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Effects of static magnetic fields on human osteoblast-like cell differentiation

조선대학교 대학원

치의학과

나 명 수

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사람 골모세포분화에 미치는 정자계의 효과

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Effects of static magnetic fields on human osteoblast-like cell differentiation

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Major Advisor

나명수의 박사학위논문을 인준함

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ABSTRACT

Effects of static magnetic fields on human osteoblast-like cell differentiation

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Various treatment modalities to enhance the bone defect healing are introduced such as bone morphogenic protein, growth factors or ultrasound. The aim of this study was to investigate the effects of static magnetic fields (SMFs), as an another modality to achieve this goal, on osteoblastic differentiation, and proliferation using a human osteosarcoma cell line (MG63).

The magetic fields showed an average flux of 53mT, 73mT, 180mT, 330mT respectively. Each magnetic fields were set apart, so that the influence of the fields of the adjacent magnets would be excluded. The cells were subjected to continuous SMF exposure.

To determine cell proliferation by MTT test, a human osteogenic sarcoma cell line MG63 was plated at a density of $2X10^4$ cells per well in 96 well plates. And, to analyse the bone differentiation markers by RT-PCR, total RNA was extracted from cells by homogenizing with Trizol Rreagent on days 1, 7, and 14 of culture. Four bone differentiation markers, collagen type-1(COL-1), alkaline phosphatase (ALP), osteocalcin (OC), and osteopontin (OPN) were examined by RT-PCR.

The response of SMFs on the rate of proliferation of MG63 cell were flux

density-dependent. Among the 4 bone differentiation markers examined, two markers of osteoblastic phenotype (ALP and OPN) showed a increase in 330 mT through RT-PCR analysis. The effects of SMFs on ALP mRNA in MG63 cells was twice as high as control in 330 mT at only days 14 exposure after, and the effect on OPN mRNA was 6 times as high as control exceptionally in 330 mT at day 1 exposure after. On the other hand, the expression of COL-1 mRNA almost remained unchanged compared to control, and The expression of OC mRNA showed decreased tendency compared to control, irrespective of magnetic flux densities.

Within the limited results, the local regulatory factors produced by SMFs-treated cells were higher than those of the control cultures, especially ALP and OPN. And, author proposes that time course of the SMF-stimulation is very critical, suggesting that events in bone formation may be modulated by SMFs. Although, animal studies and clinical trial are needed to understand the real process in the whole body, SMFs might be a good method as inducer for bone differentiation. In the future, animal studies will be needed to enhance the bone regeneration based on the this experiment.

Ⅰ. Introduction

The proliferation and/or differentiation of osteoblasts is modulated by several extracellular factors such as cytokines, hormones, pulsed or static electromagnetic fields (EMFs) and static magnetic fields (SMFs)[1].

Magnetic fields are widely distributed in environment and their effects are increasing by the development of electrical machines[2]. It has been reported that EMFs affects calcium ion transport and specific gene transcription[3,4], and cell growth[5]. On the other hand, Cohly et al.[6] reported the effects of static EMFs (average field intensity of 0.618 mT) on MG63 cells. Reverse-transcription -polymerase chain reaction (RT-PCR) revealed that collagen type-1 (COL-1), alkaline phosphates (ALP), parathyroid hormone receptor, and osteocalcin (OC) mRNA were down regulated with the low intensity static EMFs. Exposure to very low Static EMFs affects the MG63 osteoblasts in a manner that may be detrimental to bone formation. Although pulsed EMFs yield both a magnetic field and an electric current, no definite conclusion can be drawn as to which factor is more responsible for bone formation[1].

The effects of SMFs at the various cells also have been studied including living mouse[7], erythrocytes[8], human gingival fibroblasts[9], human skin fibroblasts[10] and periodontal membrane[11,12]. So far, the effect on exposure to SMFs varies depending on the experimental protocols. Bondemark et al.[13] reported that SMFs produced by orally placed orthodontic rare-earth magnets did not result in any change in human dental pulp or gingival tissue adjacent to the magnets. Linder-Aronson and Lindskog[14] reported that significantly and progressively impaired attachment and growth over a 5 week period was observed when human periodontal fibroblasts were cultured in a SMFs. Darendeliler et al.[15] demonstrated that both EMFs and SMFs stimulated groups showed increases in

