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Effects of Subchronic Exposure to a 1439 MHz Electromagnetic Field on the Microcirculatory Parameters in Rat Brain

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Abstract. *The aim of this study was to investigate whether repeated exposure to radio frequency electromagnetic field (RF-EMF) of 1439 MHz affects the cerebral microcirculation, including blood-brain barrier function, in a rat brain. Materials and Methods: The head of the rat was exposed for four weeks (60 min/day, 5 days/week) to RF-EMF at 2.4 W/kg of brain averaged specific absorption rate (BASAR). Three microcirculatory parameters: blood-brain barrier permeability, leukocyte behavior and plasma velocity were measured before and after RF-EMF exposure using a closed cranial window method. Results: No extravasation of intravenously injected dyes from pial venules was found at any BASAR level. No significant changes in the number of endothelial-adhering leukocytes after exposure were found. The plasma velocity remained constant within the physiological range through each exposure. Conclusion: These findings suggest that there were no effects on the cerebral microcirculation under the given RF-EMF exposure conditions.*

The possibility of radio-frequency electromagnetic fields (RF-EMF) of mobile phones causing possible adverse health effects is a subject of numerous studies and publications. One of the most remarkable findings in recent years was the permeability change in the blood-brain barrier (BBB) due to RF-EMF exposure. The early studies reported that RF-EMF exposure, which was high enough to cause a temperature increase, could change BBB permeability (1-3). In contrast to these observations, Salford *et al.* (4, 5) reported that albumin leakage sites were found in the rat brain after 915 MHz-EMF exposure even under non thermal intensity levels. However, several other studies failed to replicate their findings (6-9).

The BBB function is not the only important parameter of the cerebral microcirculation. Leukocyte behavior and blood flow velocity are also valuable parameters in the evaluation of the cerebral microcirculation (10, 11). For example, an increase in leukocyte adhesion to the endothelium in pial venules reflects inflammatory responses in the brain (12). In many cases that involve inflammation changes in BBB permeability have been reported (13). These phenomena suggest a strong relationship between BBB permeability and other parameters. Therefore, the simultaneous examination of several parameters is important in analyzing this very complex behavior.

An experiment of repeated exposure to physical or chemical factors, as well as acute exposure, is generally required to evaluate such a factor's toxicity. Our companion study found that the acute exposure (10 min) of rat brain to 1439 MHz RF-EMF did not affect BBB permeability, leukocyte behavior, or plasma velocity in the pial microcirculation. However, it is reported that repeated exposure to a low dose agent, which cannot induce acute effects, can elicit some physiologically significant effects (14). This phenomenon suggests that repeated exposure to RF-EMF may also have the potential to cause biological effects. Tsurita *et al.* (6) reported that no albumin leakages were found in the rat brain after repeated exposure for four weeks to 1439 MHz RF-EMF. However, the effects on other microcirculatory parameters remain unclear.

In the present study, we focused on the changes in three cerebral microcirculatory parameters, BBB permeability, leukocyte behavior and plasma velocity, to demonstrate whether a 4-week repeated (subchronic) exposure to 1439 MHz RF-EMF could induce any effects on the rat brain. The

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closed cranial window method modified by us was applied to evaluate the parameter changes directly *in vivo*. The three parameters were measured before and after the exposure of the rat brain to 1439 MHz RF-EMF at 2.4 W/kg of brain averaged specific absorption rate (BASAR), which is a level 1.2-fold higher than the current permissible exposure guideline value (2 W/kg) for localized RF-EMF (15).

Materials and Methods

Animals. Male Sprague-Dawley rats (10-11 weeks old, 404±36 g, Tokyo Laboratory Animals Science Co. Ltd, Japan) were used in this experiment. They were fed a standard pellet diet and water *ad libitum*, and were maintained with a 12-h light/dark cycle, at a temperature of 23.0˚C±1˚C and a relative humidity of 50%±10%. All experimental procedures were conducted in accordance with the ethical guidelines for animal experiments at the National Institute of Public Health, Japan.

