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Subchronic Effects on Leukocyte-endothelial Interactions in Mice by Whole Body Exposure to Extremely Low Frequency Electromagnetic Fields

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Abstract. *In the present study, we measured and quantified changes in leukocyte behavior in the subcutaneous microcirculation in vivo and examined serum cytokine levels in vitro while mice were exposed to continuous 50 Hz electromagnetic fields for 17 days. Mice were exposed to 50 Hz sinusoidal electromagnetic fields at magnetic densities of 0.3, 1.0 and 3.0 mT. The intramicrovascular behavior of leukocytes was evaluated by fluorescence microscopy using a dorsal skinfold chamber technique. Significantly increased endothelial-adhering leukocytes were observed only in the 3.0 mT exposure group, but no changes were detected in serum TNF-α and IL-1β levels. Subsequently, long-term continuous whole body exposure to 50 Hz electromagnetic fields elicited leukocyte-endothelium interactions, however, this effect does not appear to be controlled by cytokine levels.*

Extremely low frequency electromagnetic fields (ELF-EMF) are generated by all electrical facilities including electrical transmission lines, distribution lines and electrical appliances. There is concern as to whether ELF-EMF exposure affects public health (1). Over the past few decades, a considerable number of studies have been conducted on this subject, but the health-related effects of ELF-EMF still remain ambiguous. Results of pooled analysis of epidemiological reports have suggested that exposure to residential power frequency EMF is associated with increased health risks, particularly childhood leukemia (2, 3). However, the potential adverse effects of EMF are controversial because such epidemiological results have been largely inconsistent with *in vivo* and *in vitro* findings.

Numerous *in vitro* and *in vivo* experimental studies elucidating the biological effects induced by ELF-EMF have also been reported and it is known that *in vitro* exposure to ELF-EMF influences some biological processes: the enzymatic activity of ornithine decarboxylase (4), the expression of several genes for heat shock proteins (5, 6), micronuclei formation and sister chromatid exchange (7) and protein phosphorylation (8).

ELF-EMF are believed to affect biological immune systems (7, 9-11), although experimental models and conditions have differed greatly and direct evidence supporting this hypothesis is inconclusive. Since the immune system plays an essential role in the pathophysiology and development of disease, it is important to clearly determine how immunological functions can be influenced by ELF-EMF exposure. Jonai *et al.* (12) evaluated immune response to 50 Hz ELF-EMF exposure *in vitro* by assaying cytokines produced by human peripheral blood mononuclear cells (PBMCs) and found that some cytokine levels changed in response to exposure. Specifically, they observed a significant decrease in tumor necrosis factor α (TNF-α) and a significant increase in interleukin 1β (IL-1β) levels. The reduction of IL-1 activity by ELF-EMF was also observed in sheep (13). Generally, cytokines like TNF-α and IL-1β control specific cellular and molecular responses in the target cells. In the case of leukocytes, cytokines stimulate the up-regulation of E-selectin and L-selectin ligands, which mediate subsequent leukocyte rolling (14). To confirm these phenomena *in vivo*, we used a dorsal skinfold chamber (DSC), which enabled us to visualize the vascular system and blood circulation of mice (15, 16). Combining *in vivo* fluorescence microscopy and *in vitro* ELISA, we examined the immunological effects of long-term ELF-EMF exposure on the mouse microcirculatory system.

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Key Words: Microcirculation, low-frequency electromagnetic fields, leukocyte-endothelium interactions, subchronic effect, immunological response, mouse.

