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치의학박사 학위논문

Influences of immunosuppressants  
on the osteogenic activity of rat  
mesenchymal stem cells  
in vitro & in vivo.

면역억제제가 백서 골수 유래 줄기세포의 골형성능에

미치는 영향에 관한 연구

2017년 2월

서울대학교 대학원

치의학과 치주과학 전공

변 유 경

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## **Abstract**

# **Influences of immunosuppressants on the osteogenic activity of rat mesenchymal stem cells in vitro & in vivo.**

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**Purpose** The purpose of this study was to evaluate the influences of immunosuppressants on the proliferation and osteogenic differentiation of undifferentiated rat mesenchymal stem cells (MSCs) in vitro and bone regeneration of MSCs grafted rat calvarial defects in vivo.

**Methods** MSCs obtained from rat femurs were cultured with immunosuppressants, FK506 and cyclosporine A (CsA), 50 and 500 nM respectively. Cell proliferations during 7 days were assessed with MTT assay. For osteogenic differentiation evaluation, alkaline phosphatase (ALP) analysis and alizarin red S staining were done. RT-PCR was used for the analysis of expression of mRNA for bone-related proteins. For in vivo study, MSCs were obtained from F344 rats and seeded in collagen matrixes. Animals for experiment were divided to 5 groups; control group (defect left without any treatment), Collagen group (defect filled with collagen matrix only), MSC group (defect filled with MSC-seeding matrix), FK506 group (MSC-seeding matrix was grafted and FK506 was injected for 4 weeks), and Rapamycin group (MSC-seeding matrix was grafted and rapamycin was injected for 4 weeks). At 4 and 8 weeks after surgery, the animals were sacrificed and histologic/micro-CT analysis was done.

**Results** Cell proliferation was promoted more in FK506 groups than control or CsA groups. During the experimental period, FK506 groups showed increased ALP activity compared to other groups. Mineralization nodule formations were prominent in FK506 groups than control or CsA groups. Genes for osteopontin, osteonectin, and type I collagen were expressed more in FK506 groups, however the intensity of expression decreased over time. Runx2 and Dlx5 gene expression were up-regulate on day 7 in FK506 groups. In 500 nM CsA group, most of the

genes were less expressed compared to control. In histologic view of rat calvarial defects, FK506 group showed a prominent new bone formation. 3D-images analysis revealed that the defect fill of FK506 group was outstanding. The new bone area (%), bone mineral density, and bone volume/tissue volume (%) were greater in FK506 group than other groups. Rapamycin group did not show apparent new bone formation.

**Conclusions** These results suggest that FK506 could enhance the osteogenic differentiation of rat MSCs and stimulate osteogenic activity of rat MSCs in rat calvarial defects. Other immunosuppressants show inconsistent results in vitro and in vivo.

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Keywords: cell differentiation, cyclosporin A, FK506, rapamycin, immunosuppressants, rat mesenchymal stem cells

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## **Introduction**

In recent decades, bone regeneration has been the main topic of oromaxillofacial area. Lots of materials including membranes, grafts etc. & surgical techniques have been suggested as a better, faster way to regenerate bone (Aghaloo et al. 2016, Larsson et al. 2016). Among them, tissue engineering technology using mesenchymal stem cells (MSCs) is one of the most potential candidates (Quarto & Giannoni, 2016).

MSCs are multipotent progenitor cells that can differentiate into osteoblast, chondroblast or adipocytes. They can be easily collected from bone marrow and expanded without losing their multipotency (Caplan, 2007). Another characteristic which makes MSCs a good applicant for bone tissue engineering is their immunoprivileged property. Undifferentiated and differentiated allogenic MSCs did not elicit lymphocyte proliferative responses (Le Blanc et al. 2003). MSCs did not stimulate alloreactivity, and they could escape lysis by cytotoxic T-cells and natural killer (NK)-cells (Duffy et al. 2011, Rahimzadeh et al. 2016). The immunomodulatory ability of MSCs has made it possible to use allogenic stem cell graft in the animal experiments and clinic. Numerous studies have reported that allogenic MSCs alone can be used for regeneration of various organs (Bernet

et al. 2015, Imanish et al. 2008, De Bari et al. 2003).

For osteoblast-mediated bone deposition, grafted MSCs should proliferate and differentiate to the osteoprogenitor cells, which occur through the multiple steps regulated by various transcription factors and signaling proteins (Zhang, 2010). The main transcriptional regulators for osteoblast is *Runx2* (Komori et al. 1997, Jensen, 2010), and then the cells proliferate and express osteopontin, collagen. Differentiated osteoblasts produce alkaline phosphatase (ALP) (Hoemann et al. 2009) and collagen for maturing the extracellular matrix. As the osteoblasts mature, the mineralization of extracellular matrix occurs with osteocalcin, osteopontin and bone sialoprotein (Stein et al. 2004).

Those series of process need time, but generally, the faster bone formation would be helpful in clinical practice because the bone tissue itself must have a mechanical strength for bearing the load. So, several localized approaches-BMP (Shin JH et al. 2010), TGF- $\beta$  (Bonewald & Mundy, 1990), IGF (Gunter & Rosen, 2013) etc. - and systemic approaches – growth hormones (Andreassen TT et al. 1995), parathyroid hormone (Kroll MH, 2000) etc. - have been studied to enhance the osteoblastogenesis and bone formation.

There are a few studies reported the effects of immunosuppressants on the proliferation and osteoblastic differentiation of mesenchymal cells. Tang et al.

studied the effect of FK506, CsA, and rapamycin on osteoblastic differentiation of MC3T3E1 mouse osteoblast-like cell lines, ST2 mouse bone marrow stromal cell lines, and C3H10T1/2 mouse undifferentiated mesenchymal cell lines. Combined treatment with FK506 and BMP-4 resulted in a significant increase in ALP activity. Also, FK506 enhanced the effects of BMP on ALP/ osteocalcin mRNA expression. Isomoto et al. assessed the effects of FK506, CsA and rapamycin at 10 nM on the osteogenic differentiation of rat MSCs in vitro. Only FK506 without dexamethasone (Dex), a well-known differentiation hormone, revealed higher ALP activity, calcium content, and osteocalcin content. Rapamycin did not affect the osteogenic differentiation and inhibited the osteogenic effect of Dex. But, as far as I know, there is no in vivo experiment to test the influences of immunosuppressants with undifferentiated MSCs.

The purpose of this study is to evaluate the influences of immunosuppressants on the proliferation and differentiation of rat MSCs in vitro and the bone regeneration after grafting allogenic rat MSCs in rat calvarial defects.

