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Acceleration of Cell Proliferation and Gene Expression in Human Chondrosarcoma Cells Stimulated by Strong Pulse Magnetic Field

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For the treatment of osteoarthritis, pulsed electromagnetic field stimulus has been suggested as a useful therapeutic method in rehabilitative medicine. Most studies have been performed under low-frequency and low-energy to find out biological properties for stimulating chondrocyte with pulsed magnetic field. In this study, the effect of strong pulse magnetic field on the human chondrosarcoma cells (SW-1353) has been investigated by means of cell counting, morphologies, and gene expression of cartilage extracellular matrix genes. The SW-1353 cells were exposed under the field intensities of 270, 100, 55, 36, and 26 mTesla during 6 hours a day in 5 consecutive days. The pulse magnetic field with an LRC oscillating signal has the pulse width of 0.126 msec and stimulation period of 1 sec. For the 270 and 100 mTesla stimulation, the cell proliferation significantly increased in 21-24% as compared with the non-stimulated cells. Gene expression of cartilage extracellular matrix genes (*ACAN, COMP* and *COL2A1*) was assayed by quantitative real time-PCR method. The *ACAN* gene expression showed a significant brightness, which means the increase on gene expression, compared with the non-stimulated cells. Our results suggest that the strong pulse magnetic field stimulation can be utilized to accelerate cell proliferation and gene expression on human chondrosarcoma cells.

Keywords : pulsed electromagnetic field, *ACAN*, SW-1353, chondrocyte, gene expression

1. Introduction

An electromagnetic field can be used to stimulate muscles and nervous tissues, since changing the magnetic field induces tiny electrical currents in human tissues. Pulse electromagnetic field (PEMF) has been widely used to treat non-healing fractures and related problems in bone healing since their approval by the Food and Drug Administration (FDA) in 1979 [1, 2]. Some studies have suggested that electromagnetic fields might be useful in the treatment of chronic illnesses, such as lumbar radiculopathy, whiplash syndrome, posterior neck pain, and knee osteoarthritis [3]. Almost all the physiological effects of electromagnetic field stimulation on cells and tissues were investigated at a few tens of mTesla with a frequency range of 6.4-75 Hz [4].

The PEMF affected the cells in the form of increased activity of the phagocyte or enzyme with the movement of ions; depolarization of the cell membrane and the trans membrane potential difference; growth factor secretions; calcium ion transmission; cartilage cell synthesis, and others, although the mechanism behind these effects is still unclear.

PEMF has been used clinically to promote bone healing for un-united fractures [5]. P. Diniz *et al*. found that the stimulatory effect of PEMF with the 15 Hz pulse burst and 7 mTesla on bone-tissue-like formations was associated with the increase in the number of cells and the enhancement of the cellular differentiation [6]. Most researchers had reported the positive efficacy of exposure to electromagnetic fields on basic cellular functions such as control of cell proliferation, protein synthesis, gene transcription and expression, ion transport, ligand-receptor binding events, neurite outgrowth, and protein kinase activation, among others [7]. Most of the PEMF stimulators that were used in such studies consisted of pulse bursts that were repeated at 2-300 Hz and that had field intensities of 2-25 mTesla. Even though PEMF stimulators have been widely used and studied, there have been no reports yet on PEMF devices with strong magnetic fields of above 10^2

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mTesla and on their efficacy on cells or tissues.

Low-frequency, low-energy PEMFs are widely used to promote bone healing of un-united fractures in human [8]. Moreover some clinical observations seem to suggest the possibility that PEMF exposure might be useful for the treatment of osteoarthritis (OA). Some studies have suggested that electromagnetic fields might be useful in the treatment of chronic illnesses, such as lumbar radiculopathy, whiplash syndrome, posterior neck pain and knee osteoarthritis [9]. However, these studies have been done under relatively low intensity of magnetic field. Most of the PEMF stimulators that were used in such studies consisted of pulse bursts that were repeated at 2-300 Hz and that had field intensities of 2-25 mTesla. Even though PEMF stimulators have been widely used and studied, there have been no reports yet of PEMF devices with strong magnetic fields of above100mTesla and of their efficacy on cells or tissues.

