치의과학박사 학위논문

Comparison of Osteogenic Effects of Simvastatin on Human Periodontal Ligament Stem Cells and Bone Marrow Mesenchymal Stem Cells

사람 치주인대줄기세포와 골수줄기세포에 대한 심바스타틴의 골형성 효과 비교 연구

2016 년 2 월

서울대학교 대학원
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- Abstract –

Comparison of Osteogenic Effects of Simvastatin on Human Periodontal Ligament Stem Cells and Bone Marrow Mesenchymal Stem Cells

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The statin 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor is an anti-hyperlipidemic agent that is used worldwide. It also has pleiotrophic effects associated with antithrombotic, antioxidant, anti-inflammatory, and bone anabolic actions. In this study, the osteogenic and anti-adipogenic effects of simvastatin on human

periodontal ligament stem cells (PDLSCs) and bone marrow mesenchymal stem cells (BMMSCs) were investigated *in vitro* and *in vivo*. Direct cell counting and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that proliferation of both cell types was not inhibited by treatment with 0.01 to 0.1 μM simvastatin, although viability of both cell types decreased in a dose dependent manner. Inhibition of adipogenic differentiation by simvastatin was highly dose dependent in both PDLSCs and BMMSCs according to reverse transcription polymerase chain reaction and oil red O staining. Osteogenic gene expressions were not changed by simvastatin treatment. However, PDLSCs showed more osteogenic activity than BMMSCs, as demonstrated by alkaline phosphatase activity in cells treated for 7 days with simvastatin. PDLSCs treated with 0.01 to 0.1 μM simvastatin showed increased mineralization at a level comparable to BMMSCs by alizarin red S staining. In this study, *in vivo* transplantation of mesenchymal stem cells pretreated with simvastatin revealed that most new bone formation was found after treatment with 0.1 μM of simvastatin, although BMMSCs showed relatively more new bone formation than PDLSCs. Immunohistochemical staining with antibodies against human mitochondria proved that the newly formed mineral matrix around the

hydroxyapatite/tricalcium phosphate carrier was generated by human derived PDLSCs and BMMSCs. Mineralized tissue formations around grafted particles were also confirmed by immune-specific markers such as type I collagen and osteocalcin. We determined that *in vitro* stimulation of bone regeneration by simvastatin on human PDLSCs was comparable to that of BMMSCs. Calvarial transplantation demonstrated that PDLSCs treated with simvastatin increased mineralized tissue formation similar to BMMSCs. Simvastatin is a promising tool for PDLSCs specific periodontal regeneration.

Keywords: Hydroxymethylglutaryl-CoA Reductase Inhibitors, Osteogenesis, Bone regeneration, Mesenchymal stem cell transplantation, Periodontium

Student Number: 2012-30603

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Introduction

Statin is a lipid-lowering agent that has been used worldwide for decades. Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme reductase A (HMG-CoA) and suppress mevalonate synthesis and downstream signaling pathway[1]. Apart from their cholesterol lowering effects, statins have also drawn attention for their pleiotropic effects associated with antithrombotic, antioxidant, anti-inflammatory, and bone anabolic actions[2].

Several multicenter studies have demonstrated the pleiotropic roles of statins on primary and secondary prevention of hyperlipidemia, decreases in cardiovascular events and mortality, and cerebrovascular attacks[3-12]. These effects are primarily due to decreases in low density lipoprotein cholesterol. Upregulation of high density lipoprotein cholesterol and downregulation of triglycerides and c-reactive proteins are other effects of statins[13].

Periodontitis is an inflammatory condition that involves in the breakdown of periodontal tissues, including the tooth supporting alveolar bones, gingiva, and periodontal ligament fibers around teeth[14]. Periodontitis is the main cause of tooth loss, and a majority of adults over 18 suffer from it[15-17]. It is also associated with systemic diseases including cardiovascular disease[18], diabetes mellitus[19], metabolic syndrome[20], and adverse pregnancy

outcomes[21]. Once the periodontium is damaged, it is difficult to restore connective tissue attachment to the newly formed cementum of the root surface[22-24]. Therefore, to find effective methods against mechanical debridement and behavior modifications, regeneration of periodontal defects using cells, scaffolds, or growth factors is actively being studied[25,26].

Guided bone regeneration[27] or bone replacement grafts[28] have been successfully used for periodontal regeneration. Since the first report of bone formation by Mundy et al.[29], application of statins for periodontal regeneration has been attempted by many researchers. Although promising results were found in some *in vitro*[30] and *in vivo* studies[31-33], as well as in some clinical trials[34-42], clinical utilization of statins for the treatment of periodontitis is still controversial because most statins are administrated orally, leading to high first-pass metabolism in liver and low bioavailability in bone. Consequently, only a small portion of the ingested statin reaches the periodontium, which is a burden to effective application of statin to periodontal diseases[43,44]. Low water solubility and lack of specificity to cranial bones also deter clinical uses of statins[2,45].

There are limitations to using high dose statins; significant complications such as

hepatotoxicity and myopathy have been reported[46-49]. Increased incidence of hepatotoxicity directly correlated with dosage was reported within 12 weeks of statin uses[48]. Previous reports suggested that drug interactions should be closely monitored, because drugs that are metabolized by the liver may increase the risks of statin-induced hepatotoxicity[50-52]. Life-threatening myopathy by statins is also highly dose-dependent[47]. The risk of rhabdomyolysis is increased by drug interactions with statins. Collectively, the use of the appropriate dosage of statins is important to maximize drug effects and minimize risks of serious complications.

Several signaling pathways are associated with bone anabolic actions of statins including elevation of osteoblast differentiation, suppression of osteoblast apoptosis, and inhibition of osteoclastic differentiation[45,53-56]. Statins increase osteoblast differentiation by stimulating bone morphogenetic protein-2 (BMP-2) expression or decreasing synthesis of mevalonate and downstream isoprenoid precursors[56]. Osteoblast apoptosis is suppressed by Transforming growth factor-β/SMAD family member 3 (TGF-β/Smad3) pathway[54], and osteoclast differentiation is inhibited by the Osteoprotegerin/Receptor Activator of NF-κB/RANK ligand (OPG/RANK/RANKL) pathway[55].

Bone marrow mesenchymal stem cells (BMMSCs) are multipotent stem cells of mesenchymal origin[57] and are widely studied stromal stem cells. BMMSCs are able to differentiate into mature cells due to their osteogenic, chondrogenic, and adipogenic potential. However, some limitations of BMMSC harvesting, such as pain, morbidity, and low cell number, have led to a search for alternate sources for BMMSCs.

Cell populations resembling mesenchymal stem cells derived from dental tissues are among many other stem cells of specialized tissues that have been isolated and characterized[58]. Periodontal ligament stem cells (PDLSCs)[59] are one of five dental stem cell lines; others are post-natal dental pulp stem cells (DPSCs)[60], stem cells from exfoliated deciduous teeth (SHED)[61], stem cells from apical papilla (SCAP)[62], and dental follicle precursor cells (DFPCs)[63].

As both PDLSCs and BMMSCs are types of stem cells, they share some characteristics[64]. Both can develop and come to maturity to have characteristic morphologies and specialized functions, and both cell types are capable of self-renewal and multi-lineage differentiation.

Dental stem cells are capable of developing into at least 3 cell lineages including

osteo/odontogenic, adipogenic, and neurogenic[58]. Distinctively, dental stem cells tend to show odontogenic rather than osteogenic traits[59,61,65,66].

Dental tissues such as teeth do not show constant remodeling as occurs in other bony tissues. Dental-tissue derived stem/progenitor cells appear to be restricted in their differentiation capability compared to BMMSCs[58]. Furthermore, the dental mesenchyme is derived from ectomesenchyme, the embryonic tissues from the neural crest, although BMMSCs are mesenchymal in origin[67]. Ectomesenchyme-derived dental stem cells may show different characteristics compared to BMMSCs[58].

Currently, numerous cell-surface markers are being used to find putative stem cells[58]. Positive markers for BMMSCs include STRO-1, CD44, CD73, CD105, CD106, CD146, Oct4, and Nanog, while negative markers for BMMSCs include CD14, CD34, and CD45. Positive markers for PDLSCs include CD13, CD29, CD44, CD59, CD90, CD105, CD146, and scleraxis, whereas negative markers for the cells are not identified.

Most of previous studies for statins focused on the delivery systems[68-70] or specific pathologic conditions such as fracture healing[71,72]. Although some reports have dealt with the application of statins on PDLSCs[30,73], the bone forming abilities of statins among

different kinds of stem cells have not yet been tested.

The purpose of this study was to compare osteogenic and anti-adipogenic effects of simvastatin between human derived PDLSCs and BMMSCs for therapeutic applications. We hypothesized that PDLSCs and BMMSCs would respond differently to simvastatin. To verify this, adipogenic and mineralized tissue differentiations were tested *in vitro*, and transplanted cells treated with simvastatin were tested *in vivo*.