both the organization and amount of new bone deposited in the area of tension between the orthodontically moved maxillary incisors. Yan et al.[16] suggest that the long-term local SMF stimulation (12 weeks after implantation) on the rat femurs has a local effect to prevent the decrease in bone mineral density caused by surgical invasion or implantation. On the other hand, Nakahara et al.[17] suggested that SMFs alone do not affect cell growth, cell proliferation, cell cycle distribution pattern and apoptotic cell death of Chinese hamster ovary CHO-K1 cells, regardless of the magnetic flux density, but might potentiate DNA damage induced by other DNA-damaging factors such as X-rays. In animal studies of the effects of SMFs, SMFs of less than 100 mT induced the flow potential around the heart as acute effects, and SMFs sometimes of less than 1 mT induced the skin blood flow and arterial blood pressure as chronic effects, and there were no reliable effects of exposure to SMFs of 1 T and above on the animal reproduction and development, or on the growth and development of tumors[18]. As for the magnetic orientation of cells, Iwasaka et al.[19] reported that a high-intensity magnetic field of 14 T affected the morphology of smooth muscle cell assemblies, and the shapes of the cell colonies extended along the direction of the magnetic flux. The phenomenon was most notable under magnetic fields of more than 10 T.

Various treatment modalities to enhance the bone defect healing are introduced such as bone morphogenic protein[20], growth factors[21] or ultrasound[22]. In the present study, author investigated the expression of bone differentiation markers, such as COL-1, ALP, OC, and osteopontin (OPN) using SMFs as an another modality to achieve this goal. In this study, therefore, the effects of SMFs used clinically on differentiation of cultured human osteoblast-like cells (MG63 cells), were examined.

Ⅱ. Materials and methods

Static magnetic fields

In the present study, neodymium-iron-boron magnet disk (every 20 magnetic of 2 ㎝ in diameter: Usung magnet Co., Korea) and 60 ㎜ plastic culture plates were used. The magnet was placed below the well to expose the cultures (Fig. 1). The magnetic flux density was monitored with a Gauss meter (Kanetec co., Japan) at the bottom of each wall, where human osteogenic sarcoma cell line MG63 cells attached themselves to the culture plates. The magnetic fields showed an average flux of 53 mT, 73 mT, 180 mT, and 330 mT, respectively. Each magnetic fields were set apart, so that the influence of the fields of the adjacent magnets would be excluded. The cells of exposed to SMFs were subjected to continuous SMFs exposure. In negative control group, non-magnetic disks were placed below the wells. The flux density values of the wells of the control culture plates were no greater than 0.05 mT. And the expressions of bone differentiation marker mRNA (COL-1, ALP, OC, and OPN) were examined by RT-PCR.

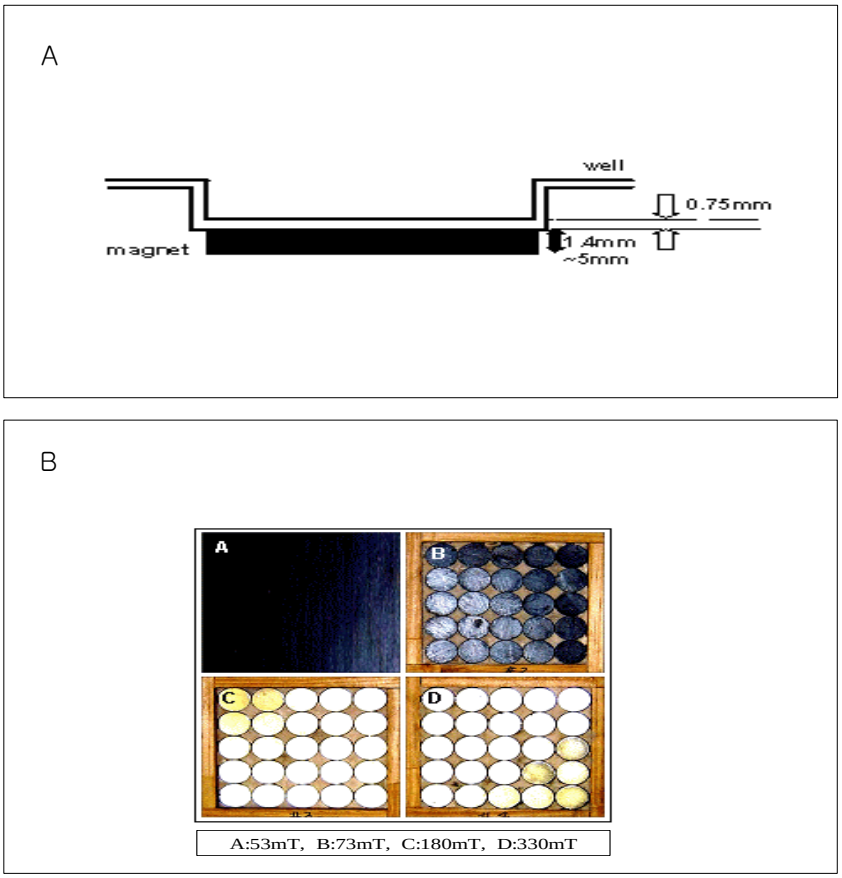

Fig. 1. (A and B) Diagram of the magnet placement and flux density used in this experiment.

