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Absence of Mutagenic Effects of 2.45 GHz Radiofrequency Exposure in Spleen, Liver, Brain, and Testis of lacZ-Transgenic Mouse Exposed in Utero

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—— A possible mutagenic effect of 2.45 GHz radiofrequency exposure was examined using lacZ-transgenic Muta™ mice. Pregnant animals were exposed intermittently at a whole-body averaged specific absorption rate of 0.71 W/kg (10 seconds on, 50 seconds off which is 4.3 W/kg during the 10 seconds exposure). Offspring that were exposed in utero for 16 hours a day, from the embryonic age of 0 to 15 days, were examined at 10 weeks of age. To minimize thermal effects, the exposure was given in repeated bursts of 10 seconds of exposure followed by 50 seconds of no exposure. Mutation frequencies at the lacZ gene in spleen, liver, brain, and testis were similar to those observed in non-exposed mice. Quality of mutation assessed by sequencing the nucleotides of mutant DNAs revealed no appreciable difference between exposed and non-exposed samples. The data suggest that the level of radiofrequency exposure studied is not mutagenic when administered in utero in short repeated bursts.

———— radiofrequency field; fetal exposure; DNA alteration; mutation

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The rapid expansion of mobile phone and microwave oven utilization has given rise to widespread concern about the safety of radio-frequency (RF) exposure. Reviews of extensive studies on many kinds of biological effects of RF exposure indicate that most of significant biological influences of RF exposure are attributable to hyperthermic effects. Effects are observed when the absorbed energy per kg body weight per second (called the specific absorption rate, SAR) exceeds about 4 W/kg for periods of about 1 hour. Such exposure is associated with an increase in body temperature of about 1°C (International Programme on Chemical Safety 1993; International Commission on Non-Ionizing Radiation Protection 1998; Brusick et al. 1998; Verschaeve and Maes 1998; Moulder et al. 1999). However, it is not clear yet whether RF exposure has a biological effect when administered repeatedly under low or non thermal conditions (Brusick et al. 1998; Moulder et al. 1999).

Several recent studies using cultured cells under artificially controlled temperature to minimize thermal effects have revealed that exposure to 1-10 W/kg of RF energy for 24 hours induced no effects on DNA structure or chromosomes (Li et al. 2001; Vijayalaxmi et al. 2001a), whereas in nematodes, a very low SAR dose of RF exposure, such as 1 mW/kg for 18 hours, was reported to induce stress-response genes (De Pomerai et al. 2000). Recent results with mammals have been complicated. Micronuclei frequencies in the peripheral blood and bone marrow cells of rats were not affected by exposure to 2.45 GHz for 24 hours at 12 W/kg (Vijayalaxmi et al. 2001b). Nor were stress-related responses induced in rats exposed to pulsed microwaves at 5 W/kg in the brain and 1.38 W/kg in the body for 2 hours (Stagg et al. 2001). In both cases, the increase in body temperature was minimal. On the other hand, Sykes et al. (2001) recently reported that repeated exposure of mice to RF energy at 4 W/kg for 25 days at 30 minute/day suppressed spontaneous mutant frequency in the spleen. Tumor-promoting activity of RF exposure is also controversial. For example, Repacholi et al. (1997) showed acceleration of lymphoma incidence in Eμ-Pim1 transgenic mice by exposure to pulsed RF exposure at 0.13-1.4 W/kg for 1.5 years at a rate of two 30-minute exposure episodes per day. On the other hand, Frei et al. (1998) reported no effect on mammary cancer incidence in C3H/HeJ mice exposed to RF radiation at 1 W/kg for 1.5 years at 20 hour/day. The more recent report of Utteridge et al. (2002) showed no induction of lymphoma in Eμ-Pim1 mice exposed to 0.25-4 W/kg for 2 years at a rate of 1 hour/day, 5 day/week.

These studies demonstrate a need to investigate the effects of long periods of exposure to RF energy in the range of 0.1-10 W/kg from different biological viewpoints. Among many biological indices, mutation of DNA is interesting because it is one of the major causes of cancer and, probably, of senescence-associated disorders (Hoeijmakers 2001). Although RF exposure is non-ionizing and is not likely to damage the chemical structure of DNA directly, the DNAs in living cells are constantly undergoing replication, spontaneous damage, and repair. The processes of DNA replication and repair are performed by many kinds of proteins at high but not perfect fidelity that results in mutations (Hoeijmakers 2001; Kunkel and Bebenek 2000). In fact, deficiency in mismatch repair proteins is known to result in a very high incidence of mutation (Prolla et al. 1998; Yao et al. 1999). Thus, it is possible that non-ionizing radiation influences protein functions and leads to alteration of DNA.

