

No adverse effects detected for simultaneous whole-body exposure to multiple-frequency radiofrequency electromagnetic fields for rats in the intrauterine and pre- and post-weaning periods

Tomoyuki Shirai^{1,2†}, Jianqing Wang^{3*}, Mayumi Kawabe⁴, Kanako Wake⁵,
So-ichi Watanabe⁵, Satoru Takahashi² and Osamu Fujiwara³

¹Nagoya City Rehabilitation Center, Yatomi-cho, Mizuho-ku, Nagoya, 467-8622, Japan

²Graduate School of Medical Sciences, Nagoya City University, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, 467-8601, Japan

³Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya, 466-8555, Japan

⁴DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya, 491-0113, Japan

⁵National Institute of Information and Communications Technology, Nukui-Kitamachi 4-2-1, Koganei, Tokyo, 184-8705, Japan

[†]The late Dr Shirai had approved publication of this work by the co-authors.

*Corresponding author. Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan. Tel/Fax: +81-52-735-5457;

Email: wang@nitech.ac.jp

Received May 9, 2016; Revised July 5, 2016; Accepted July 18, 2016

ABSTRACT

In everyday life, people are exposed to radiofrequency (RF) electromagnetic fields (EMFs) with multiple frequencies. To evaluate the possible adverse effects of multifrequency RF EMFs, we performed an experiment in which pregnant rats and their delivered offspring were simultaneously exposed to eight different communication signal EMFs (two of 800 MHz band, two of 2 GHz band, one of 2.4 GHz band, two of 2.5 GHz band and one of 5.2 GHz band). Thirty six pregnant Sprague-Dawley (SD) 10-week-old rats were divided into three groups of 12 rats: one control (sham exposure) group and two experimental (low- and high-level RF EMF exposure) groups. The whole body of the mother rats was exposed to the RF EMFs for 20 h per day from Gestational Day 7 to weaning, and F₁ offspring rats (46–48 F₁ pups per group) were then exposed up to 6 weeks of age also for 20 h per day. The parameters evaluated included the growth, gestational condition and organ weights of the dams; the survival rates, development, growth, physical and functional development, memory function, and reproductive ability of the F₁ offspring; and the embryotoxicity and teratogenicity in the F₂ rats. No abnormal findings were observed in the dams or F₁ offspring exposed to the RF EMFs or to the F₂ offspring for any of the parameters evaluated. Thus, under the conditions of the present experiment, simultaneous whole-body exposure to eight different communication signal EMFs at frequencies between 800 MHz and 5.2 GHz did not show any adverse effects on pregnancy or on the development of rats.

KEYWORDS: multi-frequency radiofrequency electromagnetic field, whole-body exposure, biological effect, rat, reproductive and developmental toxicity

INTRODUCTION

With the steep increase in ubiquitous communications, we are constantly exposed to radio waves of multiple frequencies. Under such conditions, it is important to assess possible adverse health effects of exposure to multiple-radiofrequency (RF) electromagnetic fields

(EMFs). To protect the public, several guidelines for limiting daily RF EMF exposure have been issued. Basic restrictions and the resultant reference levels (which are defined in terms of specific absorption rate (SAR), electric field, magnetic field, or power density) have been proposed. However, such restrictions are primarily

based on animal experiments with exposure to a single-frequency EMF. The increased demand to explore the biological effects of simultaneous exposure to multiple-frequency RF EMFs makes the establishment of an appropriate animal exposure system one of the most important issues in this field. Previously, Lee *et al.* [1, 2] published data from two experiments in which simultaneous combined two-frequency RF EMF exposure, consisting of single code-division multiple access (CDMA) and wideband code-division multiple access (WCDMA) exposure, was performed on pregnant mice or mature rats for the entire gestational period or for 12 weeks, respectively. The exposure to mice was performed twice per day for 45 min (with a 15 min interval) every day throughout the entire gestational period. They found no adverse effects on the mortality, growth or morphology of the fetuses in autopsy examinations on Gestational Day 18. The same group also performed a similar experiment with two RF EMFs to expose rats in order to explore adverse effects on testicular function. They observed no adverse effects on rat spermatogenesis either. Thus in the two experiments, Lee *et al.* did not find any adverse effects in mouse fetal development or rat testicular function. Other two-frequency exposure results are also mentioned in the international authority report [3].

On the other hand, Shirai *et al.* investigated the multigenerational effects of whole-body exposure of rats to 2.14 GHz W-CDMA cellular phone signals from Gestation Day 7 to weaning, and confirmed no adverse effects on functional development, behavioral function, memory function, and reproductive ability in F₁, F₂ or F₃ offspring [4].

Recently, Wang *et al.* [5] developed a whole-body exposure system in which unconstrained rats were effectively exposed to eight communication signal EMFs of between 800 MHz and 5.2 GHz simultaneously, and the SARs in the free-moving rats in the experimental system were successfully evaluated. The frequencies chosen between 800 MHz and 5.2 GHz consisted of the 800 MHz, 2 GHz, 2.4 GHz and 5.2 GHz bands. All of these frequency bands are being used for cellular phones or wireless LANs, and the resultant RF EMFs are the most common ones in our daily life.

According to current knowledge, the only possible biological effect at the investigated frequencies is a thermal effect, which would depend on the whole-body average SAR. However, the single-frequency and multiple-frequency exposures produce distinctly different distributions of electromagnetic fields inside the human body. In addition, except for two-frequency exposure, there are no reported results on multiple-frequency exposure until now; thus, there is doubt about whether or not the RF biological effect really depends upon the whole-body average SAR. To remove this doubt, experimental evidence is essential. We therefore planned this multiple-frequency RF EMF exposure experiment.

In this study, using the exposure system developed by Wang *et al.* [5], we evaluated the effect of simultaneous whole-body exposure of rats during pregnancy, and of F₁ offspring during the pre- and post-weaning periods, to eight different communication signal RF EMFs. We designed a two-level exposure: the high SAR level was based on the International Commission on Non-Ionizing Radiation Protection (ICNIRP) basic restrictions for occupational exposure, and the low SAR level was based on the ICNIRP basic restrictions for general public exposure [6]. The exposure time was set to 20 h per day in order to simulate daily exposure, as much as

possible. We believe this is the first attempt to produce experimental evidence for the presence/absence of adverse effects of multiple-frequency RF EMF exposure.

