Effects of a 4.7 T Static Magnetic Field on Fetal Development in ICR Mice

RYUJI OKAZAKI¹*, AKIRA OOTSUYAMA¹, SOSHI UCHIDA² and TOSHIYUKI NORIMURA¹

¹Department of Radiation Biology and Health, ²Department of Orthopaedic Surgery, School of Medicine, University of Occupational and Environmental Health, 1–1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807–8555, Japan

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In order to determine the effects of a 4.7 T static magnetic field (SMF) on fetal development in mice, we evaluated fetal teratogenesis and endochondral ossification following exposure in utero. Pregnant ICR mice were exposed to a 4.7 T SMF from day 7.5 to 9.5 of gestation in a whole-body dose, and sacrificed on day 18.5 of gestation. We examined the incidence of prenatal death, external malformations and fetal skeletal malformations. There were no significant differences observed in the incidence of prenatal death and/or malformations between SMF-exposed mice and control mice. Further, we evaluated the immunoreactivity for the vascular endothelial growth factor (VEGF), which is implicated in angiogenesis and osteogenesis, in the sternum of fetal mice following magnetic exposure. Our studies also indicated that on day 16.5 of gestation following SMF exposure, the immunoreactivity for VEGF was increased compared to unexposed controls. However, it was decreased in the exposed group compared to the control group on day 18.5 of gestation. DNA and proteoglycan (PG) synthesis were also measured in rabbit costal growth plate chondrocytes in vitro. No significant differences were observed in DNA synthesis between the SMF exposed chondrocytes and the control chondrocytes; however, PG synthesis in SMF exposed chondrocytes increased compared to the controls. Based on these results, we suggest that while SMF exposure promoted the endochondral ossification of chondrocytes, it did not induce any harmful effects on fetal development in ICR mice.

INTRODUCTION

Over the span of our lifetime, the chance of being exposed to magnetic fields has increased¹. While the effects of exposure are being debated, in epidemiological studies it has been reported that magnetic field exposure was related to cancer in childhood². On the other

*Corresponding author: Phone; +81–93–603–1611 (ext. 2332), Fax; +81–93–692–0559, E-mail; ryuji-o@med.uoeh-u.ac.jp
hand, Fulton et al\textsuperscript{3)} have reported that there is no relationship between magnetic fields and leukemia, and in recent scientific evaluations magnetic fields were determined to have no effect on DNA strand breaks\textsuperscript{4)}, cell proliferation or the migration of chick motoneurons\textsuperscript{5)}. Additionally, low static magnetic field (SMF) exposure has been determined to have no significant effects on amphibian development\textsuperscript{6)} or on the morphological changes that occur in guinea pig organs\textsuperscript{7)}. Intense SMF (1.5 T) also has no effect on the body temperature of normal human subjects\textsuperscript{8)}. To clarify the effects of high SMF exposure on mouse fetuses, we investigated whether teratogenesis and endochondral ossification resulted after exposure to a 4.7 T SMF \textit{in vivo}. Additionally, we evaluated the effects of the 4.7 T SMF on rabbit costal growth plate chondrocytes by measuring DNA and proteoglycan (PG) synthesis \textit{in vitro}.

The vascular endothelial growth factor (VEGF) is a 44-kDa protein well known as a potent angiogenic molecule owing to its mitogenic and permeability-causing properties\textsuperscript{9)}. Neo-vascularization in growth plates plays a fundamental role in the endochondral ossification process\textsuperscript{10)}. It has been reported that pulsing electromagnetic fields stimulate DNA and PG synthesis of rabbit costal growth plate chondrocytes \textit{in vitro}\textsuperscript{11)}. However, it has not been reported as to whether the magnetic field increases the expression of VEGF. Therefore, we investigated the immunoreactivity for VEGF in chondrocytes obtained from fetal ICR mice following a 4.7 T SMF exposure by immunocytochemistry.

**MATERIALS AND METHODS**

\textit{Exposure of pregnant ICR mice to a magnetic field and irradiation}

All experimental protocols were approved by the Ethics Committee of Animal Care and Experimentation of the University of Occupational and Environmental Health, Japan.

Twelve pregnant ICR mice received a whole-body exposure to a 4.7 T SMF using a SISCO/Varian MRI system (40 cm bore) (Spectroscopy Imaging Systems Corp., Palo Alto, CA, USA) at room temperature for gestational days 7.5 – 9.5 (organogenesis), and were subsequently sacrificed on day 16.5 or 18.5 of gestation by cervical dislocation (SMF group).

