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The blood-brain barrier (BBB) consists of tight junctions between the endothelial cells that line the capillaries in the central nervous system. This structure protects the brain, and neurological damage could occur if it is compromised. Several publications by researchers at Lund University have reported alterations in the BBB after exposure to low-power 915 MHz energy. These publications increased the level of concern regarding the safety of wireless communication devices such as mobile phones. We performed a confirmation study designed to determine whether the BBB is altered in rats exposed in a transverse electromagnetic (TEM) transmission line cell to 915 MHz energy at parameters similar to those in the Lund University studies. Unanesthetized rats were exposed for 30 min to either continuous-wave or modulated (16 or 217 Hz) 915 MHz energy at power levels resulting in whole-body specific absorption rates (SARs) of 0.0018–20 W/kg. Albumin immunohistochemistry was performed on perfused brain tissue sections to determine the integrity of the BBB. Chi-square analysis revealed no significant increase in albumin extravasation in any of the exposed animals compared to the sham-exposed or home cage control animals.

INTRODUCTION

The blood-brain barrier (BBB) plays a vital role in maintaining the environment of the central nervous system (CNS) and protecting it from noxious stimuli. It is composed of the endothelial cells of the capillaries in the CNS as well as pericytes, astrocytes and the basement membrane. The endothelial cells of the capillaries that make up the BBB have unique characteristics, including tight junctions and a paucity of transcytotic vesicles. These characteristics decrease the permeability of the BBB (*1*). While some small, lipophilic molecules pass through the BBB, larger molecules and lipophobic molecules must be transported across the barrier via transport mechanisms. Increased permeability of the BBB can result in neurological damage. Causes of permeability include disease and thermal or chemical insults.

Over the past 30 years, there has been considerable controversy about the influence of electromagnetic fields (EMFs) on the integrity of the BBB. Polyashchuk (*2*) was the first to report microwave effects on the BBB. In that study, rabbits injected with ^{32}P were exposed to 2307 MHz energy for 10 or 20 min. An increase in the penetrability of ^{32}P in various areas of the brain was observed with increasing forward power. Subsequent studies have reported varied results. Frey *et al.* (*3*) reported alteration of the BBB after exposures to relatively low levels of 1.2 GHz microwave energy. However, the results of a number of other studies revealed that effects were induced only after exposures that resulted in an increase in brain temperatures up to 42–43°C (*4–12*).

With the advent of cellular phone technology in this frequency range, a number of reviews and reports were published summarizing the literature on the effects of energy of this frequency on the BBB[2] (*13*). This controversy was reignited with the increased global use of cellular telephones and the study by Salford *et al.* (*14*) showing that exposure to low-power microwave energy altered the integrity of the BBB. In that study, unanesthetized rats were placed in a transverse electromagnetic (TEM) transmission line cell and exposed to low-power continuous-wave or pulsed (8, 16, 50, 200 Hz) 915 MHz energy. Immunohistochemical analysis for albumin showed that the CW modulation failed to produce a significant increase in albumin leakage, whereas the pulsed modulation produced significant increases in albumin leakage. These investigators published several subsequent reports showing that exposure to

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[2] National Radiological Protection Board Report: Mobile Phones and Health—an update. http://www.liv.ac.uk/radiation/mobile.htm, 2003.

low-level CW and pulsed [8 Hz (0.57 ms pulse duration), 16 Hz (0.57 ms pulse duration), 50 Hz (0.57, 4.0 and 6.0 ms pulse durations), 200 Hz (0.57 and 4.0 ms pulse durations), 217 Hz (0.57 ms pulse duration)] 915 MHz energy alters the integrity of the BBB[3] (*15–19*). With these exposure parameters, the specific absorption rates (SARs) ranged from 0.004 W/kg to 8.3 W/kg. These results caused considerable international concern regarding the health and well-being of humans exposed to low-level EMFs. The 2003 World Health Organization radiofrequency (RF) research agenda listed ''studies to assess the accuracy and reproducibility of published RF effects on the permeability of the blood-brain barrier and other neuropathologies'' as short-term or urgent needs.