Cell proliferation assay

To determine cell proliferation, the MG63 cells were plated at a density of $2X10^4$ cells per well in 96 well plates. After incubation for 24 hours, the culture medium was replaced by various SMFs. The cells were incubated at 37℃ for 7 days. The medium was replaced every other day. At 4hours before the end of incubation, the cells were washed twice with 10 mM phosphate-buffered saline (PBS, pH7.2), and then incubated with 0.5 ㎎/㎖ MTT for the last 4 hours. The medium was then decanted, the cells were incubated with 10% SDS and 0.01M

HCl for 2 hours, and the absorbance was determined at 570 nm using an enzyme linked immunosorbent assay reader (ELISA, BIO-TEK Instruments, USA).

RNA extraction and RT-PCR

Total RNA was extracted from cells by homogenizing with Trizol Rreagent on days 1, 7, and 14 of culture. cDNA was synthesized by reverse transcription of 5 ㎍ RNA in 20 ㎕ of master mix containing 200 U/ul superscript ™ II (Invitrogen), 5 mM $MgCl_2$, first strand buffer, 1 mM dNTP, 1 U/㎕ RNase inhibitor™, and 2.5 mM oligodT in DEPC-treated distilled water. The master mix was incubated at 42℃ for 50 min and 96℃ for 10 min. Synthesized cDNAs were subjected to 30 cycles of amplification under the following conditions: 94℃ denaturing for 5 min, 65℃ annealing for 1 min and 72℃ extension for 1 min. The primer sets used in this study were shown in Table 1.

Statistical analysis

Numerical values are expressed as the mean ±SD, n=3 per group. In all studies, three similar experiments were performed for each group. Statistical differences among the experimental groups were evaluated by analysis of variance followed by Kruscal-Wallis test; *, p values < 0.05, **, p values < 0.005 versus control were considered statistically significant.

Table 1. Amplification primer sets used in PCR

Primer		Sequences (5′-3′)	Product size	NCBI Accession No.
GAPDH	sense	5'-GGAGTCCACTGGCGTCTTCA-3'	182	NM_002046
	anti-sense	5'-AGCAGTTGGTGGTGCAGGAG-3'		
ALP	sense	5'-CGTGGTCACTGCGGACCATT-3'	219	NM_000478
	anti-sense	5'-GCAGACTGCGCCTGGTAGTT-3'		
COL-1	sense	5'-CTTCCTGCGCCTGATGTCCA-3'	192	NM_000088
	anti-sense	5'-CTCGTGCAGCCATCGACAGT-3'		
OPN	sense	5'-ACAGCCAGGACTCCATTGACTCGAACGACTCT-3'	198	NM_000582
	anti-sense	5'-CCACACTATCACCTCGGCCATCATATGTGTCT-3'		
OC	sense	5'-AGCGGTGCAGAGTCCAGCAA-3'	190	NM_199173
	anti-sense	5'-AGCCGATGTGGTCAGCCAAC-3'		

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALP: alkaline phosphotase; COL-1: collagen type 1; OPN: osteopontin; OC: osteocalcin.

Ⅲ. Results

Cell prolifation

To determine cell proliferation, the MG63 cells were plated at a density of $2X10^4$ cells per well in 96 well plates. After incubation for 24 hours, the culture medium was replaced by various SMFs (average flux of 53 mT, 73 mT, 180 mT, and 330 mT). The cells were incubated at 37℃ for 7 days. The effects of SMFs on the rate of proliferation of MG63 cells were flux-dependent ($p>0.05$)(Fig. 2).