Four pregnant ICR mice received a total body irradiation of 2 Gy \textsuperscript{137}Cs $\gamma$-rays at a dose-rate of 1.04 Gy/min from a Gammaxcell 40 Exactor (Nordion Intl. Inc., Canada) on day 7.5 of gestation, and were then sacrificed on day 18.5 of gestation by cervical dislocation (I group).

\textit{Staining of skeletons}

Fetal skeletons were doubly stained for cartilage and mineralized bone by the modified method of Inouye\textsuperscript{12)} and Kimmel et al\textsuperscript{13)}. Briefly, after fetuses were sacrificed in a freezer, they were immersed in a 70°C water bath for approximately 30 sec to promote rapid skinning. The skin and internal organs were then removed, prior to fixation in 100% ethanol at room temperature for 3 days, followed by transfer to acetone for one day. The fetuses were then placed in a staining solution consisting of 1 vol of 0.1% alizarin red S (nakalai tesqu, Inc., Kyoto, Japan) in 95% ethanol, 1 vol of 0.3% alcian blue 8GX (Wako Pure Chemical Indus-
tries, Ltd., Osaka, Japan) in 70% ethanol, 1 vol of 100% acetic acid, and 17 vol of ethanol at room temperature for 3 days. Alizarin red S stains mineralized bone and alcian blue stains cartilage. After rinsing with water, specimens were kept in 1% KOH for approximately one day. When the skeletons became clearly visible, they were stored in 60% glycerol with a small quantity of thymol (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Evaluation of external and skeletal malformations and deaths**

In order to evaluate the incidence of prenatal death and external or skeletal malformations resulting from SMF exposure and irradiation of 2 Gy, fetuses were examined on day 18.5 day of gestation. The number of deaths following day 10 of gestation (late death), as well as live fetuses with or without morphological malformations were recorded. The stage of late death was estimated from the size of the dead fetuses and their placentas. Post-partum survival was determined by observing the fetal movement and breathing upon stimulation. The skeletal malformations were evaluated by Morita’s classification.

**Rabbit Costal Growth Plate Chondrocyte Culture**

For an in vitro study, samples of chondrocytes could not be obtained from fetal mice, since there was only a small amount of cartilage. Accordingly, chondrocytes were isolated from costal growth plates of male Japanese white rabbits, which were six months old and weighed approximately 3 kg, as described previously. Briefly, the cartilage tissue pieces were digested with ethylenediaminetetraacetic acid (EDTA), trypsin and collagenase. Cells were seeded at $1.0 \times 10^5$ cells in 9.6 cm$^2$ collagen-coated dishes (Corning, Iwaki Glass, Tokyo, Japan) in HAM F-12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$. The conditioned medium was changed after 3 days. Dishes were cultured for a total of 6 days before SMF exposure.

**Exposure of cultured rabbit chondrocytes to the SMF**

All dishes were sealed off from outer air using Parafilm M (American Can Company, Greenwich, CT, USA). For the SMF exposed cell culture, the warm water supplied from the thermocontroller flows over the top and bottom of dishes to maintain culture conditions at 37°C. Chondrocytes were incubated in the MRI system for 6 h. For the control group dishes were incubated at 37°C in a CO$_2$ incubator without a CO$_2$ supply for 6 h.

**DNA synthesis:**

To quantify DNA synthesis, $^3$H-thymidine (TdR) incorporation was measured, as described previously. Briefly, 37 kBq of $^3$H-TdR (Amersham Japan, Tokyo, Japan) was added to each dish. After incubation for 6 h at 37°C, each dish was washed with cold phosphate buffered saline (PBS) three times, 5% trichloroacetic acid (Wako, Osaka, Japan) once, and ethyl alcohol-diethyl ether (3:1, v/v) once. Cells were then harvested using a 0.3N sodium hydroxide solution. The thus-obtained cell suspension was filtrated through a cellulose acetate
filter (0.45 mm in diameter) (Sartorius, Göttingen, Germany). After neutralization, radioactivity bound to the filter was determined by a liquid-scintillation counter (LSC-3500E Liquid Scintillation System, ALOKA, Tokyo, Japan).