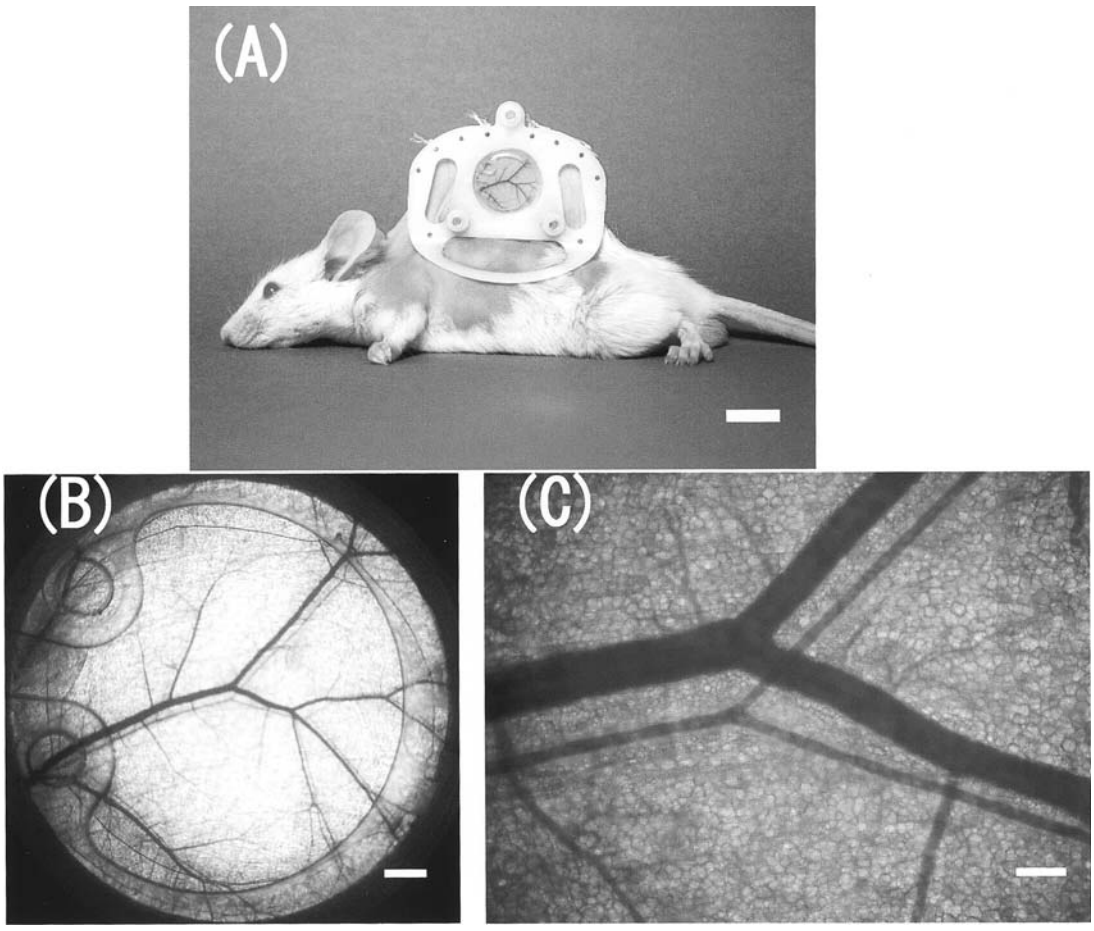

Figure 1. *Overview of mouse dorsal skinfold chamber. (A) Whole body image of a DSC implanted mouse. (Bar indicates 1 cm) (B) Intravital microscopic image of DSC. (Bar indicates 1 mm) (C) Magnified image of subcutaneous vessels in DSC. (Bar indicates 100 μm) Images of (B) and (C) were obtained by using a transparent light source.*

Materials and Methods

Animal models. The mouse dorsal skinfold chamber (DSC) model was originally designed by Algire and Legallais (17) and modified chambers have since been applied to various experimental animal models (16). The most commonly used chamber frame is made of titanium. However, in the present study, we used a non-metal chamber which we have recently developed. The chamber frame was made of Duracon™ resin, a non-metallic material specifically utilized for this purpose. This chamber prevents heat production induced by electrical current. DSCs were implanted in 8-week-old male BALB/c mice (Tokyo Zikken Doubutsu Co., Tokyo, Japan). The implantation procedure was essentially identical to that previously described (18). Briefly, mice were anesthetized by *i.m.* injection of a cocktail of ketamine hydrochloride (90 mg/kg body weight, Sigma Chemical Co., St. Louis, MO, USA) and xylazine hydrochloride (10 mg/ kg body weight, Sigma). Then the chamber frames were implanted so as to sandwich the extended double layer of dorsal skin. Using an operating microscope and microsurgical instruments, one layer of skin was removed in a circular area of approximately 15 mm in diameter and the remaining layer was covered with a coverslip incorporated into one of the chamber frames. The procedure was performed 3 days prior to EMF exposure. An overview and magnified images of the vessels in DSC are shown in Figure 1.

All animals were fed a standard pellet diet and given water *ad libitum.* The mice were maintained with a 12-h light/dark cycle and at a temperature of 23.0±1.0˚C. Animal cages, lids and water bottles did not contain any metal that might interfere with electromagnetic fields. All procedures were conducted in accordance with the ethical guidelines for animal experiments at the National Institute of Public Health, Japan.

