The effect of pulsed electromagnetic field in human intervertebral disc cells

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먼저 부족한 저에게 생물학도로써의 마음가짐과 삶의 지혜를 알게 해 주시고 바쁘신 가운데 꾸준히 specimen을 보내주신 저의 지도 교수님인 이환모 선생님과 처음 실험실에 들어와서 지금까지 많은 생물학적 지식과 연구 기법들을 알려주시고 골대사학회 일을 통해서 새로운 경험을 하게 해주신 문성환 선생님, 자주 뵐 수는 없었지만 영동 세브란스에서 언제나 저희를 후원해주시는 김학선 선생님, 부족한 제 논문의 심사를 맡아주신 윤도흠 선생님께 깊은 감사의 마음을 전합니다. 또한 학부 4년 동안 분자생물학이라는 어려운 과목을 알기 쉽게 가르쳐 주시고 실험실 생활이 어떠한 것인지를 알려주신 송민동, 박태규, 이광호, 김찬길 교수님께 감사 드리고, 힘든 실험실 생활을 재미있고 즐겁게 할 수 있도록 항상 곁에서 힘이 되어주시고 또 연세대로 들어 올 수 있는 발판을 마련해 주신 김대영 선배님께 감사 드립니다.

아무것도 모르는 학부생을 단지 후배라는 이유하나로 뽑아 아낌없는 사랑과 가르침을 주시고 때론 엄마처럼 때론 친구처럼 고민상담과 조언을 해주신 김향 선배님께 감사 드립니다. 그 고마움에 보답도 못하고 떠나는 것 같아 죄송한 맘이 크지만 사회 나가서 성공해서 갚으라고 했던 말 잊지않고 열심히 노력해서 은혜에 보답하는 후배 되겠습니다. 우리 실험실의 청일 점, 남자라고 하기엔 좀 여성스러운 면이 많았지만 그래도 남자라는 이유로 실험실의 모진 일들을 다 맡아 해주고 실험실 분위기를 띄워 주는 분위기 메이커 광일 오빠, 나보다 나이어린 후배가 들어오길

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ABSTRACT

The effect of pulsed electromagnetic field in human intervertebral disc cells

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(Directed by Professor Hwan-Mo Lee)

Objectives: To assess the effect of pulsed sinusoidal electromagnetic fields (EMF) on human intervertebral disc (IVD) cells.

Summary of literature review: EMF are known to modify some relevant physiologic parameters of cells cultured in vitro such as proliferation, synthesis, secretion of growth factors and transcription. EMF induces bone formation in delayed or non-union and spinal fusion model. Also, the exposure of EMF was shown to protect hazardous effect of smoking in rabbit IVD.

Materials and Methods: Human IVD cells were cultured three-dimensionally in alginate beads. EMF were exposed to IVD cells with 650Ω, 1.8millitesla magnetic flux density, and 60Hz sinusoidal wave. Cultures were divided into control and EMF group with various exposure times. Cytotoxicity, DNA synthesis, and proteoglycan synthesis were measured by MTT assay, [^{3}H]-thymidine, and [^{35}S]-sulfate incorporation respectively. Reverse transcription-polymerase chain reaction (RT-PCR) was performed for aggrecan, collagen type I, and II mRNA expression.

Results: There was no recognizable cytotoxicity in EMF groups. Cellular proliferation was stimulated in EMF group ($p<0.05$). Newly synthesized proteoglycan normalized by DNA synthesis was decreased with EMF group ($p<0.05$). In culture with EMF exposure, there was decreased expression of aggrecan (48 hours exposure) and type II collagen (48 hours exposure) mRNA compared to control group, while there was no difference in collagen type I mRNA expression.

Conclusion: EMF seem to be hazardous in chondrogenic matrix synthesis while marginally beneficial in cellular proliferation in human IVD cells.

Key words: electromagnetic fields (EMF), intervertebral disc (IVD), proteoglycan, matrix synthesis

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Ⅰ. INTRODUCTION

Low-frequency, low-energy pulsed electromagnetic fields (EMF) are widely used for treatment of delayed or non union of long bone.[1-4] EMF show clinical benefits particularly in the treatment of delayed union and pseudarthrosis after bone fracture.[5, 6] Studies have shown EMF to have effects on many aspects of bone formation and healing. These include EMF-induced endothelial cell proliferation and capillary formation, which are integral to angiogenesis in the revascularization of fracture healing.[7] They demonstrated that electric stimulation increased the osteoblast proliferation and induced the