**PG Synthesis:**

For quantification of PG synthesis, 74 kBq of $^{35}$S-sulfate (NEN Life Science Products, Inc., Boston, MA, USA) was added per dish. After incubation at 37°C for 4 h, each sample was separated into a solution layer and a cellular layer. For the solution layer, CaCl$_2$·2H$_2$O with Tris-HCl, chondroitinsulfate, MgSO$_4$ and cetylpyridinium chloride were added to each sample. After nuclease-free protease was added and incubated at 37°C for 24 h, the cell layer was treated in the same way. Samples were then filtered through a cellulose acetate filter, and radioactivity bound to the filter was determined by a liquid-scintillation counter.

**Immunocytochemistry in chondrocytes obtained from fetal ICR mice**

For both the SMF group and the control group, fetal mice from two respective dams were sacrificed on day 16.5 and 18.5 of gestation. Fetuses were skinned and internal organs removed in the same way as described above. The front sections of the thoracic cage were cut out, and fixed in a solution of 4% paraformaldehyde in a 0.1M phosphate buffer. Specimens were dehydrated through a graded series of alcohol, and embedded in paraffin. Approximately 5 µm-thick sections of sternum were prepared on a microtome.

The deparaffinized and hydrated serial sections were incubated in methanol containing 0.3% H$_2$O$_2$ at room temperature for 20 min to remove any endogenous peroxidase activity. Nonspecific immunoglobulin binding was blocked by incubation with PBS containing 5% normal goat serum at room temperature for 15 min. Sections were then incubated with a rabbit anti-VEGF polyclonal antibody (Santa Cruz Biotechnology, CA, USA) at dilutions of 1:100 at 4°C for 18 h. After a brief rinse with PBS, the labeled streptavidin-biotin complex method (LSAB kit; Dako, Carpinteria, CA, USA) was used for subsequent immunostaining. The peroxidase complex was visualized by a treatment with a freshly prepared diaminobenzidine tetrahydrochloride (0.1 mg/ml) solution with 0.01% H$_2$O$_2$ for 5 min. The specificity of the above mentioned immunoreactivities was confirmed by replacing the primary antibodies with either normal rabbit sera or PBS. Finally, sections were counterstained by Mayer’s hematoxylin (Muto pure chemicals Ltd., Tokyo, Japan).

**Statistical analysis**

Binomial data were subjected to a chi-square analysis (Fig. 1a, 2, 3 and table 1). If significant F values were found, a Fisher’s test for multiple comparisons was carried out (Fig. 1b). The data in Fig. 4 were subjected to a one-way analysis in the two-sample t-test. Data were expressed as mean ± SD and evaluated by an analysis of the variance. A $p$ value of < 0.05 was considered to be significant.
RESULTS

External malformations

One hundred and ninety-three fetuses were born from 13 control dams. Of the 193, 186 (96.4%) were normal, six (3.1%) had died, and one (0.5%) had external malformations, as manifested by an absence of skull bone. One hundred and seven fetuses were obtained from eight pregnant mice previously exposed to a SMF. Of the 107, 105 (98.1%) were normal and two (1.9%) had died. Thus, there were no significant difference in the incidence of malformations or prenatal death between SMF-exposed mice and the control mice. Fifty-seven fetuses were born from four dams previously irradiated with 2 Gy. Of the 57, 34 (59.6%) were normal, three (5.3%) had died, and external malformations were observed in 20 (35.1%) fetuses. The malformations were mainly a shortened or curved tail (Fig. 1a).

The body weights of fetuses to 2 Gy irradiation (1.35 ± 0.13 g) were significantly (p < 0.05) decreased compared to a control (1.56 ± 0.10 g); however, there were no differences between the control and the SMF group (1.58 ± 0.11 g) (Fig. 1b).