# **MATERIALS AND METHODS**

## **Part 1 : In vitro study**

### **Cell isolation and culture**

Rat MSCs were obtained from the femurs of 8 weeks old, Sprague-Dawley rats. Femurs were excised aseptically, cleaned off soft tissues and both ends of femur were cut away. The marrow was flushed out by 20 ml of culture medium and the collected cell suspension was centrifuged at 400 g for 5 min. After the supernatants were removed, the pellet was released into 175 flasks with 20 ml culture medium. Cultures were incubated in humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Standard culture medium which consists of  $\alpha$ -MEM, 15% fetal bovine serum and antibiotics was replaced after 24 hours to remove the non-adherent cells and then three times a week. At near confluence, marrow cells were released using 0.1% trypsin and seeded in 24, 96-well cell culture plate for subculture. All biochemical assays were carried out at least in triplicate.

### **Immunosuppressants**

FK506 (Cayman Chemical Company, Ann Arbor, MI, USA) and Cs A (Cell

Signaling Technology Inc. Danvers, MA, USA) were used from soluble sources. Each drug was diluted with phosphate buffered saline (PBS) to concentrations of 50/500 nM.

### **Cell proliferation assay**

Rat MSCs were seeded in a 96-well plate at  $1 \times 10^3$  cells/well with the standard culture medium. Each immunosuppressant of different concentration was added to each sample the next day. The culture medium and immunosuppressants were changed every other day. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Lancaster, Morecambe, England) assay was used to determine the toxicity of immunosuppressants on stem cell proliferation at 1, 3 and 7 days. Medium was replaced with 200  $\mu$ l fresh medium and 50  $\mu$ l MTT solution was added. After 4 hrs incubation at 37°C, MTT solution was removed and Dimethyl sulfoxide (DMSO) was added for the extraction of formazan. The optical density was measured with an automatic microplate reader (Thermo Max<sup>®</sup>, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 540 nm.

### **Measurement of Alkaline phosphatase (ALP) activity**

The cells seeded in a 24-well plate at  $5 \times 10^3$  cells/well were subcultured in 1 ml of the standard media supplemented with 10 mM  $\beta$ -glycerophosphate, L-ascorbic acid phosphate magnesium salt *n*-hydrate and immunosuppressants. Each assay was performed in triplicate. The medium and drugs were refreshed three times a week. After 3, 7, 14 days of subculture, the activity of alkaline phosphatase was examined using SensoLyte<sup>®</sup> pNPP Alkaline Phosphatase Assay kit (AnaSpec, Fremont, CA, USA). The culture media were removed and the cells were washed twice in 1xAssay buffer. 300  $\mu$ l of Cell lysis buffer were added to each well and the cells were scraped. The cell lysates were centrifuged at 130000rpm for 10 min. p-nitrophenyl phosphate substrate was added to the supernatants (50  $\mu$ l) and let the reaction occur at 37°C for 30 min. And then, Stop solution was added and the absorbance was measured at 405 nm. The ALP activity of each well was expressed as micromoles of p-nitrophenol/30min/ $\mu$ g. The ALP activity in each well was divided by the quantity of total protein in each well.

### **Mineralization nodule formation**

Mineralization was assessed using alizarin red S staining. Rat MSCs were

seeded in a 24-well plate at  $5 \times 10^3$  cells/well. The cells were subcultured in 1 ml of the standard media containing with 10 mM  $\beta$ -glycerophosphate, L-ascorbic acid phosphate magnesium salt *n*-hydrate and immunosuppressants. At 21 days, the cells were fixed in 95% ethanol, washed twice with distilled water and stained for 5 min with Alizarin Red S solution.

## **Real-time polymerase chain reaction (PCR) for bone related mRNA detection**

### **Total RNA extraction**

Rat MSCs were plated at a density of  $1 \times 10^6$  cells /60 mm petri dish. The next day labeled as day 0, culture media were changed to media containing with 10 mM  $\beta$ -glycerophosphate, L-ascorbic acid phosphate magnesium salt *n*-hydrate and immunosuppressants.

RNA was extracted from the cultured cells on day 1 and 7. The procedures were summarized as follows: The cultured cells were lysed with easy-Blue™ (iNtRON Biotechnology, Kyunggi, Korea). Chloroform was added to the lysates to separate the phenol layer from aqueous layer containing RNA. The upper aqueous layers were collected in a new tube and dehydrated with isopropanol. After washing

stage with 75% ethanol, RNA pellets were air-dried and re-dissolved using diethyl pyrocarbonate (DEPC) treated distilled water.

### **Real-time PCR**

1 µg total RNA was reverse transcribed for cDNA conversion using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequences of the primers used for the real-time PCR are presented in Table 1.

The amplification reactions were performed in MicroAmp® 96 well plates and each well contained SYBR® *Premix Ex Taq*™ II, each PCR primer, ROX™ Reference Dye II, dH<sub>2</sub>O and each template. Amplifications were carried out in 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and cycling conditions were as follows: The initial denaturation step at 95°C for 15 sec, 40 3-step amplification cycles of 15 sec denaturation at 95°C, 15 sec annealing at 60°C and 33 sec extension at 72°C, dissociation stage. All RT-PCR reactions were run in duplicate.

Relative gene expressions were normalized to GAPDH expression and the data were presented as fold change using the formula  $2^{-\Delta\Delta C_T}$  as recommended by manufacturer (Applied Biosystems User Bulletin No.2(P/N 4303859), Foster

City, CA). When the value of  $2^{-\Delta\Delta C_T}$  is less than 1, the negative inverse of  $2^{-\Delta\Delta C_T}$  is presented as fold change reduction in expression.

## **Part2. In vivo study**

### **Cell isolation and culture**

Rat MSCs were harvested from the femurs of 8-week-old F344 rats. Rat femurs were obtained, and both ends of each femur were excised. 20 ml of culture medium were used to flush out the marrow, and the collected cell suspensions were centrifuged at 400 g for 5 min. After removing the supernatants, the pellet was released in 175 flasks with 20 ml culture medium, made of  $\alpha$ -MEM, 15% FBS and antibiotics. Every culture was incubated in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C and the culture medium was changed after 24 hours to remove the non-adherent cells. During the incubation, the medium was changed three times a week. At near confluence, the marrow cells were released using 0.1% trypsin and seeded in collagen matrix (CollaCote™, Zimmer Dental Inc., Carlsbad, CA, USA), containing 1x10<sup>6</sup> cells per matrix. The MSCs used for experiments were in passage 2 to 5.