Intravital fluorescence microscopy. Real-time confocal laser scanning microscopy (CLSM) was used to evaluate leukocyte-endothelium interactions with the mouse DSC model. This CLSM observation system is schematized in Figure 2 and was mainly composed of a Confocal Scanning Unit (CSU 10, Yokogawa Electric Co., Tokyo, Japan), a microscope (BX-51WI, Olympus Co., Tokyo, Japan) and an Electron Bombardment CCD camera (C7190-21, Hamamatsu

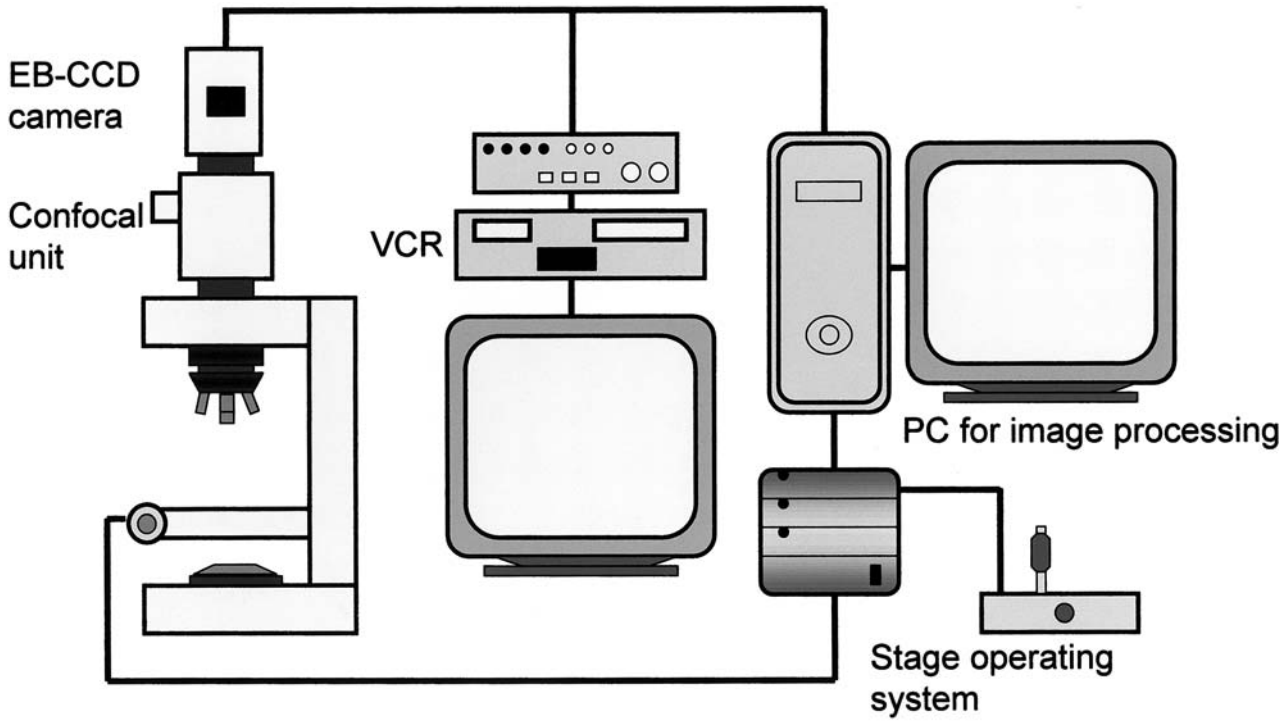

Figure 2. *Schematic diagram of the microscope and image processing system. A microscope was equipped with an excitation laser and a real-time confocal laser scanning unit. To record images, the EB-CCD camera was mounted on the microscope and images were recorded on digital video tape. The microscope stage was operated by an automatic stage controller which was connected to a computer.*

Photonics K.K., Hamamatsu, Japan). A 532 nm laser beam generated by a Diode Pumped Green CrystaLaser (model GCL-050-L, CrystaLaser Inc., Reno, NV, USA) was used as the excitation light source for CLSM. A BioPrecision stage (Ludl Electronic Products Ltd., Hawthorne, NY, USA) was set on the microscope and controlled by using IPLab/Mac software (Scanalytics, Inc., Fairfax, VA, USA) for automatic navigation in searching regions of interest. The visualized images were recorded on digital video tape using a digital video recorder (WV-DR7, Sony, Tokyo, Japan) for subsequent off-line analysis. To evaluate dynamic video images, the time code generator signal (VTG33: For.A Co., Tokyo, Japan) was superimposed on a video screen.