MATERIALS AND METHODS

Animals

Due to the limited number of animals that could be exposed to RF EMFs at one time, three identical, independent experiments were performed sequentially. For each of the three experiments, 14 pregnant Sprague-Dawley (SD) 10-week-old rats (CrI: CD) at Gestational Day 2 (GD 2) were purchased from Charles River Japan (Shiga, Japan). The animals were allowed a 5-day quarantine and acclimation period after purchase, and 12 out of the 14 rats were selected and subjected to experimentation after their normal health status was confirmed. The 12 pregnant rats were divided into three groups of 4 rats, corresponding to one control (sham-exposure) group and two experimental (low- and high-level-exposure) groups (Groups 1 to 3, respectively). Three exposure chambers were used, and one chamber was allocated to each of the groups. There were four animal cages in each chamber, as reported previously [4]. A single pregnant rat was set in each cage, with a total of four pregnant rats per group. Pregnant rats were exposed simultaneously to multiple-frequency RF EMFs for 8 weeks, from GD 7 to 21 days after delivery. The date of birth was designated as Postnatal Day (PND) 0. On PND 4, the litters were randomly culled to 8 pups (4 males and 4 females) per dam, for a total of 16 pups of each sex per exposure level (4 pups/sex/cage × 4 cages/exposure chamber) (Fig. 1). At weaning, the mother rats were removed from the cages and 8 male and 8 female pups per exposure level were randomly selected and set in the four cages in each of the exposure chambers, i.e. 4 male pups/cage in two cages and 4 female pups/cage in two cages in each of the exposure chambers. These pups were exposed simultaneously to multiple-frequency RF EMFs for 20 h/day until 6 weeks of age. After termination of RF EMF exposure, half of the pups were subjected to various biological examinations (4 males and 4 females from each exposure level); the remaining pups were used for mating, which was undertaken at 11 weeks old. Mating was undertaken between pups from different dams to generate the F₂ rats. The F₂ animals were sacrificed 4 days after birth (Fig. 1).

All of the cages contained wood-chip bedding, and the animals were allowed free access to a powder diet (MF, Oriental Yeast, Tokyo Japan) and drinking water in the exposure chambers. Dams

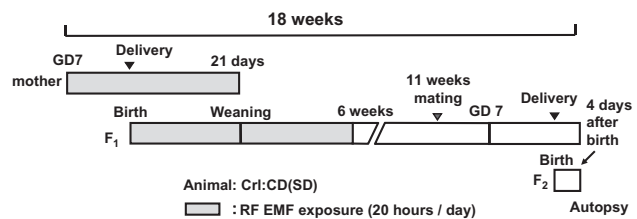


Fig. 1. Experimental design. RF EMF signals were generated for 20 h/day. [shaded box]: represents exposure to RF EMF in Groups 2 and 3 or sham exposure in Group 1.

and their offspring were observed clinically for symptoms and mortality once per day in the morning over the entire experimental period. When the offspring were 6 weeks of age, they were removed from the exposure chambers and kept in cages on wood-chip bedding in an air-conditioned animal room, maintained on a 12 h light/dark cycle at $22 \pm 3^\circ\text{C}$ and $55 \pm 15\%$ humidity, and they were allowed free access to a pelleted diet (MF, Oriental Yeast, Tokyo Japan) and drinking water. The body weight of the dams was measured on GDs 7, 14, 17 and 20, and after delivery on PNDs 0, 4, 7, 14 and 21. The body weight of the offspring was measured on PNDs 0, 4, 7, 14 and 21. After weaning, the body weight of the dams and their offspring was measured weekly until the termination of the experiment.

Exposure system

As described by Wang *et al.* in [5], the multiple-frequency and multi-generation whole-body exposure system was designed to use signal forms ranging from 800 MHz to 5.2 GHz. The first four signal forms were IMT-2000 DS-CDMA at 800 MHz band, IMT-2000 MC-CDMA at 800 MHz band, IMT-2000 DS-CDMA at 2 GHz band, and IMT-2000 MC-CDMA at 2 GHz band. The other four signal forms were wireless LAN IEEE 802.11b/g at 2.4 GHz band, mobile WiMAX at 2.5 GHz band, Next Generation PHS at 2.5 GHz band, and Wireless LAN IEEE 802.11a at 5.2 GHz band (Table 1). All of the eight signals were modulated signals with a continuous wave (CW) carrier in specified frequencies, and the frequency bandwidth was under 20 MHz. So strictly speaking, they were band-limited modulated CW signals. Table 1 summarizes the bandwidth for each signal. To generate the eight communication signals, the signals from five signal generators were mixed into a multiple-frequency signal (Fig. 2a). The multiple-frequency signal was then divided into three components and was input to three amplifiers with different gains. The three different level signals were transmitted to wideband antennas located in the three different exposure chambers, corresponding to high, low and sham exposures

(Fig. 2b). Between each amplifier and antenna, a power sensor was placed to monitor the power supplied to the antenna in order to set the required SAR level in the exposed rats.

The exposure chamber was made of metal, had dimensions of $90\text{ cm} \times 90\text{ cm} \times 58\text{ cm}$, and the insides, except for the top, were covered with a 6-cm-thick planar RF absorber with a reflection loss of $>20\text{ dB}$. The shielding effectiveness was nearly 80 dB, which was the maximum within the working frequency bands for approximating a free-space environment. The wide-band antenna, which was

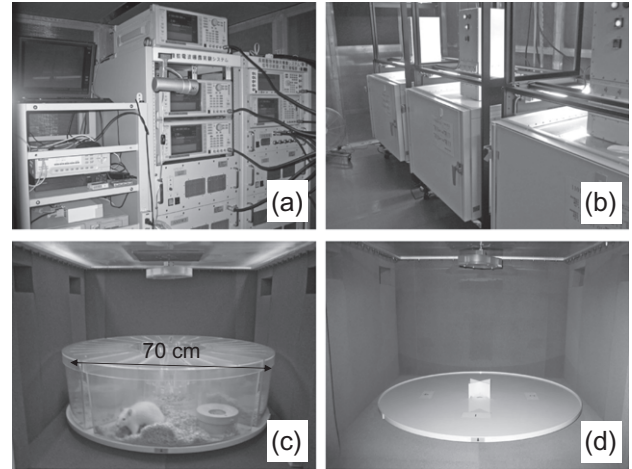


Fig. 2. Exposure apparatus for RF EMF generation. Panel (a) shows five signal generators with three amplifiers and power sensors. Panel (b) is an outside view of the three exposure chambers. Panel (c) is an inside view of an exposure chamber, with four 90° fan-shaped acrylic cages placed on the floor of the exposure chamber. There is an antenna beneath the ceiling. A mother rat and eight pre-weaning pups are in each cage. Panel (d) is an inside view of an exposure chamber without the animal cages.

