Rompun® mixture, animals were sacrificed by 100% carbon dioxide inhalation. The heart, liver, spleen, kidney, lung, brain and lymph node were excised and collected for gDNA extraction (Fig.

2). The maxilla of mice were collected for histologic and IHC observation and immersed 4% PFA for 24 hours at 4 ℃.

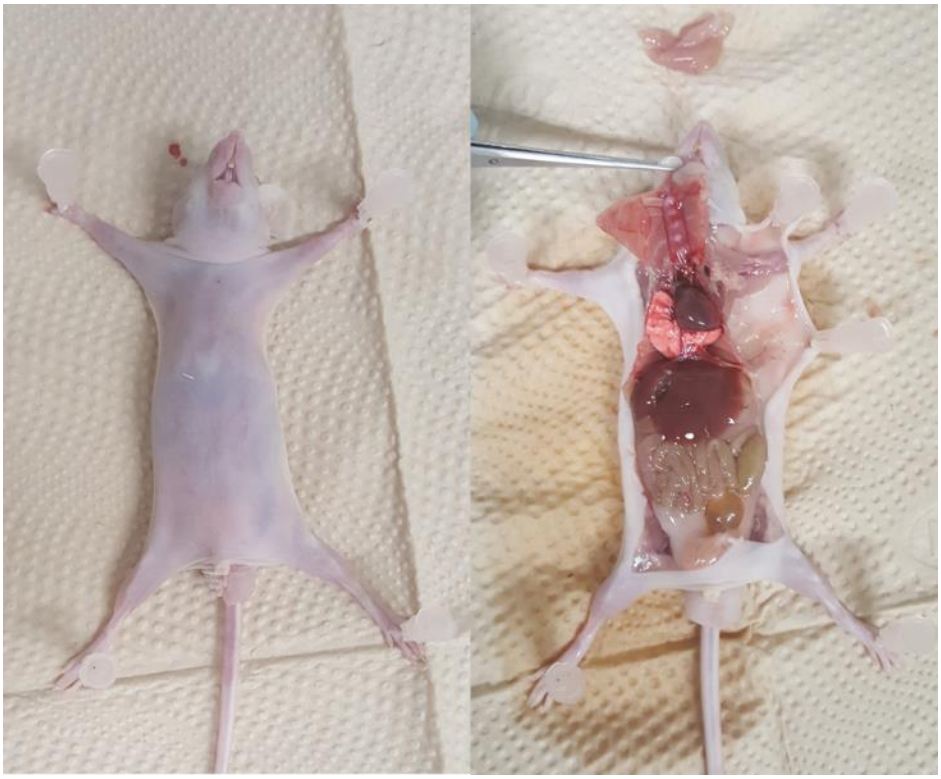

Fig. 2. (A) After anesthesia by intra-peritoneal injection of Zoletil 50® and Rompun®, mouse was fixed with needle. (B) The heart, liver, spleen, kidney, lung, brain and lymph node were excised and collected for gDNA extraction.

2.4 Quantitative Analysis of Injected hDPSCs: gDNA Extraction and PCR Analysis of β-globin

The biological samples were submitted to gDNA extraction and PCR analysis to detect the presence of human cells in mice recipients. gDNA was extracted from collected

organs and purified using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). A human β-globin element in hDPSCs gDNA amplifications were performed in accordance with the standard recommended amplification conditions (Applied BioSystems, Foster City, CA, USA) as previously described by Heid et al. (Heid et al., 1996). The value of DNA contained in each somatic cell (diploid) is 6.16 pg with two copies of non repeated gene. This value was used to calculate the number of gene copies that contain a certain amount of human DNA (measured by PCR). Therefore, DNA and copy number are proportional to the number of cells. Amplification of human β -globin gene was used to quantify the amount of human DNA in each sample of mouse tissue after DNA extraction. Absolute standard curves were generated for the human β-globin genes and used to quantify the amount of human DNA in each mouse tissue. One hundred nanograms of purified DNA from various tissues were amplified using TaqMan universal PCR master mix (Applied BioSystems). The HBB (human β-globin, Applied BioSystems) was used as primers and probe. To determine the efficiency of amplification and the assay precision, calibration curves for human β-globin gene was constructed with a 0.99 correlation (r^2) and efficiency superior to 96%. A 100% efficiency corresponded to a slope of -3.41 as determined by the following equation: efficiency = 10(-1/slope) - 1. Mouse DNA was isolated from the identical tissues of nontransplanted nude mice and used as a negative control. Also, human DNA was isolated from hDPSCs culture and used as a positive control.

2.5 Immunohistochemistry and Immunofluorescence Staining

There are limitations when performing a quantitative analysis by extracting DNA from inside a hard tissue such as the teeth, and so a qualitative analysis was performed by IHC and immunofluorescence (IF) staining of intra-pulp transplanted hDPSCs. The maxilla of mice of intra-pulpal transplantation group were dissected, rinsed in PBS for 60 min, and decalcified for 4 weeks in a 5% EDTA with 4% sucrose in PBS with pH 7.4 and agitation at room temperature. The solution was renewed every week. After decalcification, samples were dehydrated through increasing grades of isopropyl alcohol (Duksan). The dehydration process consisted of a series of isopropyl alcohol rinses starting from 70% solution for one hour and followed by 80%, 90%, 95% and 100% solution for one hour. After dehydration, samples were embedded in paraffin block according to standard procedures. After embedded in paraffin, the samples were cut with a microtome (Leica) in sections of 6 μm. For histology, slides were stained with H&E and observed by light microscope (Nikon). The unstained sections were deparaffinized and rehydrated by xylene, absolute alcohol, and 90%, 80% and 70% alcohol, and then washed with 0.05% TBS-T for 10 min. Antigen retrieval was performed using Retrieval solution (DAKO) for 30 min at 95 ℃ and then cooling. To block endogenous peroxidase activity, the slides were treated with 3.0% hydrogen peroxidase in distilled water for 10 min. After blocking non specific antigen with 1% BSA solution for 30 min, the sections were incubated for 60 min at room temperature with monoclonal anti-HLA Class 1 ABC antibody (clone EMR8-5, 1:200 dilution, Abcam Inc., Cambridge, MA, USA) was applied for IHC

analysis. For IF analysis, monoclonal anti-HLA Class 1 ABC antibody (clone EMR8-5) was applied overnight in the dark at 4℃. The slide was washed 3 times with PBS and the Goat Anti-Mouse IgG, IgM Secondary Antibody (Alexa Fluor® 488) and double-stranded nucleic acids (TO-PRO®-3) were used at a dilution of 1:10000 for 2 hours in the dark at room temperature. The slides were mounted in fluoromount and observed by the confocal microscope.

2.6 Statistical Analysis

An analysis of variance (ANOVA) with a post Bonferroni test was conducted to analyze the biodistribution of intravenous and intra-pulpal transplanted hDPSCs. All data were analyzed using IBM SPSS statistics v21.0 software (IBM Corp, Somers, NY, USA), and the significance level was established at 0.05.

III. Results

1. Animal Models for Dental Pulp Disease

1.1 Light Microscopy Observation

In the negative control group, the histology of the pulp appeared normal. Loose connective tissue composed of fibroblast and blood vessels form the dental pulp located in the dental cavity. Odontoblasts form a layer lining the periphery of the dental pulp and have a process extending into the dentin (Fig. 3).