Here, we chose SAR level of 4.3 W/kg, which was similar to the proposed threshold level of 4 W/kg, and investigated whether it had a mutagenic effect when administered repeatedly at the fetal stage. For the frequency of RF, 2.45 GHz was selected because it is used widely in microwave ovens and close to the frequencies used in mobile phones. To minimize thermal effects, we applied RF exposure at an intermittent fixed SAR. The fetal development stage was selected because it is the period in life when sensitivity is high to environmental stresses such as ionizing radia-

tion, e.g., x-rays (National Council on Radiation Protection and Measurements 1998). We used *lacZ*-transgenic mice because they enables us to study mutations in different tissues of individuals (Gossen et al. 1991; Ono et al. 1997, 1999, 2000).

Materials and Methods

RF Exposure

RF exposure of 2.45 GHz (continuous wave) was generated by a signal generator (HP8683A, Hewlett-Packard, Spokane, WA, USA) and amplified to an output of 20 W by an amplifier (GRF4025, Ophir RI Inc., Los Angeles, CA, USA). The signal was fed to a horn antenna attached from above to an exposure box of 30×40 ×26 cm, as shown in Fig. 1. The four mice were located in a two liter plastic beaker, which had been separated into four equal spaces by plastic plates, and set at the center of the exposure unit. Application of the FDTD (finite-difference time-domain) calculation based on the measured electric fields, as detailed by Wang et al. (2002), to the four mice gave a whole-body average SAR of 4.25 W/kg when the four mouse bodies were parallel to the electric field excitation (x-direction), 4.11 W/kg when the four mouse bodies were perpendicular to the electric field excitation (y-direction), and 4.50 W/kg when the mice were standing (z-direction). The average dose was determined to be 4.3 W/kg for the three situations. The exposure to mice was done continuously or intermittently. The intermittent exposures were 20 seconds of exposure followed by 40 seconds of no exposure (1.4 W/kg) or 10 seconds of exposure followed by 50 seconds of no exposure (0.71 W/kg).

Mice

Muta™ mice (Gossen et al. 1989) were purchased from Covance Research Products, Inc. (Denver, PA, USA). At 10 weeks of age they were mated, and females showing plugs the next morning were used for exposure. Starting from the evening of that day, the mothers were exposed to RF energy for 16 hours per day, from 1700 to 0900 the following day. From 0900 to 1700, they were kept in an animal room under regular conditions. The exposure was repeated daily for 15 days (i.e., until the embryo was 15 days old). Over a 16-hour exposure period, each mouse was provided with three pieces of chow, each about 3 g (CE-2, Clea Japan, Inc., Tokyo). After day 15 of pregnancy, the mice were maintained in the

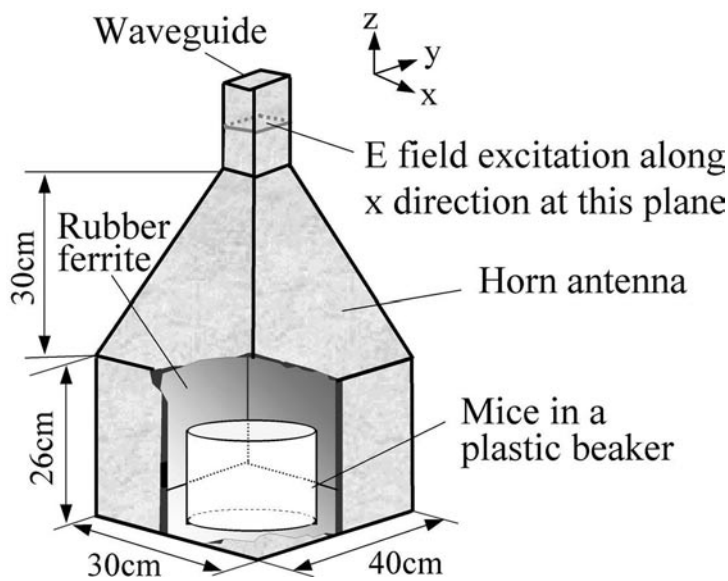

Fig. 1. An illustration of exposure box. The insides of the exposure box were inlaid with planar rubber ferrite absorber. The box was air-ventilated. The details are described by Wang et al. (2002).

animal room. When the newborn mice became 10 weeks of age, the spleen, liver, brain, and testis were removed and frozen. The studies on mice were conducted according to the Guideline for Animals Welfare and Experimentation of Tohoku University.