release of a mitogenic activity into the culture medium in embryonic chick calvarial cells.[8] In the TE85 human osteosarcoma cell line, exposed to combined magnetic fields, the same authors showed an induction cell proliferation associated with an increase in the secretion of IGF-Ⅱ and of the number of IGF-Ⅱ receptors.[8,9] Bone cell proliferation can also be induced by EMF, as is the stimulation of matrix formation and calcification.[10-13] Specific factor(s) that may mediate these bone-forming and healing EMF effects have not been identified yet. Moreover, the previous studies have shown that the exposure to EMF enhances chondrogenesis and endochondral ossification. The increase in chondrogenesis was demonstrated to be dependent upon stimulation of the progenitor cell pool. EMF induce an increase in the proliferation of human chondrocytes-measured by ^{3}H-thymidine incorporation. Both articular and nasal chondrocyte used in our experiments were maintained in vitro the expression of type Ⅱ collagen, which is a specific marker of the chondrocyte phenotype.[14] Even if the exact role of growth factors and mitogens is not clear yet, it seems that their presence in the culture medium is necessary to obtain an induction of cell proliferation after EMF exposure, suggesting that the interaction between cell membrane receptors and mitogens is one of the molecular events affected by electromagnetic fields.[15]

EMF are known to modify some important physiological parameters of cells cultured in vitro such as proliferation, transcription, synthesis, and secretion of growth factors.[16] The influence of these fields on cell cycle activity has been associated with a number of membrane-mediated signaling mechanisms, but the effects on phenotypic or genotypic expression have not been explained. In the previous report, rabbits were exposed to cigarette-smoke and EMF. The cigarette-smoke-exposed rabbits that were exposed to the EMF for 4hr/day demonstrated no change in their intradiscal pH, in contrast to those who were exposed to smoke alone. In conclusion, cigarette smoke exposure in rabbits consistently produces a lower intradiscal pH and EMF can defend against this adverse effect.[17] Despite numerous studies on intervertebral disc (IVD) metabolism, the influence of EMF on IVD has not been thoroughly studied. Accordingly, we generated, applied pulsed sinusoidal EMF on IVD cells, and analyzed possible mediators of its biologic effects.

Ⅱ. MATERIALS AND METHODS

All of the experimental protocols were approved by the human subjects Institutional Review Board of the institute.

1. Materials

Lumbar and cervical IVD tissues were obtained from ten patients (age range: 30 to 56 years) during surgical disc procedures. Two cervical IVDs and 8 lumbar IVDs were harvested from patients with disc herniation. Classification of the IVD of each patient as grade of degeneration was performed based on magnetic resonance images of each disc as described in the literature. Grade III and IV degenerations were included in this study to minimize the effect of degeneration grades on the expression of phenotype and matrix synthesis. An attempt was made by the operating surgeon (SHM, HML) to carefully obtain tissues from the central aspect of the disc to optimize harvest of only nucleus pulposus and transitional zone. The disc tissue specimens were washed with Hank's balanced salt solution (HBSS, Gibco-BRL, Grand Island, NY, USA) to remove blood and bodily fluid contaminants, and were then transported in sterile HBSS to the laboratory,

less than 20 minutes following surgical removal.

2. Intervertebral disc cell culture

Any obvious granulation tissue, dense outer anulus, and cartilaginous endplate were removed carefully from the disc tissue specimens. Disc cells were then isolated from the inner anulus and nucleus pulposus as described before. [5] Briefly the dissected specimens were minced with a scalpel into pieces of approximately two cubic millimeters in volume. Disc tissues from the nucleus pulposus and inner anulus were digested for 60 minutes at 37° C under gentle agitation in a medium composed of equal parts of Dulbecco's Modified Eagle Medium and Ham's F-12 medium (DMEM/F12, Gibco-BRL, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY, USA) with 0.4% pronase (Sigma, St. Louis, MO, USA) and 0.004% deoxyribonuclease II type IV (DNase, Sigma, St. Louis, MO, USA). The tissue was then washed 2 times with DMEM/F12 and digested overnight under the same conditions, except that the pronase was replaced with bacterial 0.025% collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA). Cells were filtered through a sterile nylon mesh filter (pore size: 70um) and then were counted in a haemocytometer and

plated in 24 well plates (Falcon, Franklin Lakes, NJ, USA) at a density of approximately 6 x 10^4 cells/ml. Primary cultures were sustained for 2 to 3 weeks in DMEM/F12 containing 10% FBS, 1% v/v penicillin, streptomycin and nystatin (all antibiotics from Gibco-BRL, Grand Island, NY, USA) in a 5% CO_2 incubator with humidity. Culture medium was changed twice a week. Cell viability was determined by trypan blue exclusion test. Secondary cultures after trypsinization of primary cultures were exclusively utilized to minimize the effect of subculture on the expression of phenotype.