Materials and Methods

Sample collection and cell culture

Impacted third molars were obtained from healthy volunteers (18-26 years old, n=3) who underwent third molar extractions at the department of oral and maxillofacial surgery in Seoul National University Dental Hospital. Informed consent was received from all volunteers and was approved by the Institutional Review Board at School of Dentistry, Seoul National University (IRB No. S-D20080009). Tissues from the periodontal ligament and bone marrow from the alveolar bone were carefully detached, chopped into pieces, and digested using 3 mg/mL type I collagenase (BioBasic Inc., Toronto, Ontario, Canada) and 4 mg/mL dispase (Gibco BRL, Grand Island, NY, USA) for 1.5 hour with shaking at 37℃ in a 5% CO_2 incubator to make a single cell suspension. The suspension was filtered with a 70 ㎛ cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) to remove debris. The cells were then cultured our culture medium: α-MEM (Gibco BRL) supplemented with 15% fetal bovine serum (EquitechBio Inc., Kerrville, TX, USA), 100 μM of L-ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2 mM of L-glutamine (Gibco BRL), and 1% antibiotic-antimycotics (Gibco BRL). The medium was replaced every other day, and

the cells were subcultured when the dishes were about 70-80% confluent. Cells passage 2 to 4 were used for all experiments.

Evaluation of cell proliferation

The number of viable cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's recommendations. In brief, PDLSCs and BMMSCs were detached and seeded on a 96-well microplate at 2 x 10^4 cells per well. The culture medium was changed to a medium containing simvastatin at 0.01, 0.1, 1, or 10 μM or to culture medium without simvastatin 24 hours after seeding. The medium with or without simvastatin was changed every other day for 1, 3, 5, and 7 days. MTT stock solution (5 mg/ml; Sigma-Aldrich) was added to each well at the end of the 1, 3, 5, or 7 day, then incubated at 37°C for 4 h. After removal of the MTT solution, dimethylsulfoxide (DMSO; BioBasic) was added to each individual well for solubilization of the converted dye. The plate was shaken gently on a platform rocker for 30 minutes to enhance dissolution. Absorbance was measured at 595 nm using an ELIZA microplate reader (Model 550, BIO-RAD, Hercules, CA, USA). For

confirmation of proliferation, the cells were stained by 0.4% trypan blue (Gibco BRL) and counted using a hemocytometer. All experiments were repeated in triplicate.

Osteogenic and adipogenic differentiation

For osteogenic induction, cultured PDLSCs and BMMSCs were seeded into 6-well plates at a density of 5,000 cells/cm^2 and incubated overnight. When the cultures reached 40-50% confluency, the culture medium was changed to osteogenic differentiation medium; α-MEM containing 10% fetal bovine serum (Gibco BRL), 100 μM ascorbic acid (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich), 5 mM β-glycerol phosphate (Sigma-Aldrich), and 1% antibiotic-antimycotics (Gibco BRL). This osteogenic induction medium was changed every other day for 2 weeks. For adipogenic differentiation, PDSCs and BMMSCs were treated for 3 weeks with adipogenic induction medium containing 0.5 mM 3-isobutyl-1-methylxanthine (Invitrogen, Camarillo, CA, USA), 5μg/ml insulin (Sigma-Aldrich), and 60 μM indomethacin (Invitrogen).

Gene expression confirmed by reverse transcription polymerase chain reaction

Total RNA was extracted from PDLSCs and BMMSCs cultured with simvastatin (0.01, 0.1, or 1 μM) using RNeasy Mini Kit (Qiagen, Hilden, Germany) for cDNA synthesis. RNA concentration was measured using a Nanodrop 2000 spectrophotometer (ND2000, Nano Drop Technologies, Wilmington, DE, USA). Reverse transcription polymerase chain reaction (RT-PCR) was done using a SuperScript™ III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instruction. Amplification of DNA template by RT-PCR was performed in a separate tube using 1 μg of cDNA per 10 pmole of each primer in a GoTaq Green Master Mix (Promega, Madison, WI, USA). The list of primer sets and annealing temperatures are described in Table 1. RT-PCR products were analyzed by electrophoresis in 1.5% agarose gels (BioBasic).

Oil red O staining

To identify lipid droplets in mature adipocytes, cells cultured with adipogenic induction medium were fixed with 10% formalin and stained with 1% oil red O (Chemicon, Temecula, CA, USA). Stained plates were photographed. After extraction of lipid-bound oil red O with 100% isopropanol, absorbance at 490 nm was measured with a spectrophotometer

(FLUOstar OPTIMA ABS.; BMG LabTech, Oldenburg, Germany).

Alizarin red S staining

PDLSCs and BMMSCs at passage 2 to 4 were seeded and cultured on 6-well plates for 14 days. Cultured medium with or without simvastatin (0.01, 0.1, or 1 μM) was changed every 48 hours. The plates were washed twice with PBS, fixed for 20 min with 10% formalin at room temperature, and then washed again several times with distilled water. After the addition of 40 mM alizarin red S solution (pH 4.1, Sigma-Aldrich) to the fixed cells for 30 min, cells were washed several times with distilled water. Positive stained bright orange red nodules were examined under light microscopy (IX53, Olympus®, Tokyo, Japan). For quantification of calcium deposits, cells were destained with a solution of 20% methanol and 10% acetic acid on an orbital shaker for 30 min. Supernatants with dissolved calcium from the cultured cells were measured at an absorbance of 450 nm using an ELIZA multiplate reader (Model 550, BIO-RAD).

Alkaline phosphatase activity

Bone-specific alkaline phosphatase (ALP) activity was elucidated using a QuantiChrom Alkaline Phosphatase Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's recommendations. In brief, PDLSCs and BMMSCs were cultured under osteogenic medium for 7 days with different concentrations of simvastatin. The cells were washed with PBS and lysed in 0.2% Triton X-100 (Sigma-Aldrich) in distilled water at room temperature. A working solution was prepared containing 200 μl assay buffer, 5 μl magnesium acetate (final 5 mM) and 2μl para-nitrophenylphosphate liquid substrate (10 mM). Working reagent (150 μl) was promptly mixed with 50 μl of the cell lysate in separate wells of a 96-well plate. Optical density was measured at 405 nm, and the ALP activity of the sample was calculated from the formula according to the manufacturer's instructions.

***In vivo* transplantation**

PDLSCs and BMMSCs were cultured with 0.01, 0.1, or 1 μM simvastatin for 7 days. The 5.0×10^6 *ex vivo* expanded cells were then mixed with 40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic particles (Zimmer Inc, Warsaw, IN, USA). The mixture was shaken in a 5% CO_2 incubator at 37℃ for 1 hour and then

centrifuged at 2100 rpm for 5 minutes.

Immunodeficient mice (n=40) (NIH-bg-nu/nu-xid; Harlan Sprague Dawley, Charles River, Wilmington, MA, USA, 8~10-week-old) were divided into ten groups, including blank (n=4), HA/TCP carrier group (n=4), PDLSCs treated with simvastatin at individual concentrations (0, 0.01, 0.1, or 1μM) (n=4 per group) and simvastatin mixed with the HA/TCP carrier, and BMMSCs treated with simvastatin at individual concentrations (0, 0.01, 0.1, or 1μM) (n=4 per group) and simvastatin mixed with HA/TCP carrier. For each mouse, the skin was incised along the midsagittal line following a betadine scrub, then the pericranium was elevated. A critical-sized (2.7 mm diameter) calvarial defect of the right parietal bone was prepared using a low-speed trephine drill (Ideal Micro-Drill™ Surgical Drills, Harvard Apparatus, Cambridge, MA, USA) under saline irrigation. Care was taken not to damage the dura mater. After transplantation, the skin was sutured with 6-0 Dafilon® (B. Braun AG, Melsungen, Germany). The mice recovered under a heat lamp and were then moved to our breeding room. Each mouse was checked daily for 2 days and then inspected once per week to ensure healing.

Hematoxylin & Eosin staining and histomorphometric analysis

Eight weeks after transplantation, the mice were euthanized by CO_2. The calvarial specimens were fixed with 4% paraformaldehyde for 24 hours at 4℃, and then decalcified with 10% EDTA for 7 days. After embedding in paraffin wax, 4 μm thickness tissue sections were cut by rotary microtome and affixed on glass slides. Selected on midportion of each graft, two sections were deparaffinized and stained with hematoxylin and eosin (H&E). The area of newly mineralized matrix was measured from eight random selected images of each specimen using light microscopy (IX53, Olympus®) and analysis software (CellSens Dimension 1.12, Olympus®).

Immunohistochemistry

Sections were incubated with primary antibodies: mouse monoclonal anti-human mitochondria antibody (1:200 dilution; Chemicon), rabbit monoclonal antibody to type I collagen (1:200 dilution; Abcam, Cambridge, UK), or mouse monoclonal antibody to osteocalcin (1:200 dilution; Abcam). After rinsing with phosphate-buffered saline, the sections were treated with a mouse- or rabbit-specific HRP/DAB (ABC) detection kit

(Abcam).

Statistical analyses

All data are expressed as mean ± standard deviation of triplicate determinations. Statistical significance of differences between groups was assessed by Student's *t*-test. Data were considered significant when p-values were less than 0.05.