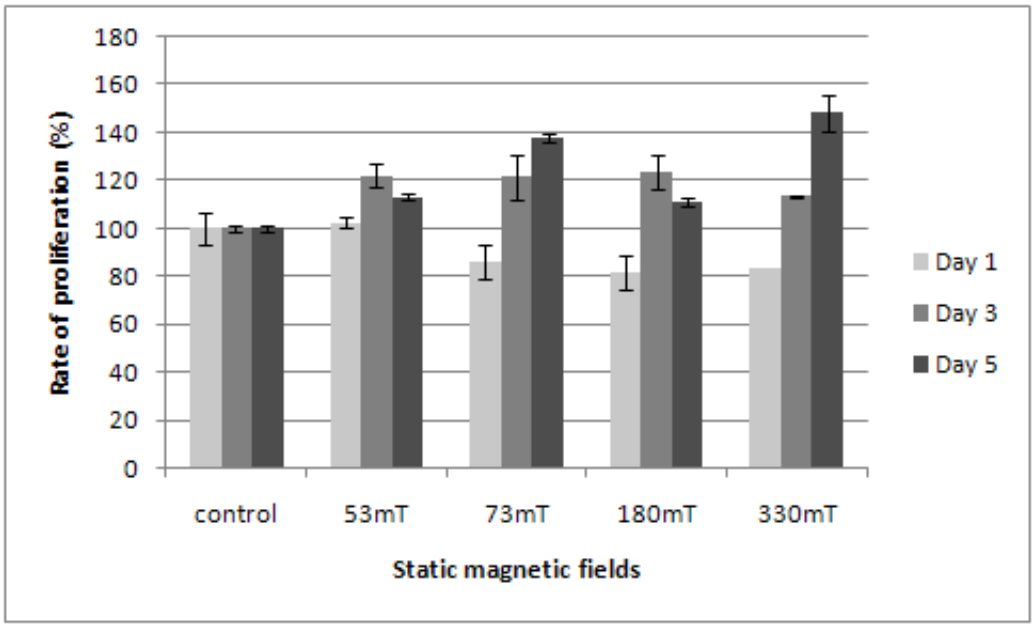

Fig. 2. Cell proliferation assay for MG63 cells exposed to SMFs of different flux densities (by MTT assay in day 7).

Expression of *ALP mRNA*

The effects of SMFs on ALP mRNA in MG63 cells were twice as high as control in 330 mT at only 14-day exposure after ($p<0.005$) (Fig. 3).