PEMFs were tested in human and animal monolayer chondrocyte cultures and tissue explants and their effects were investigated by different methodologies. Conflicting observations have been reported and some studies demonstrated a significant effect of PEMFs in increasing articular chondrocyte proliferation [10-12], ECM (extracellular matrix) synthesis and PG (proteoglycan) content in cartilage tissue explants [13] while others demonstrated that PEMFs had no effect on articular chondrocyte GAG (glycosaminoglycans) synthesis [12].

Many studies showed that chondrocyte proliferation and matrix synthesis are significantly enhanced by PEMF stimulation i*n vitro* [10, 11, 13, 14]. The physical properties of the fields used (intensity, frequency, impulse amplitude, etc.) and the exposure time, the availability of growth factors, environmental constrictions and the maintenance of the native-cell matrix interactions seem to be fundamental in driving the PEMF-induced stimulation [10, 13]. In particular, the interaction between cell membrane receptors and mitogens seems to be one of the molecular events affected by PEMFs [11]. These data are in agreement with results of *in vivo* studies with a decalcified bone matrix induced endochondral ossification model and showing that the stimulation of TGFb-1 may be a mechanism through which PEMFs affect complex tissue behavior and through which the effects of PEMFs may be amplified [10].

In this study, we have investigated the effect of strong pulse magnetic field on the human chondrosarcoma cells (SW-1353; ATCC, USA) by an observation of the changes in the number of cells and gene expression of cartilage extracellular matrix genes.

2. Materials and Methods

2.1. PEMF Device

Figure 1 shows the cell culture plate on the magnetic coil of the PEMF stimulator and the pulse signal of the

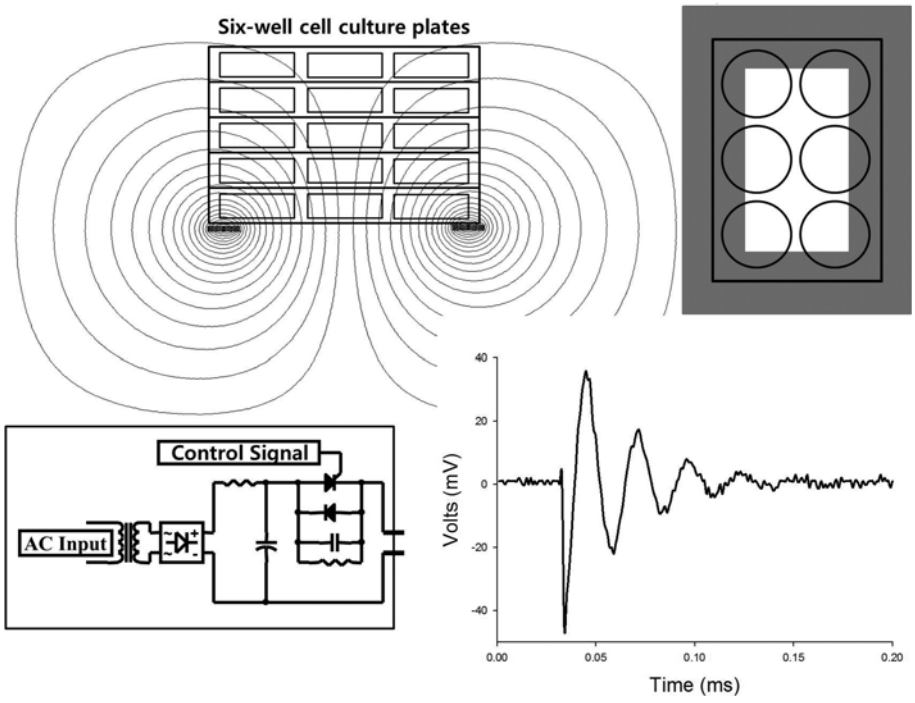

Fig. 1. The cell culture plate on the magnetic coil of the PEMF stimulator and the pulse signal of the magnetic field generated from the coil.