Results

Cell proliferation was maintained with low concentration simvastatin treatment

The number of proliferating cells indicated that simvastatin did not significantly inhibit proliferation of either PDLSCs or BMMSCs in the range of 0.01 to 0.1 μM simvastatin concentration. However, proliferation was suppressed significantly when more than 1 μM of simvastatin was applied (Figure 1); the number of viable cells of both PDLSCs and BMMSCs decreased significantly as the concentration of simvastatin increased, as measured by the MTT assay (Figure 2).

Adipogenic differentiation was inhibited by simvastatin treatment

Expression levels of peroxisome proliferator-activated receptor gamma (PPAR-γ) and lipoprotein lipase (LPL) were inhibited as the concentration of simvastatin increased (Figure 3a). Dose-dependent inhibitory effects of simvastatin on both PDSLCs and BMMSCs were also seen with oil deposits, which formed in adipogenic induction medium after 3 weeks (Figure 3b). BMMSCs exhibited more oil deposits than PDLSCs in each concentration of simvastatin treatment (Figure 3c).

Osteogenic differentiation was enhanced with low concentrations of simvastatin

The BMP-2 gene was expressed with 0.01 to 0.1 μM simvastatin treatment at days 3 and 7 in PDLSCs, whereas the gene expression pattern of BMP-2 was not clearly detected in BMMSCs at same days. At day 14, BMMSCs showed expression of the BMP-2 gene with 0.01 to 0.1 μM simvastatin treatment, whereas the expression pattern showed that BMP-2 was not clearly detected by simvastatin at day 14 in PDLSCs. The BMP-2 genes were not changed by simvastatin treatment. Likewise, the expression patterns of ALP, osteocalcin, and runt-related transcription factor 2 (RUNX2) were not changed by simvastatin treatment (Figure 4).

Both cell types exhibited significantly increased ALP activity with simvastatin treatment in the range between 0.01 and 0.1 μM after osteogenic induction for 7 days. However, suppression of ALP activity was found when 1 μM of simvastatin was applied on to both cell types. PDLSCs showed a significantly higher value of ALP activity than BMMSCs throughout all experiments. Treatment with 0.01 μM of simvastatin caused peak increase in enzyme activity on both PDLSCs and BMMSCs (Figure 5).

Mineralization was not inhibited in either PDLSCs or BMMSCs after treatment with simvastatin between 0.01 and 0.1 μM as confirmed by alizarin red S staining. However, the number of mineral nodules decreased significantly when 1 μM of simvastatin was applied. The dishes were sparsely coated with mineral deposits at 0.01 to 0.1 μM simvastatin in PDLSCs, whereas mineral nodules were more densely coated in BMMSCs (Figure 6a). BMMSCs showed significantly more mineral contents than PDLSCs following treatment with 0.01 to 0.1 μM simvastatin. These results were confirmed in sequence by quantification of absorbance of solubilized mineral contents (Figure 6b).

Simvastatin enhanced osteogenesis of PDLSCs and BMMSCs *in vivo*

H&E staining showed increased new bone deposition around grafted particles using both PDLSCs and BMMSCs treated with 0.01 to 0.1 μM of simvastatin, although BMMSCs indicated relatively more new bone formation than PDLSCs. PDLSCs showed a significant increase in new bone formation after treatment with 0.01 to 0.1 μM simvastatin compared to control (0 μM of simvastatin treatment). There was a significant difference in the mineralized area between PDLSCs and BMMSCs in the control. This result was further

confirmed by quantification of new bone area. Newly formed bone was most abundant in groups treated with 0.1 μM simvastatin for both cell types (Figure 7).

Type I collagen and osteocalcin were expressed on newly formed mineralized tissue around the calvarial defect in a mouse model

Cells stained with anti-human mitochondrial antibody around HA/TCP particles revealed that both PDLSCs and BMMSCs were involved in new bone formation. Mineral matrix formations were confirmed in tissue sections by staining with immune-specific markers: type I collagen and osteocalcin (Figure 8).

Discussion

In this study, we found that proliferation of neither PDLSCs nor BMMSCs were affected by 7 days of treatment with 0.01 to 0.1 μM simvastatin, whereas significant inhibition was seen in both cell lines following treatment with 1 and 10 μM simvastatin. The expression of osteogenesis related genes were not changed by 0.01 to 0.1 μM simvastatin treatment. PDLSCs showed more osteogenic activity than BMMSCs, as elucidated by ALP activity treated with simvastatin for 7 days. We also proved that PDLSCs treated with 0.01 to 0.1 μM simvastatin showed increased mineralization at a level comparable to BMMSCs by alizarin red S staining. Simvastatin at 0.01 μM was most effective for both PDLSCs and BMMSCs for osteogenesis *in vitro*. However, calvarial transplantation in immunocompromised mice revealed that 0.1 μM simvastatin was most effective for osteogenic differentiation with both cell types. Because experimental culture conditions differ from environmental condition in living animals, the fate of the cells might be different.

In this study, we focused on comparing the osteogenic effects of simvastatin between PDLSCs and BMMSCs in treating calvarial defects. Ectopic transplantation, filled with

human DPSCs or human PDLSCs have been studied before individually but not simultaneously[31,73]. By comparing calvarial regeneration influenced by simvastatin with PDLSCs and BMMSCs transplantation, we determined that local delivery of simvastatin with PDLSCs may be effective for future periodontal regeneration. In vivo transplantation showed that newly mineralized area of BMMSCs was significantly higher than that of PDLSCs in control. Noticeably, PDLSCs produced comparable amount of mineralized tissue to BMMSCs at low concentration of simvastatin application. This result demonstrated that simvastatin was capable of enhancing mineralization potential of PDLSCs comparable to BMMSCs. Several reports argued that BMMSCs in nature show osteogenesis superior to other stem cells from mesenchymal origins[58,74]. Limited and committed differentiation potential of PDLSCs which produce less amount of mineralized tissue than BMMSCs makes it less attractive for regeneration purpose. As simvastatin treatment enhances PDLSCs' osteogenic potential, effective and efficient utilization of PDLSCs based therapy would be possible.

There are several statins including simvastatin, atorvastatin, fluvastatin, pitavastatin, rosuvastatin, and pravastatin. Each statin has unique characteristics, such as dosage,

lipophilicity, absorption, bioavailability, hepatic extraction, elimination half-life, and metabolism[44]. Previous studies have suggested diverse signaling pathways of statins and that either mevalonate, TGF-β/Smad3, or OPG/RANK/RANKL might be related to the anabolic effects of statins[45,53].

Lee et al. reported that simvastatin concentrations less than 0.1 μM had no inhibitory effect on proliferation of human mesenchymal stem cells, whereas simvastatin concentrations of 1 μM noticeably decreased cell proliferation[75]. Other studies have reported that proliferation of PDLSCs was maintained at simvastatin concentrations between 0.01 and 0.1 μM after 5 days of treatment, similar to our results[73]. In agreement with previous studies, simvastatin treatment between 0.01 to 0.1 μM is adequate for proliferation of human PDLSCs and BMMSCs *in vitro*.

Statins are usually given orally. Oral application of 20 mg simvastatin daily resulted in a plasma concentration of 0.01 μM[76]. Interestingly, this was equivalent to the concentrations of simvastatin used in our study, in which proliferation and differentiation of both PDLSCs and BMMSCS were greatest *in vitro*. Other reports also gained their highest osteoblastic differentiation of human periodontal ligament cells at 0.01 μM

simvastatin[30]. However, it is uncertain whether serum levels of simvastatin are equal to the local concentration of the target cells. A high first-pass effect over 80% and low bioavailability less than 5% make it difficult to estimate final concentrations in peripheral sites[2,44,77].

In this study, we found opposite roles for simvastatin on osteogenic and adipogenic differentiation. The effect of simvastatin on multipotent stem cells was not only osteogenic but also anti-adipogenic. Similarly, lovastatin was shown to inhibit adipogenesis and enhanced osteoblastic gene expression in a multipotential cell line[78]. Lovastatin may move bone marrow progenitor cells from the adipocyte to the osteoblastic differentiation pathway. This pattern was also supported by other studies[79,80].

BMP-2 was suggested as a causal factor involved in bone anabolic action of simvastatin in other studies. When mouse osteoblast cells were treated with simvastatin, BMP-2 expression was elevated as seen by northern blot analysis[29]. Chen et al. demonstrated that simvastatin increased osteogenic differentiation via the Ras/Smad/Erk/BMP-2 signaling pathway in cytoplasmic and membrane bound proteins[81]. Other reports supported this notion by *in vitro*[82] and immunohistochemical staining[83]. However, we did not find any

relationship between BMP-2 expression and simvastatin treatment.

The periodontal ligament is easily obtainable from the tissues on impacted third molars. Simvastatin-based periodontal regeneration using PDLSCs is therefore a promising tool for cost benefit and minimal invasive procedures. Further studies with other statins are required because each statin shows unique characteristics including bioavailability and lipophilic traits, as previously described[44]. Studies under various conditions using different statins will be helpful for determining the most appropriate applications of statins.