Body temperature

Rectal temperature was obtained with a digital thermometer TD-300/320 (Shibaura Denshi Co. Ltd., Tokyo) equipped with a 2 mm-diameter probe. The measurement was performed right before exposure at 1700 and right after exposure at 0900 on the following day. Control mice located in the same room, but away from the exposure unit, underwent the same measurement.

Mutant frequency

DNA was extracted from the four types of tissue as described previously (Ono et al. 1997). For testis, seminiferous tubules were separated before DNA isolation as described in a manual provided by Stratagene for Big-Blue^{TM} assay (La Jolla, CA, USA).

The lacZ genes in the mouse genome were retrieved as a part of the lambda genome in phage particles. The total number of phage was assayed as plaques on E. coli C (lacZ$^-$, galE$^-$), and the number of mutant phage was determined on the same bacteria in the presence of P-gal. The mutant phage were confirmed by the absence of X-gal digestion (Ono et al. 1997).

DNA sequencing

DNA was extracted from each mutant phage plaque by use of phenol, and the lacZ gene DNA was amplified by PCR as 6 overlapping fragments of DNA, each about 600 base pairs (bp) long. Each DNA fragment was sequenced by use of the PRISM 377 (PE Applied Biosystems, Foster City, CA, USA). Sequencing was performed in accordance with the manufacturer's manual. The DNA sequence thus deduced was compared to that of the wild type lacZ gene. Details were described previously (Ono et al. 2000).

Statistical analysis

Mutant frequencies in each tissue with or without exposure to RF energy were analysed by Student's or Welch's *t*-test. The spectra of frequencies of different types of mutations were analysed by χ^2-test. A $p<0.05$ was considered statistically significant.

Results

Selection of exposure condition

We selected a rather high dose rate of 4.3 W/kg (whole-body average). Since a long period of exposure to this SAR was expected to raise body temperature, we examined rectal temperature before and after 16 hours of exposure at various intervals of exposure (Table 1). The continuous exposure raised body temperature by 1.38°C, whereas intermittent exposure of 20 seconds separated by 40 seconds of no exposure (1.4 W/kg) raised body temperature by

Table 1. *Rectal temperature rise (2.45 GHz)*[a]

Exposure condition	SAR	Exposed	Non-exposed	Net
Continuous	4.3	0.95±0.17	–0.43±0.36	1.38
20 sec exposure +40 sec no exposure	1.4	0.33±0.53	–0.28±0.38	0.61
10 sec exposure +50 sec no exposure	0.71	0.23±0.22	–0.20±0.36	0.43

[a] Rectal temperature after 16 hours of exposure was subtracted from that observed before the exposure. The average values for 4 mice and the standard deviations are shown.

0.61°C. A reduction of exposure time to 10 seconds followed by 50 seconds of no exposure (0.71 W/kg) resulted in a temperature increase of 0.43°C. The minus values observed in non-exposed mice, shown in Table 1, were assumed to reflect the circadian rhythm in body temperature. We assumed that a 0.43°C rise in temperature would not be harmful to mice (International Programme on Chemical Society 1993), and set exposure at a rate of 10 seconds per minute.

Mutant frequency

The pregnant mothers were subjected to 15 daily exposures, and were brought back to the animal room after each exposure period. They gave birth normally, and the pups developed without any noticeable abnormalities. At 10 weeks of age, 3 males were randomly selected and the liver, spleen, brain, and testis were removed for analysis of mutant frequency in the lacZ gene. The number of genes examined, the number of mutants found, and the mutant frequencies calculated are shown in Table 2. In the Table, the mutant frequencies of unexposed mice (previously determined from 3 to 4 individuals [Ono et al. 1997, 2000]) are also shown. The levels of spontaneous mutant frequencies in the young unexposed mice were examined routinely in the past nine years in our lab and they always fell in the same ranges as were shown in the Table. The data in Table 2

