Demonstration of the Static Magnetic Field's Inability to Improve the Bone Mineral Density in an Osteoporotic Rat Model

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Abstract

Objectives: Osteoporosis is a raising public health issue. There are several medications in the treatment of osteoporosis and osteoporotic-related fractures involving either enhancement of the osteogenesis or inhibition of the osteoclastogenestis. However, each medication has side effect. Therefore, we try to look for an alternative therapy for the treatment of osteoporosis, which increases bone mineral density without accompanying side effects.

Methods: In the current study, we used a Static Magnetic Field (SMF) to evaluate the hypothesis of improvement of the bone mineral density after an SMF exposure. *In-vitro*, we tested the capacities of osteogenesis and osteoclastogenesis on Mesenchymal Stem Cells (MSCs). *In-vivo*, we implanted a permanent magnet adjacent to the vertebrae in an osteoporotic rat model to examine the effects on the treatment of osteoporosis

Results: In cell culture studies, a positive osteogenesis effect of Mesenchymal Stem Cells (MSCs) was observed under an SMF exposure; this osteogenesis effect was correlated to the SMF intensity. However, there was no effect on the osteoclastogenesis of MSCs under the same condition. In an osteoporotic rat model, there was no positive effect on alteration of the bone mineral density after a persistent 6-week exposure by an implanted SMF.

Conclusion: An *in-vitro* study showed that an SMF exposure of MSCs had a positive osteogenesis effect. However, there was no effect in terms of improvement of the bone mineral density when the SMF was applied on the osteoporotic rat model.

Introduction

Osteoporosis is a systemic disease, characterized by a decreased bone mineral density, leading to risks of fractures in the elderly population. According to the Yang's report [1], the estimated prevalence of osteoporosis from the Nationwide Health Insurance (NHI) database in Taiwan for people aged more than 50 years was 1.63% for men and 11.35% for women. However, it was underestimated in this report.

The pathogenesis of age-related osteoporosis mainly involves alteration of the bone mineral density [2]. Following osteoporosis, osteoporosis-related fracture is a major public health issue. In the year of 2000, there were (estimated) 9 million new osteoporotic fractures worldwide, of which 1.6 million were at the hip, 1.7 million at the forearm, and 1.4 million were clinical vertebral fractures [3]. There are several medications in the treatment of osteoporosis and osteoporotic-related fractures involving either enhancement of the osteogenesis through e.g., recombinant human parathyroid hormone [4] or inhibition of the osteoclastogenesis through e.g., bisphosphonate [5]. However, both treatments have side effects [4,5].

The use of magnets in medical treatments could be traced back to the fifteen century. With the advancement of the technology, the magnets are mainly used to provide Pulsed Electromagnetic Field (PEMF) and Static Magnetic Field (SMF). Bassett et al. [6,7] applied PEMF in fracture healing in 1989. Since then, the applications of PEMF have been widely expanded, including anti-inflammation, enhancement of fracture healing, treating osteoarthritis, improvement of wound healing, and prevention of osteoporosis [8]. The PEMF is generated by electric current, which is a major disadvantage in long-term clinical applications, as an external energy is required to generate the magnetic field. In addition, the electric current may cause tissue damage from the heat or electric hazards [9].

SMF is generated from a permanent magnet. The SMF creates no detectable electrical potential in the blood and hemodynamic flows at field levels below 5 T [10,11]. In addition, a permanent magnet does not need an external power to generate a magnetic field, which makes the SMF promising and potentially advantageous in orthopedic applications, which always require a mid-term or even life-long implantation.

Studies on the enhancement of fracture healing with an SMF have been reported [12-17]. The SMF in bone healing leads to the induction of angiogenesis, formation of collagen, promotion of hyperplasia, and differentiation of osteogenic cells [12-16]. These findings inspired us to investigate whether magnetic field can be used to treat osteoporosis. Therefore, in this study, we investigated the influences of the magnetic field on the treatment of osteoporosis. We started with a hypothesis that "a static magnetic field regulates Mesenchymal Stem Cell (MSC) differentiation, and thus can increase the bone density and be used in treatments of osteoporosis".