Based in part on the data from the Swedish studies, a number of research groups have conducted recent experiments to determine the effects of EMF exposure on the BBB. Fritze *et al.* (*20*) used a central antenna in a carousel system to expose restrained rats to 900 MHz GSM at brain SARs from 0.3 to 7.5 W/kg. Although these authors did not examine the effects of the extremely low SARs used in the Swedish studies, using albumin immunohistochemistry, these authors found significant increases in albumin extravasation only in the highest exposure groups. Tsurita *et al.* (*21*) also used a central antenna in a carousel system to expose restrained rats to 1439 MHz TDMA at a brain SAR of 2 W/kg (whole-body SAR of 0.25 W/kg). Rats were exposed for either 2 or 4 weeks for 1 h per day for 5 consecutive days with 2 days rest. Tissue evaluation by Evans-Blue staining or albumin immunohistochemistry failed to reveal significant changes in BBB permeability. Finnie *et al.* (*22*) exposed mice to 898.4 MHz GSM for 60 min at 4 W/kg. Albumin histochemistry did not reveal significant changes in the permeability of the BBB. A chronic study by the same group in which female mice were exposed to 900 MHz (modulated at 217 Hz) at whole-body SARs from 0.25 to 4.0 W/kg for 1 h per day, 5 days a week for 104 weeks also failed to reveal significant changes in BBB permeability at any of the exposure levels (*23*). Tore *et al.*[4] exposed both intact and sympathetectomized rats while the rats were restrained, using a loop antenna for 2 h to 900 MHz GSM at an average brain SAR of 2.0 W/kg. Rather than evaluating endogenous albumin leakage, these researchers infused bovine serum albumin via a venous catheter 15 min prior to the end of the exposure. Albumin immunohistochemistry revealed extravasation in the exposed groups in the dura mater and the deeper cortical layers. Albumin extravasation was greater in the sympathetectomized rats.

Due to the lack of agreement in the literature and the concerns raised by the Swedish BBB studies, we designed a confirmation study of the Swedish experiments. The present study should not be considered to be a attempt to replicate any of Salford's studies since the range of some parameters was adjusted to exclude possible confounding variables such as sex, size, age of subjects, and placement within the exposure system. We visited Drs. Salford, Persson and Brun at Lund University while designing this confirmation study to ensure an understanding of the experimental protocol and technical procedures used in their studies. As a result of this visit, TEM cells were constructed for us by Dr. Lars Malmgren, who built the TEM cells used in the Swedish experiments. Our immunohistochemistry and tissue analysis techniques were refined during the visit to Lund University.

METHODS

Animals

Male rats of the Fischer 344 CD-VAF strain were obtained from Charles River, Portage, MI. The animals were procured, maintained and used in accordance with the Animal Welfare Act and the ''Guide for the Use and Care of Laboratory Animals'' prepared by the Institute of Laboratory Animal Resources–National Research Council. Rats weighed 250–300 g at the time of the experiment. Each animal was housed individually in standard plastic cages (26 × 23 × 20.5 cm) with *ad libitum* access to food (Formulab 5008, Purina Meals, St. Louis, MO) and water. Rooms were maintained at 24 ± 1°C with 60 ± 10% relative humidity. The air-flow rate was between 10 to 15 exchanges per hour. A time-controlled lighting system provided 12-h/12-h light/dark cycle (lights on at 0600).

TEM Cell

Exposures to 915 MHz electromagnetic energy and sham exposures were conducted in two identical TEM cells. Each cell consisted of an outer conductor and a center septum contained in a wooden box. The outer conductor was made of brass mesh (50 mesh) while the center septum was made of aluminum. The ends of the cell were removable, allowing access to the inside. The animals were held in the cells in Plexiglas™ holders (14 cm wide × 14 cm deep × 6 cm high). Holes in the animal holder and wooden box allowed ventilation. As in the Swedish experiments, a Plexiglas™ rectangular shell that was 21 cm in length was placed perpendicular to the outside of the wooden box. An electric fan (Sinwan, Model S109 AP-11-1; 50/60Hz) was mounted on the end of the rectangular shell that was furthest from the wooden box and provided air flow through the TEM cell during both the habituation and exposure periods.