## **Immunosuppressants**

For animal experiments, FK506 (Cayman Chemical Co., Ann Arbor, MI, USA) and rapamycin (Cell signaling technology, Danvers, MA, USA) were selected as an immunosuppressant. Drug dosage used in this study was 1 mg/1 kg body weight of FK506 and rapamycin.

## **Surgical procedure**

Guidelines established by the Seoul National University Institutional Animal Care and Use Committee (Approval No SNU-131023-2) have been followed during the experiment.

40 male Sprague-Dawley rats (8 weeks old) were used for this study. The animals were anesthetized by an intramuscular injection of ketamine and xylazine. The dorsal field of cranium was shaved before surgery and prepared with betadine scrub. A linear incision over the scalp was made at midline and the skin/musculature and the periosteum were reflected. An 8 mm diameter critical size defect was made using trephine bur (Osstem, Seoul, Korea) under sterile saline irrigation. The experimental animals were divided into 4 groups as follows: (1) control group ; the defects were remained untreated. (2) Collagen group ; the

defects were filled with the collagen matrix only, (3) MSC group ; the defects were filled with the MSC soaking collagen matrix, (4) FK506 group ; the defects were filled same as MSC groups, and FK506 was injected every day for 2 weeks, and then every two days for 2 weeks. (5) Rapamycin group ; the defects were filled same as MSC groups, and rapamycin was injected every day for 2 weeks, and then every two days for 2 weeks. The periosteum over the defect was closed with 5-0 chromic gut and the skin was sutured with 4-0 silk.

In each group, four rats were sacrificed by CO<sub>2</sub> at 4 and 8 weeks after surgery. The calvarias including surgical defects were obtained and fixed in 10% neutralized-buffered formalin solution

### **Histologic/histomorphometric analysis**

The specimens were decalcified with 10% ethylenediaminetetraacetic acid (EDTA) solution for 2 weeks and dehydrated through a series of ethanol solutions of increasing concentrations and embedded in paraffin. 5 µm-thick coronal sections through the center of the defects were obtained and stained with hematoxylin-eosin stain.

After microscopic examination, a photograph of each slide was taken.

Measurement of new bone area was carried out using an automate image analysis system (Tomoro Scope Eye 3.5 Image Analyzer, Techsan Digital Imaging, Seoul, Korea). Regeneration of bone was quantified by measuring the percentage of the regenerated bone area in the whole defect area.

### **Micro-CT analysis**

Micro-CT was taken using Skyscan 1172 (Bruker, Kontich, Belgium) at 70 kV, 141  $\mu$ A. Reconstructions were performed with NRecon software (Bruker, Kontich, Belgium) and the region of interest of each specimen was analyzed with CtAn program (Bruker, Kontich, Belgium) for bone mineral density (BMD), bone volume fraction (bone volume/tissue volume, BV/TV).

### **Statistical analysis**

One-way analysis of variance with Tukey's test was used to evaluate the differences among the experiment groups. To compare the results between 4 and 8 weeks, Student t-test was used. Statistical significance was set at  $p < 0.05$  and the statistical analysis were performed using SPSS software (SPSS, Chicago, IL,

USA).

# Results

## Part 1 : In vitro study

### Analysis of cell proliferation

Cell proliferation assessment was done on days 1, 3 and 7. All groups showed evidently increased cell proliferation over the whole examination period (Figure 1). There were significant differences in optical density (OD) over time ( $p < 0.05$ ).

On day 1, there was no significant difference among all groups. However, FK 506 groups showed greater OD value than control and CsA groups on day 3 and 7. No significant difference was found between control and CsA groups. The concentration of FK506, Cs A did not show any significant difference in cell proliferation.

### Analysis of ALP activity

Figure 2 shows the effects of immunosuppressants on the ALPase activity of MSCs. ALP activity was assessed at 3, 7 and 14 days after treatment with immunosuppressants. On day 3, ALP activity of control group was slightly greater than other groups, but not significantly different ( $p > 0.05$ ). Fifty nM and 500 nM FK506 (F50 and F500) groups showed higher ALP activity than other groups on day 7 and the differences between FK506 groups (F50 and F500) and

500 nM CsA group (C500) was significant ( $p < 0.05$ ). At later time point, the increase of ALP activity level was most remarkable in 50 nM FK506 (F50) group and significantly different to control, C500 group. The ALP activity of CsA groups did not differ from control group in all assessments ( $p > 0.05$ ).

### **Analysis of cultured cell mineralization**

In the groups with FK506, Alizarin red S-(+) nodules were most prominent (Fig 3). Control group showed more mineralization nodule formation than CsA groups. In experimental groups, the concentration of drug made no difference to nodule formation macroscopically.

### **Analysis of the expression of mRNA for bone-related proteins**

Quantifying the relative changes in gene expression was done on day 1 and 7. Immunosuppressants showed diverse outcomes depending on drug concentrations, genes of interest and time (Fig 4).

Expression of osteopontin, extracellular bone protein, was most markedly increased when treated with 500 nM FK506 on day 1. Lower concentration (50 nM) also increased the osteopontin mRNA expression over time. CsA decreased the expression of osteopontin relative to control on day 1, however, the expression was increased over time.

Treatment with FK506 had a clear positive effect on osteonectin mRNA expression on day 1 even though the expressions were diminished on day 7. Contrary to FK506, CsA did not show the decrease in mRNA expression on day 7.

The fold changes of RUNX2 mRNA expression in all experimental groups were negative on day 1, and it implies the immunosuppressants had an adverse effect on RUNX2 expression. However, at later time points, the fold changes were reversed, which meant that RUNX2 mRNA was more expressed than control group.

Type I collagen (Col-I), mid bone marker protein, showed an increased expression when treated with 50, 500 nM FK506 and 50 nM CsA. Higher concentration of CsA (500 nM) decreased the Col-I expression during experiment period.

Immunosuppressants treatment induced a down-regulation of DLX5 gene expression on day 1, but on day 7, the expression was up-regulated compared to control group.

## **Part2. In vivo study**

### **Histologic findings**

At 4 weeks, most of the regenerated bone was observed along the margin of the

defect and the amount of new bone was limited in control, MSC and Rapamycin group. On the other hand, in Collagen group and FK 506 group, the regenerated bone was revealed in the middle of the defect and the amount of new bone was much more than control, MSC, Rapamycin group. There were no inflammatory cells infiltrations in all groups (Fig 5).