Techniques have to be refined and sophisticated with high specificity to target cells to overcome low bioavailability and to increase efficiency to the periphery. The simvastatin prodrug was recently used in combination with free simvastatin and introduced into fracture sites in a mouse model for specific targeting and sustainability[45]. This prodrug-induced simvastatin consolidated to the target area, resulting in more calcified callus formation. Local drug delivery systems are helpful to escape first-pass effect in liver. For example, local application of simvastatin using methylcellulose gel on rat mandibles or tooth extraction sockets showed both increased bone quantity and quality[68,84]. Soaking gelatin sponge with simvastatin or polylactic acid/polyglycolic acid copolymer also resulted in

increased bone formation[32,71]. Compared to previous local delivery techniques, our study has an advantage that we intended not to improve recipient bed but to activate the cell itself. Thus, transplanted stem cells with high osteogenic potentials are expected to enhance mineralization even on recipient site with poor situation.

Conclusion

Within the limitation of this study, stimulation of osteogenic differentiation by simvastatin on human PDLSCs was comparable to that on BMMSCs. The expression of osteogenesis related genes were not changed by 0.01 to 0.1 μM simvastatin treatment. ALP activity and alizarin red S staining revealed that osteogenic differentiation of PDLSCs by simvastatin were as effective as that of BMMSCs *in vitro*. Calvarial transplantation demonstrated that PDLSCs treated with low concentrations of simvastatin could increase osteogenic differentiation comparable to BMMSCs. Taken together, PDLSCs specific therapy with low concentration of simvastatin may be a promising tool for periodontal regeneration.

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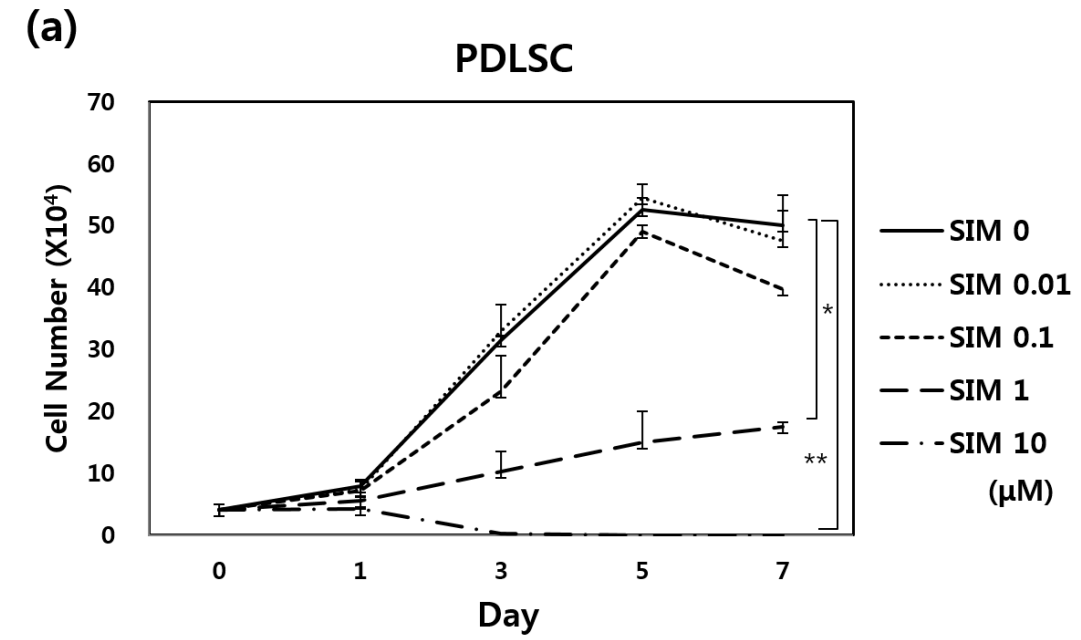
Table and Figures

Table 1. Primary sequences used for RT-PCR

Genes	Genebank Accession No.	Primer sequence (5′-3′)	Size (bp)	Tm (℃)
PPARr2	NM_005037	F: *CTCCTATTGACCCAGAAAGC* R: *GTAGAGCTGAGTCTTCTCAG*	347	57
LPL	NM_000237	F: *ATGGAGAGCAAAGCCCTGCTC* R: *GTTAGGTCCAGCTGGATCGAG*	564	61
ALP	NM_000478	F: *TGGAGCTTCAGAAGCTCAACACCA* R: *ATCTCGTTGTCTGAGTACCAGTCC*	454	61
RUNX2	NM_001024630	F: *GTGGACGAGGCAAGAGTTTCA* R: *CATCAAGCTTCTGTCTGTGCC*	135	59
OC	NM_199173	F: *GTGCAGAGTCCAGCAAAGGT* R: *TCAGCCAACTCGTCACAGTC*	175	59
GAPDH	NM_002046	F: *AGCCGCATCTTCTTT* TGCGTC R: *TCATATTTGGCAGGTTTTTCT*	816	57

Abbreviation: PPARγ2; Peroxisome proliferator-activated receptor gamma-2, LPL; Lipoprotein lipase, ALP; alkaline phosphatase, RUNX2; runt-related transcription factor 2, OC; osteocalcin, GAPDH; glyceraldehyde 3-phosphate dehydrogenase.


(a)
PDLSC
Cell Number (X10⁴)
70
60
50
40
30
20
10
0
0
1
3
5
7
Day
*
**
SIM 0
SIM 0.01
SIM 0.1
SIM 1
SIM 10
(μM)

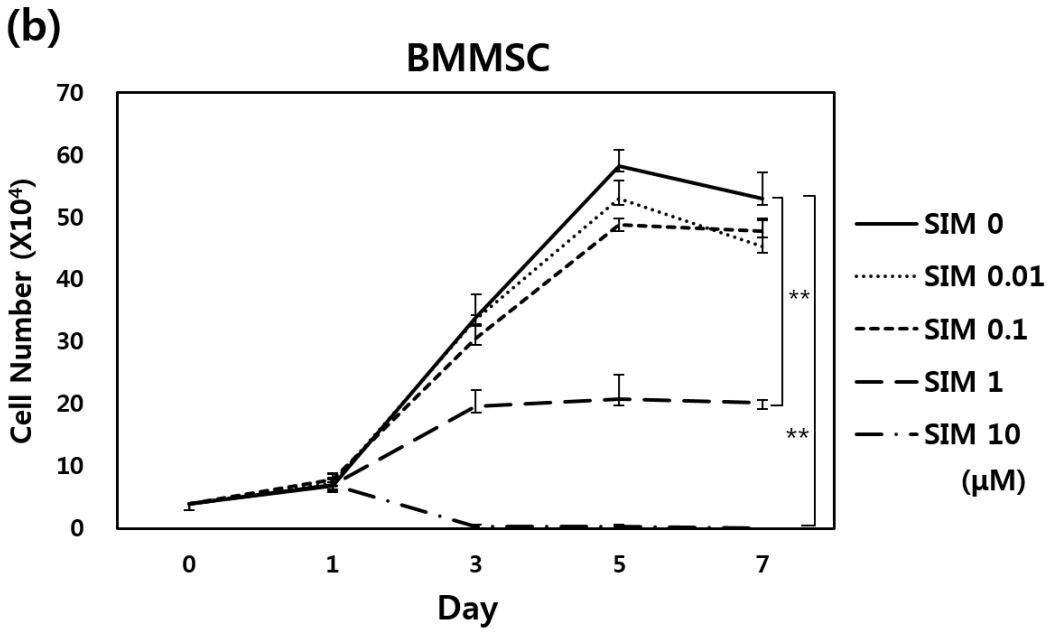
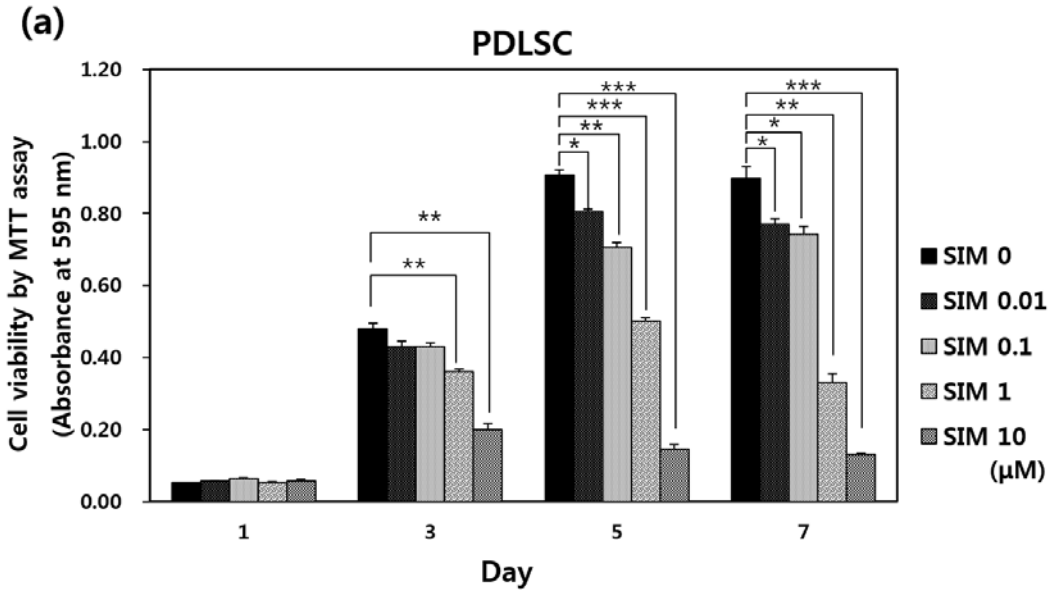