TABLE 1. Clinical features of the cases studied

Case	Age	Sex	Diagnosis	Operation level
1	53	F	HNP (Grade 4)	L4/L5
2	35	F	HNP (Grade 3)	L4/L5
3	37	M	HNP (Grade 4)	L3/L4
4	42	M	HNP (Grade 4)	L3/L4
5	56	F	HNP (Grade 3)	C5/C6
6	49	F	HNP (Grade 3)	L3/L4
7	52	M	HNP (Grade 4)	L4/L5
8	32	F	HNP (Grade 4)	C4/C5
9	49	M	HNP (Grade 4)	L3/L4
10	36	F	HNP (Grade 3)	L4/L5

* HNP: herniated nucleus pulposus

3. Incorporation of isolated cells into alginate beads

The preparation of IVD cells in alginate beads was performed as described elsewhere. Briefly, isolated cells from primary culture with trypsinization were resuspended in sterile 0.15M NaCl containing 1.2% low-viscosity alginate (Sigma, St. Louis, MO, USA) at a density of two million cells per milliliter, then slowly expressed through a 22-gauge needle in a drop-wise fashion into 102mM $CaCl_2$ solutions. After gelation, the beads were allowed to polymerize further for a period of 10 minutes in the $CaCl_2$ solution. After wash in 10 volume of 0.15M NaCl and 3 washes in 10 volumes of DMEM/F12 medium. The beads were finally placed in complete culture medium. Ten beads were cultured in each well of a 24-well plate.

4. Depolymerization of alginate bead

To remove cells from the alginate bead, wells were rinsed twice with 0.15M NaCl with gentle pipetting into the well. The rinse solution was incubated for 1 minute and was aspirated off. Three times the volume of alginate in dissolving buffer (55mM sodium citrate and 0.15M NaCl) was

added to the wells and plates were incubated at 37 ˚C for 10 minutes with shaking.

5. Characteristics of EMF and exposure condition

EMF is designed similarly like a McLeod. Winded with copper line tightly, the cylinder of polyethylene tube, which is the diameter of 12cm and height of 30cm, was connected with electric power supply. The cultured cell was placed on the bottom of three-dimensional middle position in cylinder shaped tool. The copper line was designed for the resistance of 650 ohme and the output of 1.8militesla(mT) magnetic flux density - measuring by Walker mode MG2A Gaussmeter, when the source of electric power was connected. The wave generation was designed to generate the sinusoidal wave of 30Hz and connected with the electric power supply, which was designed for generating the sinusoidal of 30Hz in the EMF of 1.8mT. Cultures were divided into control and EMF group with various exposure times (6, 24, 48, and 72 hours) for quantification of dose response.