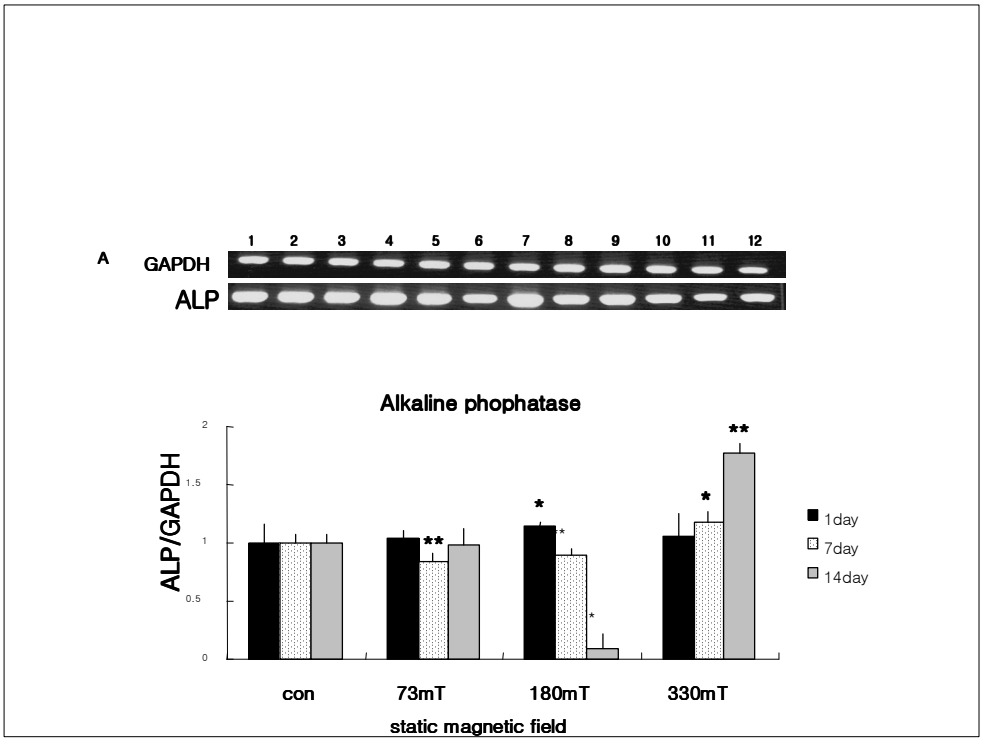

Fig. 3. Expression of ALP mRNA after the exposure of various flux densities of SMFs in MG63 cells with differentiation culture media. 1: Day 1 - control group, 2: Day 1 - 180 mT exposure, 3: Day 1 - 330 mT exposure, 4: Day 1 - 73 mT exposure, 5: Day 7 - control group 6: Day 7 - 180 mT exposure, 7: Day 7 - 330 mT exposure, 8: Day 7 - 73 mT exposure, 9: Day 14 - control group, 10: Day 14 - 180 mT exposure, 11: Day 14 - 330 mT exposure, 12: Day 14 - 73 mT exposure. The upper is representative expression of the mRNA for ALP assayed by RT-PCR. The lower is quantitative analysis of the mRNA for ALP analyzed by scanning densitometry. Values are means ± S.D. (n=3, ALP/GAPDH). *P<0.05 Vs. control group, **P<0.005 Vs. control group.

Expression of COL-1 mRNA

The expression of COL-1 mRNA almost remained unchanged compared to control irrespective of magnetic flux densities ($p>0.05$)(Fig. 4).

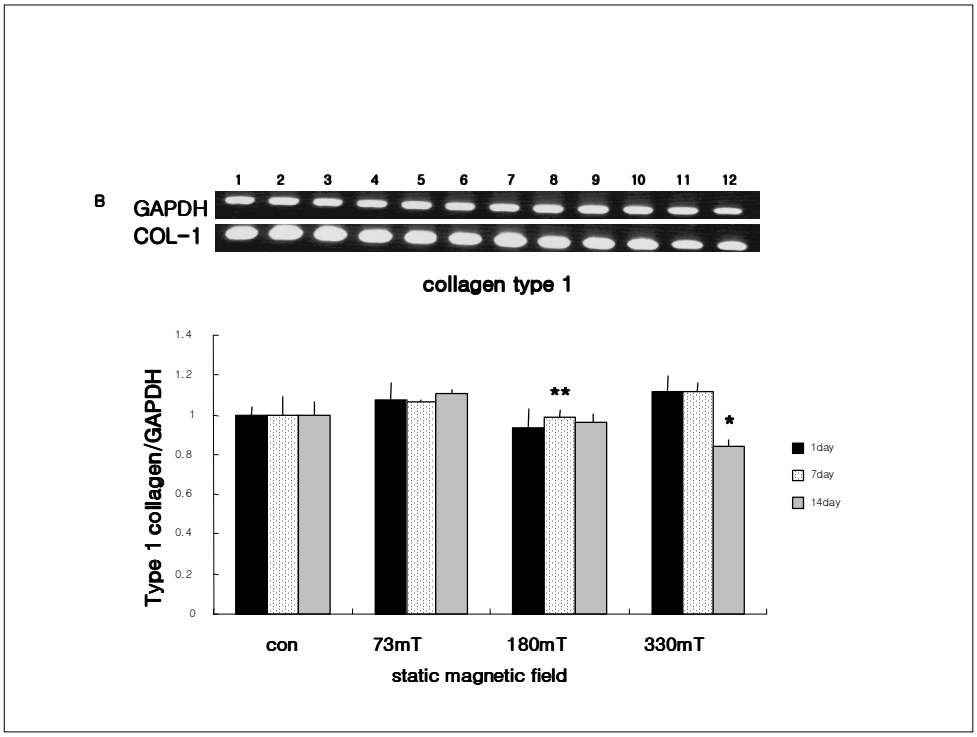

Fig. 4. Expression of COL-1 mRNA after the exposure of various flux densities of SMFs in MG63 cells with differentiation culture media. 1: Day 1 - control group, 2: Day 1 - 180 mT exposure, 3: Day 1 - 330 mT exposure, 4: Day 1 - 73 mT exposure, 5: Day 7 - control group 6: Day 7 - 180 mT exposure, 7: Day 7 - 330 mT exposure, 8: Day 7 - 73 mT exposure, 9: Day 14 - control group, 10: Day 14 - 180 mT exposure, 11: Day 14 - 330 mT exposure, 12: Day 14 - 73 mT exposure. The upper is representative expression of the mRNA for COL-1, assayed by RT-PCR. The lower is quantitative analysis of the mRNA for COL-1 analyzed by scanning densitometry. Values are means ± S.D. (n=3, ALP/GAPDH). *P<0.05 Vs. control group, **P<0.005 Vs. control group.

Expression of OC mRNA

The expression of OC mRNA showed significantly decreased compared to control irrespective of magnetic flux densities ($p<0.005$) (Fig. 5).