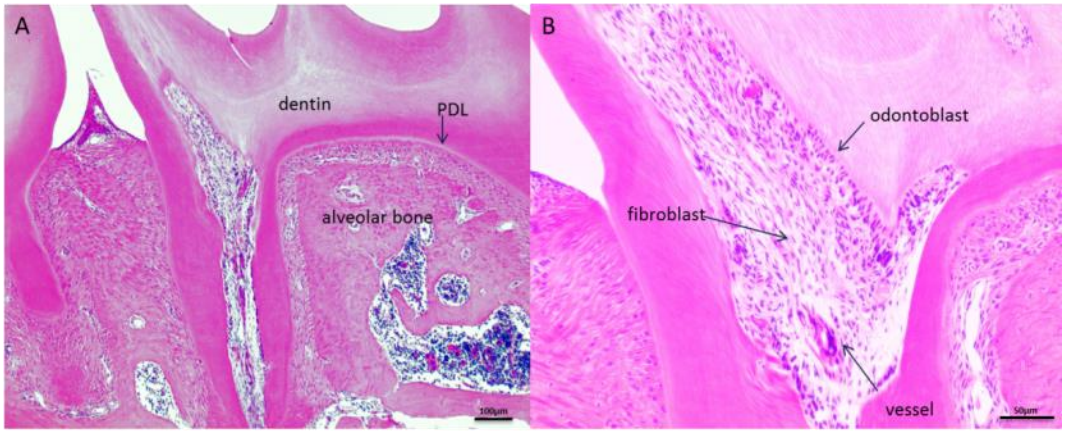

Fig. 3. Normal pulp (A) Fibroblast, blood vessels and loose connective tissue form the dental pulp located in the dental cavity (x 40). (B) Odontoblasts form a layer lining the periphery of the dental pulp and have a process extending into the dentin (x 100).

In the experimental group, until the one week postoperatively, the below of the pulp chamber orifice tissue showed normal. The remaining coronal portion of the pulp tissue is

inflammatory and necrotic (Fig. 4-7). The specimens obtained after the one experimental day showed hyperemia. Dilation of the pulp blood vessels were observed, and large numbers of red blood cells were seen around fibroblasts (Fig. 4). The specimens at postoperative day two showed a pattern of inflammation, with deposition of inflammatory cells such as neutrophils and lymphocytes in the pulp chamber superior to the orifices (Fig. 5). At postoperative day four, pulp chamber showed a microabscess in the distal pulp horn (Fig. 6A, 6B). The specimen of postoperative day four, severe accumulation of chronic inflammatory cells was observed in the mesial pulp horn.

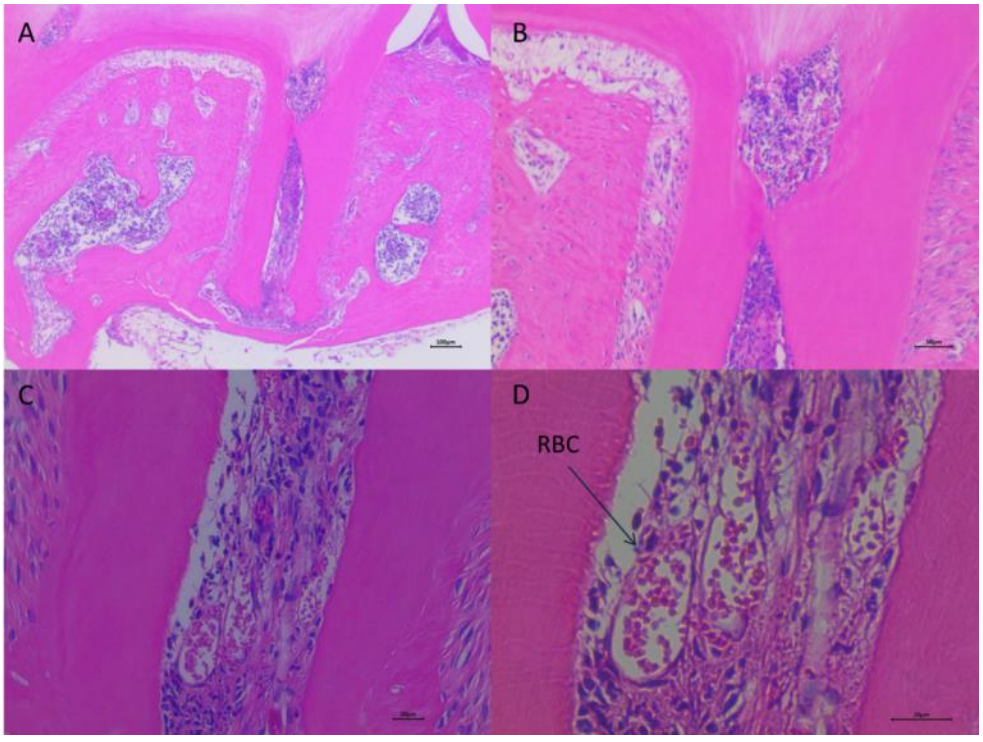

Fig. 4. One day after pulp exposure. (A, B) Dilation of the pulp blood vessels was observed (x 40, x 100). (C, D) At higher magnification of box, dilation of the pulp blood vessels and large numbers of red blood cells were seen (x 200, x 400).

At postoperative day seven specimens showed well-defined caseous necrosis surrounded by chronic inflammatory cells in the center of pulp chamber (Fig. 7).

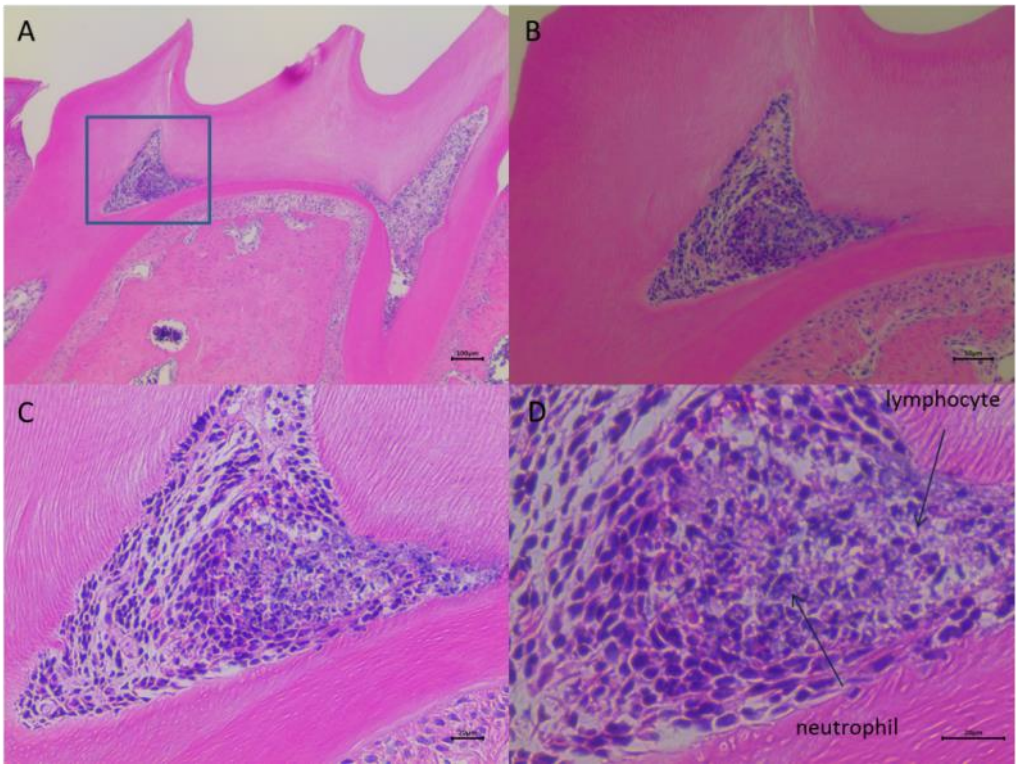

Fig. 5. Two days after pulp exposure. (A) Low magnification (x 40). (B, C, D) At higher magnification, deposition of inflammatory cells such as neutrophils and lymphocytes were observed in the pulp chamber superior to the orifices (x 100, x 200, x 400).