magnetic field generated from the coil. The magnetic field intensity is measured 10 mm away from the surface of the generating coil, and calibrated using the standard searching coil and integrated circuits. The 10 mm away from the coil is similar with the distance between patient skin and the coil. The pulse magnetic field with an LRC oscillation has a maximum intensity of 203 mTesla at the first peak. The change in the magnetic field (ΔH) between the first upper peak and the bottom peak is 330 mTesla, and its transition time is 126 μsec. Therefore, the magnetic flux change (ΔH/Δt), which induced an electric current in the tissues, is 2,620 Tesla/sec in air. The time duration of the pulse oscillation signal was almost 0.1 msec and its frequency is 8 kHz. The repetition time of stimulation is 1 sec.

The coil is wound with a hollow 2.5 × 5.5-inch rectangle for 15 turns. The cool water plate below the coil was used to prevent the increasing temperature by strong magnetic field. The change in the internal temperature of the incubator with cell plate and magnetic coil did not exceed ±1.0°C.

2.2. PEMF Exposure and SW-1353 Cell Proliferation Analysis

SW-1353 cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Gibco BRL, Germany) with 10% FCS (fetal bovine serum; Gibco BRL, Germany) in humidified atmosphere 5% CO_2 in air at 37°C. Monolayer cells were washed in phosphate buffered saline (PBS) three times and stimulated with 1 ng/ml interleukin-1β (IL-1β) (Roche Diagnostics, Germany) in DMEM/F12 containing 0.5% lactalbumin enzyme hydrolysate (Sigma, Germany).

After the cells attached at culture plate and was cultured during 12 hours, the PEMF stimulation on the cells was acted. The SW-1353 cells were exposed by strong PEMF with different intensity (270, 100, 55, 36 and 26 mTesla) during 5 days and 6 hours per a day. The morphology and cell count were performed with a 400-magnification microscope to investigate strong PEMF's stimulatory effect on SW-1353 cells.

2.3. Gene Expression

Total RNA was prepared from each experiments using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. To synthesize cDNAs, 3 μg of RNA was reverse transcribed in a 20 μL reaction volume using standard methods [9]. The synthesized cDNA samples were diluted in 80 μL of diethylenepyrocarbonate (DEPC)-treated water.

Gene expression of cartilage extracellular matrix genes was assayed by DDRT-PCR (differentially display reverse transcriptase-polymerase chain reaction). The mRNA levels were analyzed DDRT-PCR using gene-specific primer set (Table 1) including aggrecan (*ACAN*), cartilage oligomeric matrix protein (*COMP*), collagen, type II, alpha 1 (*COL2A1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *GAPDH* gene was used as an internal control. The PCR amplification was performed for one cycle at 94°C for 5 min, 50°C for 3 min, and 72°C for 1 min; this was followed by 40 cycles at 94°C for 40 s, 55-58°C for 40 s, and 72°C for 40 s; a final cycle was run at 72°C for 5 min. The PCR products were separated by electrophoresis on 2.0% agarose gel.

We also validated gene expression by quantitative real-time PCR assay. Real-time PCR amplification mixtures (20 μl) contained 1 μl cDNA, 2 × SYBR Green I Master Mix (10 μl) (Qiagen., GmbH, Germany), and 10 pM forward and reverse primers. The real-time PCR reactions started at 95°C for 15 min for pre-denaturation, the reaction was then set at 95°C for 10 s, 56°C for 20 s and 72°C for 30 s, and 40 PCR cycles were performed. The PCR was conducted in ABI 7500 realtime PCR system (Ap-

Table 1. Primer information used for DDRT-PCR and *quantitative real-time* PCR.