To visualize leukocytes *in vivo* the fluorescent marker rhodamine 6G (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) was used (19). Rhodamine 6G was dissolved in physiological saline. Each solution was freshly diluted with saline until a final concentration of 0.02 mg/ml on the day of experiment. To remove undissolved substances, the solution was filtered through a 0.22 μm membrane filter (Syringe-driven filter unit Millex-GN, Millipore Co., Billerica, MA, USA) before each experiment. The prepared solution (100 μl) was injected into the caudal vein 5 min before image recording. During the data recording under CLSM, animals were anesthetized with the same dosage of ketamine and xylazine cocktail used in the DSC procedure and then placed in an acrylic resin tube of 26 mm inner diameter. The tube was fixed to an acrylic resin plate that was then set on the microscope. For repeated observations of the same vascular bed at different time points, we programmed a script, which works on IPLab/Mac Software and makes it possible to find the regions of interest. Venules with diameters between 20 μm and 40 μm were chosen for the observation of leukocyte-endothelium interactions. Seven to 14 animals were prepared for each group and three venules per animal were observed on the first day of exposure (Day 1), 8th day and the 15th day of exposure.

ELF-EMF exposure. The exposure apparatus used throughout these experiments was comprised of the exposure system setup shown in Figure 3. The power supply unit and coils were obtained from Takano Giken, Inc. (Isegahara City, Japan). Typically, experimental magnetic fields are generated by circular Helmholtz coil, however, this exposure system consisted of four spaced square coils due to difficulties in establishing a uniform magnetic field with as large a volume as possible within limited space. Three axis coils were crossed at right angles to each other, independently regulating the input current. Spatio-temporally, the magnetic fields were approximately constant at the center of the incubator space. At the centers of all 18 animal cages, fluctuation was within ±5 percent when the output intensity was set to 3.0 milliTesla (mT). The intensity of the magnetic field was measured using an EM field analyzer EFA-2 (Wandel and Goltermann Co., Germany).

The animal rack surrounded by the coils was made of Bakelite. The temperature inside the coils was stable (23.0±1.0˚C) and no vibrations from the coils were detected in the animal rack during the exposure period.

Magnetic field exposure was produced at a frequency of 50 Hz. Magnetic flux densities applied to this experiment were set to 0.3,