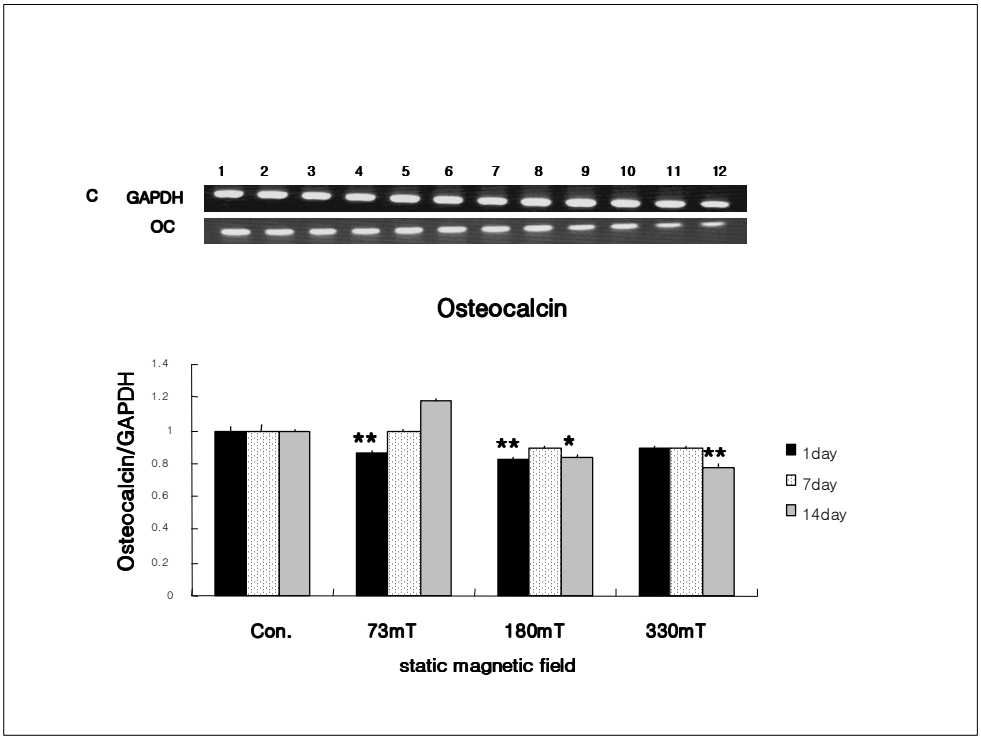

Fig. 5. Expression of OC mRNA after the exposure of various flux densities of SMFs in MG63 cells with differentiation culture media. 1: Day 1 - control group, 2: Day 1 - 180 mT exposure, 3: Day 1 - 330 mT exposure, 4: Day 1 - 73 mT exposure, 5: Day 7 - control group 6: Day 7 - 180 mT exposure, 7: Day 7 - 330 mT exposure, 8: Day 7 - 73 mT exposure, 9: Day 14 - control group, 10: Day 14 - 180 mT exposure, 11: Day 14 - 330 mT exposure, 12: Day 14 - 73 mT exposure. The upper is representative expression of the mRNA for OC assayed by RT-PCR. The lower is quantitative analysis of the mRNA for OC analyzed by scanning densitometry. Values are means ± S.D. (n=3, ALP/GAPDH). *P<0.05 Vs. control group, **P<0.005 Vs. control group.

Expression of OPN mRNA

The expression of OPN mRNA was increased at 1 day after exposure of SMFs compared to control in all 4 magnetic flux densities. The expression of OPN mRNA is 6 times as high as control exceptionally in 330 mT at day 1. With the lapse of time, the expression of OPN mRNA showed decreased trend ($p>0.05$) (Fig. 6)