Gene name[1]	Primer sequence (5'-3')	Product size (bp)	GenBank accession no.
ACAN	F-CCAGTGCACAGAGGGGTTG	146	BC036445
	R-TCCGAGGGTGCCGTGAG		
COMP	F-GGGTGGCCGCCTGGGGGTCTT	116	AB086984
	R-CTTGCCGCAGCTGATGGGTCTC		
COL2A1	F-CCGGGCAGGGGCAATAGCAGGTT	128	BC007252
	R-CCATGATGGGGAGGCGTGAG		
GAPDH (control)	F-GGCGATGCTGGCGCTGAGTAC	149	BC001601
	R-TGGTTCACACCCATGACGA		

[1]*ACAN*, aggrecan; *COMP*, cartilage oligomeric matrix protein; *COL2A1*, collagen, type II, alpha 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; F, forward primer; R, reverse primer.

plied Biosystems, USA). Following amplification, a melting curve analysis was performed to verify the specificity of the reactions. The end point used in the real-time PCR quantification, Ct, was defined as the PCR threshold cycle number. The ΔCt value was determined by subtracting the *GAPDH* Ct value for each sample from the target Ct value [15]. To confirm whether the *GAPDH* gene was suitable for internal housekeeping gene in this experiment, we tested gene suitability of this gene. The gene expression stability value is less than 0.05, which met the stability requirement to be a housekeeping gene [16]. The real-time PCR data were used to calculate the normalized expression values ($2^{-\Delta Ct}$) for statistical analysis.

2.4. Statistical Analysis

Statistical analyses of cell proliferation, gene expression levels, and SW-1355 cell numbers in each PEMF group were carried out with the Microsoft Excel data analysis program for t-test analysis, and a P-value < 0.05 was considered statistically significant. Experiments were performed at least twice. Results are expressed as the mean standard deviation (SD).

3. Results and Discussion

3.1. Effect of PEMF on SW-1353 Cell Proliferation

Figure 2 showed the cell morphologies of the SW-1353 exposed to the field intensities of 270, 100, 55, 36, and 26 mTesla during 6 hours a day in 5 consecutive days. These photos were taken after the lapse of 6 days. There were no significant morphological transformations in all groups, but it was observed that the numbers of SW-1353 cell were changed depending on the intensity of PEMF. The groups exposed to relatively strong PEMF stimulation, 100 and 270 mTesla, showed the increase of cell counting as compared with the groups exposed to non-PEMF and weak PEMF.

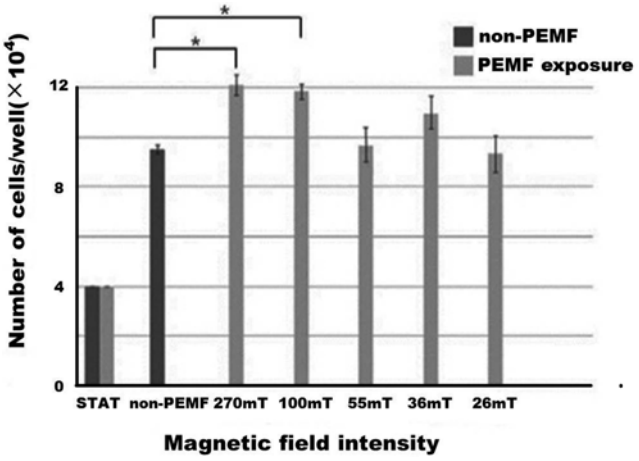

Fig. 3. The numbers of SW-1353 cells exposed to the field intensities of 270, 100, 55, 36, and 26 mTesla by 6 hours a day during 5 days were counted. At the field intensities of 270 and 100 mTesla, the cell proliferation significantly increased in 21-24% rather than the non-stimulated cells. The data points represent the mean of cell numbers and standard deviation (n = 5). STAT and * in figure means the number of cells before proliferation, 4×10^4 and significant level at $P < 0.05$, respectively.