After one week postoperatively, inflammation and necrosis were apparent in the root canals inferior to the orifices (Fig. 8-11). In specimens at postoperative day nine, all tissue above the orifices showed necrosis, while inflammatory cells and normal tissue were observed inferior to the orifices (Fig. 8). The specimens at postoperative day 12 showed necrosis up to one-third of the way to the apex (Fig. 9). The specimens obtained

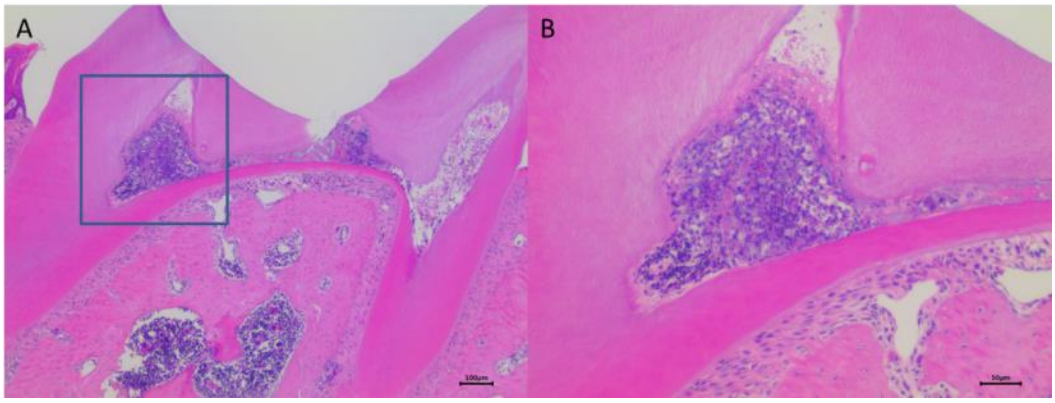

Fig. 6. Four days after pulp exposure. (A) Low magnification (x 40) and (B) high magnification (x 100) show a severe accumulation of chronic inflammatory cells in the mesial pulp horn.

after the experimental day 14 showed necrosis of all tissue in the root canals, and inflammatory cells were observed inferior to the apex (Fig. 10). The specimens obtained after the experimental day 28 showed complete pulpal necrosis and could not detect any cells (Fig. 11A, 11B). Apical lesions were observed in specimens at postoperative day 35 (Fig. 11C, 11D).

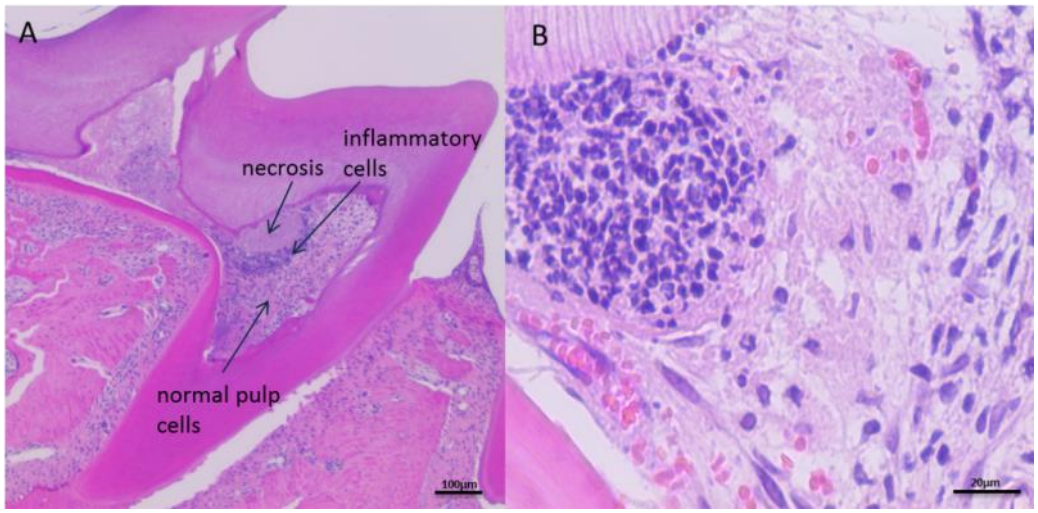

Fig. 7. Seven days after pulp exposure. (A) Low magnification (x 40) and (B) high magnification (x 200); Well-defined caseous necrosis surrounded by chronic inflammatory cells was observed in the center of pulp chamber.

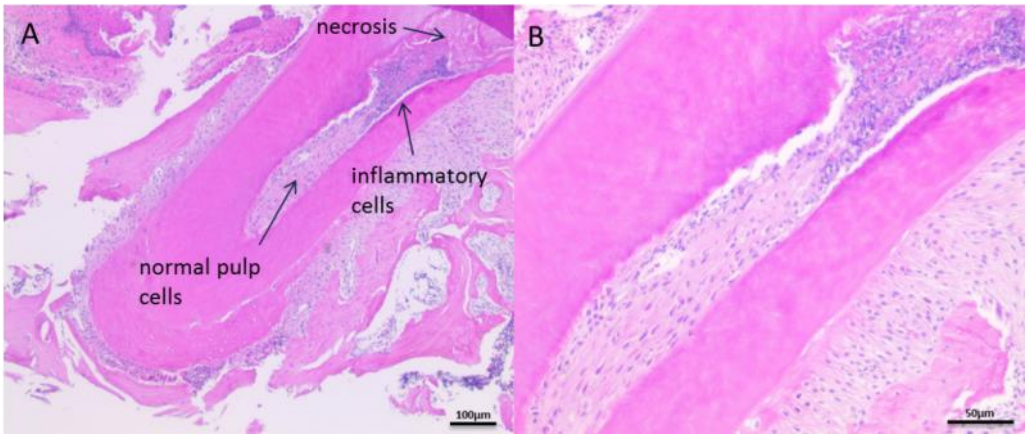

Fig. 8. Nine days after pulp exposure. (A) Low magnification (x 40) and (B) high magnification (x 100); All tissue above the orifices showed necrosis, while inflammatory cells and normal tissue were observed inferior to the orifices.

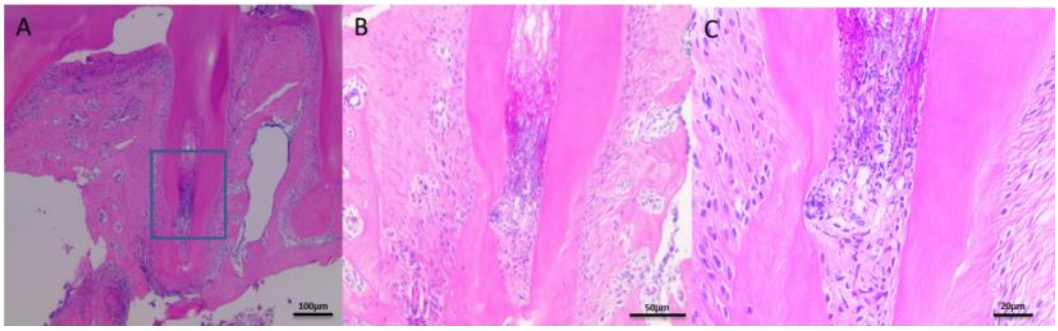

Fig. 9. Twelve days after pulp exposure. (A) Necrosis up to one-third of the way to the apex (x 40). At higher magnification, (B) necrotic tissue and inflammatory cells were observed (x 100) and (C) normal pulp cells were observed inferior the chronic inflammatory cells (x 200).