Materials and Methods

In order to investigate our hypothesis, we applied a SMF on an osteoporosis animal model. Two specific sub-hypotheses and two specific aims were used according to our main hypothesis.

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First sub-hypothesis

Different magnetic flux densities of the SMF may cause different effects on the osteogenesis and osteoclastogenesis of MSCs.

First specific aim

We isolate MSCs from Sprague-Dawley (SD) rats. By evaluating different SMF exposure durations, we reveal the optimal duration of SMF exposure for the stimulation of osteogenesis and inhibition of osteoclastogenesis of the Rat MSCs (RMSCs). In addition, the osteogenic and osteoclastogenic capacities of the RMSCs are evaluated after stimulations with two different SMF intensities. Based on the results, we determine the optimal intensity of the SMF to stimulate MSC differentiation and proliferation.

Second sub-hypothesis

SMF may increase the bone density in osteoporotic rats.

Second specific aim

We create an osteoporosis model of SD rat. After tying a magnetic rod on the osteoporotic rat, we observe the change of the bone density by micro-Computed-Tomography-(CT) scanning, serum analysis, and histomorphology analysis, and compare the results with those of a control group.

MSC isolation from an SD rat: RMSCs are isolated as described previously [18,19]. In order to identify relevant patterns, cells are harvested under standardized conditions from rats with the same gender and age range (male, age: 6 weeks, weight: 250-300 g). The process of euthanasia is performed by CO_2 asphyxiation according to the Institutional Animal Care and Use Committee (IACUC) guidelines. The tibiae and femora are aseptically removed and an adherent soft tissue is thoroughly described. An 18-gauge needle is used to bore a small opening through the growth plate on the distal end of the femora and proximal end of the tibiae. A small volume of the medium (Modified-Eagle's-Medium-Low-Glucose (MEM-LG) supplemented with 10% Fetal Bovine Serum (FBS)) is ejected to expel the bone marrow from the medullary canal. Marrow samples are collected and mechanically disrupted. Disaggregated marrow is centrifuged at $500 \times g$ for 15 min and re-suspended in the serum-supplemented medium. An aliquot of the cell suspension is combined with an equal volume of 4% (v/v) acetic acid to lyses red blood cells; nucleated cell numbers are determined with a hemacytometer. Cells are seeded at $5 \times 10^7/10$ cm culture dishes. The dishes are incubated at 37°C in a humidified 5%-carbon-dioxide atmosphere. On the fourth day of the culture, the non-adherent cells are removed by replacing the medium, and the cells are cultured until near confluence for further investigation. The RMSC cultures (primary culture) at 80-90% confluence are trypsin-released and sub-culture.

Quantification of the magnetic flux density of the magnetic plate: In this study, special ordered magnetic plates and 48-well plastic culture plates were used (Figure 1). We control each magnet plate with stable 0.1-T and 0.05-T magnetic flux densities using a standard Gauss meter (Hirst Magnetic Instruments, United Kingdom). The magnetic plates are placed under the plastic culture plates, with a diameter of 10 mm and height of 1.5 mm, to expose the cultures to the North Pole. The magnet flux density is then monitored to ensure stable quantified magnet intensity.