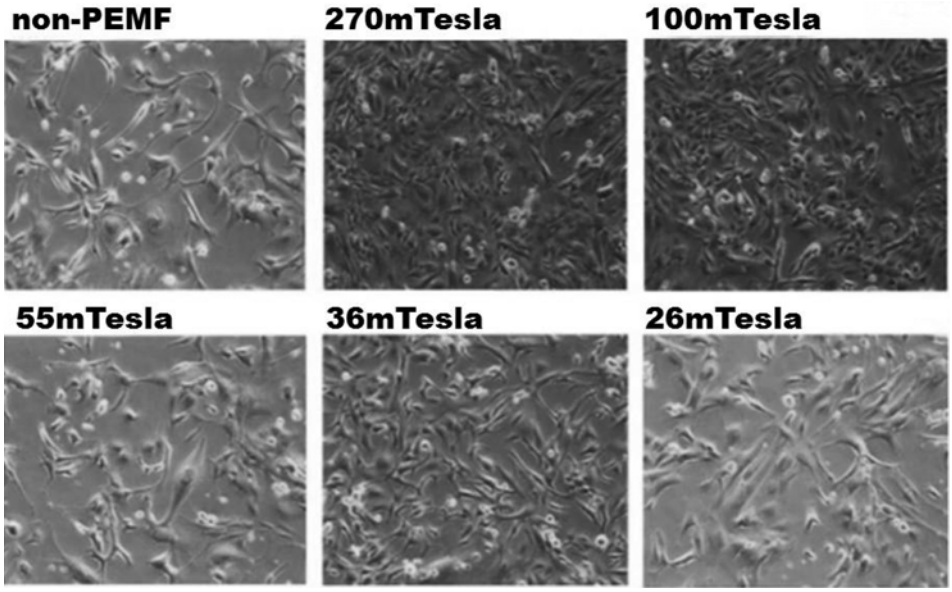

Fig. 2. The cell morphologies of the SW-1353 exposed to the field intensities of 270, 100, 55, 36, and 26 mTesla during 6 hours a day in 5 consecutive days. These photos were taken after the lapse of 6 days.

Figure 3 showed the results of comparative analysis of reproductive rate in cells exposed to PEMF and non-PEMF stimulation. The number of cells significantly increased in the group exposed to strong PEMF of 270 and 100 mTesla ($p = 0.021$). For the group exposed to PEMF stimulation of 270 mTesla, the cell proliferation increased from 1×10^4 to 1.21×10^5 which means increasing in 24% compared with the non-stimulated cells of 9.77×10^4. Also for the group exposed to PEMF stimulation of 100 mTesla, the cell proliferation increased from 1×10^4 to 1.18×10^5 which means increasing in 21% compared with the non -stimulated cells of 9.77×10^4. On the other hand, for the group exposed to relatively weak PEMF stimulation of 55, 36 and 26 mTesla, the number of cells remained nearly same or a little decreased and p-value was higher than 0.05. Therefore it might be concluded that strong PEMF stimulation of above 100 mTesla to the human chondrosarcoma cells affects cell proliferation, but weak PEMF stimulation of 55 mTesla or less did not.

3.2. Expression of Cartilage Extracellular Matrix Genes

We observed in cell level the activation of chondrocyte exposed to the strong PEMF stimulation so far. In this study in order to elucidate in mRNA level the effect of PEMF stimulation on chondrocyte, gene expression of cartilage extracellular matrix was investigated by DDRT-PCR method. *ACAN*, *COMP* and *COL2A1* among cartilage extracellular matrix perform a vital role in cartilage.