Preparation of closed cranial window. The closed cranial window (CCW) setup used in the present study was developed with acryl, plastic and glass, but no metal materials, because of the RF-EMF exposure. The CCW had a convex shape which allows it to be inserted into the skull hole. The bottom of the window had a circular cover-glass of 8.0 mm diameter. The CCW was implanted into the parietal region of the rats. The animals were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The head of each rat was fixed in a stereotaxic apparatus. After removal of hair, skin and connective tissue from the parietal region, a 10 mm circular skull hole was made using a dental drill with cold saline drip to prevent heat generation during the drilling. Subsequently, the dura mater and arachnoid were carefully removed from the cerebral surface to expose the pia mater. The window was inserted into the hole of the skull and fixed with cyanoacrylate glue. All animals were used for the experiment at least one week after the window implantation to allow for recovery (16).

RF-EMF exposure. The exposure system consisted of a small anechoic chamber, with a carousel type rat holder, a monopole antenna and a fan for air supply (Figure 1a) (17). The non-anesthetized rat was held in a custom made acrylic holder (Figure 1b) with its head positioned toward the antenna placed at the center of the chamber. The animals were exposed for four weeks (60 min/day, 5 consecutive days/week) to an RF-EMF of 1439 MHz near-field TDMA (time division multiple access) signal for PDC (Personal Digital Cellular, Japanese cellular telephone standard) system to simulate the exposure from mobile phones. The PDC signal has pulsed 6.67 ms waveforms at repetition intervals of 20 ms, *i.e.* the peak power is three-fold higher than the temporal average. The field intensity was adjusted to provide 2.4 W/kg in BASAR. Whole-body exposure occurs concomitantly, with a whole-body averaged SAR of 0.64 W/kg. Throughout the 60 min exposure, fresh air was circulated in the chamber using a fan. The rats were randomly divided into three groups: exposed group (with RF-EMF), sham-exposed group (without RF-EMF), and cage-control group (just breeding in cages), consisting of ten rats each.

Whereas acute exposure is defined as exposure to chemical or physical factors for less than 24 h, the period for subchronic exposure generally refers to four to twelve weeks (18). Our CCW method does not affect the three parameters we measured for at least for four weeks (16). Thus, a 4-week repeated exposure to RF-EMF was chosen as a subchronic exposure in the present study.

Intravital-microscopic observation. The rat pial microcirculation within the CCW was monitored using an intravital-microscopic system prior to, two and four weeks after the beginning of RF-EMF exposure. Each observation was performed at least for 24 h after the last 60 min-exposure in five consecutive days to avoid estimating the acute effects which would be occurred by the last exposure. The rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and with a subcutaneous injection of pentobarbital (25 mg/kg). The animal, whose head was fixed in the stereotaxic apparatus, was placed under an intravital-microscopic monitoring system (Figure 2a). This system consisted of a fluorescent microscope (BX50WI, Olympus Optical Co. Ltd., Tokyo, Japan) and an image-intensified camera (C2400-80, Hamamatsu Photonics K.K., Hamamatsu, Japan). The light source was a mercury lamp (U-ULH, Olympus Optical Co. Ltd.). The filter cube has two types of excitation filter of 524 nm and 490 nm. The images of the pial microvascular bed through the system (Figure 2b) were recorded at 30 frames per second to a video cassette recorder (WV-DR7, Sony, Tokyo, Japan) with a video timer (VTG-33, FOR.A Co., Ltd., Tokyo, Japan). All the images were digitized and later analyzed off line.