At 8 weeks, newly formed bone at the bony defects in FK506 group was dramatic and obvious compared with other groups. The sponge-like immature bones observed at 4 weeks in FK506 group changed to more mature bone with large blood vessels (Fig 6).

### **Histomorphometric analysis**

Regenerated bone area (%) of the control, Collagen, MSC, FK506 and Rapamycin group, was  $16.85 \pm 1.53\%$ ,  $28.62 \pm 11.90\%$ ,  $24.52 \pm 7.99\%$ ,  $44.48 \pm 8.29\%$  and  $24.51 \pm 10.25\%$ , respectively, at 4 weeks. New bone area was significantly greater in the FK506 group than control, MSC and Rapamycin group (Table 2).

At 8 weeks, regenerated bone area (%) of the control, Collagen, MSC, FK506 and Rapamycin group, was  $31.48 \pm 2.14\%$ ,  $31.69 \pm 2.58\%$ ,  $31.59 \pm 14.63\%$ ,  $54.21 \pm 11.17\%$ , and  $23.08 \pm 13.28\%$ , respectively. FK506 group showed the greatest new bone formation than other groups with statistical significance.

The increase of regenerated bone area between 4 and 8 weeks was larger in MSC and FK506 group than other groups, but statistically not significant.

### **Micro-CT image**

The bone formation of rat skull defect was evaluated using 3-dimensional micro-CT images (Fig 7). New bone formation was prominent in FK506 group only. Other groups except FK506 group showed limited bone formation along the defect margin. In FK506 group, newly formed bones were observed in entire defect including central area and the bone thickness was increased at 8 weeks compared to 4 weeks (the sagittal view not shown here).

### **Qualitative Micro-CT analysis - BMD**

At 4 weeks, BMD was  $0.049 \pm 0.057 \text{ g/cm}^3$  for control,  $0.188 \pm 0.072 \text{ g/cm}^3$  for Collagen group,  $0.056 \pm 0.031 \text{ g/cm}^3$  for MSC group,  $0.191 \pm 0.099 \text{ g/cm}^3$  for FK506 group,  $0.091 \pm 0.036 \text{ g/cm}^3$  for Rapamycin group. Collagen group and FK506 group had greater BMD value than other groups, however in most cases, there was no statistical significance between groups. There was statistical significance between control - FK506 group only.

At 8 weeks, control group ( $0.05 \pm 0.026 \text{ g/cm}^3$ ) did not show any statistical difference compared to 4 weeks. BMD of MSC group ( $0.111 \pm 0.067 \text{ g/cm}^3$ ) and

Rapamycin group ( $0.070 \pm 0.054 \text{ g/cm}^3$ ) increased slightly, and Collagen group ( $0.235 \pm 0.124 \text{ g/cm}^3$ ) and FK506 group ( $0.307 \pm 0.107 \text{ g/cm}^3$ ) showed a larger increase than 4 weeks. There is a statistical significance between FK506 and other 3 groups (control, MSC, Rapamycin) (Table 3).

BMD value increased from 4 to 8 weeks in all groups except Rapamycin group which BMD was slightly decreased, but the differences were not significant statistically.

#### **Quantitative Micro-CT analysis -BV/TV**

Bone volume / tissue volume (%) was defined as ratio of new bone area to entire defect created with trephine bur (Table 4). BV/TV of all groups was increased during the experiment period and FK506 group showed the highest percentage than other groups. At 4 weeks, BV/TV of control group ( $6.95 \pm 0.58\%$ ) was significantly lower than Collagen group ( $15.46 \pm 2.26 \%$ ), MSC group ( $10.37 \pm 3.97\%$ ), FK506 ( $26.35 \pm 4.83\%$ ) and Rapamycin group ( $15.64 \pm 2.94\%$ ). BV/TV of FK506 group was significantly higher than other groups.

At 8 weeks, FK506 group ( $39.09 \pm 1.46\%$ ) exhibited the largest increase compared with 4 weeks and significantly more new bone area than control group ( $8.14 \pm 1.37\%$ ), Collagen group ( $19.76 \pm 2.82\%$ ), MSC group ( $17.38 \pm 1.34 \%$ ) and Rapamycin group ( $15.66 \pm 4.17\%$ ). Control group showed significantly lower

percentage than other groups.

Compared with the percentage of 4 weeks, MSC and FK506 groups showed significantly higher percentage at 8 weeks. There was no statistical significance between 4 and 8 weeks in control, Collagen and Rapamycin group.

## **Discussion**

The present study designed the experiment to assess the influence of immunosuppressants itself, without any other reagents or compounds, on the osteogenic activity of rat mesenchymal stem cells.

In this experiment, it was demonstrated that immunosuppressants could affect osteogenic differentiation of rat MSCs in vitro. FK506 promoted cell proliferation and ALP activity than CsA in vitro. Also, bone-related protein mRNA expressions had elevated in FK506 treated group. These results mean that FK506 may stimulate cell proliferation and differentiation to osteoblasts. However, the other tested immunosuppressant, CsA, did not reveal any evident effect on cell growth or differentiation.

In animal experiment, FK506 showed a positive effect on the new bone formation in rat skull defect that was grafted with allogenic rat MSCs. In histologic view and 3D micro-CT view, FK506 treated group had shown pronounced defect fill and new bone formation. BMD and bone volume/tissue volume was higher in FK506 group than other groups. However, the other immunosuppressant, rapamycin, did not show apparent effect on allogenic rat MSCs in calvarial defect.

There are several in vitro, in vivo studies those results are similar to this study

even though the used cell types or experiment conditions were slightly different from this experiment. Kugimiya et al. applied FK506 and CsA to C<sub>17</sub> and C<sub>2</sub>C<sub>12</sub> cells to induce the osteogenic differentiation. In their experiment, the concentration of each drug was 1000 nM, which was higher than the concentration used in this study, and they demonstrated that FK506 was able to induce osteocalcin mRNA expression.

In vivo study of Yoshikawa et al. used the cultured bone construct in porous hydroxyapatite and tested the effect of FK506 on the bone formation of implanted isogenic/allogenic bone construct. Subcutaneously implanted isografts showed extensive bone formation and higher ALP activity/osteocalcin content with FK506 than without FK506. In the allografts, without FK506 administration, there was no bone formation and osteocalcin mRNA expression.

In this animal study, the inflammatory infiltrations were not observed in allogenic MSC graft group even though the immunosuppressants were not injected. It can be explained by hypoinmunogenic and immunosuppressing properties of MSCs (Jacobs SA et al. 2013). There are numerous studies which report that mesenchymal stem cells possess low levels of MHC class I and co-stimulatory CD40, CD80, and CD86 and no MHC class II molecules (Tse WT et al. 2003) and they do not elicit allogenic lymphocyte responses (Bartholomew A. 2002, LeBlanc et al. 2003).