ACAN known as cartilage-specific proteoglycan core protein or chondroitin sulfate proteoglycan 1 is a protein that in humans is encoded by the *ACAN* gene. This gene is a member of the aggrecan/versican proteoglycan family. The encoded protein is an integral part of the extracellular matrix in cartilagenous tissue and it withstands compression in cartilage [17]. Cartilage oligomeric matrix protein is a protein that in humans is encoded by the *COMP* gene [18, 19]. The protein encoded by this gene is a noncollagenous extracellular matrix protein [20]. Collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital), known as *COL2A1*, is a human gene that provides instructions for the production of the pro-alpha1(II) chain of type II collagen. This gene encodes the alpha-1 chain of type II collagen, a fibrillar collagen found in cartilage and the vitreous humor of the eye.

Figure 4 showed gene expression of cartilage extracellular matrix by DDRT-PCR in SW-1353 cells exposed to the field intensities of 270, 100, 55, 36, and 26mTesla. In the *ACAN* gene, the groups exposed to PEMF stimulation of 270 and 100 mTesla showed a significant increasing on gene expression ($P < 0.05$) compared with non-PEMF and weak PEMF stimulated groups. On the other

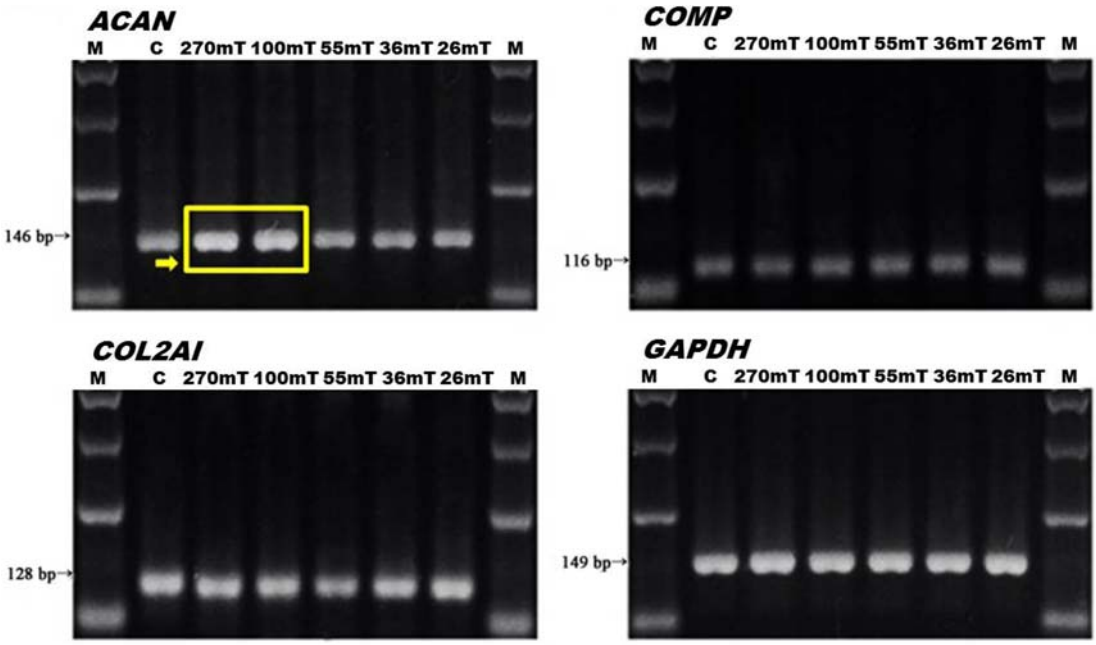

Fig. 4. (Color online) Gene expression of cartilage extracellular matrix genes was assayed by DDRT-PCR. The SW-1353 cells were exposed to the field intensities of 270, 100, 55, 36, and 26 mTesla by 6 hours a day during 5 days. In the *ACAN* gene, the groups exposed to PEMF stimulation of 270 and 100 mTesla showed a significant increasing on gene expression ($P < 0.05$) compared with non-PEMF and weak PEMF stimulated groups. In this figure, M means 100bp DNA ladder, and C means non-PEMF stimulation as a control.