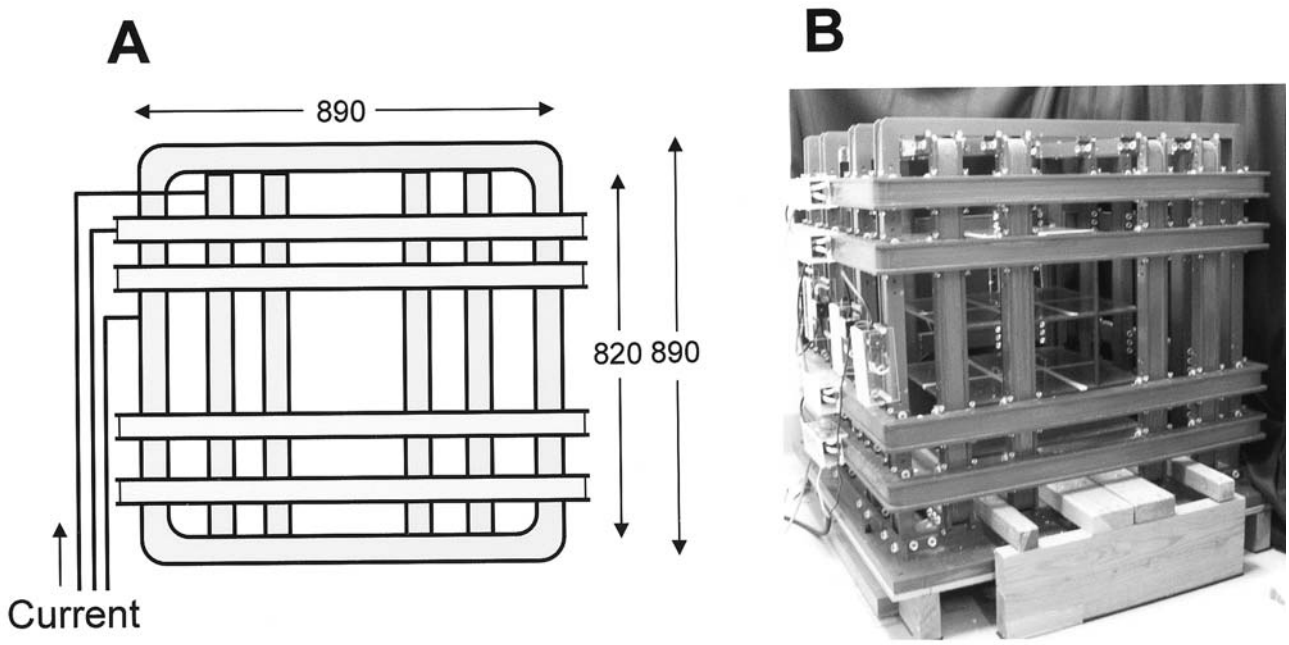

Figure 3. *Design of ELF-EMF generating coils. (A) Front view of Helmholtz coil. Coils were connected to the power supply and the current in each coil was regulated independently. Unit of numeral in figure is mm. (B) Photograph of the exposure set-up. The animal rack is located at the center of coils, and is separated from the coils to prevent vibration.*

1.0 and 3.0 mT (rms) (linear field Bx=By=Bz). The animals were continuously exposed to EMF for 20 hours/day (2 p.m. to 10 a.m.) for a total of 17 days.

ELISA detection of IL-1β and TNF-α serum levels. Blood specimens were collected immediately after the 17th day of exposure and serum was isolated by centrifugation. Immunoreactive levels of IL-1β and TNF-α in serum were quantified using sandwich ELISA (Biotrak™ ELISA system; Amersham Biosciences Corp., Piscataway, NJ, USA). Experimental procedures were conducted according to the manufacturer's instructions.

Statistical analysis. Results were presented as mean values ± SEM. Values for each group were compared using the Student's *t*-test. Significance was assumed at $p<0.05$.

Results

Animal model and intravital fluorescence microscopy. In this study we used a mouse DSC made of Duracon™ resin, which is not affected by electromagnetic fields (*e.g.*, EMF can produce thermal effects on commonly used metal frame chambers). An earlier study we recently conducted showed that mice tolerate this dorsal skinfold chamber well and exhibit no adverse physiological effects (unpublished data). To obtain clear images of leukocytes *in vivo,* CLSM was used. Figure 4 illustrates a series of fluorescent images of rhodamine 6G-labeled leukocytes rolling or adhering to the endothelium of subcutaneous venules. This labeling technique with rhodamine 6G enables the quantitative evaluation of dynamic leukocyte behavior.

Leukocyte endothelium interaction. The numbers of rolling and adherent leukocytes were determined by off-line image analysis. The numbers of flowing leukocytes (NF) and rolling or adhering leukocytes (NR) were counted independently and rolling counts indicating the ratio of rolling cells to total leukocyte fluctuation were calculated as: rolling counts (%) = NR / (NF+NR) x 100. The data presented in Figure 5 show that leukocyte-endothelial interactions could be seen even under conditions without any EMF exposure. However, slightly higher numbers of adherent leukocytes were found in the 3.0 mT group on Day 8 and Day 15 ($p<0.05$) compared with the first day of exposure (Day 1). The rolling counts in the sham exposure group and the 0.3 mT and 1.0 mT exposure groups were not affected by the exposure conditions.

Serum levels of IL-1β and TNF-α. After evaluating leukocyte endothelium interactions, exposure to mice was continued for a total of 17 days. On the 17th day, blood was collected and serum levels of IL-1β and TNF-α were determined using an ELISA assay. As shown in Figure 6, no statistical difference in concentration was observed between any of the