BBB permeability. BBB permeability was evaluated using two methods which were widely used in the previous studies (12, 19). One was aimed at examining the appearance of BBB disruption, while another was to observe the time-dependent changes in BBB permeability. The transient extravasation of sodium fluorescein (MW: 376, Sigma-Aldrich Inc, Saint Luis, MO, USA), as an indicator of low molecular leakage, was monitored prior to, two and four weeks after the beginning of the experiment using three rats in each group. Sodium fluorescein (2%, 100 μl/kg) was intravenously injected and the image of the pia mater including pial venules and the extravascular region was recorded under the intravital-fluorescence microscope with a fluorescent excitation wavelength of 490 nm. The extravascular accumulation of FITC-dextran (Mean MW: 250 kDa, Sigma-Aldrich Inc.), as an indicator of large molecule leakage, was monitored through the CCW prior to and four weeks after the beginning of the experiment using six rats in each group. FITC-dextran (50 mg/kg) was intravenously injected and the image of pia mater was recorded through intravital-fluorescence microscope with a fluorescent excitation wavelength of 490 nm. The averaged fluorescence in the arbitrary area of the pia mater was measured off line every five minutes.

Leukocyte behavior. The changes in leukocyte behavior were evaluated using the number of leukocytes having interactions with the endothelium of pial venules prior to, two and four weeks after the beginning of experiment using four rats in each group. Two states of interactions were identified: rolling or sticking. The rolling leukocytes were defined as cells having weak interactions with the endothelium, and thereby capable of rolling. The sticking leukocytes were defined as cells attached to the same endothelial area for more than 30 sec. The leukocytes were labeled with rhodamine 6G (0.1

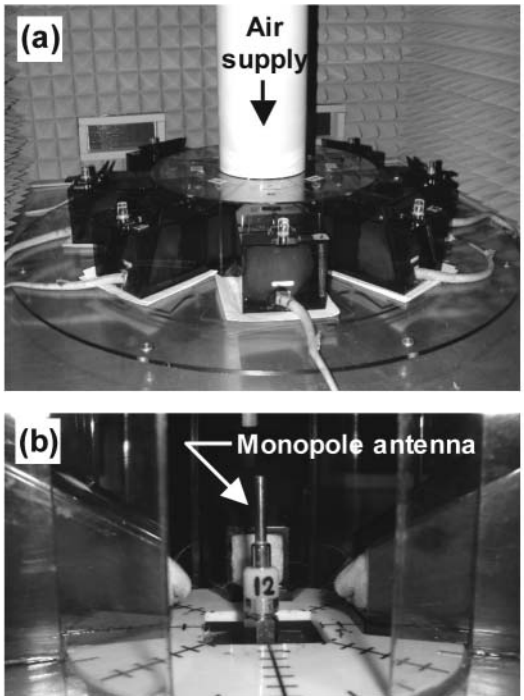

Figure 1. *Exposure system. The exposure system consisted of a small anechoic chamber (a) and a monopole antenna (b). The anesthetized rat was held in a custom-made acrylic holder and its head was positioned toward the monopole antenna placed at the center of the small anechoic chamber.*

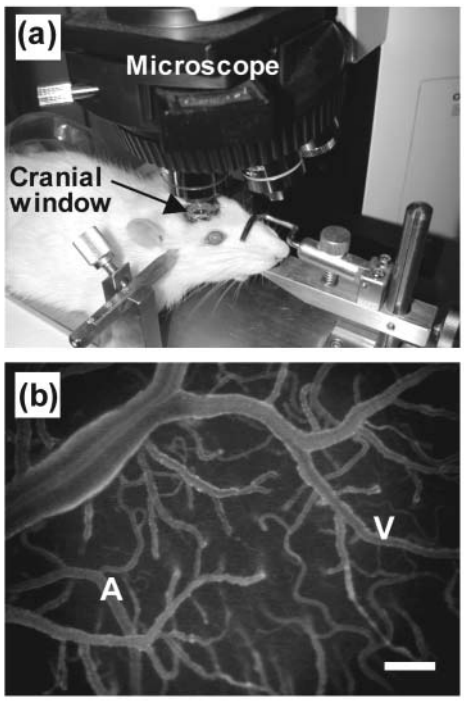

Figure 2. *Intravital-fluorescence microscopic observation. The rat parietal region equipped with a cranial window for long-term observation was placed under a fluorescence microscope (a). After the injection of fluorescent dye (FITC-dextran, 250 kDa), fluorescence images of the pial microvasculature, arterioles (A) and venules (V), were obtained through the cranial window. Bar: 200 μm.*

mg/kg, Wako Pure Chemical Industries, Ltd, Osaka, Japan) injected intravenously and were examined under the intravital-fluorescence microscope with a fluorescent excitation wavelength of 524 nm. The numbers of sticking leukocytes were counted in a 100 μm-length of four pial venules in each rat. In addition, we compared the numbers between postcapillary venules (8-30 μm) and collecting venules (31-50 μm) to investigate the detailed changes in each pial venule.