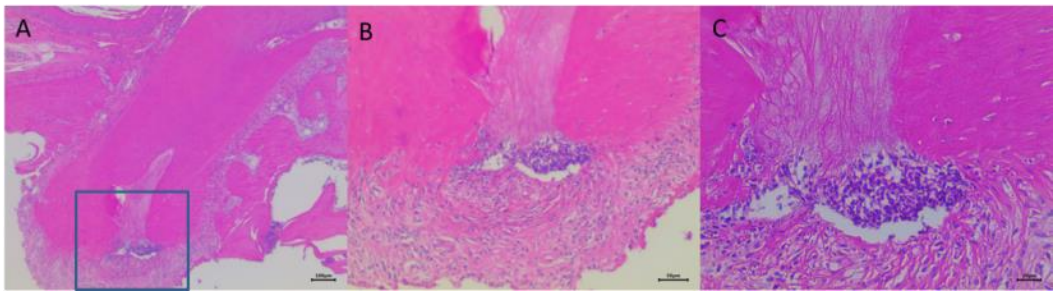

Fig. 10. (A) The specimens obtained after the 14 experimental days showed necrosis of all tissue in the root canal (x 40). (B, C) At higher magnification, inflammatory cells were observed inferior to the apex (x 100, x 200).

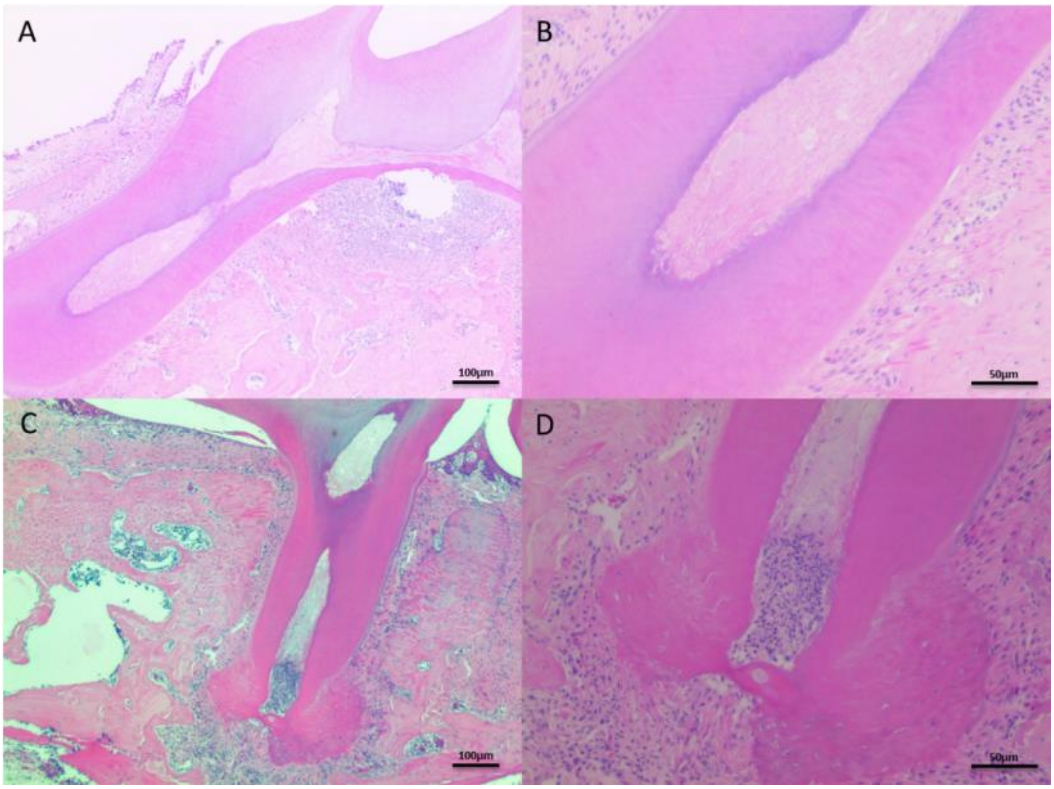

Fig. 11. (A, B) Twenty-eight days after pulp exposure. Caseous necrosis was observed in the whole root canals (x 40, x 100). (C, D) 35 days after pulp exposure. Chronic inflammatory cells accumulations were observed from root apical area (x 40, x 100).

2. Biodistribution of Intravenous and Intra-pulpal Transplanted hDPSCs in Mouse

2.1 Characterization of hDPSCs

hDPSCs were identified on the basis of stem cell markers, differentiation potentials (Fig. 12, 13). A flow cytometry analysis revealed that the cells were positive for CD73 (98.4%), CD90 (99.3%), CD105 (98%) and negative for CD45 (0.1%) (Fig. 12).

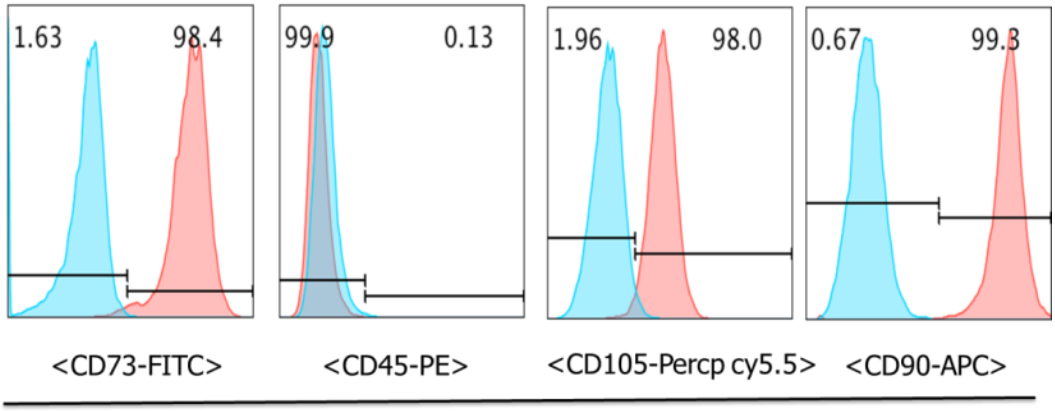

Fig. 12. Flow cytometric analysis of the surface marker expression profile of DPSCs. A flow cytometry analysis revealed that the cells were positive for markers CD73, CD90, CD105 and negative for marker CD45.

Compared with bone marrow MSCs (Fig. 13A), the osteogenic differentiation potentials of hDPSCs was verified by Von kossa staining (Fig. 13B). Nodular structures which were stained black were found, four weeks after osteogenic induction. Compared with bone marrow MSCs (Fig. 13C), the adipogenic differentiation potentials of hDPSCs was verified by Oil Red O staining (Fig. 13D). hDPSCs accumulate neutral lipid vacuoles which were stained red. Compared with bone marrow MSCs (Fig. 13E), the chondrogenic differentiation potentials of hDPSCs was verified by Alcian Blue staining (Fig. 13F).