Dosimetry

Determination of whole-body SARs was made using two methods: calorimetry and differential measurements of transmitted and reflected power. For calorimetry[5] (*24*), rats (n = 4/group) were killed humanely

[3] L. G. Salford, A. Brun, J.L. Eberhart and B.R.R. Persson, Permeability of the blood-brain barrier induced by 915 MHz electromagnetic radiation, continuous and modulated at 8, 16, 50 and 200 Hz. In Abstracts of the First World Congress for Electricity and Magnetism in Biology and Medicine, 14–19 June 1992, Lake Buena Vista, FL, 1992.

[4] F. Tore, P-E. Dulou, E. Haro, B. Veyret and P. Aubineau, Two-hour exposures to 2-W/kg, 900-MHz GSM microwaves induce plasma protein extravasation in rat brain and dura mater. Presented at the 2002 meeting of the European BioElectromagnetics Association.

[5] S. Allen and W. D. Hurt, Development and use of a calorimeter to measure specific absorption rates in small laboratory animals. Presented at the URSI International Symposium on the Biological Effects of Electromagnetic Waves, Airlie, VA, 1977.

TABLE 1
Exposure Parameters and Whole-Body SARs for each Experimental Group

16 Hz modulated (0.57 ms) (0.0091 duty factor)	Continuous wave	217 Hz modulated (0.57 ms) (0.124 duty factor)
	0.002 W/kg	0.0025 W/kg
0.0018 W/kg	0.02 W/kg	0.025 W/kg
0.018 W/kg	0.2 W/kg	0.248 W/kg
0.18 W/kg	2.0 W/kg	2.48 W/kg
	20.0 W/kg	

Notes. Power values for the pulse-modulated groups were chosen to produce SARs similar to those in the CW groups so that the effects of the modulation itself could be evaluated. The modulation rates (16 and 217 Hz) were selected from those used in the Swedish studies.

with an overdose of sodium pentobarbital (0.6 ml) and a non-perturbing EMF temperature Vitek probe was inserted 4 cm beyond the anal sphincter. Each carcass was placed in a Plexiglas™ holder and allowed to come to room temperature overnight. Calorimeters were filled with room-temperature water and were also left overnight. On the following day, the Plexiglas™ holders and carcasses were placed in the TEM cell and irradiated in the K or H orientation at a forward power of 14 W for 6 min. Using leather gloves to reduce heat transfer, each carcass was quickly removed from the TEM cell and placed in its own water-filled calorimeter. The difference in the water temperature before and for 6 h after placement of the irradiated carcass in the water was used for calculating whole-body SAR.

For estimation of whole-body SAR using differential power measurements, input power and the orthogonal transmitted and reflected powers were measured. Power absorption was taken as the difference between the input and the sum of the transmitted and reflected powers. The TEM cell input power and the orthogonal reflected and transmitted powers were measured, the quantity absorbed being the difference between the input and the sum of the reflected and transmitted powers. Because some loss occurs in the waveguide and plastic holder, absorption was measured with and without a rat carcass present in the TEM cell. The difference was the power absorbed by the animal. Power levels were measured with Hewlett Packard 432B power meters at constant transmitter output of 14 W.

Animal Exposures

Prior to exposure, rats were habituated to the exposure system on three occasions, for 30 min on each occasion, to minimize stress-related effects. For the exposures, rats (n = 27–42) were placed individually in a Plexiglas™ holder, and each holder was then placed in the top compartment of the TEM cell. Rats were sham-exposed or exposed to 915 MHz energy for 30 min. Both continuous-wave and pulsed modes of 16 and 217 Hz were used. These pulse parameters were chosen based on the data of Persson *et al.* (*26*); the 16 Hz modulation showed the most pronounced effect at the lowest SAR (0.004–0.08 W/kg) and 217 Hz showed the most pronounced effects at 0.11–0.95 W/kg. For the present study, approximate whole-body SARs for each group are reported in Table 1. Power values for the pulse-modulated groups were chosen to result in SARs similar to those in most of the CW groups so that effects of the modulation itself could be evaluated. The protocol for the sham exposures was similar to that for the EMF exposures, with the exception that the TEM cell was not activated. In addition, home cage controls, which were never removed from their housing facility, were included in the study.