Table 1. Specifications of eight different RF EMFs

	Signal	Frequency	Bandwidth
1.	IMT-2000 DS-CDMA System (ARIB STD-T63)	880 MHz	5 MHz
2.	IMT-2000 MC-CDMA System (ARIB STD-T64)	870 MHz	1.25 MHz
3.	IMT-2000 DS-CDMA System (ARIB STD-T63)	2.14 GHz	5 MHz
4.	IMT-2000 MC-CDMA System (ARIB STD-T64)	2.12 GHz	3.8 MHz
5.	Wireless LAN (IEEE 800.11b/g) (ARIB STD-T66)	2.437 GHz	20 MHz
6.	Mobile WiMAX (ARIB STD-T94)	2.61 GHz	10 MHz
7.	Next Generation PHS (ARIB STD-T95)	2.56 GHz	10 MHz
8.	Wireless LAN (IEEE 802.11a) (ARIBSTD-T71)	5.18 GHz	20 MHz

Subjects: pregnant SD rats and offspring. Exposure period:dams; gestational Day 7 to the birth (Stage 1) and weaning (Stage 2), and then F1 rats for three weeks after weaning (Stage 3). Exposure: whole body, 20 h per day. Stage 1: 1. High-exposure group: dam's whole body SAR = 0.4 W/kg; 2. Low-exposure group: dam's whole body SAR = 0.08 W/kg; 3. Sham-exposure group. Stages 2 and 3: 1. High-exposure group: offspring's whole body SAR < 0.4 W/kg; 2. Low-exposure group: offspring's whole body SAR < 0.08 W/kg; 3. Sham-exposure group.

designed to have an elliptic disc dipole structure with a major radius of 5.2 cm and a minor radius of 4.8 cm, was placed in the center of the ceiling (Fig. 2b). The voltage standing-wave ratio (VSWR) of the antenna was <2.2 at all of the considered frequencies, indicating that >80% of the signal power could be supplied to the antenna for exposure. The antenna was rotated slowly (one turn per second) to stir the internal field distribution and improve the uniformity of the EMF inside the exposure chambers (Fig. 2c and d). Because the distance between the rats in the cages and the antenna was 38–44 cm, i.e. more than one wavelength, even for the lowest frequency of 800 MHz, the exposure could be considered to be ‘far-field exposure’ (Fig. 2c). From the electric field measurement results in the rats’ activity area, using an electric field probe (HI-6005, ETS-Lingren Japan, Tokyo) at 860 MHz, 1.95 GHz and 5.2 GHz, the electric fields were found to follow a normal or Gaussian distribution. The normal or Gaussian distribution is a very common probability distribution. Its probability density function $p(x)$ of random variable x with mean μ and variance σ^2 is expressed as $p(x) = 1/\sqrt{2\pi}\sigma \exp[-(x - \mu)^2/2\sigma^2]$. All of the measured electric field values fell within the range of the mean value $\pm 60\%$, suggesting an acceptable uniformity of field distribution in the rats’ activity area in view of the variation of RF EMF intensity in the actual environment.

Eleven-week-old pregnant rats were simultaneously exposed to 8 RF EMFs for 20 h/day starting on GD 7 and continuing until weaning of the newborn pups; pups, selected as described above, were simultaneously exposed to the 8 RF EMFs for 20 h/day until they were 6 weeks old. Thus, rats were exposed to RF EMFs in three stages [Stages 1 to 3: (1) pregnant and lactating dams, (2) pre-weaning period pups, and (3) post-weaning period pups] until 6 weeks of age (Fig. 1). Since the biological effect is linked to the whole-body average SAR, not the electric field, we only paid attention to the whole-body average SAR in designing the exposure experiment. The rats’ SAR in the exposure system was evaluated using the finite difference time domain (FDTD) method, in conjunction with anatomically based numerical rat models. The pregnant rat model in the first exposure stage was developed from magnetic resonance imaging data, and the young rat models in the second and third exposure stages were developed from computer tomography data. To obtain the statistical characteristics of the rats’ positions in the exposure chamber, we set a camera in each exposure chamber and took photos of the rats’ positions inside their cages every five minutes for 20 h per day throughout the exposure period. The cameras were controlled by a personal computer, and the recorded photos were classified based on the rat’s positions in the cage. Using these photos, we derived each rat’s stay frequency w_n for each position. Second, we employed a self-developed software tool to produce 30 different rat positions in the exposure chamber, extracted from typical rat positions in the recorded photos. Third, we calculated the whole-body average SAR at each typical rat position. The FDTD-calculated whole-body average SAR is a function of rat position, frequency and exposure level. It is denoted as SAR(f_m, P_n, S_l), where f_m denotes the signal frequency, P_n denotes the rat’s position, and S_l denotes the exposure level. The exposure level for each frequency was assumed to be one-eighth of the total exposure level; therefore, for rats exposed to 0.4 W/kg, each frequency

was assumed to contribute 0.05 W/kg. Since the rats stayed in each position with a stay frequency of w_n , the whole-body average SAR in that position has the value of SAR(f_m, P_n, S_l) with a probability w_n during the exposure period. Thus, we obtained the whole-body average SAR at each frequency for each exposure level. The total SAR due to the multiple-frequency RF EMF exposure was obtained by totaling the SARs at each single frequency. We first calculated the whole-body average SAR at each frequency and then added them together to obtain the whole-body average SAR of the multiple-frequency exposure. This was based on current knowledge, that the biological effect of RF EMF is mainly a thermal effect. It is, therefore, reasonable to investigate the biological effect under a well-defined SAR. This is why we linked the whole-body average SAR with the observed biological effects. As seen in the SAR definition for the single-frequency exposure, SAR is proportional to the square of the electric field, not the electric field itself. For the simultaneous exposure of different frequencies, adding the SAR value for each exposure at different frequencies can yield the total SAR because of the linear addition feature of time-averaged absorbed power for the various frequency sources. The validity of this consideration was experimentally confirmed in our previous study [5].

Exposure protocol

All of the exposures were controlled with a personal computer. The 20 h/day exposure was started between 10:00 a.m. and 10:30 a.m., depending on animal care, and the exposure was stopped 20 h later (between 6:00 a.m. and 6:30 a.m. on the following day). During the 20 h exposure period, a 12 h light/dark cycle (light, 7:00 a.m. to 7:00 p.m.) and environmental conditions of $22 \pm 3^\circ\text{C}$ and $55 \pm 15\%$ humidity were maintained. Median whole-body average SARs were maintained at ~ 0.1 W/kg or 0.4 W/kg in each stage (SAR for dams in the first stage, and for offspring in the second and third stages), and the variation in the whole-body average SAR was within the range of the median value plus 83% and minus 67%. In light of the varying level of exposure to humans in a daily electromagnetic environment, such a variation was acceptable for unrestrained exposure of rats. As a result, the corresponding mean value, median value and mode value for the whole-body average SAR of multiple-frequency RF EMF exposure were 0.433, 0.407 and 0.406; 0.384, 0.415 and 0.414; and 0.389, 0.401 and 0.424 W/kg for the high exposure group at Stages 1 to 3, respectively. The corresponding values for the low-exposure groups at Stages 1 to 3 were one-fifth of the values for the high-dose groups.