TABLE 2. *Comparison of mutant frequencies in 4 tissues after exposure to 0.71 W/kg to those in unexposed samples*

Tissue	Individual	Total number of phage examined	Number of mutant phage	Mutant frequency (10^{-5})	Average ±S.D. (10^{-5})	Unexposed Average± S.D. (10^{-5})
Liver	1	584 480	31	5.30		
	2	606 840	38	6.26		
	3	818 025	38	4.64	5.40±0.81	6.66±2.65[a]
Spleen	1	1 114 750	49	4.39		
	2	962 000	61	6.34		
	3	839 474	56	6.68	5.80±1.24	6.51±2.48[a]
Brain	1	454 480	37	8.14		
	2	670 410	47	7.01		
	3	1 204 613	32	2.66	5.94±2.89	7.06±1.11[b]
Testis	1	1 796 762	89	4.95		
	2	956 800	42	4.38		
	3	752 538	70	9.30	6.21±2.69	6.95±0.94[b]

[a]Data reported previously (Ono et al. 1997).
[b]Data reported previously (Ono et al. 2000).

TABLE 3. *The numbers of mutants sequenced and of mutations found*

	Liver	Spleen	Brain	Testis
Sequenced mutant	38	20	15	20
Mutant with sequence alteration	37	20	15	20
Independent mutant[a]	28	12	12	14

[a]Independent implies that the mutant is not a copy of a previous mutation arising by DNA replication. Independence was ensured by eliminating the redundant mutants found in one set of tissue DNA preparation.

TABLE 4. *List of molecular changes in independent mutations*

Tissue	Location[a]	Sequence[b]	Change[c]
Liver	188–194	CGAA….CGCT	del (–7)
	192	TGGCG	G → A
	303	TACGA	C → G
	455	CGTTA	T → C
	625	TGTGG	T → C
	637	GCGGC	G → A
	840	TTCGG	C → A
	969–1233	ACCG…ACGG	del (–265)
	1072	TTCGA	C → T*
	1090	ACGAG	G → A*
	1187	TTCGC	C → T*
	1196	TCCGA	C → T
	1584	AAATG	del (–1)
	1601–1617	TGGA…TGAT	del (–17)
	1627	GCGAA	G → A*
	1832	CGGTC	G → A
	2041	CACAA	C → T
	2340	AAA•CAA	ins (+A)
	2392	ACGAC	G → A
	2545	GGCAG	C → T
	2586	TACCG	C → G
	2713	TCGGA	G → A
	2740	CCGAC	G → A
	2840	GACGC	C → A
Spleen	637	GCGGC	G → A
	764–813	CGAG…CGCA	del (–50)
	928	CCGAA	G → A
	1072	TTCGA	C → A
	1187	TTCGG	C → T
	1388	GTCGC	C → T
	1627	GCGAA	G → A
	1831	ACGGT	G → A
	2286	TGGCA	G → C
	2374	CCCGT	C → T
	2392	ACGAC	G → A
	2455	CGGCG	del (–1)
Brain	577	TGA•TGG	ins (+A)
	598	ACGGC	G → C
	1018	GCGAG	G → T
	1072	TTCGA	C → T
	1187	TTCGC	C → T
	1584	AAA•TGG	ins (+A)

TABLE 4. *Continued*

Tissue	Location[a]	Sequence[b]	Change[c]
	1627	GC<u>G</u>AA	G → A
	1636	CC<u>C</u>AC	C → A
	1898	TTT•CCA	ins (+T)
	2375	CC<u>G</u>TG	G → A
	2392	AC<u>G</u>AC	G → A
	2813	CC<u>C</u>GA	C → T
Testis	292	GG<u>C</u>AG	C → T
	367	CG<u>G</u>AG	G → T
	636	AG<u>C</u>GG	C → G
	1072	TT<u>C</u>GA	C → T
	1090	AC<u>G</u>AG	G → A
	1187	TT<u>C</u>GC	C → T
	1527	TA<u>C</u>GC	C → A
	1627	GC<u>G</u>AA	G → A
	2391	AA<u>C</u>GA	C → A
	2659	CG<u>C</u>GG	C → T
	2743	AC<u>C</u>GC	C → T
	2805	TA<u>C</u>GT	C → A
	2813	CC<u>C</u>GA	C → T
	3067	AC<u>C</u>AG	C → T

[a] Numbering of nucleotides started at the first base of the initiation codon.