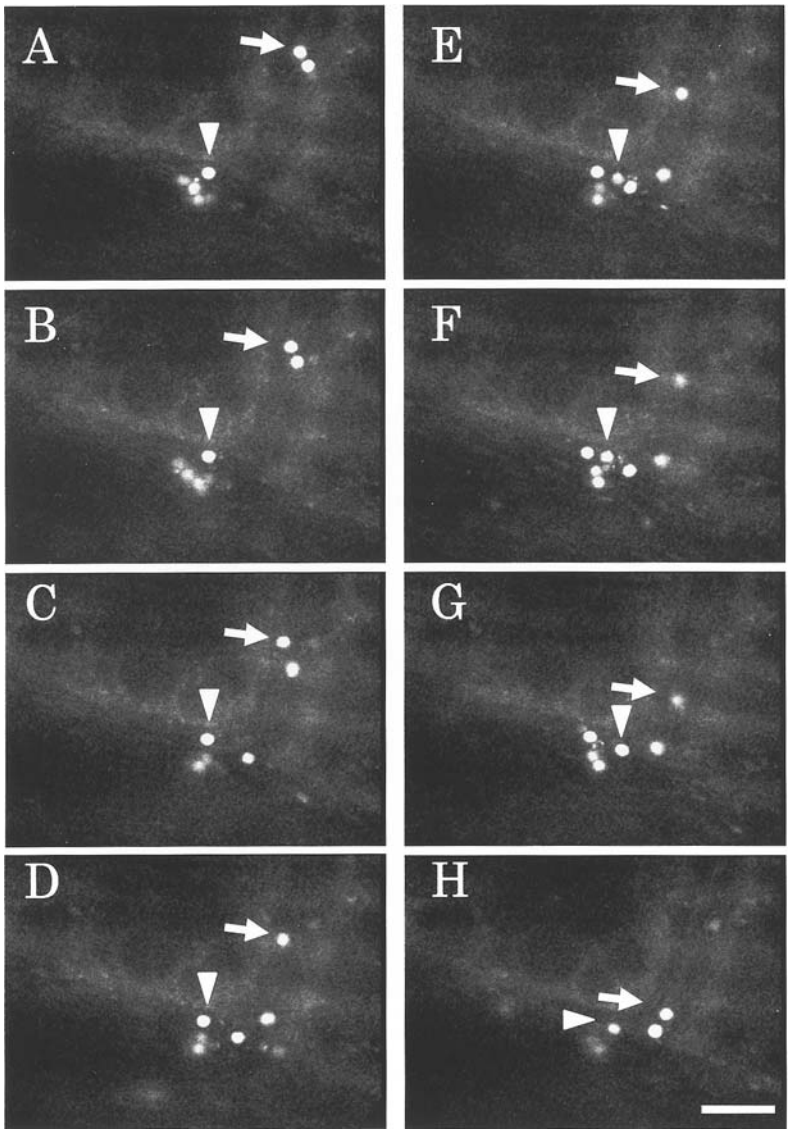

Figure 4. *Video images of intramicrovascular leukocytes in vivo. Leukocytes were stained with rhodamine 6G and observed by real-time CLSM. Figures (A-H) illustrate the time-sequence. Some cells showed slow flow compared to blood flow resulting from weak endothelial interactions with (e.g. arrow) or adhesion to (e.g. arrowhead) vessel walls due to robust interaction. Scale bar indicates 100 μm.*

groups. Therefore, whole body exposure to 50 Hz ELF-EMF for 17 days did not affect the serum levels of IL-1β and TNF-α.

Discussion

There is increasing interest in the biological effects associated with exposure to electromagnetic fields. To identify the effects, various *in vitro* studies have been examined, however little information is available from *in vivo* experiments. In the present study, to identify the *in vivo* effects from ELF-EMF exposure, we used a dorsal skinfold chamber (DSC), which enabled us to clearly visualize the subcutaneous microcirculation in mice by intravital microscopy. The DSC technique is widely applied to experiments with mice, rats and hamsters (15). Also, methodologies in fluorescence microscopy are well established for observing several physiological parameters (20). Our previous experimental data showed that short-term exposures to static magnetic fields (SMF) and ELF-EMF affect some circulatory parameters. One of our recent studies using the DSC has shown that the continuous and