In-vitro **osteogenesis of the RMSCs:** The *in-vitro* osteogenic capacity of the MSCs is evaluated by alkaline phosphatase (ALP), matrix calcium deposition, and total DNA content once induced with an osteogenic
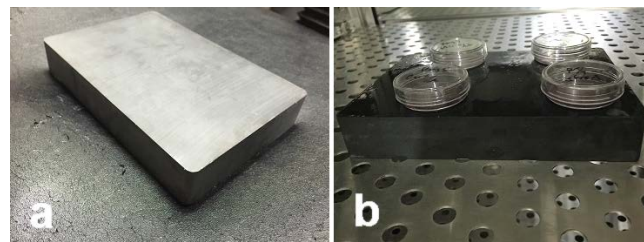
Figure 1: (a): The magnet generates a Static Magnetic Field. (b) The magnetic plates are placed under the plastic culture plates, with a diameter of 10 mm and height of 1.5 mm, to expose the cultures to the North Pole.
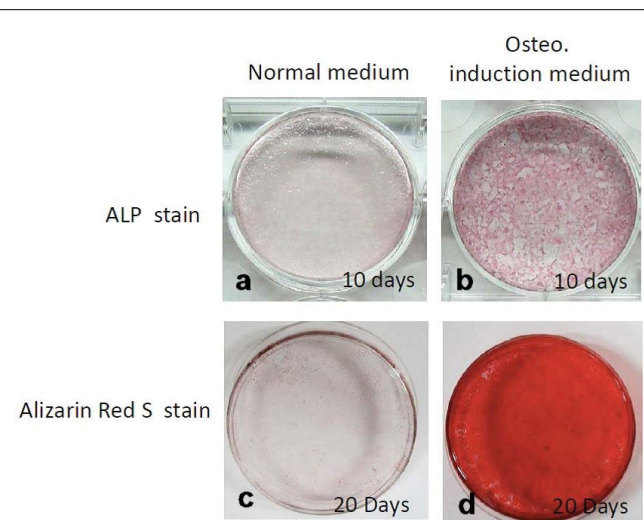
Figure 2: Evaluation of in-vitro osteogenic capacity of the Mesenchymal Stem Cells (MSCs). (a), (b): ALP stain of MSCs in normal medium and osteogenic induction medium. (c), (d): Alizarin red S stain of MSCs in normal medium and osteogenic induction medium.

induction medium (Dulbecco's Modified Eagle's Medium with 10% FBS, 0.1 mol/L dexamethasone (Sigma), 0.05 mmol/L ascorbate-2-phosphate (WAKO, Richmond, VA), and 2 mmol/L glycerol phosphate (Sigma)), referred to as OS medium. The ALP activity is assayed at day 10, while the matrix calcium deposition/mineralization is assayed at day 20 after the culture in control or OS medium (Figure 2). The results for the ALP activity and calcium content are presented per well, normalized to the DNA content. For the ALP assay, 1 mL of a 1 mg/mL solution of ALP substrate (p-nitrophenyl phosphate, Sigma) in a 50 mmol/L glycine buffer and 1 mmol/L $MgCl_2$-$6H_2O$ is added per well of a six-well dish. After 3 min, the solution is removed and transferred to a tube containing an equal volume of 1 mol/L NaOH. The absorbance of the resulting solution is read at 405 nm, and compared with those of a series of dilutions of p-nitrophenyl (Sigma).

Once the ALP assay solution is removed, the cultures are stored at -80 °C until DNA quantification. For the calcium assay, calcium in the osteogenic human MSC culture is extracted with 0.6 mol/L HCl on day 20. Aliquots of the extract are mixed with reagent from a commercial calcium assay kit (Biotron, Hemet, CA), and the absorbance is read at 575 nm. The calcium concentration is determined with a standard curve generated from a series of dilutions of $CaCl_2$.

Osteoporotic rat model: SD female rats (age: 8 months, weight: 200-320 g) are housed in an environmentally controlled animal

laboratory. They are fed with normal calcium diet (or phytoestrogen reduced food) and free to access to water. The rats are anesthetized by 1% isoflurane inhalation in a chamber. Osteoporosis is induced by bilateral ovariectomy (OVX) (Figure 3). The control rats were subjected to sham surgery exposure, but without removal of the ovaries. After 4 weeks of OVX treatment, the bone specimens are assessed by a micro-CT scan for Bone Mineral Density (BMD); a serum sample was collected to assess biochemical parameters, and then the osteoporosis diagnosis was confirmed by a significant change compared to the baseline measurements.