Biological parameters

The biological parameters examined were the same as those described in our previous studies [4, 7–10]. The biological parameters evaluated were: growth, gestational condition, and organ weight for the dams; and survival rate, physical development, functional development, behavioral function, memory function, and reproductive ability of the F₁ rats. In addition to changes in the body weight of the dams and their offspring (F₁ and F₂), food consumption was measured. At weaning, the dams were sacrificed, and their major organs were weighed and examined macroscopically. To assess reproductive function, the number of implantations in the

uteri of the dams was examined, and the live birth index and number of live and dead offspring were recorded.

All the examinations were undertaken in an examination room outside the exposure chamber. We also measured the small amount of RF EMFs leaking out of the exposure chamber during the tests, which was almost equivalent to the amount of background RF waves already in the examination room.

Physical and functional development

Physical development of the offspring (F_1 pups) was investigated by checking seven markers: pinna unfolding, emergence of hair, eruption of incisors, eyelid opening, opening of vagina for females, and cleavage of the balanopreputial gland and descent of testes for males. These examinations were carried out unblindly within the 4-h non-exposure periods by two people.

Tests of the functional development of offspring included response to pain, pinna reflex, Preyer's reflex (ear 'jump'), corneal reflex, pupillary reflex, surface righting reflex, negative geotaxis reflex and mid-air righting reflex. An open-field test was used as a behavioral assay, and a water-maze test was used to assess memory function. The details of these tests have been previously described in [8].

The pups underwent the surface righting reflex test once per day on PNDs 6–10. A maximum of 5 s per trial was allowed. The pups underwent the negative geotaxis reflex once per day on PNDs 6–12. A maximum of 30 s per trial was allowed. Unsuccessful pups were given a score of 30 s. The pups underwent the mid-air righting reflex once per day on PNDs 13–19. The pups were dropped from a height of 30 cm in the supine position onto a cushion with a flat surface. A positive response was assigned when the pup landed on all four feet.

Behavior and memory function

Open-field test: The open-field test was conducted when the pups were 7 weeks old. The open field apparatus used was a square $60 \times 60 \times 30$ cm. The color of the apparatus was black. The lighting of the open field was ~200–300 lux, provided by a fluorescent lamp at the center of the field. During the test, white noise was produced at the level of ~60 dB in the center of the field. The animals were placed in the center of the field in a dark box. The numbers of ambulation, latency, rearing and grooming movements, and the incidences of urination and defecation were recorded. The observations were performed on three consecutive days for 10 min per day.

Water-maze test: 9-week-old rats were trained in a water maze. The apparatus consisted of a circular tank (1.5 m in diameter) of water (depth, 0.3 m; temperature, $25 \pm 2^\circ\text{C}$) made opaque by the addition of black ink. A transparent platform (12 cm in diameter) for escape from the water was submerged 1 cm below the water surface. Several salient cues were placed around the testing room to enable the rats to learn the location of the platform. A video camera was placed above the center of the pool and was connected to a color video tracking system (CAT-10; Muromachi Kikai, Tokyo, Japan) and a video recorder that allowed for on- and off-line automated tracking of the swim path of the rat in the pool. During the hidden platform test, the system measured the time, swim distance,

and swim path of each rat until the rat climbed onto the platform. These data were all recorded with a computer (Model DVT-1; Muromachi Kikai). The rats were first trained to find the hidden platform and to escape the water by climbing onto the platform fixed in the center of one of the four quadrants of the pool. They performed three trials per day with an interval of 1 min over five consecutive days. For each trial, the rats were placed into the pool facing the sidewall. The start positions were selected semi-randomly from three of four equally spaced wall locations, excluding the point nearest the platform. The rats were allowed to swim until they climbed onto the platform. If a rat successfully reached the platform, it was left on the platform for 30 s and was then returned to its cage. If a rat did not reach the platform within 120 s, it was placed on the platform by hand and was left there for 30 s. A probe test was administered 30 min after the last training trial. For this test, the platform was removed from the pool, and the rat was allowed to swim freely for 60 s from the side opposite where the platform used to be. The time spent in each of the quadrants and the swim path was measured by the tracking system.

Reproductive function

The estrus cycle and fertility were used as markers of reproductive function. The fertility of male and female F_1 offspring was examined to determine the number of successful copulations (copulation index). The number of days required for copulation was also recorded.

All of the organs from the F_1 dams were examined macroscopically, and the number of implantations was recorded. The other items examined were gestation period, delivery index, live birth index, number of offspring delivered per litter, number of live offspring per litter, number of dead offspring per litter, sex ratio, and number of live offspring with external abnormalities.

Data analysis

Because the experiments were performed in triplicate, the data on the body weight, number of implantations, and number of pups delivered per litter for the dams in each experiment in the sham-exposed groups were analyzed by the *F*-test. If homogeneous, the data were analyzed with Student's *t*-test, and if not, they were analyzed with Welch's test. Bartlett's test at $P < 0.05$ was used to analyze the body weight, gestation period, number of implantations, and organ weight of the dams and F_1 pregnant dams; the number of delivered offspring, number of live or dead offspring, body weight, physical development, functional development, open-field test, water-maze test, organ weight, and number of days for copulation of the F_1 animals; and the body weight of the F_2 animals. If data distribution was homogeneous, the data were analyzed using one-way analysis of variance; when a significant difference was observed, Dunnett's multiple parametric comparison test was applied. If data distribution was not homogeneous, the data were analyzed using the Kruskal–Wallis test; when a significant difference was observed, Dunnett's multiple non-parametric comparison test was applied.

The Kruskal–Wallis test was used to assess the significance of intergroup differences between the live birth index of the dams and

F₁ pregnant dams; sex ratio, external abnormalities, viability index, weaning index, and the physical and functional development of the F₁ animals; and the sex ratio, external abnormalities and viability index of the F₂ animals. When a significant difference was observed, Dunnett's multiple non-parametric comparison test was applied.

The significance of intergroup differences in the incidence of the delivery index of the dams and the mating and fertility index of the F₁ pregnant dams was analyzed using the Chi-square test. The significance of intergroup differences in the incidence of gross pathological lesions was assessed using Fisher's exact test, and the significance of the grade of lesions was evaluated using Wilcoxon's rank-sum test. In all cases, significance was set at $P < 0.05$.