Skeletal malformations

Detailed skeletal malformations are shown in Table 1. The data show the percentage of each malformation per examined fetuses. These were observed in the vertebral arches, ribs
Table 1. Incidences of skeletal malformations

<table>
<thead>
<tr>
<th>Fetuses examined</th>
<th>Absence of skull</th>
<th>Splitting of cervical vertebral arches</th>
<th>Cleft of cervical ribs</th>
<th>Wavy cervical ribs</th>
<th>Cleft of lumbar ribs</th>
<th>Cartilage in rib bone</th>
<th>Cleft of sternebrae</th>
<th>Accessory of sternebrae</th>
<th>Asymmetry of sternebrae</th>
<th>Splitting of sternebrae</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>N 193</td>
<td>1</td>
<td>22</td>
<td>103</td>
<td>1</td>
<td>4</td>
<td>18</td>
<td>0</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>% *</td>
<td>0.5</td>
<td>13.5</td>
<td>53.4</td>
<td>0.5</td>
<td>2.1</td>
<td>9.3</td>
<td>43.5</td>
<td>0.3</td>
<td>3.1</td>
</tr>
<tr>
<td>4.7T</td>
<td>N 107</td>
<td>0</td>
<td>18</td>
<td>28</td>
<td>2</td>
<td>2</td>
<td>9</td>
<td>25</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>% *</td>
<td>0</td>
<td>16.8</td>
<td>26.2**</td>
<td>1.9</td>
<td>1.9</td>
<td>8.4</td>
<td>23.4**</td>
<td>0.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*: Incidence of skeletal malformations in examined fetuses.
**: p < 0.5 vs. control (chi-square analysis)

and sternum. In the white columns in Table 1, skeletal malformations are classified into four types: i.e. lacking bone, having excessive bone, being separated in two, and being abnormally shaped. These are relatively severe. No significant difference was observed in each malformation between the control group and the SMF group.

In the gray columns in Table 1, each bone included the cartilage, which was stained by alcian blue and would develop mineralized bone. Cleft of cervical vertebral arches, cartilage in the rib bones and splitting of sternebrae were observed in the control group statistically more than the SMF group: those incidences in the control group were 2.0, 1.9 and 3.0-times greater than the SMF group, respectively.

Forty-eight fetuses (24.9%) showed skeletal malformations in the control group. Thirty-one (29.0%) fetuses were observed to have skeletal malformations in the SMF group (Fig. 2). The data show the percentage of fetuses with skeletal malformations in the examined fetuses. There was no significant difference between the control group and the SMF group. Alterna-

![Fig. 2. Effects of SMF exposure and irradiation on skeletal malformations in ICR mice. Data show the percentage of fetuses with skeletal malformations in the examined fetuses. *: p < 0.05 vs. control (chi-square analysis).](image-url)
Effect of SMF on ossification

Fig. 3 shows the incidence of ossification. Completely ossified bone, which was not observed clef of cervical vertebral arches, cartilage in the rib bones and splitting of sternebrae, were only stained by alizarin red S. The control group (51.3%, n = 99) was significantly (p < 0.005) less than in the SMF group (72.9%, n = 78). These data show the percentage of the fetuses with completely ossified bone in the examined fetuses.

![Graph showing incidence of ossification](image_url)

Fig. 3. Effects of SMF on ossification in rib, cervical vertebral arch and sternum in ICR mice. Data show the percentage of fetuses with completely ossified bone in examined fetuses. *: p < 0.005 vs. control (chi-square analysis).

**Fig. 4.**

a: Effects of SMF exposure on \textsuperscript{3}H-TdR incorporation into DNA of cultured rabbit costal growth plate chondrocytes. b: Effects of SMF exposure on \textsuperscript{35}S-sulfate incorporation in PG of rabbit costal chondrocytes. cpm = count per minute: means and 1SD shown. *: p < 0.05 vs. control (t-test).
The incorporations of $^3$H-TdR and $^{35}$S-sulfate in cultured chondrocytes obtained from rabbit costal growth plates

There were no differences in the incorporations of $^3$H-TdR between the SMF exposed rabbit chondrocytes and unexposed control cells. The data (count per minute; cpm) in the control and in the SMF group was 300.0 ± 42.4 and 267.1 ± 51.3, respectively (Fig. 4a). However, the incorporation of $^{35}$S-sulfate was significantly increased by SMF exposure much more than the control (Fig. 4b). The data (cpm) in the SMF group was 1.2-times greater than those in the control.

Immunoreactivity for VEGF in the sternum of fetal ICR mice

On day 16.5 of gestation, the immunoreactivity for VEGF in sternum chondrocytes from fetal ICR mice was evaluated. The results showed that the immunoreactivity for VEGF increased in the SMF group compared to the control group (Fig. 5a, b). In contrast, on day 18.5 of gestation, immunoreactivity for VEGF was detected on chondrocytes in both the control and the SMF groups. It appeared that at this stage of gestation, the number of cells expressing VEGF decreased following SMF exposure. Sections c and d were not counter stained by hematoxylin, since we could clearly observe the differences of immunoreactivity for VEGF between the control group and the SMF group (Fig. 5c, d).