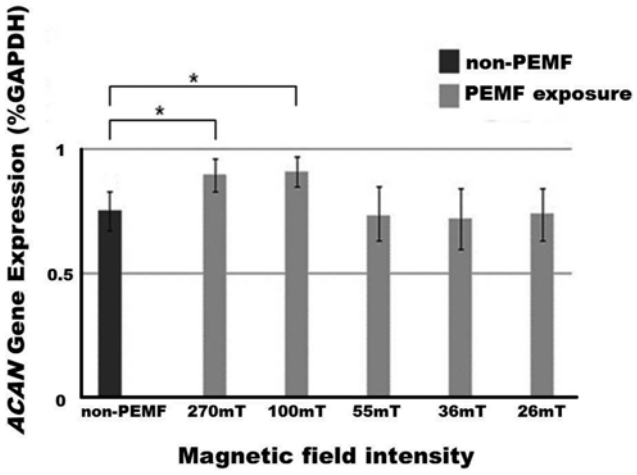

Fig. 5. Gene expression of *ACAN* gene was assayed by *quantitative real-time* PCR. In the *ACAN* gene, the groups exposed to PEMF stimulation of 270 and 100 mTesla showed a significant increasing on gene expression ($P < 0.05$) compared with non-PEMF and weak PEMF stimulated groups.

hand, for *COMP* and *COL2A1* genes, there were no changes of gene expression under PEMF stimulation.

The chondrocytes are responsible for synthesizing, organizing, and regulating the extracellular matrix of the articular cartilage. The most common proteoglycan in articular cartilage is the aggrecan–a very large proteoglycan that plays a pivotal role in the function of articular cartilage. During weight bearing, the aggrecan molecules, which are already very tightly packed together, become further compressed. During this compression water molecules (that were attracted to the negatively-charged aggrecan molecules) are forced from the extracellular matrix of the cartilage, and all of the negatively charged branches of the aggrecan molecule repel each other similar to the ends of a magnet. That is, the bones are protected by this layer of shock-absorbing articular cartilage, and the load is transmitted from one bone to another.

The significant increase of mRNA expression in *ACAN* gene shown in Fig. 4 means the activation and proliferation of *ACAN*, proteoglycan in articular cartilage. In order to testify quantitatively the difference of gene expression in *ACAN* between strong PEMF and non-PEMF stimulated groups, *quantitative real time* PCR was done. Figure 5 showed the result of quantitative analysis on gene expression of *ACAN* using *quantitative real time* PCR under PEMF stimulation with the intensities of 270, 100, 55, 36, and 26 mTesla, and non-PEMF. The gene expression of *ACAN* significantly increased in 17.3% and 18.7% with 270 and 100 mTesla stimulation, respectively. This result showed very similar to the result of Fig. 3, which was the increase in the numbers of cell with PEMF stimulation of strong field intensities, 270 and 100 mTesla. Therefore, the data presented in this study showed that strong PEMF induced an acceleration of cell proliferation and gene expression of cartilage extracellular matrix genes on human chondrosarcoma cells *in vitro*.

4. Conclusions

Although there were no significant morphological transformations in all human chondrosarcoma cell groups stimulated by PEMF, it was observed that the cell proliferation significantly increased in 21-24% at strong field intensities of 270 and 100 mTesla, rather than weak PEMF and non-stimulated cells. By means of DDRT-PCR and quantitative real time-PCR methods for elucidating the effect of PEMF stimulation on chondrocyte in mRNA level, the *ACAN* gene, exposed to PEMF stimulation of 270 and 100mTesla showed a significant increasing of the 17.3% and 18.7% compared with non-PEMF and weak PEMF stimulated groups. Our results in mRNA and cell level exactly agreed, which means an acceleration of cell proliferation and gene expression of cartilage extracellular matrix genes on human chondrosarcoma cells with PEMF stimulation of strong field intensities, 270 and 100 mTesla, rather than weak PEMF stimulation of 55 mTesla or less.

Acknowledgements

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