External use of an SMF on osteoporotic rats: The rats were anesthetized by general intraperitoneal anesthesia with maintenance of sedation by a 1% isoflurane inhalation. In the study group, we tied a magnetic rod with a stable 0.05-T magnetic flux density on the back of the osteoporotic rat with the north pole pointed ventrally (Figure 4a). In the control group, we tied a stainless rod with the same size and following the same procedure into the rat (Figure 4b). Two rats were housed together in one cage after the implantation procedure using the method described in the literature. At the 12th week after the implantation, all of the rats were sacrificed and L4 vertebrae were taken out. Examinations of the vertebrae were performed by micro-CT scan, serum analysis (Tartrate-Resistant Acid Phosphatase (TRAP), Procollagen type I N Propeptide (PINP)), and histomorphology analysis.
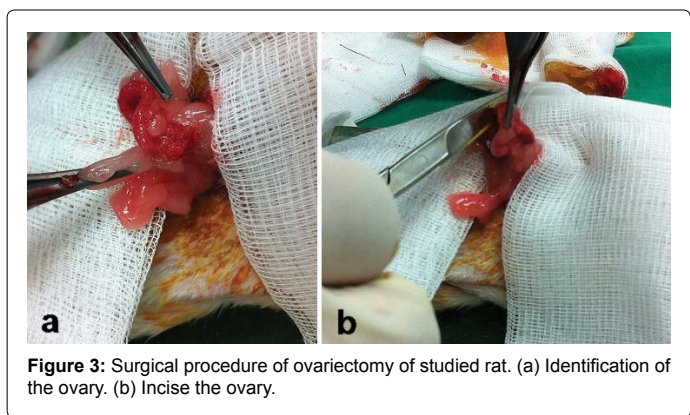
Histomorphologic analysis: Hematoxylin and Eosin (HE) stain of lumbar specimens was performed in an orthodox manner, and used to

Figure 3: Surgical procedure of ovariectomy of studied rat. (a) Identification of the ovary. (b) Incise the ovary.

Figure 4: Surgical procedure of implantation of a magnet (a) or a stainless steel (b) on the back of the osteoporotic rat.

measure BMD to confirm the osteoporotic state. The HE staining of the OVX vertebrae showed thinner and more widely separated bone trabeculae with a decreased density compared to those in the control and sham groups.
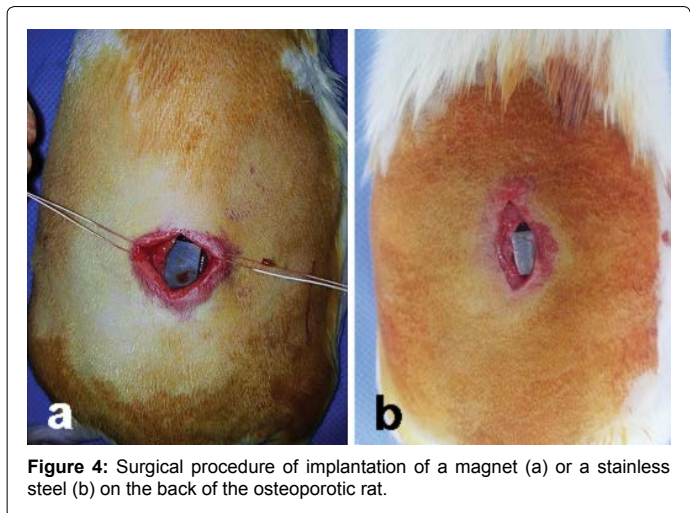
Results