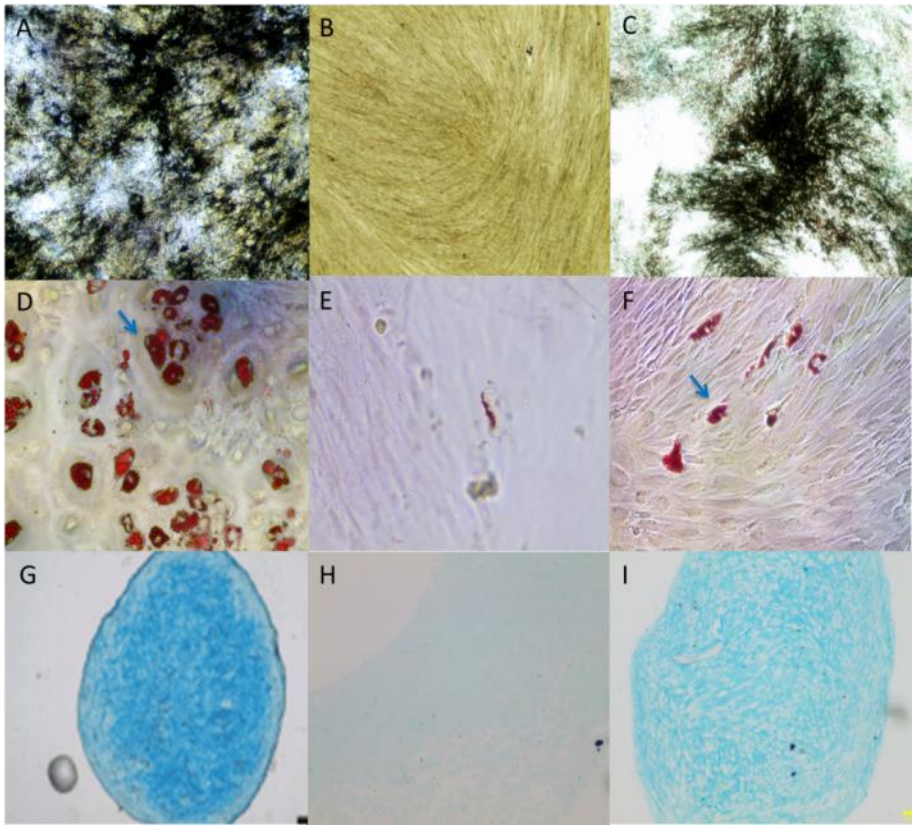

Fig. 13. Differentiation potential: Positive control; hMSC (A, D, G), negative control; hDPSCs (B, E, H), experimental group; hDPSCs (C, F, I). Osteogenic differentiation (von Kossa staining) of (A) hMSC positive control and (B) hDPSC negative control. (C) Histochemical staining with von Kossa showed the presence of a mineral associated with the matrix in the osteogenically differentiated hDPSCs, 4 weeks after osteogenic induction. Adipogenic differentiation (Oil Red O staining) of (D) hMSC positive control and (E) hDPSC negative control. (F) Histochemical staining with Oil Red O showed that adipogenically differentiated hDPSCs were positive for the stain when tested 4 weeks after adipogenic induction. Neutral lipid vacuoles which were stained red were observed (*arrow*). Chondrogenic differentiation (Alcian Blue staining) of (G) hMSC positive control and (H) hDPSC negative control. (I) Histochemical staining with Alcian Blue showed that chondrogenically differentiated hDPSCs were positive for the stain when tested 4 weeks after chondrogenic induction.

2.2 Biodistribution of hDPSCs in Mouse

2.2.1. Quantification of Transplanted hDPSCs

Using human-specific β-globin as a detection marker, hDPSC numbers in samples were quantified by reference to a standard curve of β-globin cycle threshold (Ct) value in qPCR assays. The primer set used in this study showed a wide range of detection (0.01 – 100 ng of template) (Fig. 14).

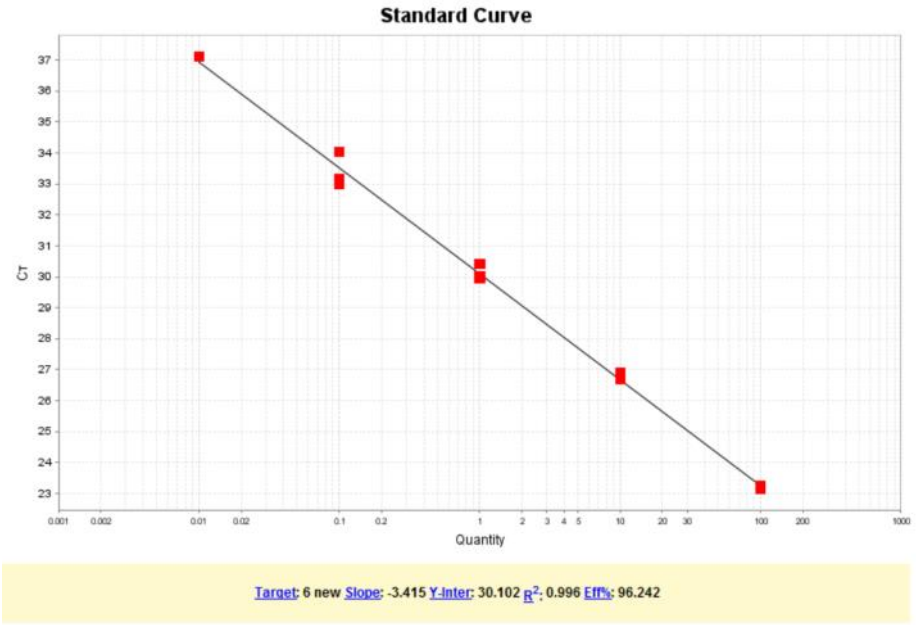

Fig. 14. A standard curve of β-globin cycle threshold (Ct) value in qPCR assays.

2.2.2. Intravenous Transplantation Group

All animals (n=20) survived after hDPSCs transplantation. The time-course detection of the human β-globin gene in mouse tissues was studied at different time intervals after hDPSCs transplantation. The mean values and standard deviations of biodistribution of each organs are presented in Table 1. Figure 15 (A) shows the detection of the human specific β-globin gene in heart, liver, spleen, kidney, brain, lymph node and lung. At 15 min post dose, intravenously injected hDPSCs showed a greater distribution to lung than other organs. At 30 min, 1 hour, 6 hours and 12 hours after intravenous transplantation, the distribution of hDPSCs were gradually decreased in lung tissue. A small amount of human DNA was detected in heart, spleen, kidney and brain sporadically. The human DNA was not detected from liver and lymph node across all time periods.

Table 1. Biodistribution of intravenously transplanted hDPSCs.

	heart	liver	spleen	kidney	brain	lymph node	lung	Significance*
15min	0	0	1.53±2.01	0	0	0	31.44±6.92	
30min	0	0	0	0	0.35±0.70	0	25.47±13.27	
1hr	0.92±1.84	0	0	0	0.54±1.07	0	22.78±3.52	lung>other organs
6hr	0	0	0	0.36±0.72	0	0	9.29±5.92	
12hr	0	0	0.79±0.92	0	0.39±0.79	0	5.97±3.92	

*$P < 0.05$ compared with other groups

2.2.3. Intra-pulpal Transplantation Group

All animals (n=20) survived after hDPSCs transplantation. The mean values and standard deviations of biodistribution of each organs are presented in Table 2. Figure 15

(B) shows the detection of the human specific β-globin gene in heart, liver, spleen, kidney, brain, lymph node and lung. Unlike intravenously injected hDPSCs, intra-pulpal transplanted hDPSCs exhibited very low distribution in lung only at 6 hours. A small amount of human DNA was detected in heart, liver, spleen, brain and lymph node sporadically. The human DNA was not detected from kidney across all time periods.