The bone fill of MSC graft group was not satisfactory although there was no inflammatory cell infiltration. The possible reason to explain those results is that the immunoprivileged properties of MSCs would not persist as they are differentiated. Chatterjea et al. implanted the allogeneic MSCs that have been pre-differentiated to the osteoprogenitors and evaluated the immune response and bone formation on rat subcutaneous pockets. They found that implantation of pre-differentiated allogeneic MSCs in an immunocompetent rat led to a T cell- and B cell- mediated immune reaction that resulted in an abolition of bone formation, while the allogeneic osteoprogenitors could form bone in a nude mouse. Then they administered immunosuppressants-FK506, and in the immunosuppressed environment, the allogeneic osteoprogenitors can generate bone as syngeneic grafts do.

FK506 treated group showed a remarkable new bone formation compared to other groups in both in vitro and in vivo experiments. Two possible hypotheses can be considered to explain the better results of FK506 groups. The first hypothesis is that immunosuppressants are needed for allogeneic cell grafts even though the stem cells are immunoprivileged. Kotobuki et al. implanted hydroxyapatite (HA) ceramic/MSCs composite subcutaneously in rat. They reported that none of the allogeneic MSCs survived or showed osteogenic differentiation. But when the recipient rats were treated with FK506, implanted

allogenic MSCs showed osteogenic differentiation. The second hypothesis is that FK506 itself could affect the bone metabolism. Darcy et al. did in vitro study using MC3T3 pre-osteoblast cells to examine the effect of FK506 on osteoblastogenesis. When FK506 alone was added to MC3T3 cell-culture, phosphorylations of Smad 1/5/8 and Osx /Runx2 mRNA levels were elevated. With or without BMP2, FK506 showed potentials for promoting osteoblast differentiation.

In addition, there is a report that FK506 can inhibit IL-17-inuced osteoclastogenesis from human monocytes. Yago et al. applied FK506 on human CD14+ monocytes which were cultured in the presence of macrophage-colony stimulating factor (M-CSF) and IL-17 and found that FK506, even at 5.0 ng/ml, strongly inhibited osteoclastogenesis. Taken together, it can be speculated that FK506 could exert a positive influence on bone formation and further studies are necessary to ascertain the mechanisms of FK506 on bone metabolism.

In the present in vivo study, rapamycin did not show a positive effect on new bone formation. That can be interpreted because rapamycin inhibits the mammalian target of rapamycin (mTOR) which was thought to involve in osteoblast differentiation, and there are several other studies which reported the similar results. Singha et al. reported that the rapamycin significantly reduced osteoblast-specific osteocalcin, bone sialoprotein and osterix mRNA expression

when applied to MC3T3-E1 preosteoblastic cells and primary mouse bone marrow stromal cells. More recently, Chen and Long found that mTORC1 had a critical role in promoting osteoblast differentiation. They first deleted Raptor, a unique and essential component of mTORC1, in primary calvarial cells. Under the osteogenic culture condition, the deletion of Raptor reduced the matrix synthesis and mineralization.

In conclusion, the results of this study suggest that FK506 can stimulate the osteogenic differentiation of rat MSCs and bone regeneration in rat calvarial defects. Allogenic MSC alone did not make a sufficient bone in rat skull defect. Therefore, the use of FK506 could be considered as a supplement in xenogenic or allogenic stem cell transplantation for the treatment of bone defects. Further studies are needed to determine the duration or dosage of immunosuppressants for the augmentation of bone graft and the risk-benefit ratio related to the systemic effects of immunosuppressants.

## Figures and Legends

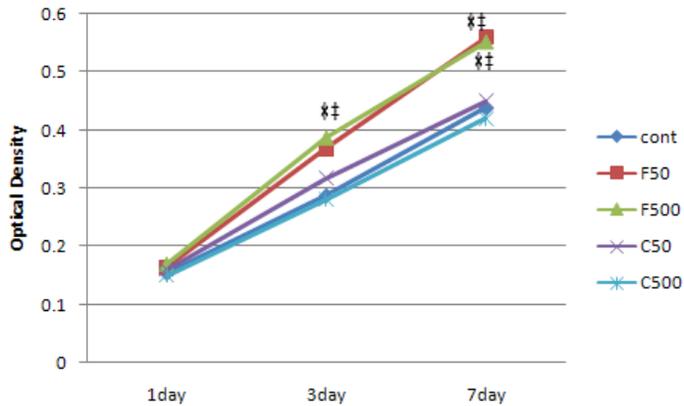


Figure 1. Effects of FK506 and CsA on proliferation of rat MSCs. Cell viability was assessed with MTT assay and the results were presented as optical density at 540 nm. FK506 groups showed increased cell proliferation compared to control and CsA groups. Significant difference from control (\*) and C500 (†) ( $P < 0.05$ ).

F50: 50 nM FK 506 group, F500: 500 nM FK506 group, C50: 50 nM CsA group, C500: 500 nM CsA group.

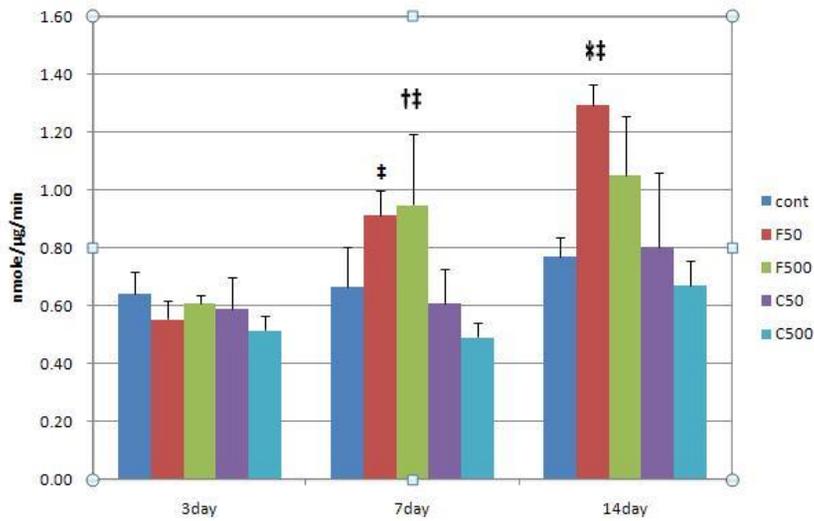


Figure 2. Effects of FK506 and CsA on alkaline phosphatase (ALP) activity of rat MSCs. FK506 groups showed higher ALP activity than other groups. There were no significant differences between control and CsA groups. Significant difference from control (\*), C50 (†) and C500 (‡) ( $P < 0.05$ ).