(b)
BMMSC
Cell Number (X10⁴)
70
60
50
40
30
20
10
0
0
1
3
5
7
Day
**
**
SIM 0
SIM 0.01
SIM 0.1
SIM 1
SIM 10
(μM)

Figure 1. Cell proliferation was maintained with low concentration simvastatin treatment

Cells were counted directly for 7 days. Simvastatin was replaced every other day. (a) The number of PDLSCs did not changed significantly with 0.01 or 0.1 μM simvastatin application. However, cells treated with 1 μM simvastatin showed a significant decrease in cell number. (b) BMMSCs showed a similar pattern as PDLSCs. The cells were not decreased greatly at 0.01 or 0.1 μM of simvastatin, although a significant decrease was shown when 1 μM of simvastatin was applied. The data represent mean ± standard deviation with statistical significance (*$p < 0.05$, **$p < 0.01$). Data were analyzed by Student's *t*-test. Abbreviation: SIM; simvastatin.


(a)
PDLSC
Cell viability by MTT assay
(Absorbance at 595 nm)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1
3
5
7
Day
SIM 0
SIM 0.01
SIM 0.1
SIM 1
SIM 10
(μM)

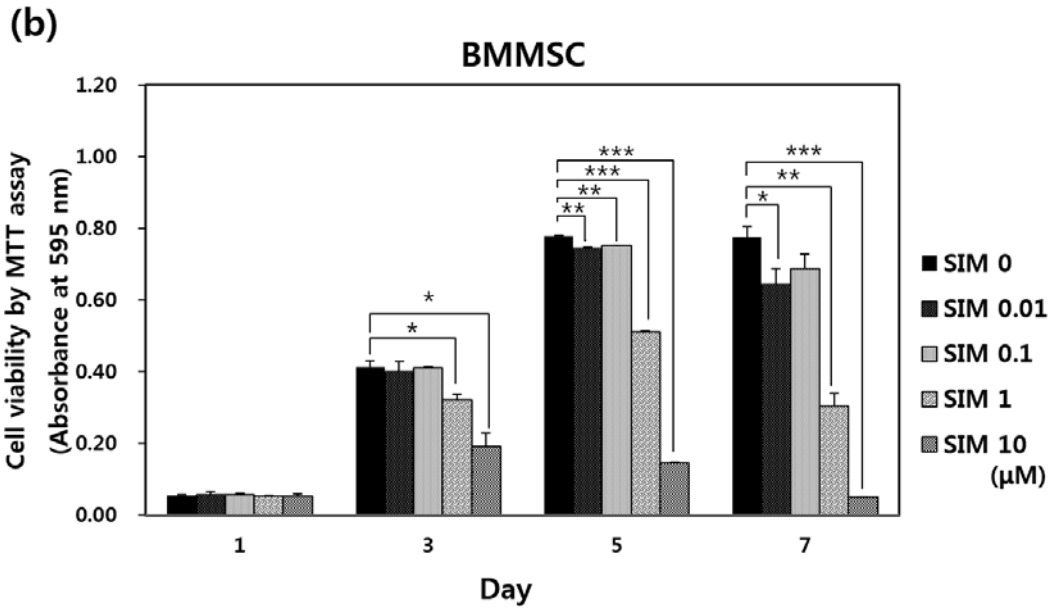
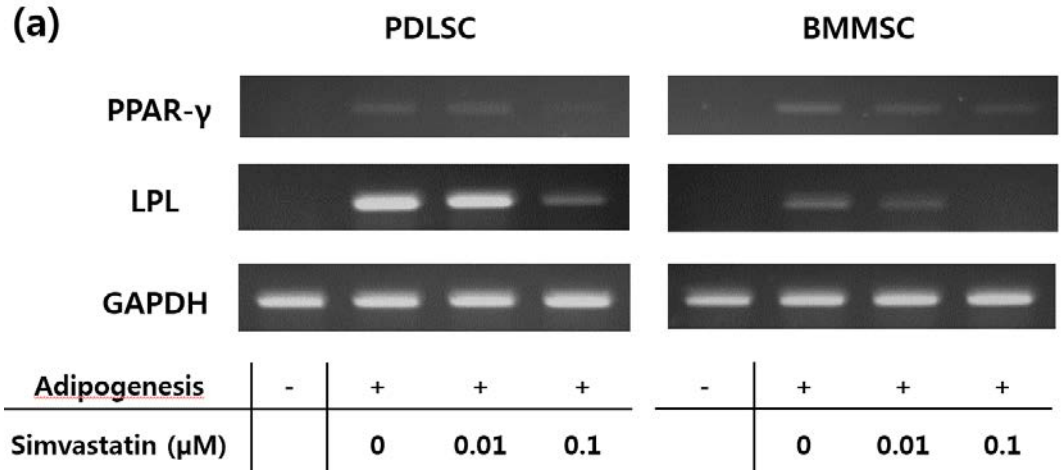
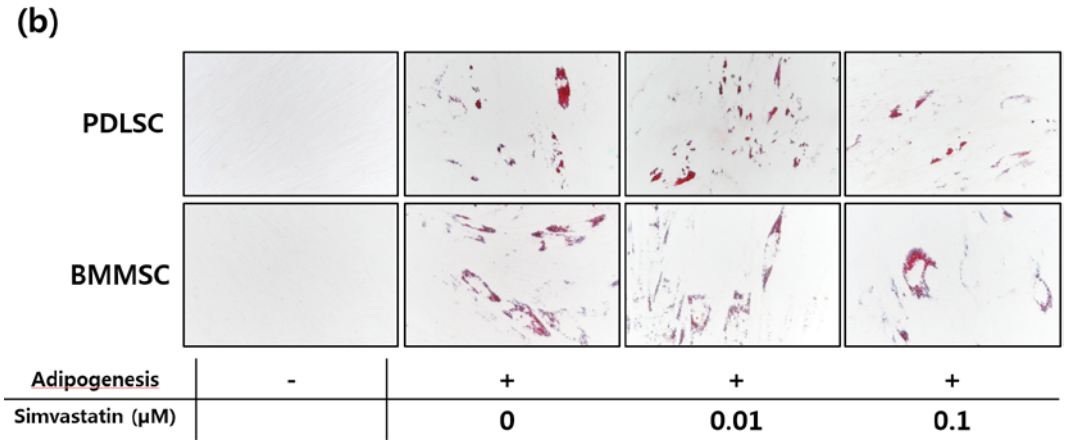

(b)
BMMSC
Cell viability by MTT assay
(Absorbance at 595 nm)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1
3
5
7
Day
SIM 0
SIM 0.01
SIM 0.1
SIM 1
SIM 10
(μM)

Figure 2. Viability of PDLSCs and BMMSCs decreased significantly in a dose dependent manner

Simvastatin was applied to the cell culture medium every other day for 7 days, and the MTT assay was performed according to manufacturer's instructions. The cytotoxic effects were not detected on day 1 with a low concentration of simvastatin. However, the toxicity of the administered drug was obvious at days 3, 5, and 7 on both (a) PDLSCs and (b) BMMSCs. High concentrations of simvastatin significantly suppressed viability of both PDLSCs and BMMSCs. The data represent mean ± standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t*-test). Abbreviation: SIM; simvastatin.


(a)
PDLSC
BMMSC
PPAR-γ
LPL
GAPDH
Adipogenesis
-
+
+
+
-
+
+
+
Simvastatin (μM)
0
0.01
0.1
0
0.01
0.1


(b)
PDLSC
BMMSC
Adipogenesis
Simvastatin (μM)
-
+
+
+
0
0.01
0.1

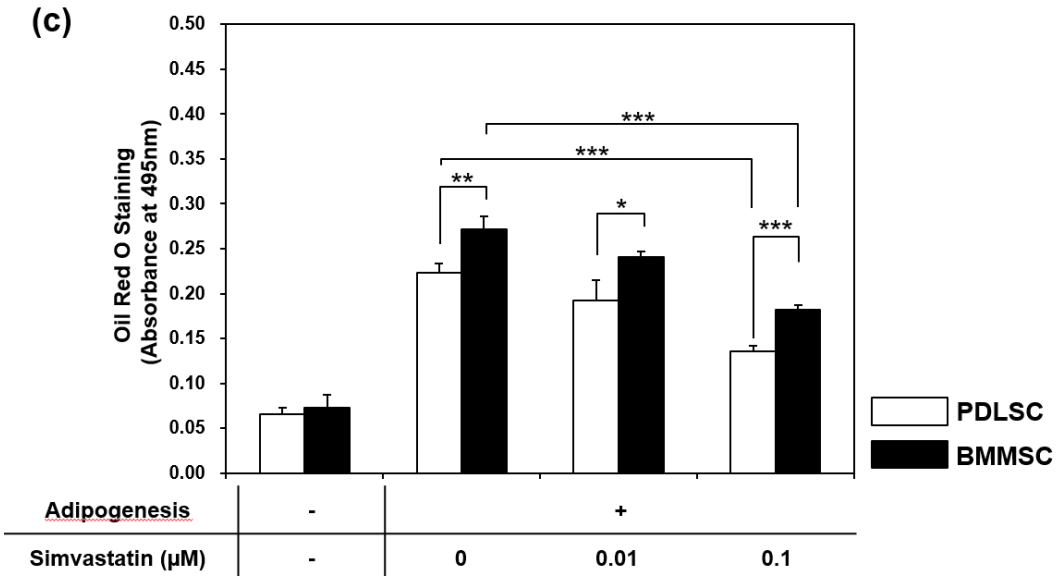

(c)
Oil Red O Staining
(Absorbance at 495nm)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
***
*
***
***
PDLSC
BMMSC
Adipogenesis
Simvastatin (μM)
-
-
+
0
0.01
0.1