The statistical analysis used here is limited in determining whether or not a true difference exists when small differences are observed between small groups ($n \approx 10$ per group). However, analyses based on the null hypothesis that 'a group difference does not exist' are passively accepted with a significance level of α [(Type I error): incorrect rejection of a true null hypothesis].

Stat Light 2000 software (Yukms, Tokyo, Japan) was used for the statistical analysis.

Animal care and protocols

The experimental design was planned by the Committee for the Study of Human Exposure to EMFs in Japan, which was established in 1997 with the aim of clarifying scientifically the effects of radio waves from mobile telephone terminals on the human body.

The animal facilities at the DIMS Institute of Medical Science, Inc., where the tests were conducted, have been fully accredited as compliant with the Good Laboratory Practice Standards by the Ministry of Health and Welfare of Japan, and the Ministry of Agriculture, Forestry, and Fisheries of Japan.

This study was performed according to the 'Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain' (Notice No. 88 of the Ministry of the Environment, 28 April 2006) and the 'Standards for Care and Use of Laboratory Animals at DIMS Institute of Medical Science' (1 September 2010). The protocol was approved by the animal experiment committee in the DIMS Institute of Medical Science, Inc.

RESULTS

Because there were no interim deaths of pregnant rats, no replacements were undertaken due to death in order to maintain constant exposure conditions.

Homoscedasticity of the three experiments

To confirm the reproducibility of the three sequential independent experiments, the experiments were compared using the data for the body weight of the dams, number of implantations, and number of pups delivered per litter. There was a significant difference in the body weight in sporadic cases: higher body weights were observed at GD 14 in the second experiment compared with in the first experiment; at PND 0 and 7 in the third experiment compared with in the second experiment; and at PND 0, 7 and 21 in the third experiment compared with in the first experiment. Lower body weights were observed at GD 14 in the third experiment compared with in the second experiment and at GD 20 in the third experiment compared with in the second experiment. However, no significant differences were noted in the number of implantations or number of pups delivered per litter. Based on these findings, it was concluded that there were no appreciable variations among the three experiments. Therefore, all of the data presented here are from Experiments 1 to 3 combined.

Table 2. Effects of EMFs on physical development of the offspring (F₁ pups)

Exposure level:	Sham	Low	High
No. of litter examined	11	12	11
Pinna unfolding(%)			
Day 2	67.0 ± 8.5	68.1 ± 9.6	61.5 ± 8.5
Day 3	100 ± 0.0	98.0 ± 1.3	97.1 ± 1.9
Day 4		100 ± 0.0	100 ± 0.0
Emergence of hair ^d	8.8 ± 0.1	8.9 ± 0.1	9.0 ± 0.1
Eruption of incisors ^d	10.6 ± 0.2	10.9 ± 0.1	11.0 ± 0.2
Eyelid opening ^d	13.8 ± 0.2	13.7 ± 0.1	13.9 ± 0.1
Opening of vagina ^d	33.9 ± 0.4	33.6 ± 0.3	33.9 ± 0.4
Cleavage of the balanopreputial gland ^d	41.9 ± 0.3	41.2 ± 0.4	40.9 ± 0.2
Descent of testis ^d	21.5 ± 0.2	21.5 ± 0.2	21.3 ± 0.1

Data were analyzed using Bartlett's test, and since all data was homogeneous, one-way analysis was applied. ^ddays (mean ± SE).

Dams (F0)

No adverse effects were observed in general condition or body weight of the rats. There were significantly higher values for delivery index and live birth index in the higher-exposed group compared with the sham-exposed group. No significant differences, however, were noted for gestational period, number of implantations, litter size, number of live offspring per litter, number of dead offspring per litter, sex ratio of the offspring, or external abnormalities. No macroscopic abnormalities, except for a few cases of dilatation of the uterus, were found in the higher-level exposure group. None of the organs showed significant changes in their weight (brain, pituitary, thyroids, lungs, heart, thymus, liver, kidneys, spleen, adrenals, ovaries or uterus).

F1 rats

During both the lactation and weaning periods, no effects were observed in the general conditions of the F₁ rats, except for a female in the low-level-exposure group that died on PND 8. No significant intergroup variations were found for body weight or viability indices at PND 4. As shown in Table 2, there were no significant intergroup variations in any of the seven physical development markers investigated (pinna unfolding, emergence of hair, eruption of incisors, eyelids opening, opening of vagina for females, and cleavage of the balanopreputial gland and descent of testes for males). The numbers of offspring tested for functional development in Groups 1–3 were 88, 96 and 88, respectively (equal numbers of males and females); all of the rats demonstrated good responses to pain, pinna reflex, Preyer's reflex, corneal reflex, and pupillary reflex. Figure 3 illustrates the age-dependent responses in the righting reflex on a surface, mid-air righting reflex and negative geotaxis tests; there were no significant differences between the groups, except for a significantly longer response time for negative geotaxis in the RF EMF-exposed groups compared with the sham-exposed group on PND 7 ($P < 0.05$ or $P < 0.01$).

None of the six items in the open-field test demonstrated an EMF exposure-associated change at any of the time points examined (data for ambulation and rearing only are shown in Table 3).

The completion time of the water-maze test tended to shorten over time in all of the animals tested (Fig. 4). No significant intergroup differences were present either in males or females. In the prove test, the time spent in the training quadrant was significantly decreased ($P < 0.01$) and time spent in the adjacent left was significantly increased ($P < 0.01$) for males exposed to the high-level EMF (Fig. 5). No differences were found in the females.

Autopsy found only one case of spleen discoloration in the male high-level-exposure group, but no particular lesions showed any statistically significant differences between the sham and exposed groups. No cause of death was identified by autopsy for the female offspring that died on PND 8. Organ weights measured at autopsy showed that absolute and relative prostate weights were lower in the high-dose-exposure group, and the absolute weight of the prostate in the low-level-exposure group was less than in the sham-exposed group. The relative weight of the female lungs was higher in the high-dose-exposure group compared with in the sham-exposed group. However, including the prostate weights, no organs demonstrated significant intergroup differences.