Visualization of Native Albumin

Rats were anesthetized with sodium pentobarbital a maximum of 10 to 15 min after the exposure was terminated, because the time required to remove the animals from the holder, transport them to the surgical suite, and wait for the anesthetic to produce the desired effect. The rats were then perfused intracardially with heparinized saline for 3–4 min, followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer with 0.01% thimersol. Animals were decapitated and the brains were removed and immersed in 4% formaldehyde solution for 12 h. The brains were embedded in paraffin and cut on a microtome into 4-μm-thick sections. Endogenous albumin was visualized by reaction with an IgG fraction of rabbit anti-rat albumin as described by Salford *et al.* (*18*).

Using light microscopy, immunolabeled brain sections were examined by two independent investigators who were blind to the exposure condition of each rat. Brain slices were examined for both extracellular and intracellular albumin labeling. Intracellular staining was defined as albumin staining within the neuronal structure. Extracellular staining is staining within the neuropil. Coronal sections from a minimum of three regions along the rostro-caudal extent of the brain [labeled as B (~0.0 mm bregma), C (~−3.0 mm posterior to bregma), and D (~−5.0 mm posterior to bregma), respectively] were examined for each animal. Section B contains neuronal structures such as the caudate putamen and piriform cortex. Neuronal structures including anterior hippocampus cortex and ventromedial thalamus are found in section C. Section D includes structures such as the posterior hippocampus, entorhinal cortex and substantia nigra. Representations of brain regions immunolabeled for extra- and/or intracellular albumin were marked on a corresponding drawing of that section from a rat brain atlas (*26*). Albumin leakage at or near circumventricular organs (CVOs) was also noted and marked. Subsequently, a score ranging from 0 to 5 was assigned to designate the amount of extracellular leakage in each section. The same score range was used to designate the amount of intracellular labeling observed in each section. Labeling of albumin leakage and absorption in the CVOs was not used in the score assignment. A score of 0 for extracellular leakage indicated that no immunolabeling was observed, while a score of 5 indicated numerous areas of extracellular leakage. Similarly, a score of 0 for intracellular albumin labeling indicated no cells with intracellular label, while a score of 5 indicated many cells with intracellular labeling spread throughout the cortical and subcortical regions of the brain tissue section.

Positive Control

Two types of positive controls were used to validate the albumin immunohistochemistry staining technique. A positive control brain section was included during each assay for quality control. The first type of positive control was produced by implanting a catheter into the right internal carotid artery of a rat anesthetized with sodium pentobarbital. Urea (10 *M*) was infused at a dose of 0.1 ml/100 g body weight over 10 s. This technique resulted in massive opening of the BBB and subsequent albumin leakage and staining on the ipsilateral side of the brain. The second type of positive control was produced by exposing a rat in the K orientation to 2060 MHz until core and tympanic temperatures rapidly reached 43°C. The rat was immediately removed from the exposure chamber and rapidly cooled by placing it on a room-temperature laboratory table. The animals were killed and brain tissue was prepared in the same manner as described above. Both the urea and high-temperature positive controls showed large amounts of intracellular and extracellular albumin immunostaining (see Fig. 1A–C).

Statistics

Each subject was assigned a final intracellular score (the highest intracellular score assigned to any of the three sections analyzed) and a final extracellular score (the highest extracellular score assigned to any of the three sections analyzed). To follow an analysis technique similar to that used by Dr. Brun at Lund University, subjects were placed in one of three intracellular groups or one of three extracellular groups based on the