Table 2. Biodistribution of intra-pulpally transplanted hDPSCs.

	heart	liver	spleen	kidney	brain	lymph node	lung	Significance
15min	0.47±0.94	0.45±0.90	1.79±3.58	0	0	0.55±1.10	0	
30min	0	0	0	0	0	0.38±0.69	0	
1hr	0	0	0	0	0.33±0.66	1.38±0.99	0	
6hr	0	0	0.36±0.72	0	0	0	0.46±1.01	
12hr	0	0	0	0	0	0	0	

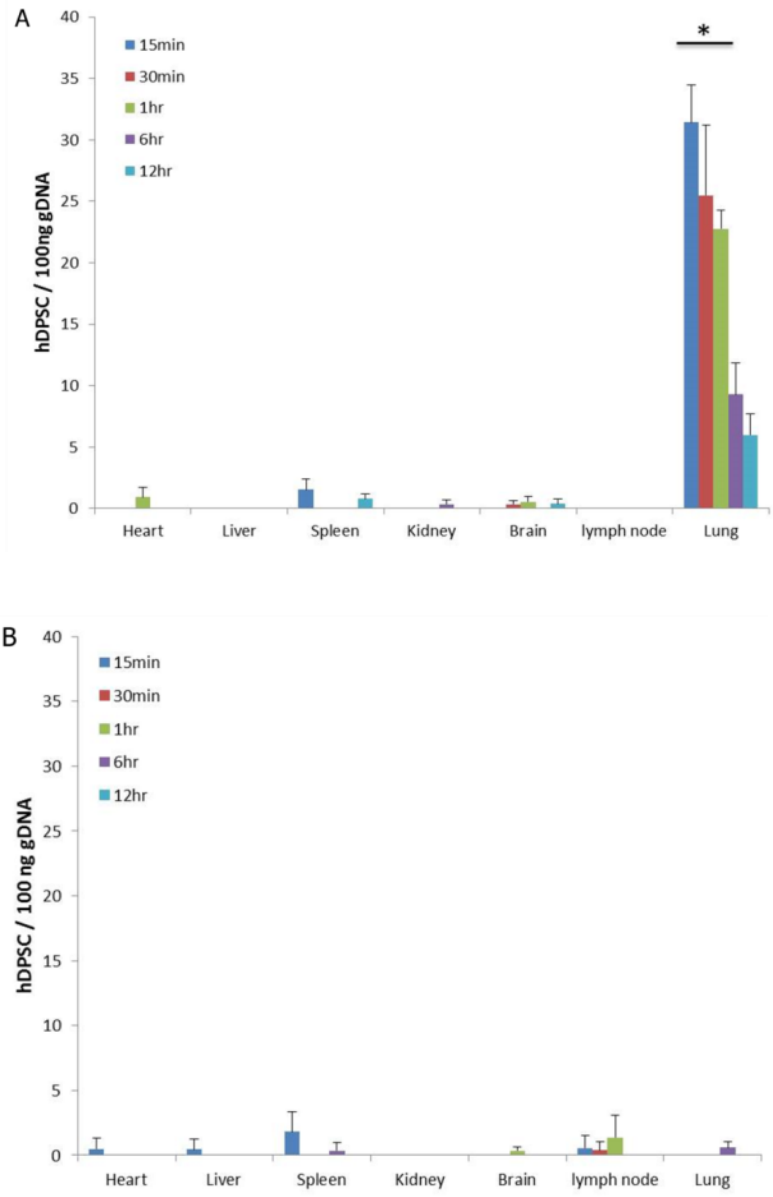

Fig. 15. (A) Biodistribution of hDPSCs following intravenously injection. The number of hDPSCs was quantified by qPCR of β-globin in 100 ng of gDNA extracted from the collected organs (*P < 0.05 compared with other organs). (B) Biodistribution of hDPSCs following intra-pulpal transplantation.

2.3 Immunohistochemistry and Immunofluorescence Staining

Figure 16 shows hDPSCs were detected in pulp chamber at 12 hours after transplantation. Numerous HLA-ABC-positive cells were observed in the pulp chamber. A large number of stained (HLA-ABC-positive) cells were observed in the pulp chamber (brown color), but not in the root canal, alveolar bone and periodontal tissue (Fig. 16A, 16C). Human cells cytoskeleton and nucleus were observed under confocal microscopy (Fig. 16B)