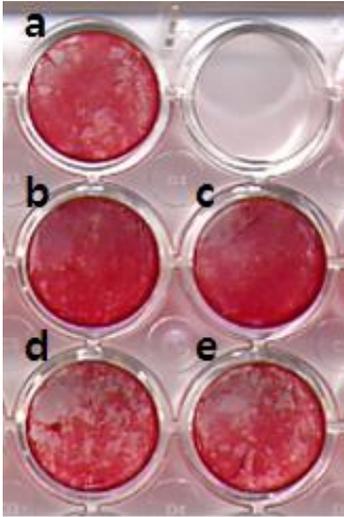
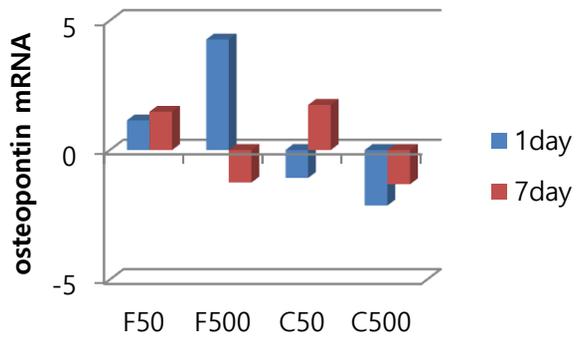
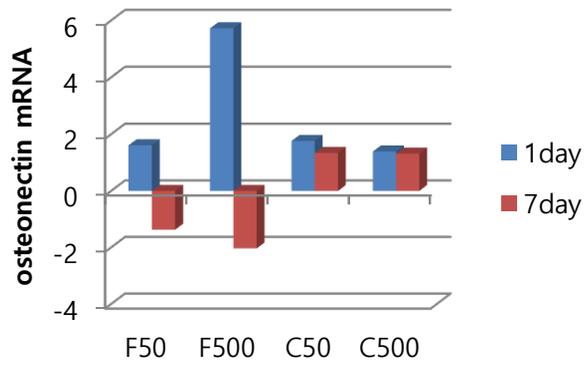


Fig 3. Alizarin red S staining of rat MSCs after 21 days of subculture. Mineralization nodule formations were most prominent in FK506 groups.

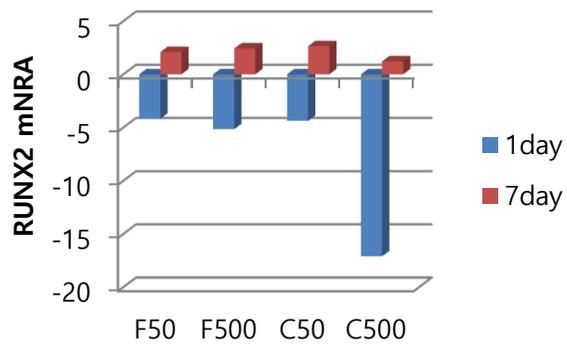
- (a) Control
- (b) 50 nM FK506
- (c) 500 nM FK506
- (d) 50 nM CsA
- (e) 500 nM CsA



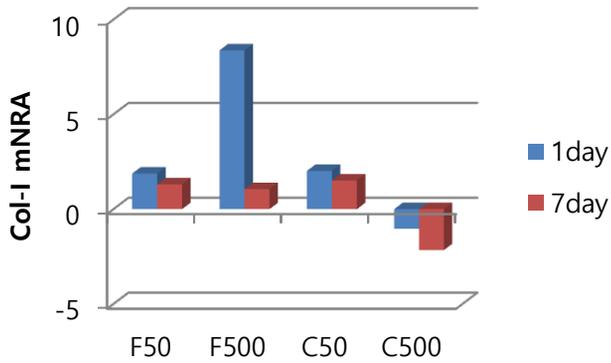
(a)



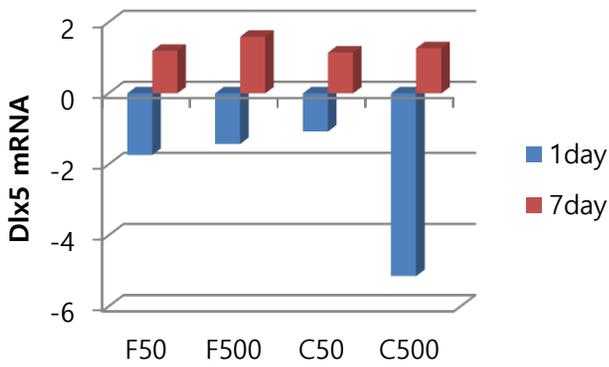
(b)



(c)



(d)



(e)

Fig 4. Osteopontin (a), Osteonectin (b), Runx2 (c), Col-I (d) and Dlx5 (e) mRNA expression during cell differentiation of rat MSCs. Relative mRNA levels were plotted as fold change of bone-related gene over control and normalized to GAPDH level.