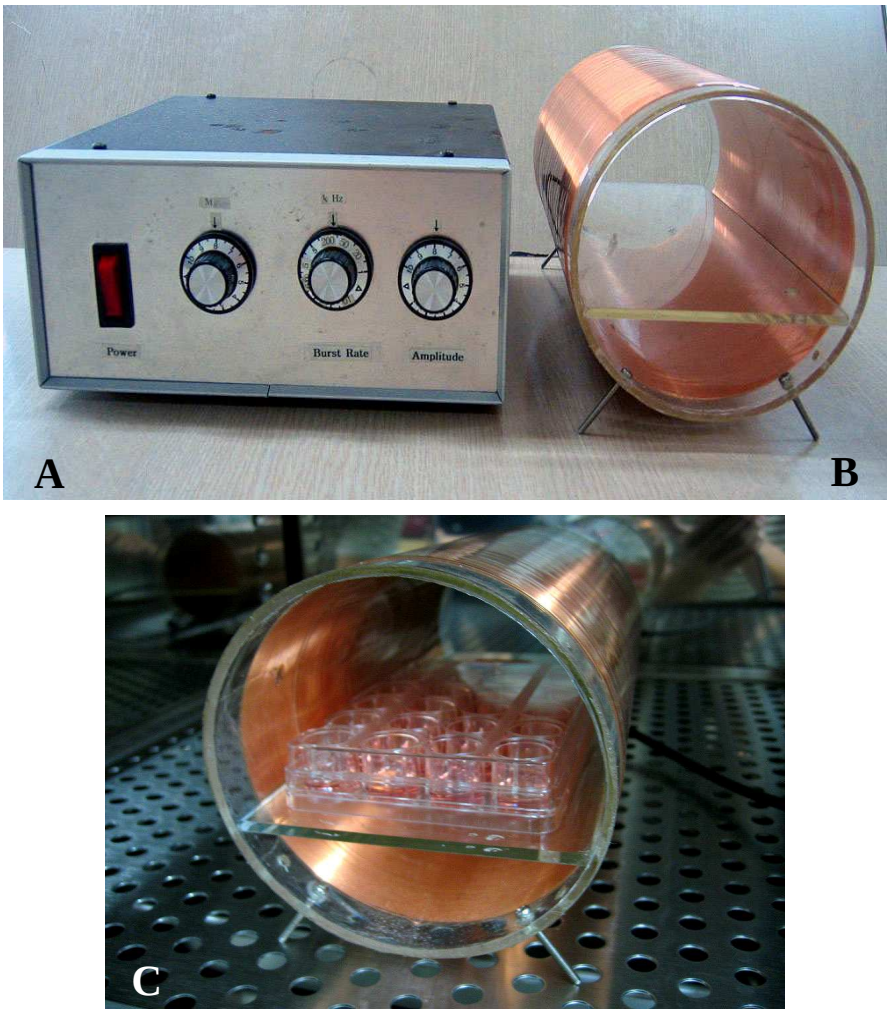


Figure 1. Electromagnetic fields (EMF) generator A; Power source B; EMF generator C; Culture plate in EMF generator. EMF was exposed to IVD cells with 650Ω, 1.8millitesla magnetic flux density, 60Hz sinusoidal wave. Cultures were divided into control and EMF group with various exposure times.

6. Cellular proliferation

DNA synthesis was measured by the [^{3}H]-thymidine incorporation. 5 μCi/ml of [^{3}H]-thymidine (Amersham Biosciences, Uppsala, Sweden; 25Ci/mmol specific activity) was added to control and treated cultures for 24h. The medium was then discarded and the cells were trypsinized with trypsin/EDTA. The trypsinized cells were filtered onto glass fiber filters (Whatman, Maidstone, England), and transferred to scintillation vial. Filters were dried and counted in 3ml of scintillation cocktail solution (Beckman Coulter Inc, Fullerton, CA, USA) in a Packard scintillation counter (Packard #1900 TR, Mariden, CT, USA). The results of each experiment, expressed as cpm/well, are the means of three parallel cultures.

7. Newly synthesized proteoglycan

5 μCi/ml of [^{35}S]-sulfate (Amersham Biosciences, Uppsala, Sweden; 25Ci/mmol specific activity) was added to control and treated cultures for 24h. At the end of culture the medium was collected and the beads were dissolved with 28mM EDTA/0.15M NaCl. The cells were then placed in an extraction media (8M guanidine HCl solution, 5mM sodium acetate (pH5.8), proteinase

inhibitor) at 4℃ for 48hours. Aliquots (200μl) of the cell extracts were eluted on Sephadex G-25M in PD-10 columns (Amersham Biosciences, Uppsala, Sweden) under dissociative condition. Fractions (1ml) were colleted in scintillation vial and mixed with 6ml scintillation cocktail solution (Beckman Coulter Inc, Fullerton, CA, USA). Five fractions were collected per sample, and three meddle fractions were counted in a Packard liquid scintillation counter (Packard #1900 TR, Mariden, CT, USA)

8. Reverse transcription-polymerase chain reaction (RT-PCR) for aggrecan, collagen type I, and collagen type II

Total cellular RNA was eluted by selective binding to a silica gel-based membrane using an RNeasy mini kit (QIAGEN, GmbH, Germany). Reverse transcription of RNA into cDNA was performed incubating 1μl of RNA in a reaction mixture containing 0.5mg/ml cDNA reaction product and was used as the template to co-amplify β-actin, aggrecan, collagen type Ⅰ, Ⅱ. PCR was performed using a DNA thermal cycler. The same reaction profile was used for all primer sets: an initial denaturation at 94℃ for 1 minute, followed by 25~40 cycles of: 94℃ for 5 seconds; 47~50℃ for 5 seconds; and 72℃ for 30 seconds; and an additional 2 minutes extension step at 72℃ after the last

cycle. Amplification reactions specific for the following cDNAs were performed: β-actin, aggrecan, collagen type I, and collagen type II. Primer sequence of each cDNA was listed on Table 2. PCR products (5μl) were analyzed by electrophoresis in 2 % agarose gels, and detected by staining with ethidium bromide. The intensity of the products was quantified using the BioImage Visage 110 system (BioRad, Hercules, CA, USA).

9. Statistical analysis.

One-way analysis of variance with Fisher's protected LSD post-hoc test was performed to test difference in densitometric data and [^{35}S]-sulfate labeled proteoglycan. Significant level was set as $p<0.05$.