[b] … indicates a row of nucleotides in the deleted sequence, and • indicates a position at which an insertion took place.

[c] Del indicates a deletion-type mutation and ins indicates insertion mutation. The numbers in the parentheses indicate the number of nucleotides altered.

[*] Indicates that the type of mutation was observed twice in tissue samples from two individual mice.

show similar levels of mutant frequencies in exposed and unexposed mice in the four types of tissue studied (p=0.47-0.67).

Quality of mutation

Although the mutant frequency was statistically not different between the two groups, the quality of mutation in exposed mice might differ from that in non-exposed mice. To compare the quality of mutation in exposed and non-exposed mice, we isolated mutant phage from exposed tissue DNAs and determined the nucleotide sequences. Table 3 shows the numbers of mutants sequenced for each type of tissue. After the sequencing, the mutants showing the same molecular nature in terms of alteration of nucleotides and position of mutation were counted as one independent mutation; thus, mutants that arose via replication of mutated DNA were not considered. The remaining mutants were classified as independent mutants (Table 3). Table 4 lists the alterations for nucleotides in the independent mutants.

Frequencies of the various mutations in exposed mice were compared to those of unexposed mice, as indicated in Fig. 2. The spectra indicate that the most predominant form of mutation in DNA was a G:C to A:T transition at the CpG site.