Plasma velocity. The changes in the plasma velocity were evaluated as the velocity of microspheres flowing in the pial venules prior to, two and four weeks after the beginning of the experiment using four rats in each group. This was performed simultaneously with the evaluation of leukocyte behavior using the same four rats. Fluorescence microspheres (2.5% solids-latex, 1.0 μm YG, Polysciences, Inc., Warrington, PA, USA) were intravenously injected at each experimental period and their motion was observed under the intravital-fluorescence microscope with a fluorescent excitation wavelength of 490 nm. The drifting distance of microspheres flowing at the centerline of pial venules was measured from the video image frame by frame. The velocity was calculated using the distance traveled for 1/30 second of the video frame. The plasma velocity expressed as the average of three measurements was compared between postcapillary venules and collecting venules.

Cranial window temperature. Changes in the temperature in the CCW were measured using an infrared thermograph (TVS-5301, Nippon Avionics Co. Ltd., Tokyo, Japan) with or without RF-EMF exposure for six rats. Before and immediately after 60 min exposure, the parietal region of rat brain equipped with the CCW was placed under the thermograph and the temperature of the CCW was recorded.

Statistical analysis. All results are presented as means ± standard errors. The statistical analysis was carried out using Mann-Whitney *U*-test or Kruskal-Wallis test followed by Scheffe test and $p<0.05$ values were considered statistically significant.

Results

Overview. The animal body weight was measured every week. Figure 3 shows the time course of those changes during the experiment. There were no significant differences in the average body weight between the sham and RF-EMF-exposed groups, whereas the weight in the control group significantly increased in the third and fourth weeks compared with that in the RF-EMF-exposed group. The CCW maintained the architecture of the pial vasculature for at least four weeks after the window implantation. The intravital microscopic observation allowed the images of pial microvessels including arterioles, venules, and capillaries to be obtained even four weeks after the implantation of CCW in all groups. There was no inflammation, regrowth of the

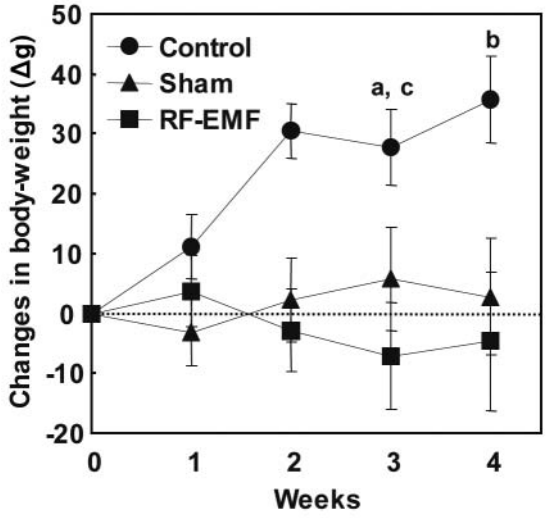

Figure 3. *Body-weight changes. Body-weights of 10 rats in each group were monitored throughout the experiment. There were no significant differences between sham and RF-EMF-exposed groups, whereas significant increases were seen in the control group; a: $p<0.05$ vs. sham exposed group; b: $p<0.05$ and c: $p<0.01$ vs. the RF-EMF-exposed group.*