TABLE 2. Sequences of the RT-PCR primers used

Primer	Sequence	Length	Size
Human β-actin	5’-GGCGGACTATGACTTAGTTG-3’	20	238bp
	5’-AAACAACAATGTGCAATCAA-3’	20	
Human aggrecan	5’-GAA TCT AGC AGT GAG ACG TC-3’	20	541bp
	5’-CTG CAG CAG TTG ATT CTG AT-3’	20	
Human collagen type Ⅰ	5’-CCT GTC TGC TTC CTG TTA AC-3’	20	182bp
	5’-AGA GAT GAA TGC AAA GGA AA-3’	20	
Human collagen type Ⅱ	5’-CAG GAC CAA AGG GAC AGA AA-3’	20	328bp
	5’-TTG GTC CTT GCA TTA CTC CC-3’	20	

TABLE 3. RT-PCR conditions

Primer	Conditions			Cycle
	Denaturation	Annealing	Polymerization	
Human β-actin	94℃ 5 sec	53℃ 5 sec	72℃ 30 sec	24
Human aggrecan	94℃ 5 sec	47℃ 5 sec	72℃ 30 sec	26
Human collagen type Ⅰ	94℃ 5 sec	48℃ 5 sec	72℃ 30 sec	21
Human collagen type Ⅱ	94℃ 5 sec	48℃ 5 sec	72℃ 30 sec	40

Ⅲ. RESULTS

1. Time course of EMF-stimulated DNA synthesis

Human IVD cell was cultured in three-dimensional alginate beads with various times of EMF exposure. Human IVD cells cultured in full medium demonstrated increase in DNA synthesis with the course of time. In culture with EMF exposure (48 hours) showed significant increase in DNA synthesis compared to control culture without EMF exposure (Figure 2) ($p<0.05$). However cultures with EMF exposure (6, 24, 72 hours) demonstrated no significant change in DNA synthesis compared to control.

2. Newly synthesized proteoglycan normalized by DNA synthesis

Human IVD cell was cultured in three dimensional alginate beads with various times of EMF exposure. Human IVD cell cultures with EMF exposure (48 hours) showed significant decrease in [^{35}S]-sulfate incorporation compared to control (Figure 3) ($p<0.05$). However cultures with EMF exposure (6, 24, 72 hours) demonstrated no significant change in proteoglycan synthesis compared to control.

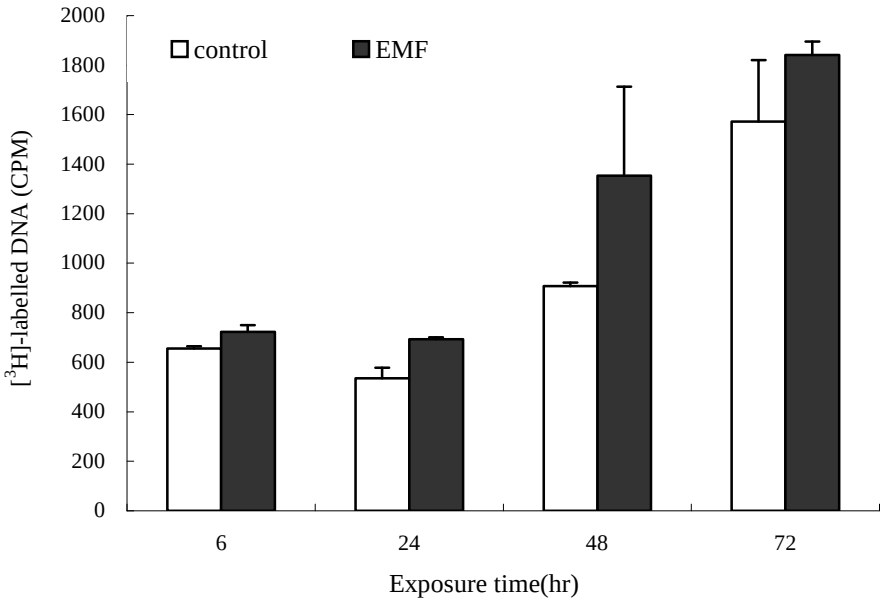


Figure 2. Percent control of DNA synthesis measured by [^{3}H]-thymidine incorporation (CPM). Control; the cultures without EMF exposure, EMF; with EMF exposure for 6, 24, 48, 72 hours.