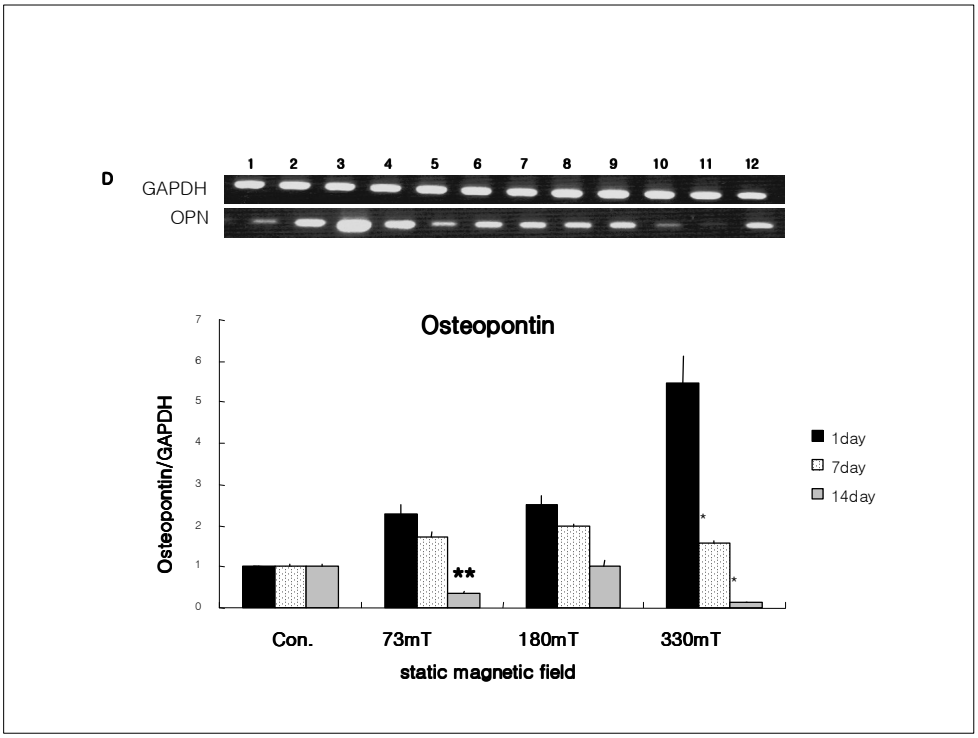

Fig. 6. Expression of OPN mRNA after the exposure of various flux densities of SMFs in MG63 cells with differentiation culture media. 1: Day 1 - control group, 2: Day 1 - 180 mT exposure, 3: Day 1 - 330 mT exposure, 4: Day 1 - 73 mT exposure, 5: Day 7 - control group 6: Day 7 - 180 mT exposure, 7: Day 7 - 330 mT exposure, 8: Day 7 - 73 mT exposure, 9: Day 14 - control group, 10: Day 14 - 180 mT exposure, 11: Day 14 - 330 mT exposure, 12: Day 14 - 73 mT exposure. The upper is representative expression of the mRNA for OPN assayed by RT-PCR. The lower is quantitative analysis of the mRNA for OPN analyzed by scanning densitometry. Values are means ± S.D. (n=3, ALP/GAPDH). *P<0.05 Vs. control group, **P<0.005 Vs. control group.

Ⅳ. Discussion

Magnetic fields of sufficient magnitude have been shown to affect various biologic systems at organ, tissue, cellular, and subcellular levels. It is said that physical forces like magnetic fields may play an important role in the regulation of bone cellular function[4]. According to Owen et al.[23], modifications in gene expression define a developmental sequence that has three principle periods-proliferation, extracellular matrix maturation, and mineralization. Actively proliferating cells produce a fibronectin/type I collagen extracellular matrix. A reciprocal and functionally coupled relationship between the decline in proliferative activity and the subsequent induction of genes associated with matrix maturation and mineralization is supported by 1) a temporal sequence of events in which there is an enhanced expression of ALP immediately following the proliferative period, and later, an increased expression of OC and OPN at the onset of mineralization.

Author used the SMFs instead of pulsed EMFs. When a permanent magnet is used for stimulation, this makes SMF stimulation more suitable for long-term local healing because power device supplied by external energy is not necessary[24].

Author undertook the present investigation to study the effects of SMFs on osteoblastic proliferation and differentiation on days 1-14 using a MG63 cell line. The effect of SMFs on the rate of proliferation of MG63 cells were flux density-dependent. Among the 4 bone differentiation markers examined, 2 markers of osteoblastic phenotype (ALP and OPN) showed a increase in 330 mT by RT-PCR analysis. The effects of SMFs on ALP mRNA in MG63 cells were twice as high as control in 330 mT at only days 14 exposure after. The effect on OPN mRNA was 6 times as high as control exceptionally in 330 mT at day 1. On the other hand, the expression of COL-1 mRNA almost remained

unchanged compared to control, and The expression of OC mRNA showed decreased tendency compared to control, irrespective of magnetic flux densities. Huang et al.[24] suggested that the local regulatory factors, such as transforming growth factor-beta1, COL-1, OPN, and ALP, produced by 0.4 T SMFs-treated cells were greater than those of the control cultures. The cells were stimulated continuously to 0.4-T SMFs for 12, 24, 48, and 72 hours. In comparison with this results, author examined the response of MG63 cells to a SMFs signal used clinically.