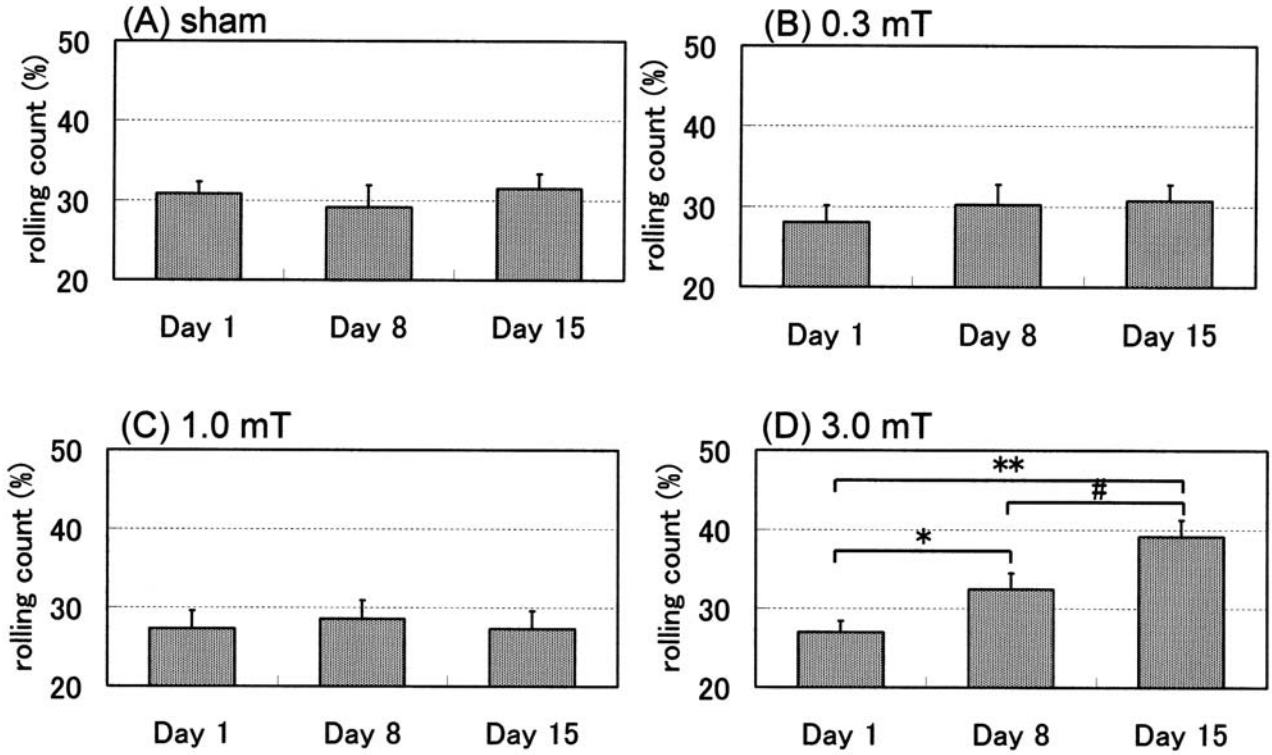

Figure 5. *Leukocyte-endothelial interactions with the dorsal skinfold chamber: effects of long-term exposure to 50 Hz electromagnetic fields. (A) Leukocyte rolling counts at each time point as without exposure (Sham). (B-D) Leukocyte rolling counts at each time point with continuous electromagnetic field exposure at magnet flux densities of 0.3mT (B), 1.0 mT (C) and 3.0mT (D). Values are expressed as means ± SEM. *p<0.05 versus Day 1; **p<0.01 versus Day 1; #p<0.05 versus Day 8.*

whole body exposure of 50 Hz EMF (30 mT) for 30 minutes significantly influenced cell to cell interaction between venular endothelial cells and leukocytes in the mouse subcutaneous microvasculature, although 3 mT or 10 mT exposure did not affect leukocyte behavior. Although microcirculatory vessels in DSC can be observed for up to 4 weeks under optimal conditions, the effects of long-term EMF exposure have not been examined thoroughly. Therefore, in this study we investigated the effects of several weeks of continuous ELF-EMF exposure by focusing on free flowing leukocytes in venules to elucidate the interactions between leukocytes and endothelium. Cell counts of adherent leukocytes to the endothelium are one good indicator for estimating pathophysiological conditions, particularly increased rolling leukocyte counts are indicated when the immune system is activated. To visualize this phenomenon by intravital microscopy, we used real-time confocal laser-scanning microscopy (CLSM) which permits real-time observation of fast movement with improved spatial resolution and depth discrimination (20, 21).

Leukocyte dynamic processes of leukocyte rolling and adhesion on the venular endothelium are also considered to be affected by the microenvironment between leukocytes and the endothelium. The leukocyte-endothelium interaction has recently been shown to be mediated by a number of adhesion molecules as well, such as selectins and integrins. However, most of these recent findings on the action of adhesion molecules have been obtained by *in vitro* experiments. Leukocyte-endothelium interactions mainly occur under conditions of inflammation, in regions where leukocytes secrete the cytokines IL-1 and TNF, which induce expression of the cell adhesion molecules ICAM-1 and VCAM-1 on endothelial cell surfaces (22-24). Under inflammatory conditions this process permits leukocytes to adhere to tissues.

Several *in vivo* investigations into the effects of ELF-EMF exposure on cytokine production have been published. Cossarizza *et al.* (25) demonstrated that the exposure of human peripheral blood mononuclear cells (PBMCs) to extremely low frequency pulsed electromagnetic fields increased both spontaneous and phytohemaggutinin (PHA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced production of IL-1β and IL-6. Jonai *et al.* (12) reported decreases in the spontaneous production of TNF-α in the intensity range of 1 mT to 30 mT and in that of interferon γ