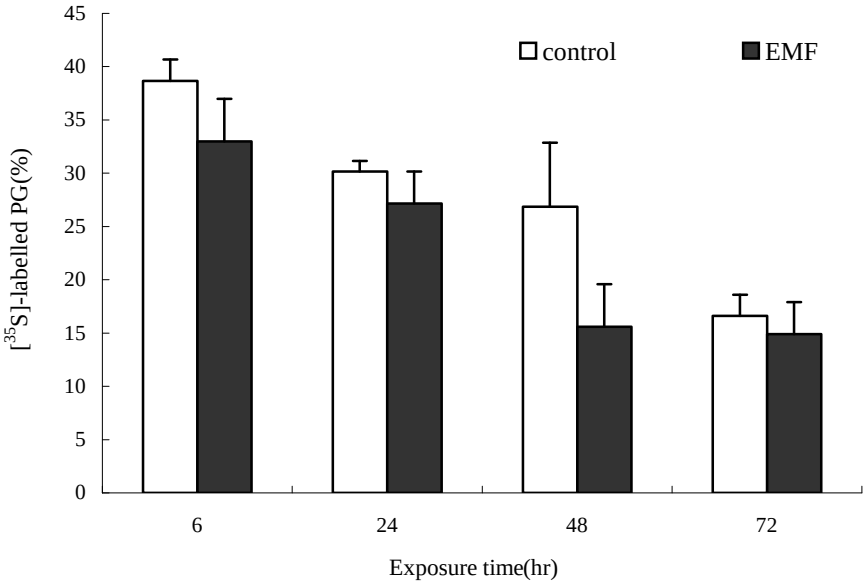
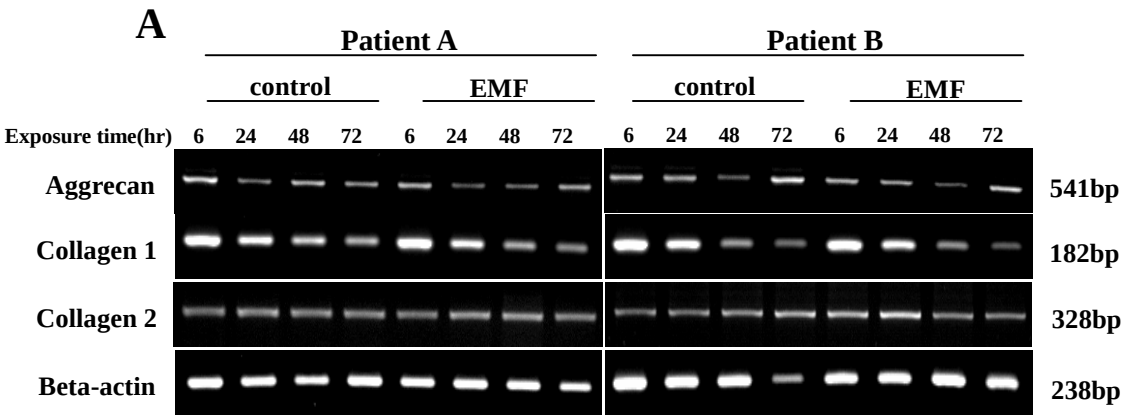


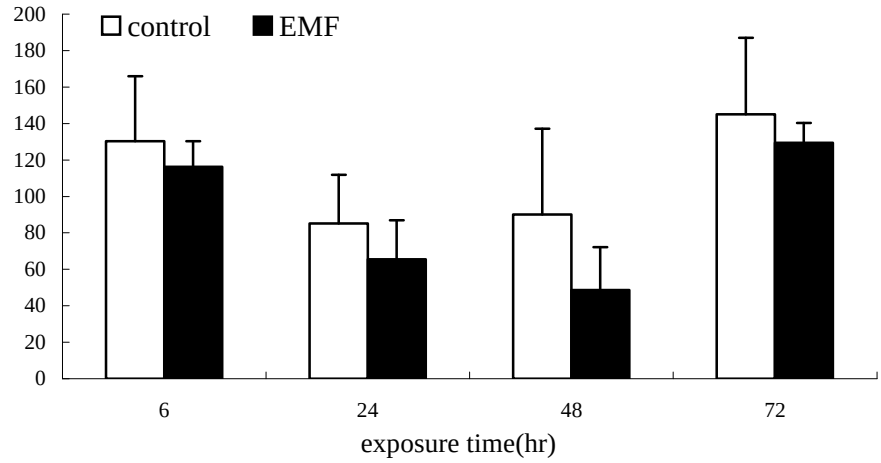
Figure 3. Percent control of proteoglycan synthesis measured by [^{35}S]-Sulfate incorporation (CPM). Control; the cultures without EMF exposure, EMF; with EMF exposure for 6, 24, 48, 72 hours.