Fig. 5. Effects of SMF exposure on immunoreactivity for VEGF in sternum from ICR mice. a: Controls were sacrificed on day 16.5 of gestation. b: SMF exposed animals were sacrificed on day 16.5 of gestation. e: Control sacrificed on day 18.5 of gestation. d: SMF animals sacrificed on day 18.5 of gestation. Scale bar = 50 µm. Sections a and b were counter stained by hematoxylin.
DISCUSSION

In this study, we demonstrated that following SMF exposure fetal ICR mice did not develop any significant external or skeletal malformations compared to controls. However, SMF did stimulate fetal endochondral ossification in vivo. Additionally, PG synthesis was observed to increase in cultured chondrocytes in rabbit costal growth plate in vitro. Furthermore, at day 16.5, immunoreactivity for VEGF in fetal sternum was increased by SMF exposure.

A significantly high incidence of congenital malformations was observed following γ-irradiation with 2 Gy as compared to the control or the SMF group. Furthermore, the body weight of the fetuses decreased. It has been reported that γ-irradiation exposure (at the same dose and dose-rate) has a mortality rate of 60%, and external malformation incidence of 20% for p53 (+/+) 129/SvJ mice. In this study, 35.1% of the fetuses displayed external malformations and 5.3% of the fetuses died. These discrepancies in results may relate to mouse strain specificity. However, 100% of the fetuses developed skeletal malformations in this study. We hypothesize that fetal chondrocytes may be highly radiosensitive, and suggest that observations of skeletal malformations are an important factor in teratology.

Our results showed that a 4.7 T SMF did not have an effect upon the external or skeletal malformations in fetal mice during the period corresponding to major organogenesis. Previously, it had been reported that neither high (6.3 T) nor low (30 mT) SMFs had any effect on fetal development in mice and rats. However, Ramirez et al. reported that SMF exposure decreased oviposition and adult viability in drosophila. We support the hypothesis that SMF exposure does not exert any harmful effects during organogenesis in mice and rats.

We also observed the cleft of cervical vertebral arches, cartilage in rib bone and splitting of sternebrae in fetal skeletons, which we hypothesize to be due to the delay of ossification. It is well known that pulsing electromagnetic fields are used clinically for congenital pseudoarthrosis or ununited fractures. Recently, it has been reported that SMF exposure also stimulates bone formation. Furthermore, Mevissen et al. reported that forelimb endochondral ossification in rat fetuses was accelerated by 30 mT SMF exposure. While we could not detect endochondral ossification in forelimbs, our results, as shown in the ribs, vertebral arches and sternebrae, support the hypothesis that SMF exposure does influence endochondral ossification.

In this study, SMF increased the incorporation of 35S-sulfate in cultured chondrocyte in rabbit costal growth plates; however, it did not increase the incorporation of 3H-TdR into DNA in vitro. In addition, SMF exposure has been reported not to increase DNA synthesis in Chinese hamster V79 cells, or affect cell proliferation of human fetal lung fibroblasts. One possibility is that SMF exposure could potentially influence the processes of cell differentiation in the cerebellum during development. Together, these data suggest that a SMF can have a stimulant effect on cell differentiation and/or PG synthesis in endochondral ossification, but not at the level of DNA synthesis. Generally speaking, endochondral ossification was observed in fetal cartilage development and growth plate chondrocytes. Though our
results were detected by using rabbit costal growth plate chondrocytes in vitro, these data might reflect the events of endochondral ossification in fetal mice in vivo.

Lastly, we observed that immunoreactivity for VEGF in sternebrae was found earlier in gestation following SMF exposure. VEGF is expressed in human fetal growth cartilage\(^{29}\), and induces neovascularization and chondrocyte differentiation during endochondral bone formation\(^{30}\). Angiogenesis is essential for replacing cartilage by bone during endochondral ossification. We suggest that SMF may induce the expression of VEGF, which in turn promotes endochondral ossification.

In summary, these results suggest that exposure to a 4.7 T SMF does not exert any harmful effects on the fetal development of ICR mice. We further hypothesize that SMF exposure may promote endochondral ossification.

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