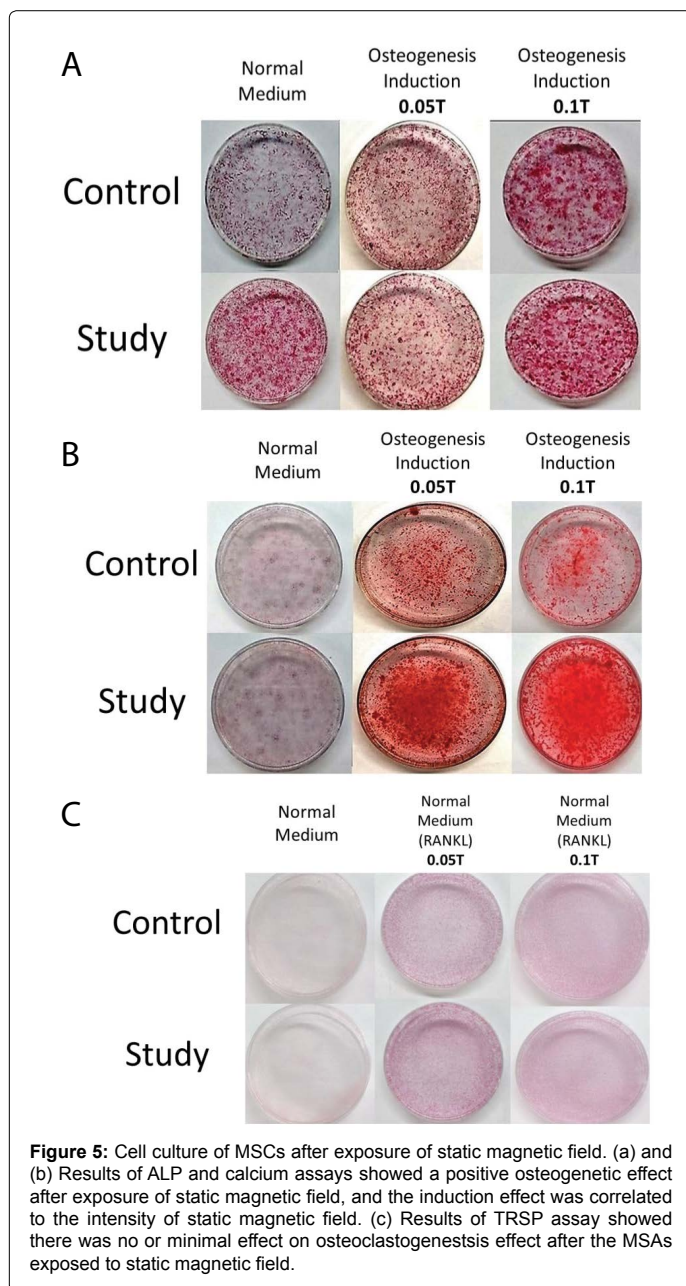### Influences on *in-vitro* bone-marrow-stem-cell differentiation

Under the presence of the SMF, the results of the ALP and calcium assays showed positive effects on the osteogenesis. In addition, the influence on the osteogenesis depended on the SMF intensity. The osteogenic capacity for the exposure with the magnetic flux density of 0.1 T demonstrated a larger enhancement of the cell culture than those of the cell culture for the magnetic flux density of 0.05 T and control group (Figures 5a and 5b).

Figure 5: Cell culture of MSCs after exposure of static magnetic field. (a) and (b) Results of ALP and calcium assays showed a positive osteogenetic effect after exposure of static magnetic field, and the induction effect was correlated to the intensity of static magnetic field. (c) Results of TRSP assay showed there was no or minimal effect on osteoclastogenestsis effect after the MSAs exposed to static magnetic field.

In addition, in order to evaluate the influence of the SMF on the osteoclastogenic capacity, we used TRAP as a marker of the osteoclastogenesis capacity. The results showed that under different intensities of the SMF, there was no (or minimum) influence on the osteoclastogenesis (Figure 5c).

In-vivo study of the effect of the SMF on osteoporosis

According to the result of the first sub-hypothesis, a magnet with an intensity of 0.1 T was used as the implanted magnet. The X-ray examinations following the implantation procedure showed that both implanted metal and magnet had some migration cranially or caudally after the implantation procedure (Figure 6). Twelve weeks after the ovariectomy, all of the tested rats were proved into the standardized osteoporosis model. Based on the micro-CT results, there was no statistical difference between the study and control groups (Figure 7).