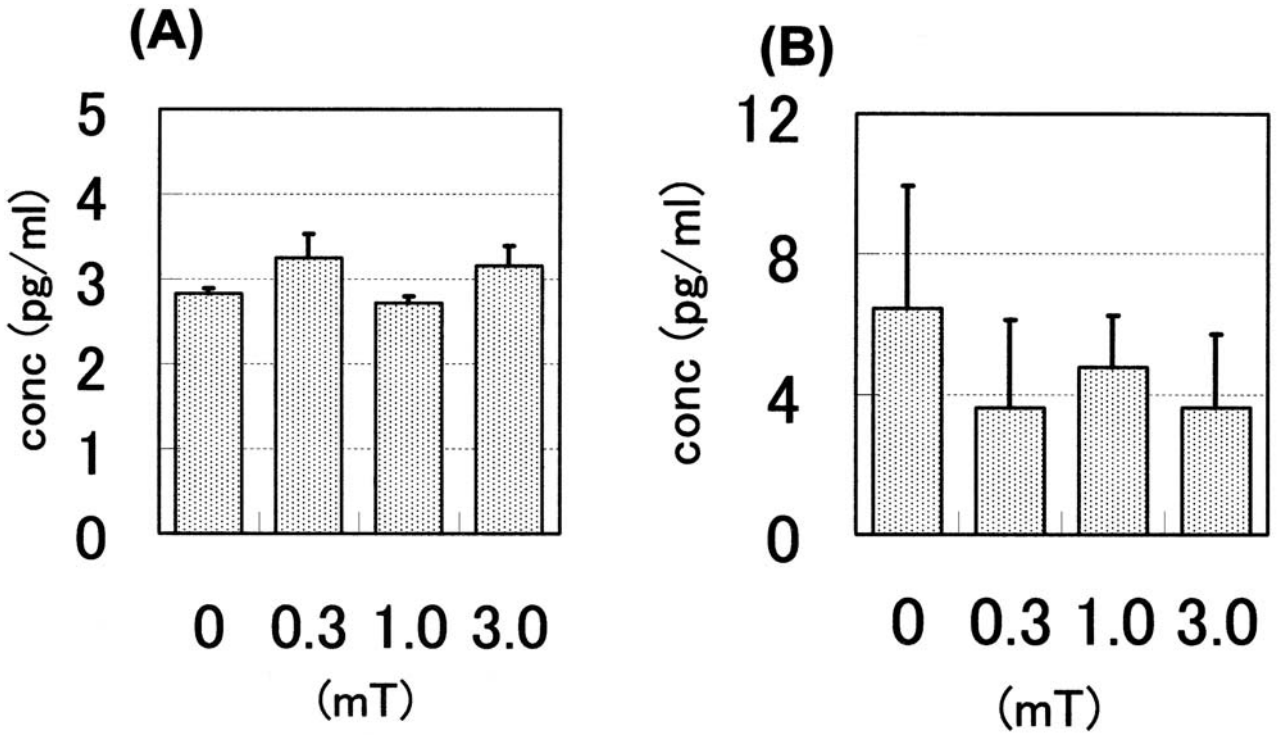

Figure 6. *Serum levels of cytokines after long-term exposure to 50 Hz electromagnetic fields. IL-1β (A) and TNF-α (B) levels in serum collected after 17 days of exposure. Values are expressed as means ± SEM.*

(IFN-γ) at 10 mT in human PBMCs. Perini *et al.* (26) reported that sinusoidal 50 Hz EMF at 1 mT suppresses TNF-α production in human PBMCs. In contrast, Ikeda *et al.* (27) reported no effects from 50 Hz or 60 Hz EMF exposure either in cytotoxic activity or cytokine production in human PBMCs.

Therefore, we examined serum cytokine levels to confirm whether ELF-EMF exposure influences the secretion of cytokines *in vivo*, in particular focusing on IL-1β and TNF-α, which have been shown to be significantly affected *in vitro* as previously mentioned. As shown in Figure 6, our results indicate that there were no differences in the levels of these cytokines under the conditions examined. Thus, we can conclude that increased numbers of rolling leukocytes are independent of regulation by these cytokines. In a separate experiment, our data indicated that the blood plasma concentration of carboxypeptidase R (CPR) precursor was decreased by long-term ELF-EMF exposure suggesting active CPR production (unpublished data), whereas it is reported that CPR activity in plasma increases during the inflammatory process (28). In this study we confirmed that inflammatory response is induced by ELF-EMF exposure by a possible pathway, but that cytokines IL-1β and TNF-α do not contribute to this effect. Nonetheless, it is still difficult to conclude that ELF-EMF are hazardous for health, since significant effects have only been observed in our studies with 3.0 mT exposure, which is several ten thousand times higher than the average environmental exposure level.

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