As for the effect of magnetic fields on the cellular proliferation, almost studies reported that decreased proliferation or no significant difference between the wells exposed the SMFs and the controls. Chiu et al.[25] suggested that SMFs affect osteoblastic maturation by increasing the membrane rigidity and reducing the proliferation-promoting effects of growth factors at the membrane domain. Lohmann et al.[26] reported that the net effect of pulsed EMFs on MG63 cells indicated the enhanced differentiation, as evidenced by decreased proliferation and increased ALP-specific activity, OC synthesis, and collagen production. McDonald[27] demonstrated that the effect of a SMFs of a neodymium magnet on cellular behavior using fibroblast- and osteoblast-like cells of the neonatal rat calvarium, which were exposed to north and south poles with a pole-face flux density of 0.61 T. They concluded that a statistically significant magnetic stimulation of turnover rate and synthesis of fibroblasts was found, but stimulation of osteoblasts did not occur. Yamamoto et al.[1] suggested that SMFs (flux density of 160 mT) stimulated bone formation by promoting osteoblastic differentiation and/or activation (high level of the number and average size of bone nodule, and significant increased ALP and OC in the presence of SMFs using rat osteosarcoma osteoblast-like cells (ROS 17/2.8 and UMR 106). Also, Shimizu et al.[28] reported that application of 300 and 800 Gauss SMF increased bone sialoprotein mRNA levels after 24 hours stimulation.

Recently, Zhao et al.[29] demonstrated that continuous SMF-stimulation of magnetic attachments (12.5, 125, 250 mT SMF) could not change rat osteoblasts proliferation activity, cell cycle distribution, and apoptosis ratio. On the other hand, Qiu et al.[30] reported that rat calvarial osteoblasts were sensitive to 0.062T SMF stimulation, and SMF induced the expression of BMP-2 and stimulated secretion of COL-I by Western blot and immunohistochemical staining.

This result was in contrast to other reports, especially cell proliferation. The one possible explanation of conflicting results will be the type cells or experimental protocols used for studies. As McDonald described, author should interpret the results carefully with understanding both variability and diversity of cellular behaviour.

The aim of this study was to investigate genes expression related with osteoblast differentiation after treatment with a various flux densities used clinically in MG63 cells. Within the limited results, the local regulatory factors produced by SMFs-treated cells were higher than those of the control cultures, especially ALP and OPN. And, author proposes that time course of the SMF-stimulation is very critical, suggesting that events in bone formation may be modulated by SMFs. Although animal studies and clinical trial are needed to understand the real process in the whole body, SMFs might be a good method as inducer for bone differentiation. In the future, animal studies will be needed to enhance the bone regeneration based on the this experiment.

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ABSTRACT in KOREAN

사람 골모세포분화에 미치는 정자계의 효과

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외상이나 치주질환에 의해 발생된 골 결손부에 대한 창상치유를 촉진시키기 위하여 현재, 골형성단백질, 성장인자 및 초음파 등을 이용한 많은 치료법 등이 소개되고 있다. 이 연구에서는 골 결손부에 대한 창상치유를 촉진하는 또 다른 방법으로서 정자계(static magnetic fields, SMFs)를 사람 골모세포 (MG63 세포)에 조사하여 세포증식과 골분화인자의 표현에 미치는 효과를 조사하였다.

정자계에 대한 MG63세포의 증식률을 평가하기 MTT 분석을 시행하였는데 이 때 사용된 평균 선속밀도(flux density)는 53 mT, 73 mT, 180 mT, 330 mT였다. MG63 세포를 각각의 정자계에 1일, 7일, 14일 동안 지속적으로 노출시킨 후, RT-PCR 법을 이용하여 4개의 골분화인자, collagen type-1(COL-1), alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OPN) 각각의 mRNA 발현정도를 정량적으로 분석하였다.

정자계가 MG63세포의 증식률에 미치는 반응은 선속밀도에 좌우되었으나 유의성은 없었다. 그리고 검사된 4개의 골분화인자들 중, ALP mRNA는 대조군에 비해 330 mT에서 14일군에서만 높게 발현되었으며, OPN mRNA은 1일군의 330 mT에서 대조군에 비해 6배 높게 발현되었다. 반면, 선속밀도에 무관하게 COL-1 mRNA은 대조군과 유사한 양상을 나타냈으며, OC mRNA는 감소되는 경향을 나타냈다. 이 제한된 연구결과, 정자계가 골분화를 유도할 수 있는 또 다른 방법이 될 수 있음을 시사한다.

(별 지)

저작물 이용 허락서

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	영문 :Effects of static magnetic fields on characteristics of human osteoblasts differentiation in culture				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) **반대**( )

2008년 2월 일

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