Figure 3. Adipogenic differentiation was inhibited by simvastatin treatment

(a) PPAR-r and LPL gene expression were decreased as the concentrations of simvastatin increased. (b) and (c) Dose-dependent inhibitory effects of simvastatin on PDSLCs and BMMSCs were also demonstrated by formation oil deposits in adipogenic induction medium over 3 weeks. BMMSCs exhibited more oil deposit formations than PDLSCs in each simvastatin treatment concentration. The data represent mean ± standard deviation (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$; Student's *t*-test).

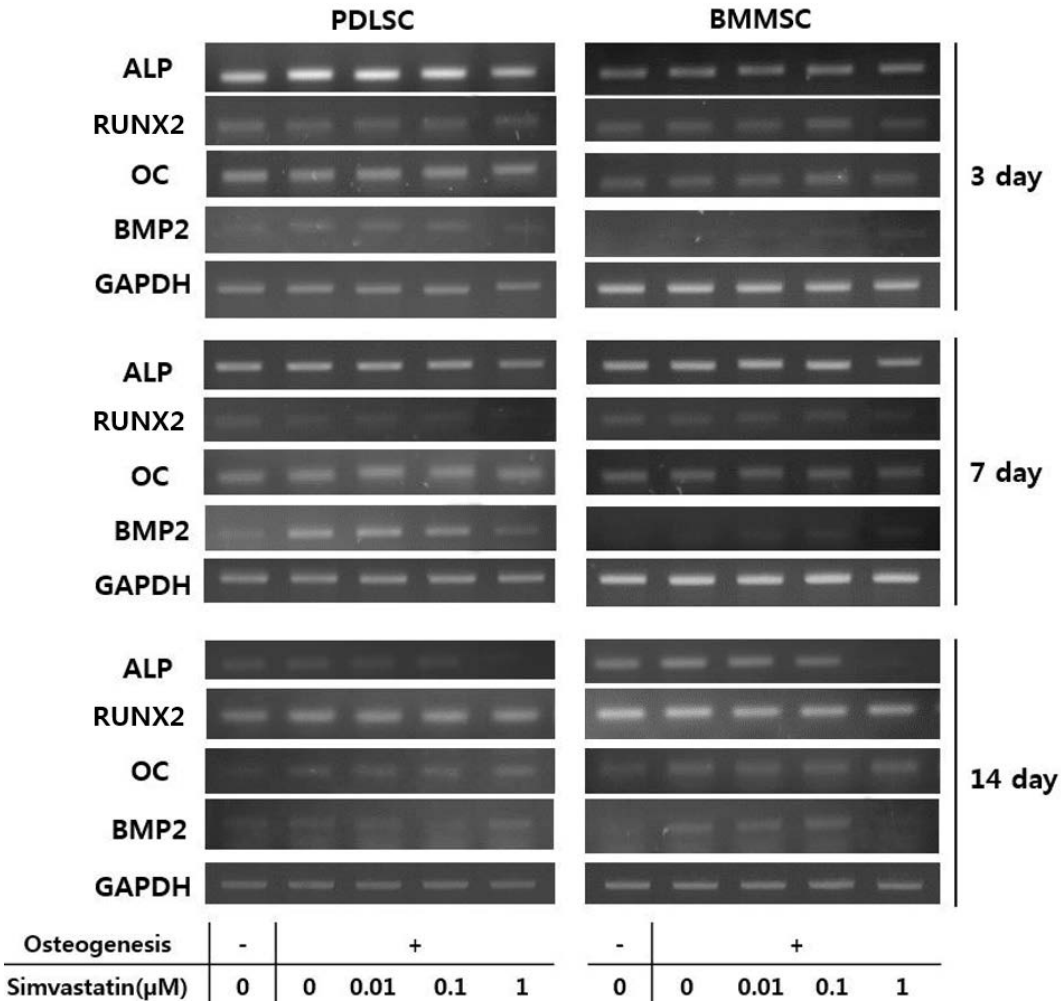

PDLSC
BMMSC
ALP
RUNX2
OC
BMP2
GAPDH
3 day
ALP
RUNX2
OC
BMP2
GAPDH
7 day
ALP
RUNX2
OC
BMP2
GAPDH
14 day
Osteogenesis
-
+
-
+
Simvastatin(μM)
0
0
0.01
0.1
1
0
0
0.01
0.1
1

Figure 4. Low concentrations of simvastatin stimulated osteogenic gene expression on PDLSCs and BMMSCs in different patterns

BMP-2 gene expression was expressed in PDLSCs at days 3 and 7 by treatment with 0.01 or 0.1 μM simvastatin, whereas the expression was not clearly detected in BMMSCs at same days. At day 14, BMMSCs showed BMP-2 expression with 0.01 or 0.1 μM of simvastatin treatment, whereas the expression pattern showed that BMP-2 was not changed at day 14 in PDLSCs. The expression patterns of ALP, osteocalcin and RUNX2 were not shown to be affected by simvastatin. Abbreviations: PPARγ2; Peroxisome proliferator-activated receptor gamma-2, LPL; Lipoprotein lipase, ALP; alkaline phosphatase, RUNX2; runt-related transcription factor 2, OC; osteocalcin, GAPDH; glyceraldehyde 3-phosphate dehydrogenase.

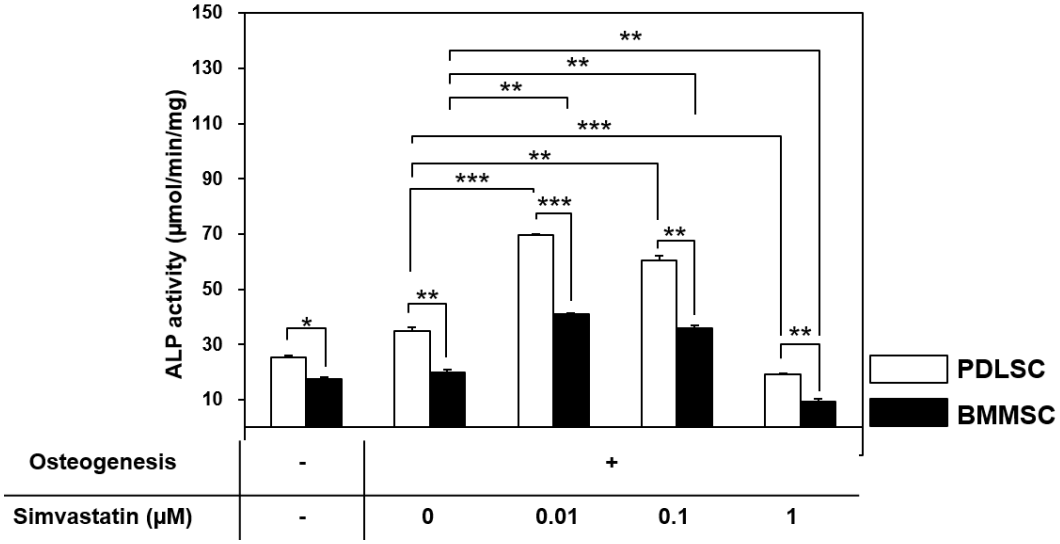
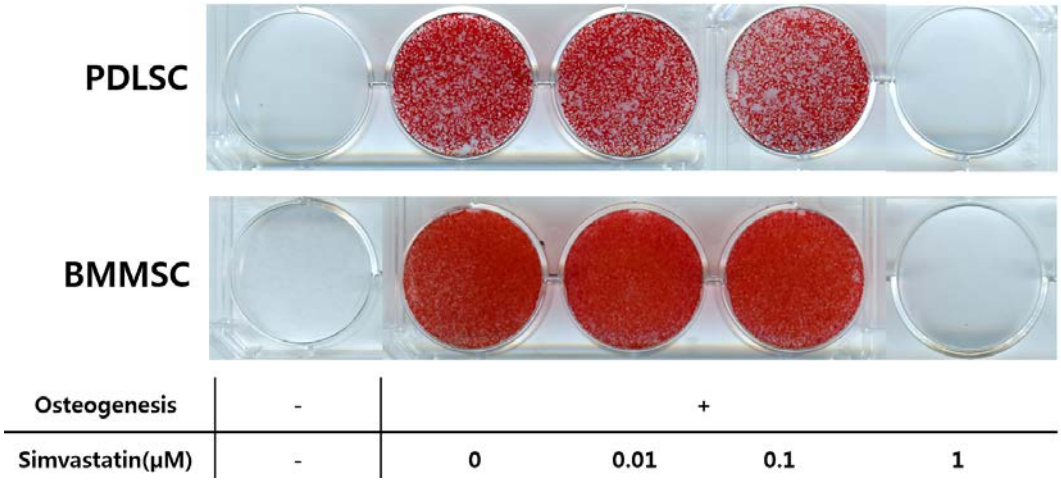


Figure 5. Simvastatin increased ALP activity more in PDLSCs than in BMMSCs

Both cell types showed increased ALP activity with 0.01 to 0.1 μM simvastatin treatment after 7 days of osteogenic induction. Treatment with 0.01 μM simvastatin showed peak increase in enzyme activity on both (a) PDLSCs and (b) BMMSCs. Baseline expression of ALP activity was significantly higher in PDLSCs than in BMMSCs. PDLSCs showed significantly higher ALP activity than BMMSCS when treated with simvastatin. The data represent mean ± standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t*-test).