3. Effect of EMF on the expression of aggrecan, collagen type Ⅰ, and Ⅱ

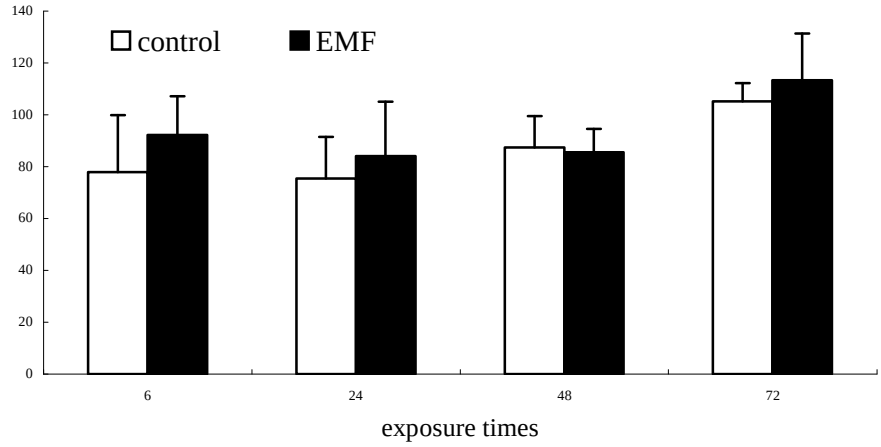
In densitometry assay of reverse transcription-polymerase chain reaction, human IVD cells cultured in three dimensional alginate beads with various times of EMF showed no statically significant changes in mRNA expression of collagen type Ⅰ, collagen type Ⅱ, and aggrecan compared to control (Figure 4).



B


aggrecan
control
EMF
200
180
160
140
120
100
80
60
40
20
0
6
24
48
72
exposure time(hr)

C


collagen type1
control
EMF
140
120
100
80
60
40
20
0
6
24
48
72
exposure times

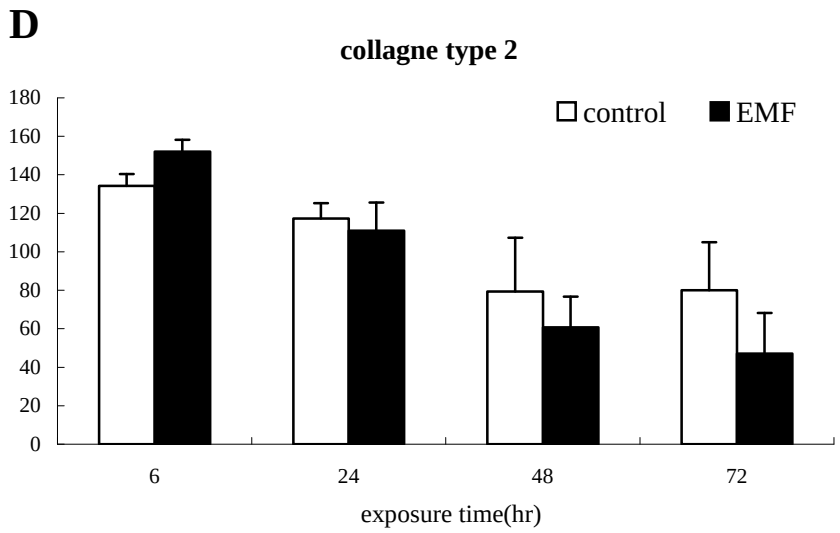


Figure 4. Effect of EMF on the expression of aggrecan and collagen type Ⅰ, Ⅱ. A; The IVD cells were exposed to various times of EMF. Total RNA was isolated from cells and subjected to RT-PCR. A; The PCR products were separated on 2% agarose gels containing ethidium bromide, and then observed on an ultraviolet transilluminator. B, C, D; the expression of each band seen in A was quantified using an image analyzer. The results are presented as the percentage of the mRNA level relative to β-actin for each band. The results showed that there was no statically significant changes in mRNA expression of collagen type Ⅰ, however there were significant decrease in mRNA expression of aggrecan and collagen type Ⅱ in 48 hours exposure group compared to control.

Ⅳ. DISCUSSION

Biological reaction of human tissue to EMF is widely divided into two categories: First, in hazardous way, focusing on a possibility of tumorogenesis by cellular proliferations; Secondly, in beneficial way, applying EMF as proliferating cells and stimulating matrix synthesis for regenerating tissues. EMF induces cellular proliferation of articular cartilage cells and nasal cartilage cells and stimulates synthesis of extracellular matrix and GAG.[16] Furthermore, EMF affects osseous tissue in proliferation, differentiation, and expression of osteogenic phenotypes. Hence, these results have a significant implication in effective fracture healing with EMF.[16] Although there are numerous studies of EMF, there have been few studies of EMF on the intervertebral disc cell. Furthermore, there has been no such a study for validating EMF effects on intervertebral disc cells in vitro. Therefore, for the first time, this study systematically examined biological effect of EMF on the intervertebral disc cells; i.e., DNA synthesis, matrix synthesis, manifesting chondrogenic phenotypes and so on.