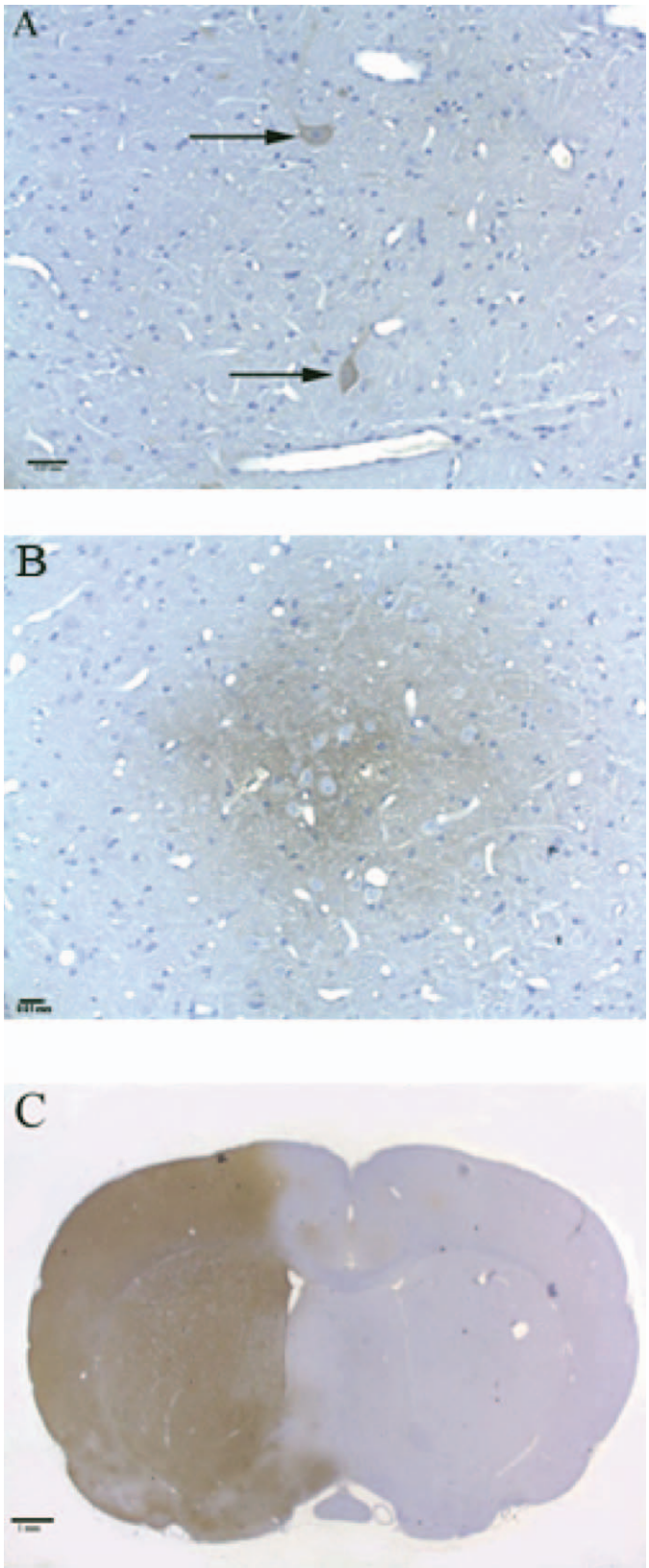

FIG. 1. Albumin Immunohistochemistry positive controls. Panel A: Intracellular staining after microwave exposure that produced high core and tympanic temperatures. Panel B: Extracellular staining after same exposure as above. Panel C: Extracellular staining after internal carotid infusion of 10 *M* urea.

immunolabeling scores. Those with a score of 0 or 1 were considered to have no or minimum extravasation, those with a score of 2 or 3 were considered to have moderate extravasation, and those with a score of 4 or 5 were considered to have extensive extravasation. Chi-square analyses were performed on both intracellular and extracellular scores of these groups.

RESULTS

Dosimetry

In the H and K orientations, calorimetry (14 W forward power) revealed whole-body SARs of 22.3 ± 3.2 and 12.8 ± 0.7 W/kg, respectively. In the H and K orientations, the differential power measurements revealed whole-body SARs of 22.1 ± 1.8 W/kg and 13.0 ± 2.0 W/kg, respectively. In the H and K orientations, differential power measurements revealed the whole-body normalized SARs in the bottom compartment to be 48% and 36% of those in the top compartment, respectively. Due to this variation, only the top compartment was used for the experiment.