Histological examination and serum analysis of the osteoporosis model after the SMF exposure

The 4th-6th lumbar vertebrae were obtained after a euthanized procedure for the histological examination. The histology result showed that there was no influence on the osteoporotic vertebrae after the SMF exposure. There was no significant improvement of the bone density in the study group, compared to the control group (Figure 8). Serum of the tested rats was obtained for the analysis of PINP and TRAP. Although a
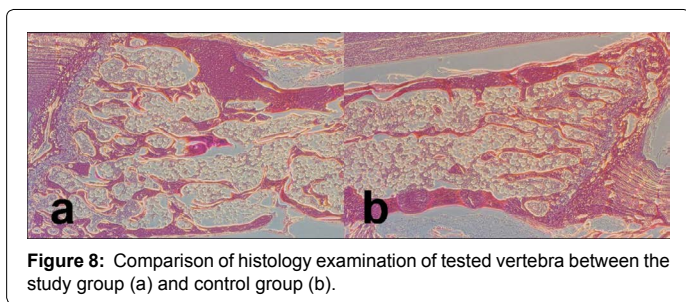

Figure 6: Radiographic follow up of the studied rats after implantation or a magnet or a stainless steel.

Figure 7: Comparison of bone mineral density by micro-CT scan. The statistical result showed there was no difference on improvement of bone mineral density after 12 weeks exposure of static magnetic field.

Figure 8: Comparison of histology examination of tested vertebra between the study group (a) and control group (b).
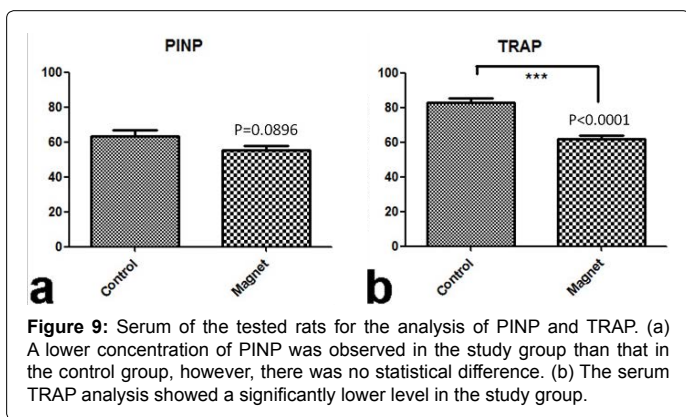

Figure 9: Serum of the tested rats for the analysis of PINP and TRAP. (a) A lower concentration of PINP was observed in the study group than that in the control group, however, there was no statistical difference. (b) The serum TRAP analysis showed a significantly lower level in the study group.
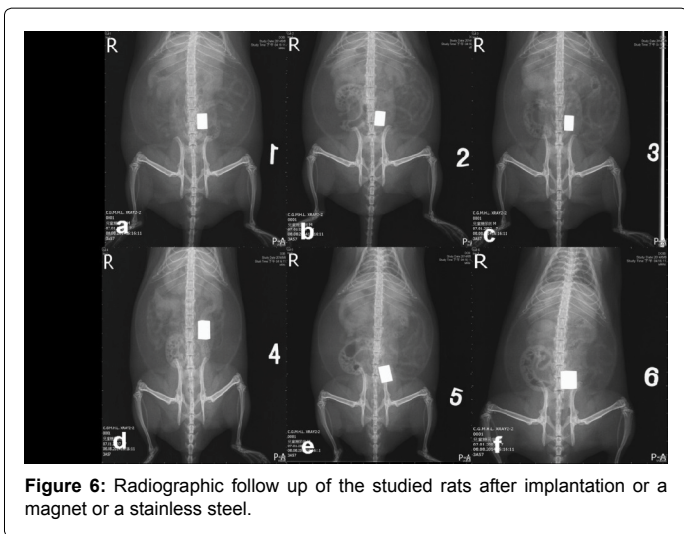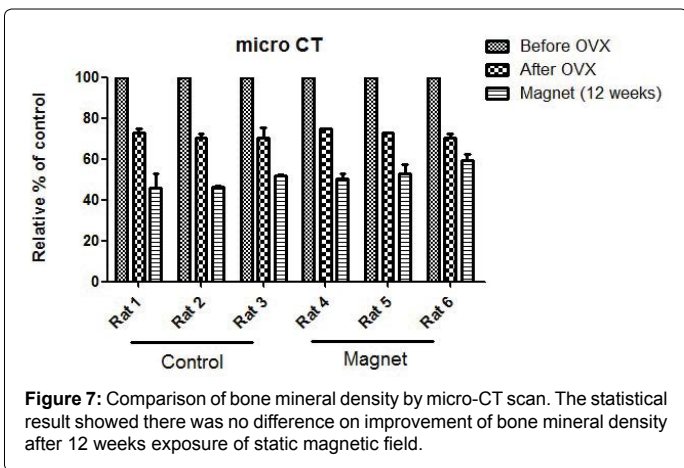
lower concentration of PINP was observed in the study group than that in the control group (Figure 9a), there was no statistical difference. On the other hand, the serum TRAP analysis showed a significantly lower level in the study group, which implied that there was some inhibitive effect on the osteoclast function after the SMF exposure (Figure 9b).