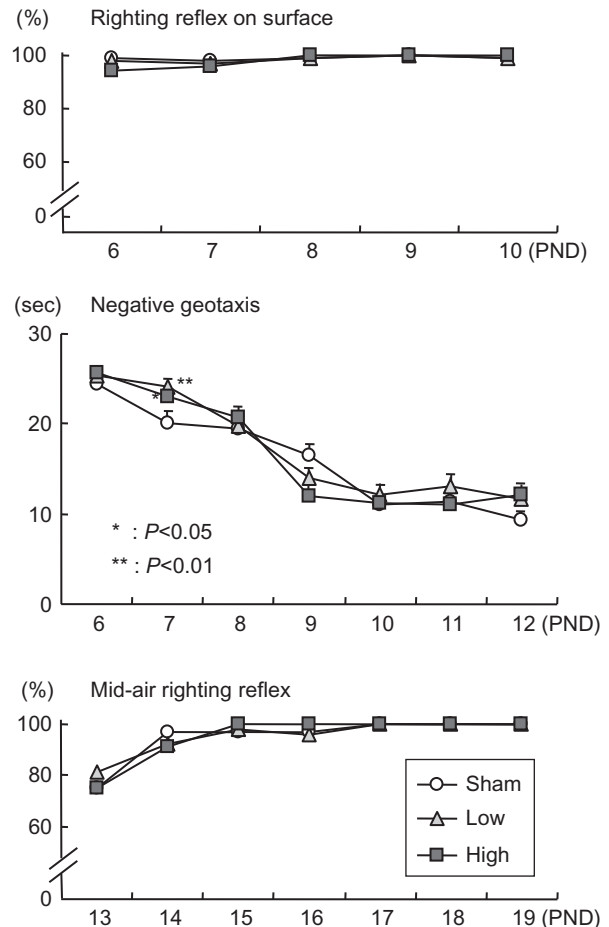


Fig. 3. Functional development of the F₁ animals. Eight tests of the functional development of the F₁ offspring were performed. This figure shows the data on the righting reflex on a surface, negative geotaxis, and mid-air righting reflex. Negative geotaxis testing at PND 7 showed a significantly slower response in the low-exposure-level ($P < 0.01$, Dunnett's test) and high-exposure-level groups (* $P < 0.05$, Dunnett's test) compared with the sham-exposure group. No significant differences were observed in either the righting reflex on the surface or the mid-air righting reflex tests. The data are expressed as means and SDs. Data were analyzed using Bartlett's test, and if data distribution was homogeneous, one-way analysis was used; when a significant difference was observed, Dunnett's multiple parametric comparison test was applied. If data distribution was not homogeneous, the data were analyzed using the Kruskal–Wallis test; when a significant difference was observed, Dunnett's multiple non-parametric comparison test was applied.**

There were no significant differences in the copulation index, fertility index or number of days for copulation between the F₁ offspring groups (Table 4). Furthermore, there were no significant

Table 3. Effects of EMFs on behavior function of F₁ animals (open field test)

	Level	Ambulation ^a			Rearing ^b		
		First day	Second day	Third day	First day	Second day	Third day
Female	Sham	280.3 ± 15.6	230.4 ± 14.8	209.9 ± 15.3	30.2 ± 4.0	19.5 ± 3.6	18.6 ± 3.9
	Low	320.0 ± 20.4	244.1 ± 19.4	211.4 ± 22.9	36.4 ± 2.3	22.1 ± 2.6	20.3 ± 2.3
	High	302.6 ± 18.0	206.4 ± 16.5	160.8 ± 15.7	27.1 ± 2.4	18.6 ± 2.9	13.0 ± 2.9
Male	Sham	320.8 ± 17.7	305.8 ± 18.0	288.6 ± 13.2	46.2 ± 2.8	34.6 ± 4.5	32.6 ± 4.3
	Low	334.2 ± 17.7	302.2 ± 19.8	306.0 ± 18.1	51.5 ± 2.9	38.7 ± 3.0	34.6 ± 3.9
	High	353.7 ± 21.6	303.7 ± 19.5	296.2 ± 22.5	52.0 ± 3.8	38.3 ± 5.5	35.7 ± 5.5

Number of animals examined; 12 rats in each group.

^aNumber of squares crossed in 10 min.

^bNumber of times. Data were analyzed using Bartlett's test, and if homogeneous, the data was analyzed using one-way analysis. If not homogeneous, the data was analyzed using the Kruskal-Wallis test.

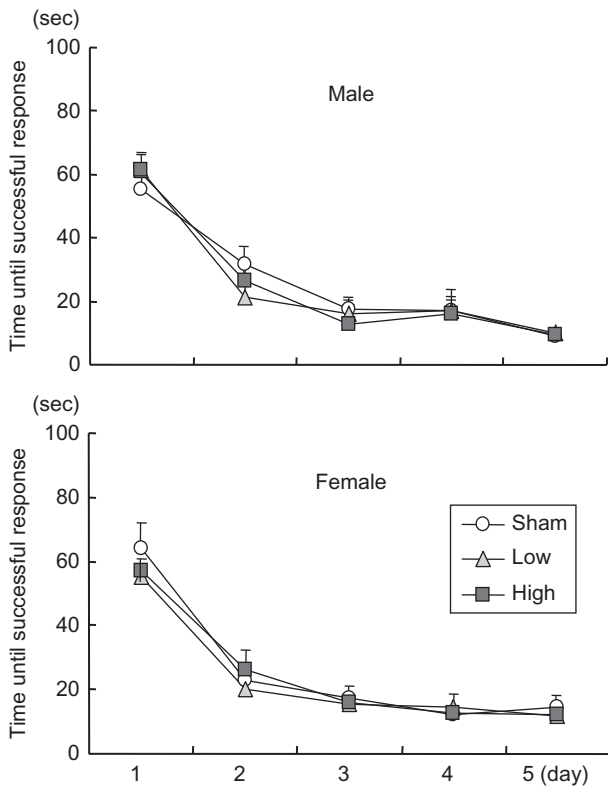


Fig. 4. Water maze test of the F₁ animals (hidden platform test). Detailed methods are described in the Materials and Methods section. Neither males nor females demonstrated any effects of RF EMF exposure in the hidden platform test. Data were analyzed using Bartlett's test, and since all data was homogeneous, one-way analysis was applied.

differences in gestation period, number of implantations, delivery index, live birth index, number of delivered F₂ offspring per litter, number of live and dead F₂ offspring per litter, sex ratio of F₂

offspring, or number of live F₂ offspring with external abnormalities (Table 4).

There were no significant differences in the body weights of the F₂ offspring at birth or at PND 4. In addition, the survival rate of the F₂ offspring did not show intergroup differences.

DISCUSSION

Contemporary society has developed ubiquitous communication networks worldwide. Consequently, human beings are increasingly being exposed to multiple-frequency RF EMFs. The possible adverse effects of local and/or whole-body RF EMF exposure, however, have been examined only at a single frequency. Animal experiments are considered to be an essential method for evaluating the potential effects of numerous agents on humans. Therefore, the establishment of satisfactory models of exposure to RF EMFs is essential for evaluation of the health effects on humans of simultaneous exposure to multiple-frequency RF EMFs.