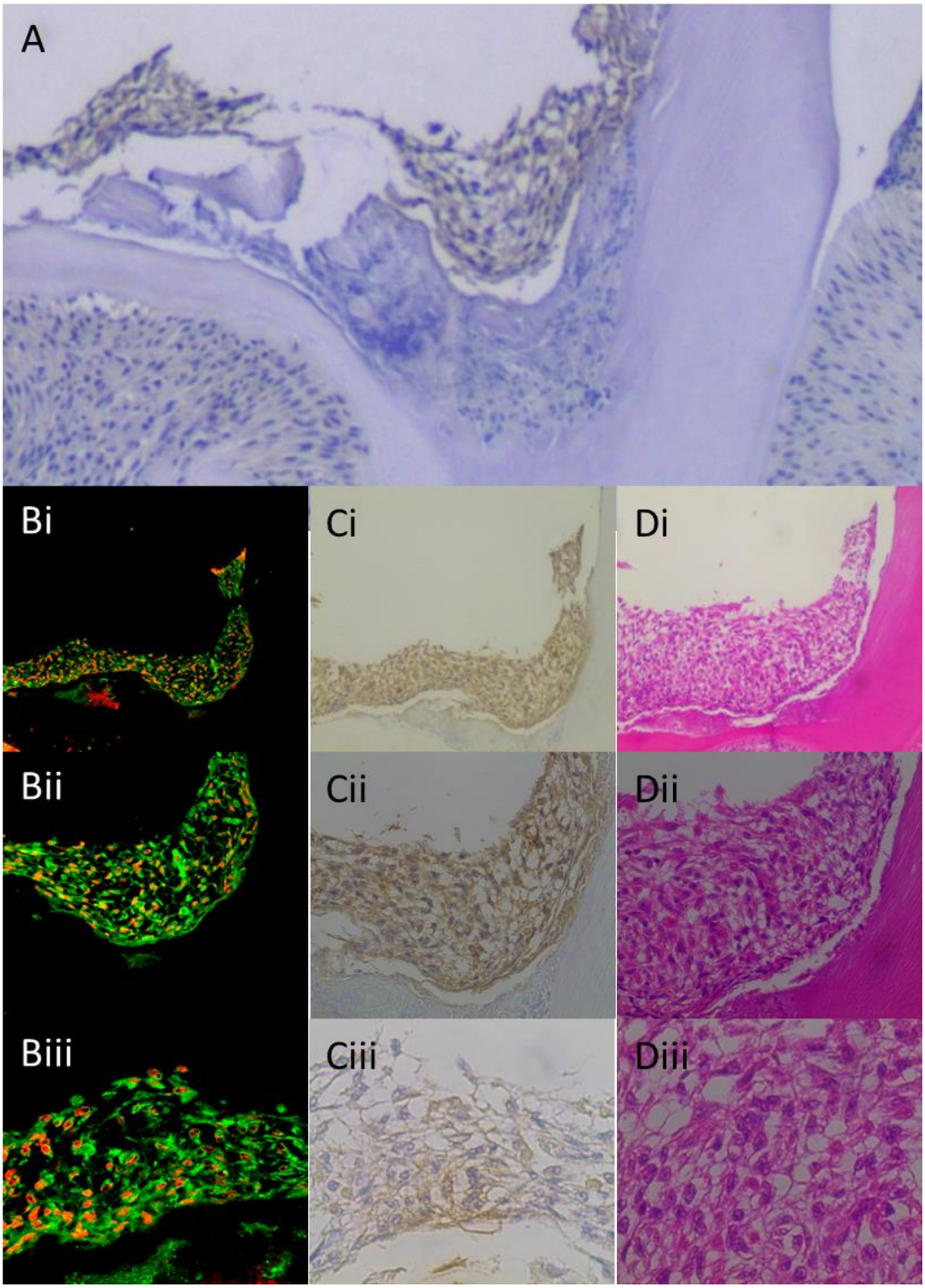

Fig. 16. IHC and IF staining show disposition of hDPSCs in the pulp chamber. (A) A large number of hDPSCs were deposited on the mouse pulp cells (IHC staining, x 100). (B) IF staining shows cytoskeleton (green color) and nucleus (red color) of hDPSCS (Bi) x 100 (Bii) x 200 (Biii) x 400. (C) High magnification of IHC staining (Ci) x 100 (Cii) x 200 (Ciii) x 400. (D) H&E staining (Di) x 100 (Dii) x 200 (Diii) x400.

IV. Discussion

The aims of this study were (1) to develop the mouse model for pulp-dentin complex regeneration research, and (2) to investigate the distribution of DPSCs in organs over time in a group receiving DPSC transplantation into the teeth and a group receiving an intravenous transplantation using the developed the mouse model.

The dental pulp may be exposed to a number of irritant that are noxious to the health of the pulp. Bacterial infection due to pulp exposure is the most common cause of pulp disease (Yu and Abbott, 2007). Thus, in order to make the mouse model, the pulp was exposed as a method of inducing pulpitis and pulp necrosis.

The pulp status was observed histologically for 14 days after pulp exposure. At seven days, inflammation was restricted to above the orifice level. By 14 days after pulp exposure, necrosis had progressed as far as the root apex, and all cells and tissue within the root canals were found to have undergone necrosis. These results are consistent with previous studies reporting that when dental pulp is exposed to bacteria due to caries or trauma, infection and pulp necrosis start at the exposure site and progress gradually in an apical direction (Ricucci D, 2013).

Looking at specimens obtained more than a day after pulp exposure, hyperemia and red blood cells were observed throughout the pulp, from the pulp horn to the root apex (Fig. 4). This sort of vasodilation is a feature of acute inflammation. Acute inflammation is characterized by marked vascular changes, increased permeability and increased blood

flow, which are induced by the actions of various inflammatory mediators. The first vascular reaction during acute inflammation is vasodilation which may increase the blood volume in the inflamed area (Heyeraas and Berggreen, 1999). In specimens at postoperative days 2–4, inflammation was observed in the pulp chamber superior to the orifices (Fig. 5, 6). A high concentration of chronic inflammatory cells, such as lymphocyte and macrophage, and a microabscess were observed in the pulp horn (Fig. 5). Postoperative seven days specimens showed that a considerable part of the pulp in the middle chamber appears caseous necrosis. Nevertheless, there is a distinct transition to the surrounding relatively normal pulp tissue. A clear demarcation line can be observed between the tissue disorganized by inflammatory infiltration and the surrounding normal tissue (Fig. 7). Within the first week after pulp exposure, necrosis and inflammatory cell accumulation was restricted to above the orifices, and the pulp in the root canals remained in a normal state. It is thought that this result could be useful in future vital pulp therapy and regenerative endodontics. Prior to advances in pulp biology, it was believed that pulp inflammation could not be reversed once it had started, and that it would inevitable progress to apoptosis and pulp necrosis (Goldberg, 2014). However, the advances of pulp biology combined with the clinical evidence of treating deep caries, have started to modify the traditional view of pulp inflammation (Hahn and Liewehr, 2007; Renard et al., 2016; Yu and Abbott, 2007). Based on the results of this study, pulp-dentin complex regeneration could be expected if only the pulp that has undergone necrosis and inflammation is selectively removed, before transplantation of DPSCs and scaffold

complex onto normal pulp cells that have retained their blood supply.

One week after pulp exposure, inflammation and necrosis progressed inferior to the orifices into the root canals, and after two weeks, all the tissue within the root canals had undergone necrosis, and inflammatory cells were observed inferior to the apex. If blood supply and removal of bacteria are considered to be essential to cell survival, once 14 or more days have passed since pulp exposure, DPSC and scaffold complex transplantation would have to be attempted after removing all the tissue with the root canals as far as the apex. In addition, further research into blood supply will be required, since this is essential for cell survival.

In this study, *in vivo* bio-distribution of intravenously injected and intra-pulp transplanted hDPSCs were also investigated. Stem cells that have been transplanted into the body have the potential for tumor mutation when they are distributed outside of the teeth and into other organs. In addition, if more cells are distributed in other organs, the number of remaining cells that can aid pulp-dentin complex regeneration will be fewer. Therefore, there is a need to analyze the impact of different routes of administration of the distribution of hDPSCs in the body. The cells used in this study were isolated and cultured using the techniques of Gronthos et al (Gronthos et al., 2000). In order to determine whether these cells met the criteria for hMSC, as provided by the International Society for Cellular Therapy in 2006 (Dominici et al., 2006), cells were characterized with respect to cell surface marker expression and differentiation potential. When cell surface marker expression was verified by flow cytometry, CD73, CD90, and CD105

expression was positive, while CD45 expression was negative (Fig 12). Differentiation to osteoblasts, adipocytes and chondroblasts were confirmed by staining with Alizarin Red, Oil Red O and Alcian Blue, respectively (Fig 13). This shows that the cells used in this study met the hMSC criteria.

Based on the results of this study, the distribution of hDPSCs injected into mice differs according to the route of transplantation. Intravenously injected hDPSCs were mostly distributed to the lung. Looking at changes in concentration over time, the maximum number of hDPSCs in the lung showed at 15 minutes after hDPSC transplantation. After the initial distribution to the lung, intravenously injected hDPSCs were rapidly decreased from the lung up to 12 hour after administration. Apart from the lungs, very small amount of hDPSCs were detected in the heart, spleen, kidney, brain sporadically. These results are consistent with other studies observing hMSC distribution in rodents after intravenous transplantation (Fischer et al., 2009; Schrepfer et al., 2007; Shim et al., 2015). One possible reason why most of the intravenously injected hDPSCs were detected in the lungs could be the size discrepancy between the mean diameter of the hDPSCs and that of the pulmonary capillaries. Fischer et al. (Fischer et al., 2009) reported that bone marrow–derived mononuclear cells (approximately 7 μm) pulmonary passage was 30-fold higher compared to MSCs (approximately 18 μm). This result shows that the diameter of the cells is an important factor affecting their passage through the pulmonary capillaries. The mean diameter of the hDPSCs (15~16 μm) is larger than the size of pulmonary capillaries (approximately 5.5 μm) (Downey et al., 1990; Suchanek et al.,

2009). Hence, hDPSCs injected into the tail vein are thought to become trapped in the pulmonary capillaries as they pass through the lungs, meaning that they are mostly detected in the lungs and not in other organs.