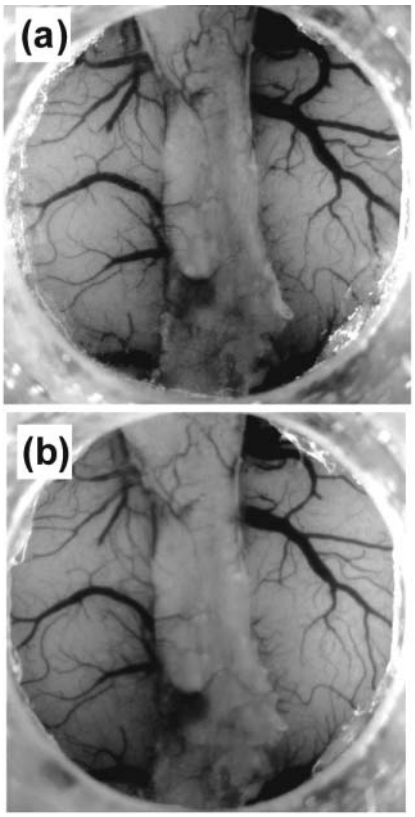

Figure 4. *Overview of the pial microvasculature. The light microscopic images of the pial microvasculature in the rat were obtained (a) before and (b) after four weeks' RF-EMF exposure in the same rat. There was no inflammation, regrowth of the dura or tortuosity of the blood vessels.*

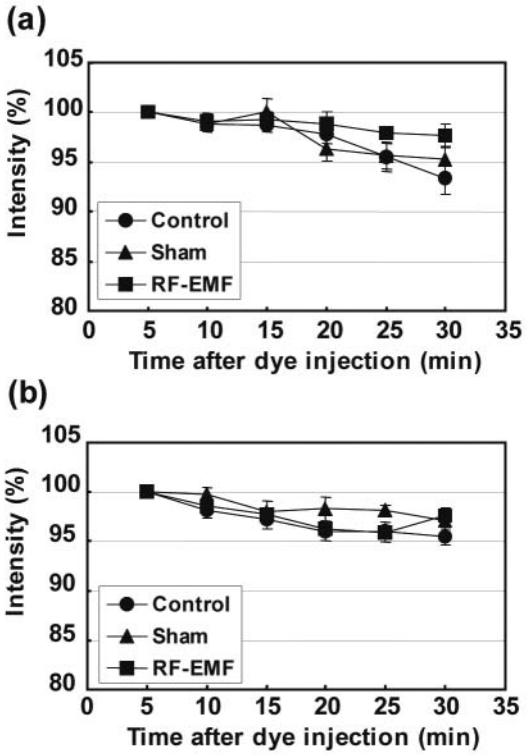

Figure 5. *Time-course of fluorescence of FITC-dextran in the pia mater region. After intravenous injection of FITC-dextran (250 kDa), the intensity, indicating accumulation of extravasated FITC-dextran, was monitored for 30 min. The time-course of the intensity change in each group (six rats each) was compared (a) before and (b) after the four week exposure. No significant differences between the three groups were found in either period.*

dura or tortuosity of the blood vessels, even in the RF-EMF-exposed group (Figure 4).

BBB permeability. The BBB permeability in the rat brain after the RF-EMF exposure was examined using two types of fluorescent dyes. No sites of leakage of sodium fluorescein in the pia mater were observed in any groups at two or four weeks after the beginning of the experiment. Although the intensities of FITC-dextran in the pia mater region decreased time-dependently, before and four weeks after the beginning of the experiment, the intensities at each measurement period showed no significant differences between the three groups (Figure 5).