The present report is the first report in the literature of animal exposure to multiple-frequency RF EMFs using a multigenerational approach. The exposure system used in the present study was established by Wang *et al.* [5]. In this system, antenna performance, electric field distribution and SAR were evaluated. In addition, after experimental validation of the FDTD modeling of the exposure system, the data were used for analysis of the SAR for anatomical rat models. Using photographs of rat activity inside the exposure apparatus throughout the experimental period to identify rat positions, the frequencies of the various positions occupied by the rats were obtained. The stay frequency was then used as a weighting factor in the calculation of the whole-body average SAR. This system offers a high-quality method for testing the biological effect of exposure to RF EMFs.

Using this exposure system, rat mothers and offspring were exposed simultaneously to eight different communication signal EMFs at average SARs of 0.08 and 0.4 W/kg.

Exposure to these levels of RF EMFs did not have any notable adverse effects on either the dams or the offspring. The parameters evaluated included the growth, gestational condition and organ

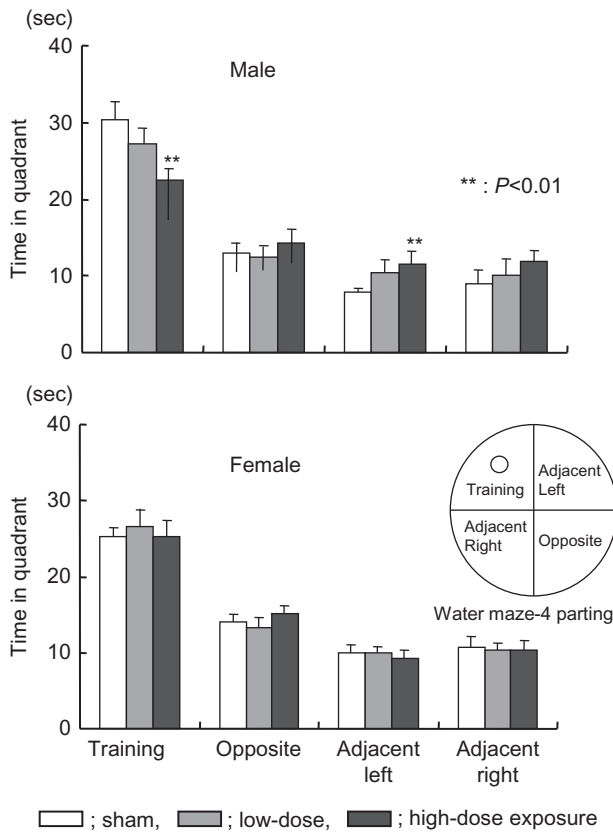


Fig. 5. Water maze test of the F₁ animals (prove test). In the prove test, a slight but significant ($P < 0.01$, Dunnett's test) increased time in the left adjacent quadrant was observed in the males from the high-exposure-level group compared with those in the sham-exposure group. The data are average times (s) and SDs. Data were analyzed using Bartlett's test, and if data distribution was homogeneous, one-way analysis was used; when a significant difference was observed, Dunnett's multiple parametric comparison test was applied. If data distribution was not homogeneous, the data were analyzed using the Kruskal-Wallis test; when a significant difference was observed, Dunnett's multiple non-parametric comparison test was applied.**

weights of the dams; the survival rates, growth, physical and functional development, memory function, and reproductive ability of the F₁ offspring; and the embryotoxicity and teratogenicity in the F₂ rats. Thus, under the conditions of the present study, whole-body exposure to multiple-frequency RF EMFs did not cause any adverse effects on pregnancy or on the development of rats. Note that positive findings were seen in the negative geotaxis test (Fig. 3) and the prove test (Fig. 5) in F₁ rats. Statistical significance was observed in the negative geotaxis in the low- and high-exposure groups at PND 7, but this was not dose dependent, and the difference disappeared later. In addition, in the prove test of the water-maze test, significant differences in staying time

were observed in the male high-exposure group, whereas the number of times for crossing the platform was similar to that of the sham-exposed group. Thus, it was considered that the changes observed in the negative geotaxis test and the prove test were incidental.

The RF EMF exposure levels used in our study are based on guidelines set by the ICNIRP and the Institute of Electrical and Electronics Engineers (IEEE). Animal studies [10, 11] have demonstrated a threshold effect for the occurrence of behavioral changes and alterations in core body temperature of $\sim 1.0^{\circ}\text{C}$ at a whole-body average SAR of $\sim 4\text{ W/kg}$. ICNIRP and IEEE guidelines use a value that is a factor of 10 lower than this whole-body SAR (i.e. 0.4 W/kg) as the basic restriction on whole-body SAR for workers in a controlled environment, and they set the restriction for exposure of the general public to a level 5 times lower than that for workers, i.e. 0.08 W/kg [6]. Thus, the exposure levels used in our study are relevant to humans.

Our group recently published multigenerational whole-body exposure experiments (on two or three generations) with a single-frequency RF EMF and did not find any adverse effects on dam fertility, offspring physical development, brain functions, behavior, or offspring fertility [4, 8]. The present experimental data are in agreement with our previous results and add the new result that simultaneous exposure to multiple-frequency RF EMFs did not seem to cause adverse effects on pregnancy or on offspring's physical or cognitive development. Our group also previously reported long-term effects of RF EMF on rat central nervous tumorigenesis initiated with N-ethyl-N nitrosourea (ENU). Two different experiments [9, 10] were conducted, in which the rats were exposed to 1.439 GHz TDMA signals and 1.95 GHz W-CDMA signals, respectively for 1.5 h/day, 5 days a week, for 2 years. Both experiments showed that those RF EMF exposures did not promote ENU-initiated central nervous system (CNS) tumorigenesis. Furthermore, development of tumors in organs other than the CNS was not enhanced. In these studies, the whole-body average SAR was set at $<0.4\text{ W/kg}$, and the experimental conditions were the same as in the present experiment, except that the irradiation site was the brain. In the present study, pups were exposed for 20 h/day for 6 weeks (approximately totaling 840 h). On the other hand, in the long-term carcinogenicity studies [9, 10], the total exposed hours were $<800\text{ h}$ in 2 years. Adey *et al.* conducted 24 months of local exposure to the brain after birth using an 836 MHz field; the maximum exposure time they studied was the highest, at 2400 h over 2 years [13]. However, biological effects of RF EMF exposure were not observed in either experiment, regardless of the exposure time/day, experimental period, or wavelength of RF EMF.