This study utilized human intervertebral disc cells cultured in three-dimensional culture in order to maintain the phenotype of intervertebral disc cells. In the result of this experiment, when we examined the EMF at a variety

of time per unit, we could not find any cytotoxicity in the human intervertebral disc. When examining the electromagnetic field in the intervertebral cell, EMF stimulated cellular proliferation as measured by increased in DNA synthesis. Cellular proliferation was strongly evident with 48 hours exposure. In detail, intervertebral cell culture after 48 hours EMF exposure showed 60% increase in DNA synthesis, compared to the control group without EMF exposure. In contrast, with time point 6, 24, and 72 hours exposure, there was no significant increase in DNA synthesis. In proteoglycan synthesis, Human intervertebral disc cells with 48 hours EMF exposure revealed decrease in proteoglycan synthesis, compared to control group without EMF exposure. In addition, we analyzed the manifestation of the intervertebral cell phenotypic expressions of aggrecan, collagen type 1, and collagen type 2 mRNA. The expression of aggrecan mRNA in culture with 48 hours exposure of EMF showed significant decrease in its expression. Also the collagen type 2 mRNA expression significantly reduced with 48 hours EMF exposure. Nevertheless, the expression of collagen type I mRNA demonstrated no meaningful changes in its expression, compared to the control group. Thus, this result means the manifestation of chondrogenic phenotypes decrease by the electromagnetic field on a certain time per unit.

The important point of this study is that the EMF can be applied to the intervertebral disc cell culture and we can obtain detailed biological effect for the first time. With the result of this study, we can suggest that EMF can be utilized in proliferating cells i.e. as an amplification process in autogenously disc cell therapy to regenerate disc tissue. In other words, it is necessary to amplify intervertebral cell by in vitro culture for tissue engineering purposes.

One of possible another adverse effects of the EMF is excessive proliferating cells resulting in a tumor formation. Although it is very short period of exposure, we could not find any transformation of metaplasia of disc cells. On the following study in the future, it will be necessary to study in detail on the mechanism of cellular proliferation with EMF exposure.

Ⅴ. CONCLUSION

EMF stimulated cellular proliferation in human intervertebral disc cells and downregulated the expression of chondrogenic phenotypes and proteoglycan synthesis.

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ABSTRACT (In Korean)

저 파동성 전자기장이 인간 추간판 세포에 미치는 영향

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연구계획 : 실험자 연구

연구목적 : 저 파동성 전자기장에 대한 인간 추간판 세포의 생물학적 반응을 알아보기 위함.

연구배경 : 전자기장은 세포 증식, 합성, 성장인자의 분비 및 세포내 전사조절 등 생체 내의 다양한 생물학적 반응을 조절한다고 알려져 있다. 또한 불유합 지연유합 혹은 척추 유합술에서의 전자기장은 효과적으로 골형을 유도하였다. 더구나 척추 추간판 조직에 대한 연구로서는 흡연에 의한 추간판 변성 변화가 전자기장에 의해 역전될 수 있음이 보고된 적 있었다.

대상 및 방법 : 인간 추간판 세포를 분리하여 alginate bead 를 이용한 3 차원 배양을 하였다. 배양한 추간판 세포에 다양한 시간별로 저 파동성 전자기장(650Ω, 1.8militesla magnetic flux

density, 60Hz sinusoidal wave)을 조사하였다. 세포 독성은 MTT assay, 당단백 생성은 [^{35}S]-sulfate incorporation 과 DNA 생성은 [^{3}H]-thymidine incorporation 으로 분석하였으며 aggrecan, 제Ⅰ형 교원질, 제Ⅱ형 교원질 mRNA 발현은 RT-PCR 에 의한 densitometric assay 로 분석하였다.

결과 : 전자기장 조사에 의한 세포독성은 나타나지 않았다. 전자기장에 의해 세포 증식력은 증가하였으나 DNA 합성으로 정량화한 당단백은 감소하는 경향을 나타내었다. 또한 연골성 표현형인 aggrecan (48 시간 조사)과 제Ⅱ형 교원질 (48 시간 조사) mRNA 발현은 대조군과 비교하였을 때 감소함을 나타내었다.

결론 : 전자기장이 인간 추간판 세포의 DNA 합성을 자극하는 효과는 가지고 있으나 연골성 기질생성은 감소시킴을 알 수 있었다.

핵심되는 말 ; 저 파동성 전자기장, 추간판, 당단백, 기질생성