Leukocytes behavior. To evaluate the leukocyte behavior in the pial microcirculation, we counted numbers of rolling and sticking leukocytes. No effects of the RF-EMF exposure on the number of rolling leukocytes were observed (Figure 6a). It can be seen that before the exposure, there were no significant differences between the three groups in the

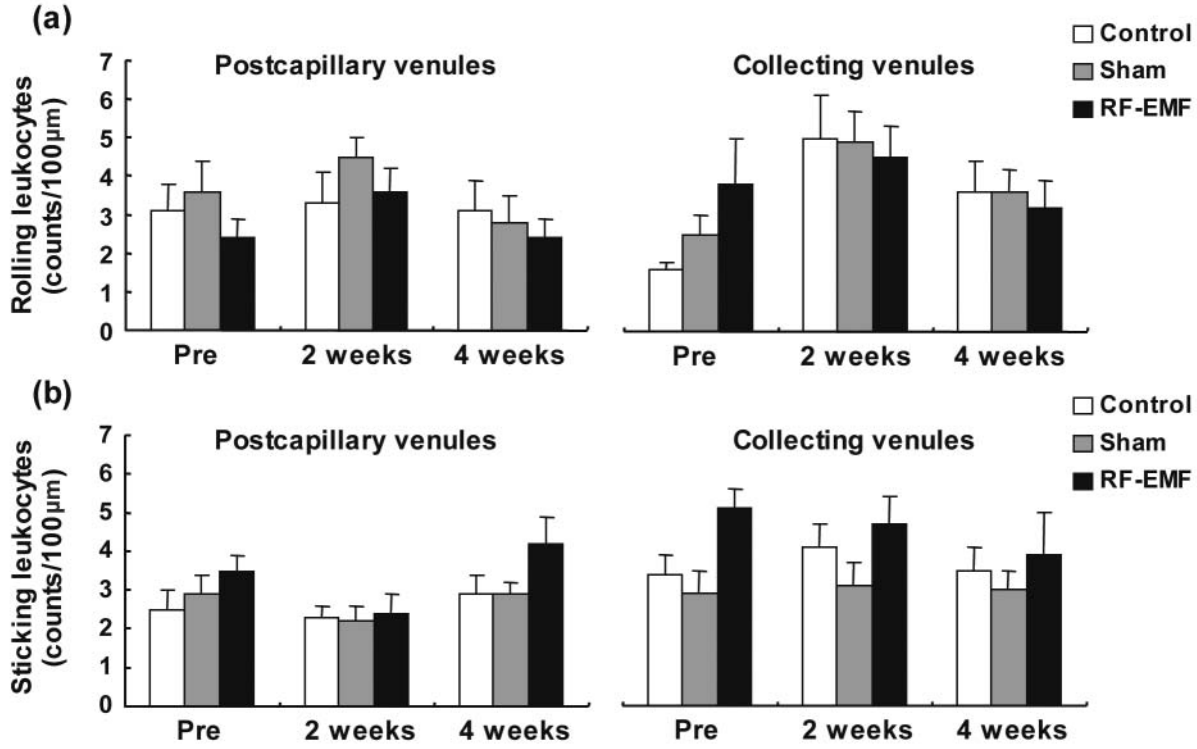

Figure 6. *Leukocyte behavior in pial venules. The interactions of leukocytes with the endothelium were classified into two state: (a) rolling or (b) sticking. The changes in each number were monitored in postcapillary venules (8-30 μm, n=8) and collecting venules (31-50 μm, n=8) in four rats prior to, two and four weeks after the beginning of the experiment. No statistically differences between the three groups were found at any time in the numbers of rolling or sticking leukocytes.*

number of rolling leukocytes in postcapillary venules or collecting venules. Moreover, two or four weeks after the beginning of the experiment, the number of rolling leukocytes in both types of venules showed no significant difference between the three groups. For the number of sticking leukocytes (Figure 6b), no significant differences in the number between the three groups were found in either type of venule at any observation period, although the number in the RF-EMF-exposed group in collecting venules tended to be higher than that in other groups.

Plasma velocity. Plasma velocities were measured for evaluation of the changes in hemodynamics after the exposure. There were no significant differences in the velocities among three groups in both types of venules at any observation periods (Figure 7).

Cranial window temperature. The temperature changes in CCW of the sham and RF-EMF-exposed groups after the 60 min exposure at BASAR 2.4 W/kg, were 0.73˚C±0.18˚C and 0.32˚C±0.34˚C, respectively, but these differences are not statistically significant.