Discussion

This study aimed to identify the influences of an SMF on the (1) enhancement of differentiation of stem cells to osteoblast and osteoclast and (2) improvement of the bone density in osteoporotic rats. The results of the ALP and calcium assays revealed that the SMF could enhance the osteogenic capacity of the MSCs. Moreover, the enhancement of the osteogenesis depended on the SMF intensity. However, only a minimum inhibition of the osteoclastogenic capacity by the SMF was obtained according to the TRAP assay. Regarding the second sub-hypothesis, the SMF had a minor effect on the change of the bone mineral density of an osteoporotic rat model.

The SMF had an up-regulation effect on osteoblast differentiation and growth [14-16]. However, these studies were focused on the differentiation ability of osteoblast or osteoblast-like cells after an SMF exposure. In this study, we used an MSC culture, a primitive cell, to evaluate our hypothesis. The results showed that under a persistent application of an SMF, the MSCs could be successfully induced to differentiate into osteoblasts. Furthermore, this osteogenetic effect had a positive correlation with the intensity of the magnetic field in the in-vitro study. However, when we investigated an osteoporotic rat model with a 6-week-interval exposure of a 0.1-T SMF, implanted next to the rat's vertebrae, there was no (or minimal) effect in terms of increase of the bone mineral density. This was attributed to two factors. First, although the 0.1-T SMF promoted the osteogenetic effect of the MSCs, this intensity might be low when applied in an animal study. Second, the ovariectomy rat exhibited a stronger negative effect on the osteogenesis

than that for the local application of an SMF. Therefore, the *in-vivo* study showed that there was no (or minimal) osteogenetic effect for the application of a local SMF on the osteoporotic model.

The effects on the osteoclastogenesis were investigated in previous studies. Iwasaka et al. reported that an inhibitive effect on osteoclast formation was observed in an osteoblast/osteoclast co-culture system under an SMF [20]. They used an SMF (horizontal: 14 T, vertical gradient: 10 T) to evaluate their hypothesis. In this study, we investigated the influences of an SMF on the osteoclastogenetic differentiation of MSCs. The *in-vitro* study revealed that there was no effect on the osteoclastogenesis under the persistent stimulation of the MSCs by the SMF. We attributed this opposite result to the different intensity of the SMF. We used an SMF with an intensity of 0.1 T, as there was an animal study following the *in-vitro* study; therefore, a higher intensity of the magnetic field might be hazardous for the studied animal. However, a negative effect on the osteoclastogenesis was obtained in the animal study after the implantation of the 0.1-T SMF. We postulated that a synergic effect could emerge from the endocrine (post-ovariectomy) and external (exposure to SMF) effects.

Our study had a major limitation. Although we observed an osteogenetic effect under the SMP exposure of the MSCs, the *in-vivo* study did not support this result. The same condition was also observed for the osteoclastogenetic effect. We postulated that the altered bone mineral density in the osteoporotic rat model was attributed to several factors. Therefore, only an SMF exposure was insufficient to change the bone mineral density of the osteoporotic rat model.

In conclusion, an *in-vitro* study showed that an SMF exposure of MSCs had a positive osteogenesis effect. However, there was no effect in terms of improvement of the bone mineral density when the SMF was applied on the osteoporotic rat model.

Acknowledgement

Disclosure

The author reports no conflicts of interest in this work.

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