It should be noted that the main differences and similarities between single-frequency RF EMF exposure and multiple-frequency RF EMF exposure are concerned with the employed frequencies, waveforms and exposure levels. The single-frequency RF EMF exposure employed only one modulation waveform at one single frequency, while the multiple-frequency RF EMF exposure employed eight different modulation waveforms at multiple frequencies. However, the exposure levels, i.e. the whole-body average SARs, were set to the same level as that in Lee *et al.*'s studies [1, 2]. In comparison with the results reported in our previous studies for

Table 4. Effects of EMFs on fertility and reproductive function of F₁ animals

Exposure level:	Sham	Low	High
No. of examined animals (males/females)	12/12	12/12	12/12
No. of couples with successful copulation	12	12	12
Copulation index (%) ^a	100	100	100
Fertility index (%) ^b	83	100	92
No. of days for copulation	2.4 ± 0.3	2.7 ± 0.4	2.4 ± 0.3
No. of F ₁ animals examined	10	12	11
Gestation period (days)	22.3 ± 0.2	22.5 ± 0.1	22.5 ± 0.2
No. of implantations	13.7 ± 1.4	14.3 ± 0.9	15.5 ± 0.7
Delivery index (%) ^c	83	100	92
Live birth index (%) ^d	84.1 ± 6.2	88.3 ± 4.1	87.7 ± 4.5
No. of delivered per litter	12.7 ± 1.5	12.9 ± 1.1	13.7 ± 0.9
No. of live offspring per litter	12.2 ± 1.5	12.8 ± 1.0	13.5 ± 0.7
No. of dead offspring per litter	0.5 ± 0.4	0.1 ± 0.1	0.3 ± 0.3
Sex ratio ^e	60.2 ± 5.6	57.3 ± 4.3	49.1 ± 5.5
No. of offspring with external abnormalities (%)	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)

^a(No. of offspring with successful copulation/No. of mated offspring) × 100.

^b(No. of pregnant offspring/No. of offspring with successful copulation) × 100.

^c(No. of dams/No. of pregnancies) × 100.

^d(No. of live offspring/No. of implantation sites) × 100.

^e(No. of live male offspring/No. of live offspring) × 100.

Data were analyzed using Bartlett's test, and if data distribution was homogeneous, the data were analyzed using one-way analysis. If data distribution was not homogeneous, the data were analyzed using the Kruskal–Wallis test. As a result, no statistically significant difference was observed between the groups.

single-frequency RF EMF exposure [4, 8], no adverse effects were detected for either single-frequency RF EMF exposure or multiple-frequency RF EMF exposure at the same whole-body average SAR of 0.4 W/kg. This finding supports the current knowledge that biological effects of RF EMFs are mainly dependent upon the whole-body average SAR, which means that the only possible effect is the thermal effect for the investigated wireless communication frequency bands.

It should also be noted that all the functional development, water-maze, and behavioral tests of offspring were conducted after the RF EMF exposure. Of course, it would be ideal to conduct the endpoint measurement in real-time exposure, but simultaneous operation of exposure and measurement were practically infeasible in our experiment. Even so, by comparing differences detected in the offspring between the exposed group and the sham-exposed group, it is still possible to observe the effects of RF EMF exposure at the endpoints of interest.

In conclusion, under the present experimental conditions, simultaneous whole-body exposure to eight different communication signal EMFs did not show any adverse effects on pregnant dams, the physical or cognitive development of F1 offspring, the fertility of F1 offspring, or the development of F2 offspring.

ACKNOWLEDGEMENTS

We are grateful to Dr Senji Kondo for his extensive help with the statistical analysis. We are also grateful to Dr David B. Alexander for critical reading of the manuscript.

FUNDING

This work was supported by a grant from the Ministry of Internal Affairs and Communications of Japan, for 2010 and 2011.

CONFLICT OF INTEREST

The authors report that there are no conflicts of interest.

REFERENCES

1. Lee H-J, Lee J-S, Park J-K, et al. Lack of teratogenicity after combined exposure of pregnant mice to CDMA and WCDMA radiofrequency electromagnetic fields. *Radiat Res* 2009;172: 648–52.
2. Lee H-J, Jin YB, Kim T-H, et al. The effects of simultaneous combined exposure to CDMA and WCDMA electromagnetic fields on rat testicular function. *Bioelectromagnetics* 2012;33: 356–64.

3. Scientific Committee on Emerging and Newly Identified Health Risks. *Opinion on Potential Health Effects of Exposure to Electromagnetic Fields (EMF)*. http://ec.europa.eu/health/scientific_committees/emerging/docs/scenih_r_o_041.pdf (9 May 2016, date last accessed).
4. Shirai T, Imai N, Wang J, et al. Multigenerational effects of whole body exposure to 2.14 GHz W-CDMA cellular phone signals on brain function in rats. *Bioelectromagnetics* 2014;35:497–511.
5. Wang J, Liao W, Kawai H, et al. Performance and validation of a broadband-multigeneration exposure system for unconstrained rats. *IEEE Trans Microwave Theory Tech* 2013;61:326–34.
6. ICNIRP. Guidelines for limiting exposure to time-varying electric, magnetic, and electromagnetic fields (up to 300 GHz). *Health Phys* 1998;74:494–522.
7. Ogawa K, Nabae K, Wang J, et al. Effects of gestational exposure to 1.95-GHz W-CDMA signals for IMT-2000 cellular phones: lack of embryotoxicity and teratogenicity in rats. *Bioelectromagnetics* 2009;30:205–12.
8. Takahashi S, Imai N, Nabae K, et al. Lack of adverse effects of whole-body exposure to a mobile telecommunication electromagnetic field on the rat fetus. *Radiat Res* 2010;173:362–72.
9. Shirai T, Kawabe M, Ichihara T, et al. Chronic exposure to a 1.439 GHz electromagnetic field used for cellular phones does not promote N-ethylnitrosourea induced central nervous system tumors in F344 rats. *Bioelectromagnetics* 2005;26:59–68.
10. Shirai T, Ichihara T, Wake K, et al. Lack of promoting effects of chronic exposure to 1.95-GHz W-CDMA signals for IMT-2000 cellular system on development of N-ethylnitrosourea-induced central nervous system tumors in F344 rats. *Bioelectromagnetics* 2007;28:562–72.
11. Stern S, Margolin L, Weiss B, et al. Microwaves: effects on thermoregulatory behavior in rats. *Science* 1979;206:1198–201.
12. Adair ER, Adams BW. Microwaves modify thermoregulatory behavior in squirrel monkey. *Bioelectromagnetics* 1980;1:1–20.
13. Adey WR, Byus CV, Cain CD et al. Spontaneous and nitrosourea-induced primary tumors of the central nervous system in Fischer 344 rats exposed to frequency-modulated microwave fields. *Cancer Res* 2000;60:1857–63.