(a)


PDLSC
BMMSC
Osteogenesis
-
+
Simvastatin(μM)
-
0
0.01
0.1
1

(b)

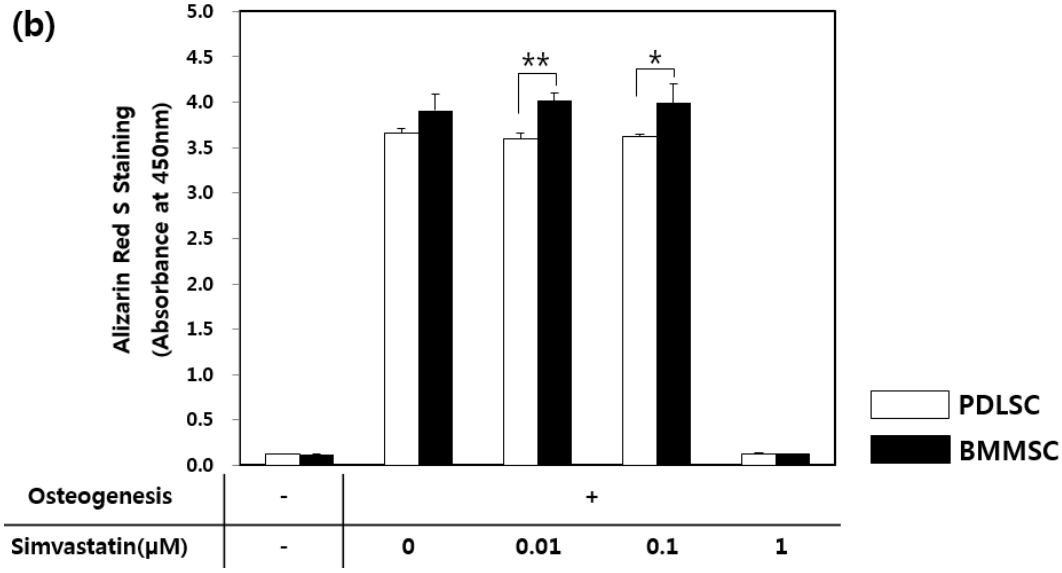
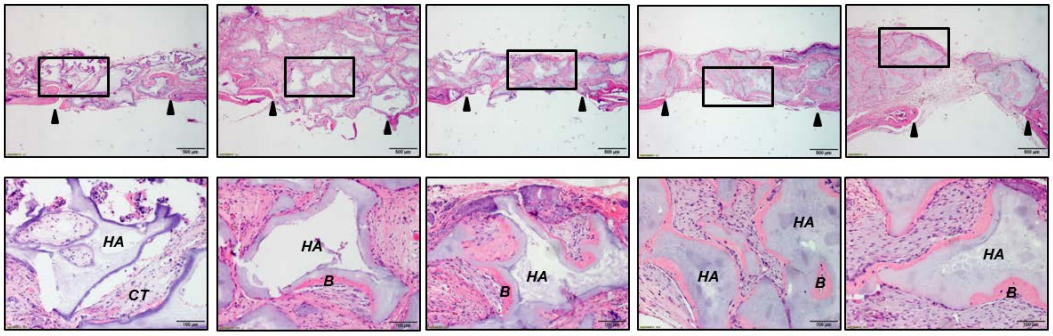

Alizarin Red S Staining
(Absorbance at 450nm)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
PDLSC
BMMSC
Osteogenesis
-
+
Simvastatin(μM)
-
0
0.01
0.1
1

Figure 6. Mineralization was not inhibited by treatment with 0.01 and 0.1 μM simvastatin on either PDLSCs or BMMSCs by alizarin red S staining

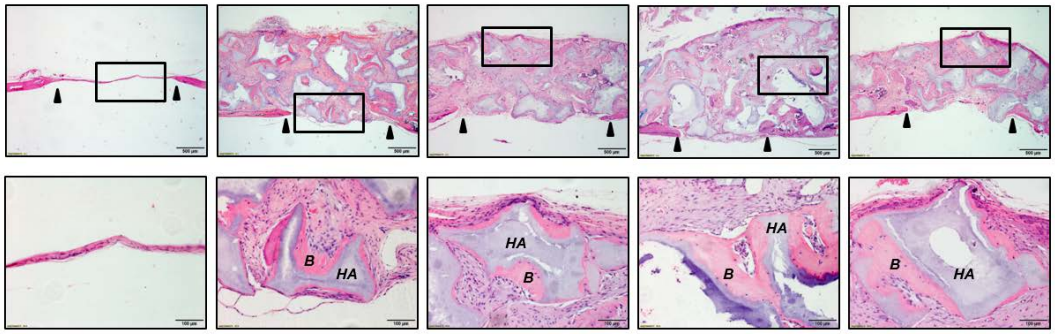
(a) Cells treated with simvastatin for 14 days were stained with alizarin red. Calcium deposits were not decreased in either PDLSCs or BMMSCs when 0, 0.01, or 0.1 μM of simvastatin was applied. However, calcium deposition decreased significantly when 1 μM of simvastatin was administered. BMMSCs showed significantly more mineral content than PDLSCs with both 0.01 and 0.1 μM simvastatin treatment. (b) Quantification of mineral contents by a colorimetric method with spectrophotometer (optical density at 405 nm). Data represent mean ± standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t*-test).

(a)



HA-TCP PDLSC only PDLSC+SIM 0.01 μM PDLSC+SIM 0.1 μM PDLSC+SIM 1 μM

(b)



Blank BMMSC only BMMSC+ SIM 0.01 μM BMMSC+SIM 0.1 μM BMMSC+SIM 1 μM

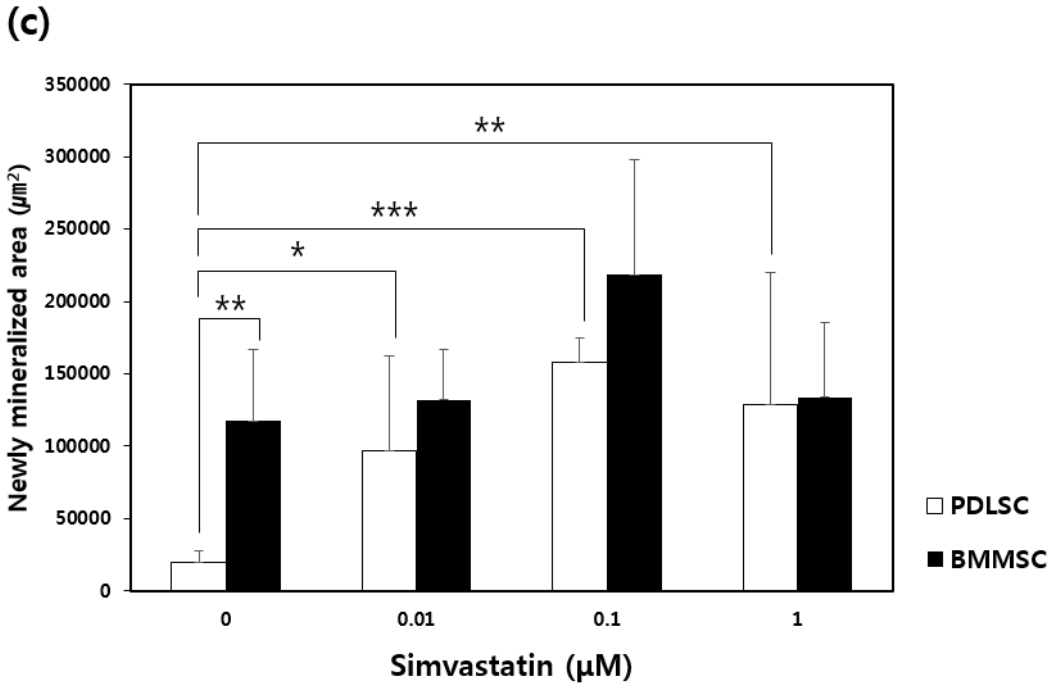
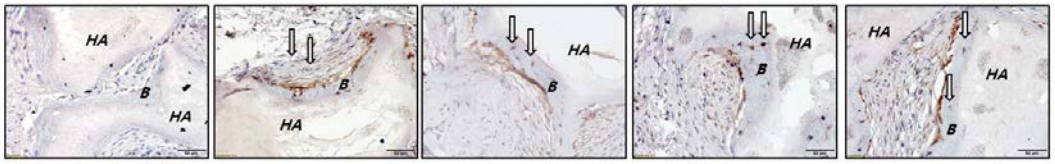


Figure 7. Simvastatin enhanced osteogenesis of PDLSCs and BMMSCs *in vivo*

Stained sections of calvarial defects by H&E staining showed significantly increased new bone formation by treatment with 0.01 and 0.1 μM simvastatin on both (a) PDLSCs and (b) BMMSCs (20x and 200x magnification). (c) Quantification of new bone area was performed. Peak increase in new bone area was found in 0.1 μM of simvastatin concentration in both PDLSCs and BMMSCs. The data represent mean ± standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t*-test). Abbreviations: HA; hydroxyapatite, B; new bone, CT; connective tissue.