Discussion

In the present study, the three cerebral microcirculatory parameters, BBB permeability, leukocyte behavior, and plasma velocity, were repeatedly measured during the four-week exposure to 1439 MHz RF-EMF in the rat. However, no noticeable changes were found in any of the three parameters under the exposure conditions.

Our closed cranial window (CCW) method allows for direct observation of the cerebral microcirculatory parameters in rat brains exposed to RF-EMF. One of the advantages of this method was the ability to observe dynamic changes in the parameters directly *in vivo*, which cannot be seen in postmortem animals. The fluorescence microscopic images of arterioles, venules and capillaries in the pia mater were sufficiently clear for detection of the parameter changes. Through the experimental period of four weeks, there was no inflammation, regrowth of the dura or tortuosity of the blood vessels, even in the RF-EMF-exposed group.

No changes in BBB permeability were found under the given conditions. BBB permeability was evaluated with two methods using two fluorescent dyes, sodium fluorescein and FITC-

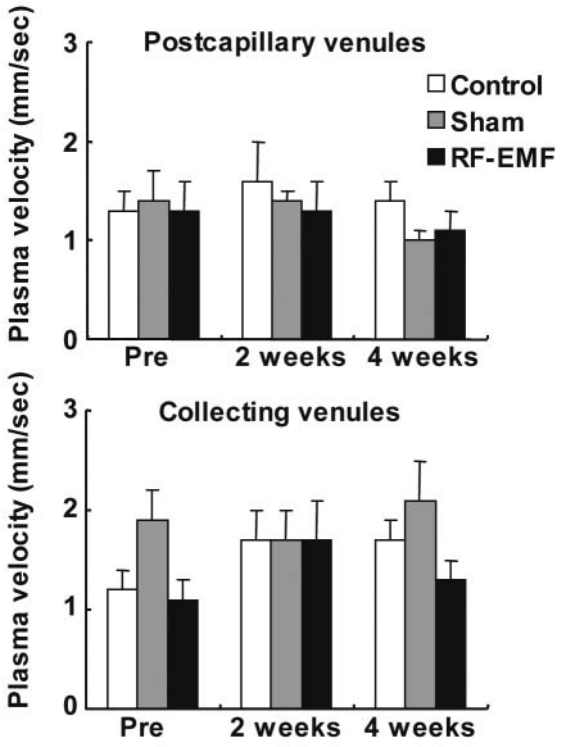

Figure 7. *Plasma velocity in the pial venules. The velocity was measured in postcapillary venules (8-30 μm, n=8) and collecting venules (31-50 μm, n=8) in four rats. There were no significant differeces in the velocities in either postcapillary or collecting venules between three groups at any time.*

dextran. Sodium fluorescein, as a small molecule, was applied to confirm the BBB disruption (19, 20). FITC-dextran, as a large molecule, is useful for measurement of the time-dependent changes in BBB permeability (21, 22). Under inflammatory conditions, both dyes extravasate from cerebral microvessels, especially venules, causing increase in fluorescent intensity around the microvessels (23). Therefore, if the subchronic exposure to RF-EMF were to induce an inflammatory response in the cerebral microvessels, a transient increase in the fluorescent intensity of sodium fluorescein or time-dependent changes in the intensity of FITC-dextran would be found around the pial venules. However, no extravasation of either dye was observed even after four weeks exposure. Tsurita *et al.* (6) used the same type of exposure system equipped with a monopole antenna and showed no albumin leakage in the rat brain after two or four weeks' exposure to 1439 MHz RF-EMF at 2 W/kg of BASAR using histological evaluation. Furthermore, in another study using mouse brains, the authors failed to find noticeable increases in albumin extravasation in the brain after prolonged (two year) exposure to 900 MHz RF-EMF (24). Those two findings were obtained with immunohistochemistry. Our result using another approach, the direct *in vivo* observation, supports their observations.