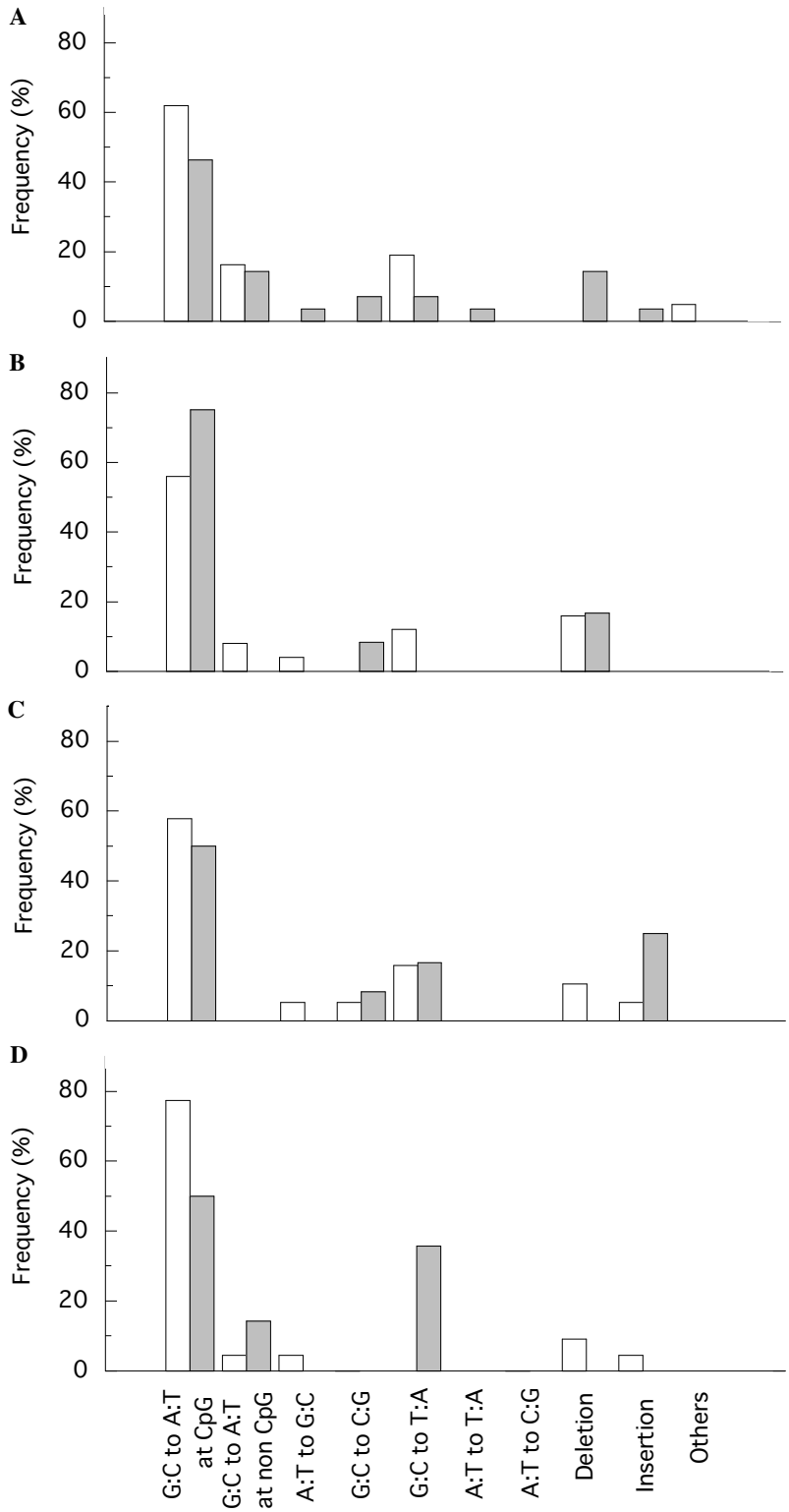

Fig. 2. Frequencies of different types of mutations found in non-exposed and RF exposed mice. The tissues examined were liver (A), spleen (B), brain (C) and testis (D). Only the independent mutations were counted for calculation. Open columns indicate non-exposed mice and the grey columns indicate exposed mice. In all tissues, there was no statistically significant difference (χ^2-test) between the spectra of non-exposed and exposed samples.

The other mutations were alterations in minor fractions. The statistical analysis of the spectra showed no significant difference between control and exposed mice in the four kinds of tissues (p-values were 0.240 for liver, 0.433 for spleen, 0.506 for brain and 0.055 for testis). Thus the spectra of mutation suggest that the quality of mutation is similar between exposed and non-exposed animals.

Discussion

It is noted that the lacZ gene used in the present study to monitor mutation is not expressed in vivo. Hence, the mutation on the gene has no effect on cellular function and would not be selected by elimination through cell death. In other words, the lacZ gene could monitor any mutation that occurred before examination. Thus, the efficiency to detect mutation could be higher in the lacZ gene than in the genes which are functioning in cells. One limitation of the present study is that the mutation assay system used can detect only small mutations; those expanding several kbp or less of DNA (Gossen et al. 1989). Larger changes can occur in vivo because of large scale deletion and recombinational events (Nakamura et al. 2000). Although small mutations, such as one base substitutions are known to be predominant among spontaneous mutations (Crow 2000), the nature and range of mutation that may be induced by RF exposure is not yet clear. Very recently, Sykes et al. (2001) found a slight anti-mutagenic effect of 4 W/kg RF exposure in response to repeated exposure over 25 days at 30 minute/day. In that system, they monitored recombinational events but did not monitor small mutations. Moreover, the effect was observed in adult mice. Thus, a comparison of their findings with ours is difficult.

Our findings here indicate that an average SAR of 0.71 W/kg and 4.3 W/kg peak (2.45 GHz) is not mutagenic in the early mouse embryo when exposure is intermittent and the increase in body temperature is limited to 0.43°C. The period of exposure, day 0 to day 15 of embryonic age, spans the beginning of embryogenesis to the end of organogenesis. This is the period in life during which the organism is most sensitive to environmental stress such as ionizing radiation, e.g., x-rays (National Council on Radiation Protection and Measurements 1998). Given that the level of RF exposure was unlikely to damage the chemical structure of DNA, the data suggest that the DNA replication and the DNA repair systems were not affected by RF exposure in utero.

It is noted that the way of exposure adopted in the present experiment was intermittent as 10 seconds of exposure to 4.3 W/kg followed by 50 seconds of no exposure. The total energy deposit in mice exposed in this way would be similar to that given by continuous exposure to 0.71 W/kg (4.3 W/kg divided by 6). Thus, the data obtained in the present study could be extrapolated to those of continuous exposure with 0.71 W/kg. The extrapolation, however, needs a caution, because biological responses against external stresses often vary depending on the way of exposure. For example, cellular transformation induced by fission-spectrum neutrons becomes more frequent when the cells are irradiated in protracted way rather than irradiated in a short period of time (Hill et al. 1984). Hence, the biological effects induced by intermittent exposure could be different from those induced by a continuous exposure even if the net energy transfer is the same.

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