Albumin Extravasation

Rating assignments for intracellular scores were analyzed by assigning the highest intracellular and the highest extracellular scores from section B, C or D to the subject and performing χ^2 analysis. The contingency table for intracellular albumin staining is shown in Table 2. For intracellular staining, the resulting 2 (rating categories: 0/1 compared to 2/3) × 14 (treatment groups) contingency table, χ^2 (13, N = 512) = 12.80, P = 0.46, indicating no significant difference in distribution of rating assignments across the groups. Note that for analysis of the intracellular scores, the 4/5 rating category was not considered, given that expected frequencies for this category were too low [<1 for all cells; see ref. (*27*)]. For similar reasons, rating assignments for the extracellular scores were not analyzed formally; expected frequencies for both the 2/3 and 4/5 rating categories were all less than 1. More specifically, in the case of extracellular scores, for each treatment group, at least 98% of all assignments were to the 0/1 category (indicative of no difference in assignments across groups). Figure 2 shows representative sections from a sham exposure and a 20 W CW exposure.

Little or no intracellular and extracellular extravasation was found in all exposure groups. The albumin immunolabeling was statistically similar in the exposed, sham-exposed and home cage control groups. Figure 3 shows the distribution of assigned scores within each exposure group.

DISCUSSION

The present study revealed no significant differences in the amount of intracellular or extracellular albumin found in the brains of EMF-exposed, sham-exposed or home-caged rats. The present study was designed to be a confirmation study rather than a direct replication of any of the

TABLE 2
Contingency Table and Expected Values for the Intracellular Scores

CW or pulsed (Hz)	Power	Mass (g)	Contingency table			Expected values		
			0/1	2/3	Total	0/1	2/3	Total
16	0.0018	272.7407	22	11	33	25.97461	7.025391	33
16	0.018	281.875	30	5	35	27.54883	7.451172	35
16	0.18	282.0194	27	6	33	25.97461	7.025391	33
217	0.0025	280.7419	24	9	33	25.97461	7.025391	33
217	0.025	278.7879	25	8	33	25.97461	7.025391	33
217	0.248	274.2258	25	10	35	27.54883	7.451172	35
217	2.48	281.4118	26	9	35	27.54883	7.451172	35
CW	0.002	279.8889	24	4	28	22.03906	5.960938	28
CW	0.02	278.7143	33	4	37	29.12305	7.876953	37
CW	0.2	278.3917	31	10	41	32.27148	8.728516	41
CW	2	280.7027	31	10	41	32.27148	8.728516	41
CW	20	280.085	32	10	42	33.05859	8.941406	42
Sham	n/a	284.3571	36	8	44	34.63281	9.367188	44
Home cage	n/a	279.3171	37	5	42	33.05859	8.941406	42
Sum			403	109	512	403	109	512