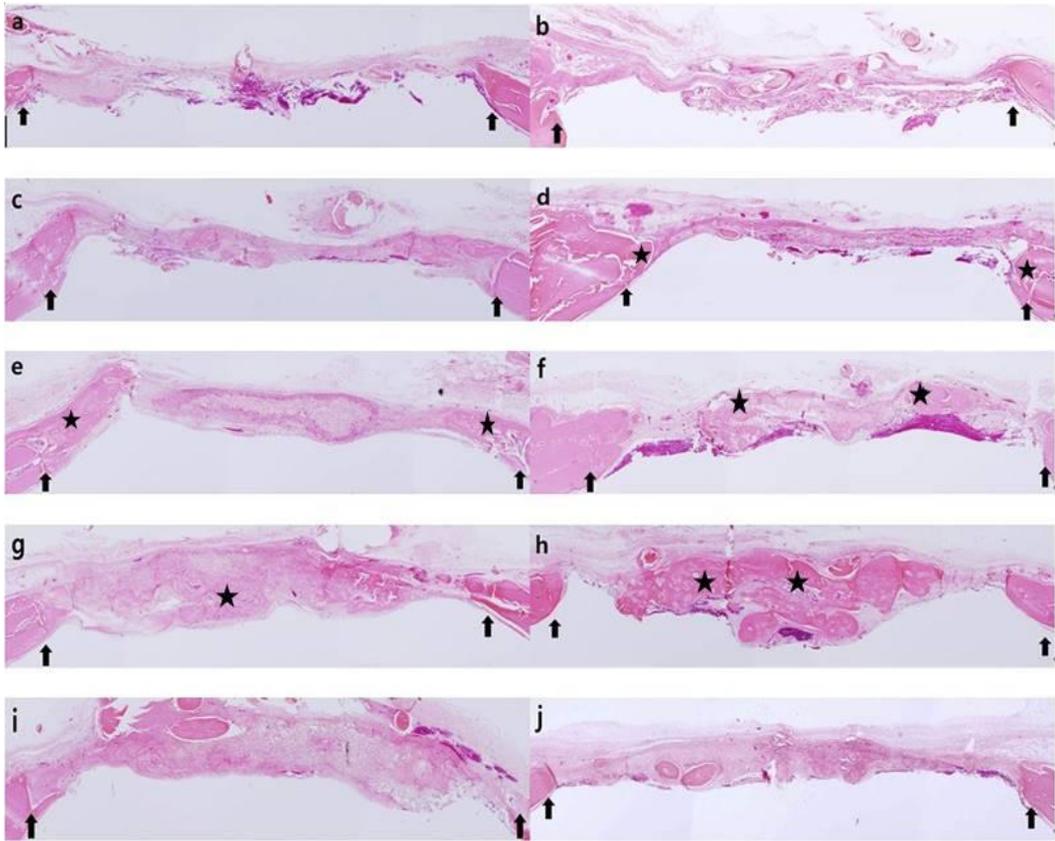


Fig 5. Histologic view of calvarial defects at 4 (left panels) and 8 (right panels) weeks (Hematoxylin-eosin stain, X4). (a,b) control group; (c,d) Collagen group; (e,f) MSC group; (g,h) FK506 group; (i,j) Rapamycin group. Arrows indicate the defect margin. New bones are marked with the asterix.

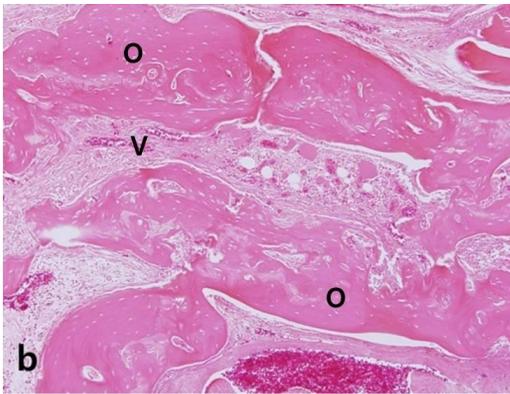
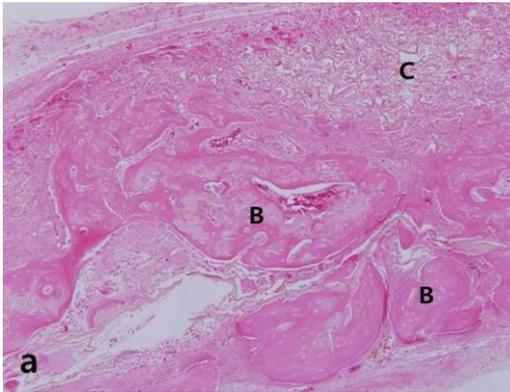


Fig 6. Histology of FK506 group at 4 (a) and 8 (b) weeks (hematoxylin-eosin stain, X10). At 4 weeks, immature bone (B) adjacent to the degraded collagen matrix (C) were observed. At 8 weeks, newly formed bones were more mature than 4 weeks. Osteocyte lacunae (O) and blood vessels (V) were clearly observed.

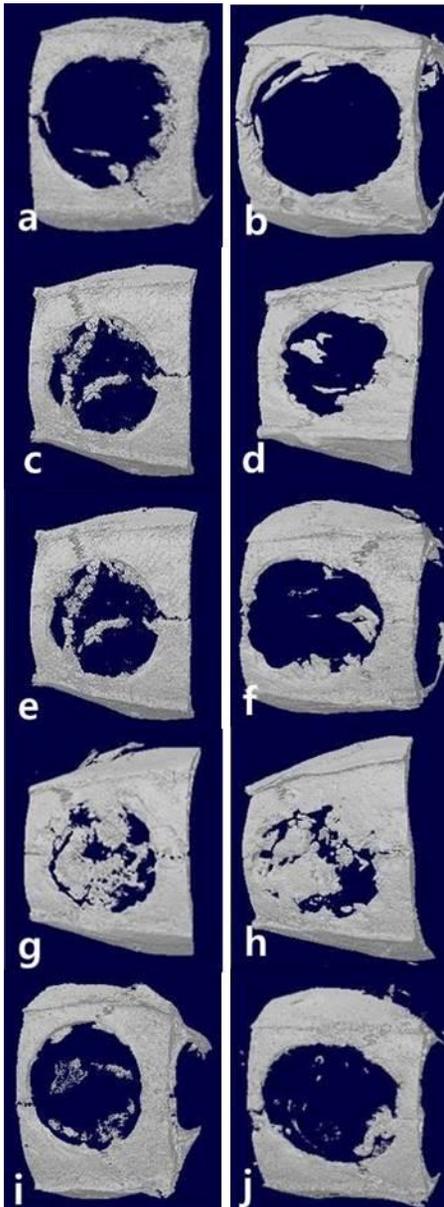


Fig 7. Micro-CT images at 4 (left panels) and 8 (right panels) weeks. (a,b) control group; (c,d) Collagen group; (e,f) MSC group; (g,h) FK506 group; (i,j) Rapamycin group. Control groups and Rapamycin groups showed the limited bone formation. FK506 groups showed a prominent bone formation compared to other groups and the most of defects were filled with the regenerated bone.