Unlike intravenously injected hDPSCs, intra-pulpal transplanted hDPSCs were hardly detected in the lung. Also, very small amount of hDPSCs were detected in any other organs apart from the teeth, throughout the whole observation period. One possible reason for the difference with intravenous transplantation in distribution is the nature of the teeth, which are one of the few organs in the body encased in hard tissue. The low-compliance of the dental pulp that is encased within non-expanding mineralized walls limits the blood circulation (Kim and Dorscher-Kim, 1989). Compared to most organs, which have an abundant blood supply through various routes, the blood supply to the teeth only consists of small holes at the root apices. Therefore, cell migration from the inside to the outside of the teeth is difficult. Furthermore, the size and structure of the vessels within the dental pulp will also have an impact. The diameter of the blood vessels within the dental pulp is very narrow. The widest venule and primary feeding arteriole have diameters of 35–50 μm, while the capillaries that are located furthest anteriorly within the vessel network, and branch directly in the subodontoblastic and odontoblastic layer to supply nutrients only have diameters of 8–12 μm (Takahashi, 1985). Due to such a structural characteristic of the tooth, hDPSCs transplanted into the pulp chamber is trapped in a tooth, thus it would be rarely observed in any other organ.

Many properties of MSC have recently raised concern about their potential for

maldifferentiation into neoplastic cells as well as the possibility to promote growth of tumor cells. In this study, intra-pulpal transplanted hDPSCs exhibited very low distribution in other organs across all time periods. Therefore, it is less likely for intra-pulpal transplanted hDPSCs to be distributed in other organs and cause unwanted effect. However, a definite conclusion about these possibilities is still awaiting and requiring further research.

The presence of hDPSCs within the teeth was evaluated qualitatively, otherwise transport of hDPSCs transplanted into the pulp chamber into other organs was evaluated by DNA extraction and quantitative PCR analysis. Although it is possible to extract DNA from the teeth (Ginther et al., 1992; Pinchi et al., 2011), there were difficulties performing a quantitative analysis of hDPSCs in the pulp chamber, since they are surrounded by hard tissue. Therefore, IHC and IF staining were performed to determine whether there were hDPSCs in the pulp chamber or not. HLA-ABC antigen is present on most nucleated cells of human. Thus, it is effective to use the HLA-ABC antibody as a marker. Also, cytoskeleton and nuclei could be confirmed by IF staining. Although hDPSCs rarely detected in other organs, a large number of hDPSCs were detected within the pulp chamber even 12 hours after transplantation.

DPSCs-based approach is a potentially useful innovative therapeutic strategy for pulp-dentin complex regeneration. Notwithstanding promising *in vitro* results with DPSCs application, moving this approach forward toward clinical application should be critically assessed by looking for unexpected, especially concerning unwanted effects. In this study,

intra-pulpal transplanted hDPSCs exhibited very low distribution in other organs until 12 hours. Through additional experiments, further research on the biodistribution of more than 12 hours is needed. Also, it was able to confirm the presence of hDPSCs in the pulp chamber, there is still a lack of research on the mechanisms of survival for cells transplanted into the pulp chamber. Within the dental pulp, the vascular system performs the roles of nutrition, oxygen supply, and removal of metabolic waste (Nakashima and Akamine, 2005). Therefore, blood supply is expected to be the most important factor in the survival of hDPSCs within the pulp chamber (Huang, 2011), and this is thought to be an area requiring additional research in the future.

V. Conclusion

1. Up to seven days after exposure of the dental pulp in mice, inflammation and necrosis was limited to the area superior to the orifice level. By 14 days after pulp exposure, necrosis had progressed as far as the root apices, and all the cells and tissues in the root canals had undergone necrosis.

2. Intravenously injected hDPSCs were mostly distributed to the lungs. The number of hDPSCs in the lungs continued to decrease at 15 minutes, 30 minutes, and up to 12 hour after hDPSCs transplantation. hDPSCs were rarely detected in organs except the lungs throughout all time periods.

3. The hDPSCs transplanted into the pulp chamber rarely migrate to other organs across all time periods. Even after 12 hours had passed, the cells were able to survive within the pulp chamber.

4. Thus, there seems to be no safety problem in cell based approach which transplants hDPSCs into pulp chamber for pulp-dentin complex regeneration.

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Abstract (in Korean)

치수질환 연구를 위한 마우스 모델 구축 및 마우스의 치수내로 이식된 인간 치수줄기세포의 체내분포 분석

김 선 일

연세대학교 대학원

치의학과

(지도교수 김 의 성)

본 연구의 목적은 (1) 치수-상아질 복합체 재생 연구를 위한 마우스 모델의 확립 (2) 확립된 마우스 모델을 이용하여 치수강 내로 이식된 치수줄기세포의 체내분포를 정맥으로 이식한 군과 비교 분석하는 것이었다.

마우스 모델 확립을 위하여 총 45마리의 마우스의 치수를 노출시키고, 5마리씩 1, 2, 4, 7, 9, 12, 14일에 걸쳐 희생하였다. 정상 치수의 상태를 확인하기 위한 대조군으로 치수를 노출시키지 않은 치아를 사용하였다. 치수괴사와 치근단 병소의 형성을 확인하기 위하여 치수를 노출시킨 후 28일과 35일에 각 5마리씩 희생하였다. 치수의 조직학적 변화는 광학 현미경을 사용하여 확인하

였다. 치수강과 정맥으로 이식한 치수줄기세포의 체내분포를 확인하기 위하여 자원자로부터 완전매복 제3대구치를 얻은 뒤 Gronthos의 방법을 이용하여 인체유래 치수줄기세포를 분리 및 배양하였다. 마우스를 심마취 한 뒤 10만개의 치수줄기세포를 마우스의 꼬리정맥이나 치수강을 통하여 이식하였다. 인체유래 치수줄기세포를 이식한 뒤 정해진 스케줄에 맞춰 마우스를 희생하였고 각 장기에 분포하는 치수줄기세포의 수를 정량적으로 분석하였다. 치수강 내로 이식된 치수줄기세포는 정성적으로 분석하였다.

치수염과 치수괴사의 진행은 마우스의 치수를 노출시킨 뒤 일주일 까지는 orifice 상방에 국한되었고, 이주일 후에는 치근단 부위까지 진행되었고 근관 내의 모든 세포는 괴사되었다. 치수노출 35일 후에는 치근단 부위의 골흡수 양상이 관찰되었다. 정맥으로 이식된 치수줄기세포는 대부분 폐에 분포하였다. 폐에 분포하는 치수줄기세포의 수는 이식 15분부터 12시간 까지 서서히 감소하였다. 치수줄기세포는 관찰 전 기간에 걸쳐서 폐를 제외한 다른 장기에서는 거의 검출되지 않았다. 치수강 내로 이식한 치수줄기세포는 관찰 전 기간에 걸쳐서 타 장기에서 거의 검출되지 않았다. 이식 12시간이 지난 이후에도 치수강 내에서 살아있는 세포가 관찰되었다.

이와 같은 결과는 치수-상아질 복합체 재생을 위하여 치수줄기세포를 치수강 내로 이식하는 세포 기반의 접근이 체내분포의 측면에서 볼 때 안전성에 문제가 없음을 시사한다.

핵심되는 말: 마우스 모델, 생체분포, 치수줄기세포, 치수, 재생