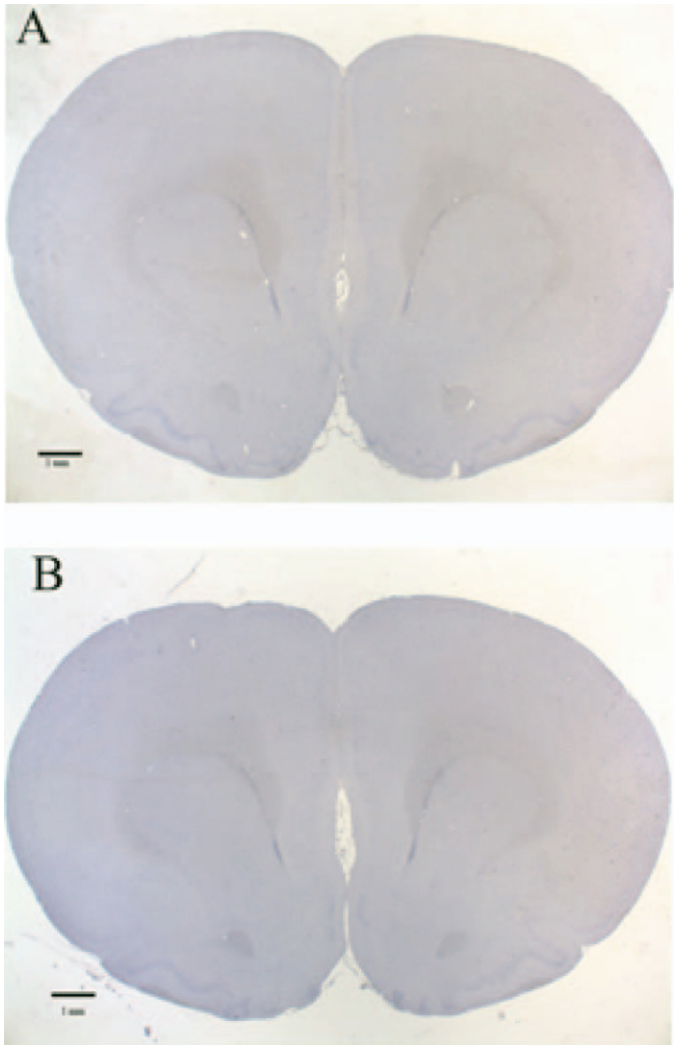

FIG. 2. Examples of albumin staining in brain sections of (panel A) a sham-exposed rat and (panel B) a rat exposed to 20W CW 915 MHz energy.

Swedish studies. The primary goal of this confirmation study was to design a well-controlled experiment that would encompass the range of exposure parameters used in the Swedish studies but that could be analyzed and interpreted more easily, due to the lack of potential confounding variables. Although we used the same type of exposure system, immunohistochemical techniques, and tissue analysis procedures as used in the Swedish study, there were a number of differences in the design of this study. These procedural differences are summarized in Table 3.

The results of the present study are contrary to those in the studies conducted at Lund University (*14, 18, 28*). The question remains as to what produced the differences between our results and those from the group at Lund University. One possible factor that may have led to these differences is that we used only the top chamber of the TEM cell instead of using both chambers. Our empirical dosimetry revealed substantial differences in the whole-body SARs of animals placed above or below the internal septum. Theoretical dosimetry performed by the Lund University group also revealed a difference in whole-body SARs produced by placing animals in either the top or bottom chambers of the TEM cell (*29*). These authors suggested that the biological effects found in the Salford studies were more prevalent in rats exposed in the top chamber compared to the bottom chamber of the TEM cell.

We do not believe that the differences we found are the result of the immunohistochemical techniques or the tissue evaluation process. A subset of adjacent tissue sections was sent to Sweden for immunohistochemistry and analysis by Dr. Brun. The two research groups obtained similar results.

To our initial surprise, the 20 W/kg exposures failed to produce a significant increase in albumin leakage across the BBB. However, the empirical dosimetry revealed that the maximum brain temperature was 40.6°C. Lin and Lin (*30*) showed that only cortical temperatures above 43.0°C al-