Leukocytes in the pial venule did not show any changes in their behavior after the subchronic exposure to 1439 MHz RF-EMF. Leukocyte behavior is related to immune function in the brain. Under inflammatory conditions, the behavior indicates augmented adhesion to the endothelial cells in microvessels. Many investigators found an enhancement of leukocyte adhesion in the cerebral microcirculation after brain disorders (25-27) or after experimental application of inflammatory mediators (12, 28, 29). For example, Yuan *et al.* (27) found an increase in the number of sticking and rolling leukocytes in rat pial venules after radiation treatment. We assume that if the repeated exposure of RF-EMF were to induce inflammatory responses around or inside cerebral microvessels, the changes in leukocyte behavior would be manifested as an increase in the numbers of sticking or rolling leukocytes. However, we found no significant increases in either number after two or four weeks' exposure. Therefore, our findings suggest that the leukocyte behavior did not change throughout subchronic exposure to RF-EMF.

Inflammatory or pathophysiological responses in the brain are also detected as changes in hemodynamics in the cerebral microcirculation. We focused on plasma velocity as an indicator of hemodynamics change. In rat pial venules, changes in plasma velocity have been recognized under inflammatory conditions (29). In addition, a recent study found that the blood flow in the human brain increased after RF-EMF exposure for 30 min (30). Therefore, some changes in plasma velocity after RF-EMF exposure would be expected in our animal study. However, no responses to RF-EMF were found, even after the subchronic exposure. We also found that acute exposure to 1439 MHz RF-EMF did not induce an acute effect on the plasma velocity in the rat pial venules (shown in our companion study). Therefore, at least in the rat brain, the 1439 MHz RF-EMF exposure did not appear to affect cerebral blood flow.

Subchronic RF-EMF exposure, not acute exposure, was the focus of the present study. Our companion study found that acute exposure (10 min) to 1439 MHz RF-EMF did not affect BBB permeability, leukocyte behavior, or plasma velocity in the rat pial microcirculation. However, Ziylan *et al.* (14) reported that the repeated exposure to a low dose agent, incapable of inducing acute effects, elicited some physiological effects. For example, a three week-treatment, but not a single injection, of ethinyl estradiol produced significant increases in the cerebrovascular permeability to sucrose and inulin. This phenomenon suggests that repeated exposure to 1439 MHz RF-EMF may also have the potential to cause biological effects. However, no changes in BBB permeability, leukocyte behavior, or plasma velocity were obtained after the four weeks' exposure to 1439 MHz RF-EMF. Therefore, our findings suggest that neither the acute nor the subchronic exposure to 1439 MHz RF-EMF modifies the cerebral microcirculation.

The current basic restriction of local exposure to RF-EMF for general environment in the ICNIRP guidelines is 2 W/kg (15). Several studies which evaluated changes in BBB permeability after RF-EMF exposure found that the exposure at an SAR level lower than the 2 W/kg did not modify the BBB permeability (6, 7, 31). In the present study, using *in vivo* observations, our result on BBB permeability supports their negative findings. Moreover, the other microcirculatory parameters showed no significant changes after subchronic exposure to 1439 MHz RF-EMF at 2.4 W/kg of BASAR. Therefore, these findings suggest that the exposure of animals to RF-EMF lower than the basic restriction of the local exposure (2 W/kg) does not induce any changes in cerebral microcirculation, if a presumption of the existence of a dose-response relationship between the intensity of the RF-EMF exposure and biological responses is accepted.

Conclusion

We introduced the CCW method for evaluating the effects of subchronic exposure to RF-EMF on the cerebral microcirculation. The three microcirculatory parameters examined, BBB permeability, leukocyte behavior and plasma velocity revealed no significant changes even after four weeks' exposure of the rat brain to 1439 MHz RF-EMF. These findings suggest that, with subchronic exposure, there were no changes in the cerebral microcirculation under the given RF-EMF exposure conditions, similar to the local permissible ICNIRP exposure limit guidelines (2 W/kg). However, further studying of other exposure conditions (*e.g.*, chronic exposure, other frequencies) is required to validate our conclusion.

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