(a)

PDLSC



SIM 0 μM SIM 0.01 μM SIM 0.1 μM SIM 1 μM

MT -

MT +

BMMSC

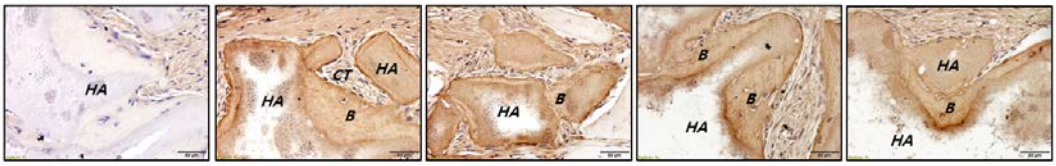
SIM 0 μM SIM 0.01 μM SIM 0.1 μM SIM 1 μM

MT -

MT +

(b)

PDLSC

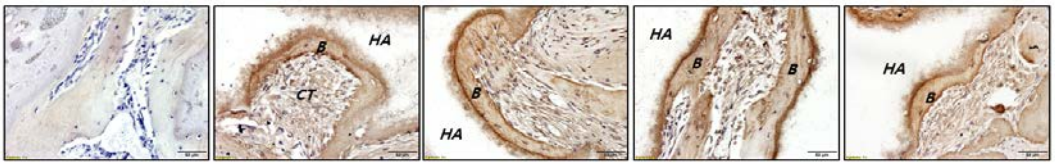


SIM 0 μM SIM 0.01 μM SIM 0.1 μM SIM 1 μM

Col -

Col +

BMMSC



SIM 0 μM SIM 0.01 μM SIM 0.1 μM SIM 1 μM

Col -

Col +

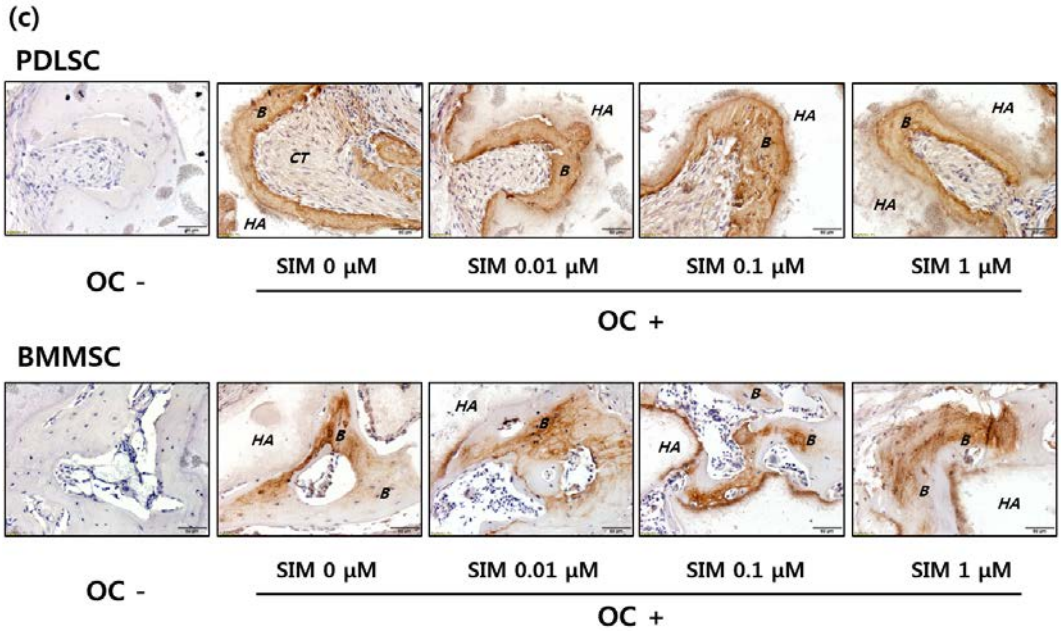


Figure 8. Type I collagen and osteocalcin were expressed on newly formed mineralized tissue around the calvarial defect

(a) Spots stained with human mitochondrial antibody around HA/TCP particles demonstrated that both PDLSCs and BMMSCs were involved in new bone formation. Mineral matrix formations around grafted bones were also confirmed by brown colored deposits stained by immune-specific bone markers, (b) collagen type I and (c) osteocalcin (400x magnification). Abbreviations: HA; hydroxyapatite, B; new bone, CT; connective tissue, MT; mitochondria, Col; type I collagen, OC; osteocalcin. SIM; simvastatin.

국문초록

효과적인 항고지혈증 약물로 수십 년에 걸쳐 안전하게 사용되어 온 스타틴 또는 HMG-CoA 환원효소 억제제는 고지혈증 치료 외에 항혈전, 항산화, 항염증, 그리고 골형성 등 다양한 효과를 가진 것으로 알려져 있다. 본 연구에서는 심바스타틴이 사람 치주인대줄기세포와 골수줄기세포에 미치는 골형성 효과를 *in vitro* 와 *in vivo* 연구를 통해 비교해 보고, 치조골재생 치료제로서의 효과를 평가해 보았다.

치주인대줄기세포와 골수줄기세포 모두에서, 비록 MTT assay 상 심바스타틴 농도에 반비례하여 세포의 생존능이 감소하지만, 0.1 μM 이하 농도의 심바스타틴을 처리시엔 세포증식능이 유의하게 감소하지 않는 것을 세포수 측정을 통해 확인하였다. 치주인대줄기세포와 골수줄기세포 모두에서 심바스타틴 처리농도와 비례하여 지질분화가 억제되는 것을 유전자발현양상과 oil red O 염색으로 확인하였다. 알카리성 인산가수분해효소 단백질 활성은 0.01, 0.1 μM 농도 심바스타틴 처리시 치주인대줄기세포에서 골수줄기세포에 비해 유의적으로 높게 발현되었다. 그러나 골표지자 발현은 심바스타틴에 의해 유의한 영향을 받지 않았다. Alizarin red S 염색 결과 두 세포 모두 무기질침착은 0.1 μM 농도 이하에서 유사하였으며, 골수줄기세포에서 치주인대줄기세포에 비해 상대적으로 다량의 침착을 보였다. 조직염색 후 정량을 통해 쥐의 두개골 이식시 두 세포 모두에서 0.1 μM 농도에서

가장 골분화가 잘 일어났으며, 치주인대줄기세포에서 골수줄기세포보다 상대적으로 적으나 비견할만한 신생골이 형성되는 것을 확인하였다. 면역조직화학염색을 통해 골형성 표지자인 제1형 콜라겐과 osteocalcin 합성을 확인하였고, 이러한 신생골은 사람 골수줄기세포 및 치주인대줄기세포에 의해 만들어지는 것을 사람 미토콘드리아 항체를 통해 확인하였다.

결론적으로, 치주인대줄기세포의 골형성 촉진능은 골수줄기세포에 비해 적은 양의 골형성을 보이지만 심바스타틴을 적정량으로 처리한 결과 골수줄기세포에 비견할 만한 정도로 골형성이 촉진되는 양상을 보였다. 세포실험을 통해 적정 농도의 심바스타틴에 의한 치주인대줄기세포와 골수줄기세포에서의 골분화 촉진을 확인할 수 있었다. 또한 쥐의 두정골 이식결과 치주인대줄기세포는 골수줄기세포와 비견할 만큼 신생골 합성을 유도할 수 있음을 확인하였다. 그러므로 적정 농도의 심바스타틴은 치주인대줄기세포에 특이적인 골조직 재생 효과를 갖고 있어 치주 조직 재생에 유용한 치료제로서 사용할 수 있을 것으로 판단된다.

Keywords: HMG-CoA 환원억제제, 골형성, 중간엽 줄기세포 이식, 치주재생

Student Number: 2012-30603