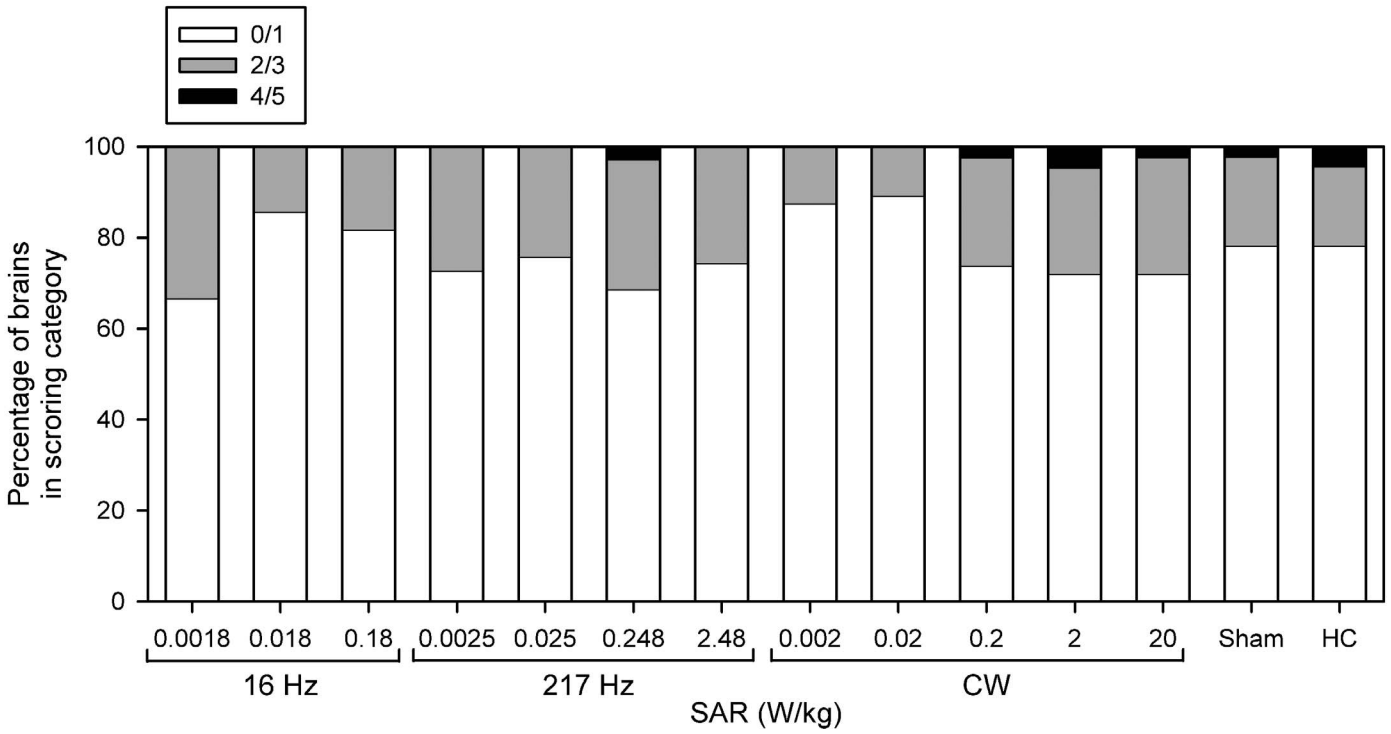

FIG. 3. Percentage of brains within each exposure group within each scoring category. For the purposes of this graph, each brain was assigned the highest score, either intracellular or extracellular, that any of the evaluated sections received.

tered the permeability of the BBB to Evans Blue. Therefore, while this 30-min exposure at 20 W/kg did produce hyperthermia, it was not sufficient to result in alteration of the BBB.

Recently, Salford *et al.* (*28*) reported that a low-level 915 MHz GSM exposure significantly increased neuronal damage. These researchers used cresyl violet staining to observe and evaluate the viability of neurons in the brains of rats that were killed 14 or 50 days after exposure. Based on this result, an international consortium was formed to examine this result. Scientists from France, Japan and the United States performed similar but independent experiments in an attempt to confirm this result. The biological results as well as extensive theoretical and empirical dosimetry reports will be reported elsewhere.

A high priority was placed on this study due to the concerns over the possible effect of low-level radiofrequency radiation on the integrity of the BBB for many years. During the past decade, the issue was reinvigorated by the dramatic results reported by Salford and his colleagues. However, using a very similar experimental approach, we have been unable to confirm their results. The resolution between their findings and ours remains to be determined; however, at this time, we see no threat to the BBB from RF-field exposure below the levels deemed permissible in the international ICNIRP and IEEE standards (*31, 32*).

TABLE 3
Primary Differences between the Swedish Studies and the Present Study

Swedish studies (1992–1997)	This study
Male and female rats, 120–555 g	Male rats, 250–300 g
N = 2–91	N = 28–46
Top and bottom chambers	Top chamber only
2–960-min exposures	30-min exposures
0.0002–8 W/kg whole body	0.002–20 W/kg whole body

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