## Tables

Table 1. Primers used in real time PCR

Gene	Sequence
GAPDH	F: 5'- ACCACAGTCCATGCCATCAC -3'
	R: 5'- TCCACCACCCTGTTGCTGTA -3'
Osteocalcin	F: 5'- AAAGCCCAGCGACTCT -3'
	R: 5'- CTAAACGGTGGTGCCATAGA -3'
Osteopontin	F: 5'- GACGGCCGAAGGTGATAGCTT -3'
	R: 5'- CATGGCTGGTCTTCCCGTTG -3'
Osteonectin	F: 5'- ACAGCTCCACCTGGACTAC -3'
	R: 5'- TCTTCTCACACGCAGTTT -3'
Runx2	F: 5'- GCTTCTCCAACCCACGAATG -3'
	R: 5'- CAACTGATAGGACGCCGACC -3'
Type I Collagen	F: 5'- TCCTGCTGATGTCGCTATC -3'
	R: 5'- CAAGTTCCGGTCTGACTCTCGT -3'
Dlx5	F: 5'- GCGCTCAACCCATACCAG -3'
	R: 5'- ACTCGGGACTCGGTTGTAGG -3'

Table 2. Histomorphometric analysis of the regenerated bone percentage in calvarial defect. (mean  $\pm$  SD in %)

<b>Group</b>	<b>4 weeks</b>	<b>8 weeks</b>
Control	16.85 $\pm$ 1.53 <sup>a</sup>	31.48 $\pm$ 2.14 <sup>d</sup>
Collagen	28.62 $\pm$ 11.90	31.69 $\pm$ 2.58 <sup>e</sup>
MSC	24.52 $\pm$ 7.99 <sup>b</sup>	31.59 $\pm$ 14.63 <sup>f</sup>
FK506	44.48 $\pm$ 8.29 <sup>a,b,c</sup>	54.21 $\pm$ 11.17 <sup>d,e,f,g</sup>
Rapamycin	24.51 $\pm$ 10.25 <sup>c</sup>	23.08 $\pm$ 13.28 <sup>g</sup>

Marks “a-g” mean significant difference between two groups (p<0.05).

Table 3. Bone mineral density (BMD) of calvarial defect (mean  $\pm$  SD in g/cm<sup>3</sup>)

<b>Group</b>	<b>4 weeks</b>	<b>8 weeks</b>
Control	0.049 $\pm$ 0.057 <sup>a</sup>	0.050 $\pm$ 0.026 <sup>b</sup>
Collagen	0.188 $\pm$ 0.072	0.235 $\pm$ 0.124
MSC	0.056 $\pm$ 0.031	0.111 $\pm$ 0.067 <sup>c</sup>
FK506	0.191 $\pm$ 0.099 <sup>a</sup>	0.307 $\pm$ 0.107 <sup>b,c,d</sup>
Rapamycin	0.091 $\pm$ 0.036	0.070 $\pm$ 0.054 <sup>d</sup>

Marks "a-d" mean significant difference between two groups (p<0.05).

Table 4. Bone volume / tissue volume (BV/TV) of calvarial defects. (mean  $\pm$  SD in %)

<b>Group</b>	<b>4 weeks</b>	<b>8 weeks</b>
Control	6.95 $\pm$ 0.58 *	8.14 $\pm$ 1.37 *
Collagen	15.46 $\pm$ 2.26	19.76 $\pm$ 2.82
MSC	10.37 $\pm$ 3.97	17.38 $\pm$ 1.34
FK506	26.35 $\pm$ 4.83 **	39.09 $\pm$ 1.46 **
Rapamycin	15.64 $\pm$ 2.94	15.66 $\pm$ 4.17

\* ; significantly lower than other 4 groups (p<0.05).

\*\* ; significantly higher than other 4 groups (p<0.05).

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# 면역억제제가 백서 골수 유래 줄기세포의 골형성능에 미치는 영향에 관한 연구

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(지도교수 이 용 무)

본 연구에서는 면역억제제가 백서의 골수에서 유래한 줄기세포의 분화 및 백서 두개골 결손부에서의 골형성능에 어떤 영향을 미치는지 알아보고자 하였다.

백서의 대퇴골에서 채취한 골수 유래 줄기세포를 50, 500나노몰 두가지 농도의 면역억제제 FK506과 Cyclosporin A(CsA)와 함께 배양하였다. 7일간 적용한 후 세포증식에 면역억제제가 미치는 영향을 확인하였다. 조골세포로의 분화 가능성을 확인하기 위하여 염기성 인산화효소의 활성화 및 알리자린 레드 에스 염색을 시행하였고 골 관련 mRNA의 발현을 RT-PCR로 확인하였다. 또, 백서의 두개골에 직경 8mm의 골결손부를 형성하고 동종의 백서에서 채취하여 배양한

줄기세포를 콜라겐 매트릭스를 이용하여 이식하였다. 대조군 (결손부에 아무런 처치를 하지 않은 그룹), 콜라겐 그룹 (결손부에 콜라겐 매트릭스만을 이식한 그룹), 줄기세포 그룹 (동종줄기세포를 부착한 매트릭스를 이식한 그룹), FK506 그룹 (동종줄기세포를 이식한 후 4주동안 면역억제제 FK506을 투여한 그룹), 라파마이신 그룹 (동종줄기세포를 이식한 후 4주동안 면역억제제 라파마이신을 투여한 그룹)으로 나누어 실험을 진행하였고, 이식 4, 8주에 각각 실험동물을 희생하여 조직학적, 삼차원 미세단층촬영 분석을 시행하였다.

세포실험에서 FK506은 대조군이나 CsA 그룹에 비해 세포증식을 촉진하는 것으로 나타났다. 또 같은 그룹에서 염기성 인산화효소 및 무기물 결정 형성이 두드러졌다. FK506 그룹에서 오스테오펀틴, 오스테오넥틴, 타입 I 콜라겐의 메신저 RNA가 더 많이 발현되었으나 시간이 지남에 따라 그 정도는 줄어들었다. Runx2 및 Dlx5 관련된 메신저 RNA의 발현은 7일째에 FK506 그룹에서 최대로 나타났으며, 고농도의 CsA 그룹에서는 대부분의 유전자발현이 억제된 것으로 나타났다. 동물 실험에서 FK506을 투여한 그룹에서 조직학적으로 월등한 신생골 형성을 확인할 수 있었으며 삼차원 미세단층촬영 영상으로도 같은 결과를 확인하였다. 조직계측학적으로 신생골의 면적을 확인하고, 미세단층촬영을 통해 골밀도 및 조직 대비 재생골 부피를 비교하였을 때 FK506 그룹이 다른 그룹에 비해 좋은 결과를 보였다. 다른 면역억제제인 라파마이신은 뚜렷한 골재생을 보여주지 않았다

결론적으로 이번 실험을 통해 FK506이 골수유래줄기세포의 조골세포로의 분화를 촉진하고 두개골 결손부에서 이식된 줄기세포의 골 형성을 증진시키는 것을 확인할 수 있었다.

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주요어 ; 세포 분화, 사이클로스포린 A, FK506, 라파마이신, 면역억제제, 백서골수유래줄기세포

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