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RESEARCH ARTICLE

Life-span exposure to sinusoidal-50 Hz magnetic field and acute low-dose γ radiation induce carcinogenic effects in Sprague-Dawley rats

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ABSTRACT

Background In 2002 the International Agency for Research on Cancer classified extremely low frequency magnetic fields (ELFMF) as a possible carcinogen on the basis of epidemiological evidence. Experimental bioassays on rats and mice performed up to now on ELFMF alone or in association with known carcinogens have failed to provide conclusive confirmation.

Objectives To study the carcinogenic effects of combined exposure to sinusoidal-50 Hz (S-50Hz) magnetic fields and acute γ radiation in Sprague-Dawley rats.

Methods We studied groups of male and female Sprague-Dawley rats exposed from prenatal life until natural death to 20 or 1000 μT S-50Hz MF and also to 0.1 Gy γ radiation delivered as a single acute exposure at 6 weeks of age.

Results The results of the study showed significant carcinogenic effects for the mammary gland in males and females and a significant increased incidence of malignant schwannomas of the heart as well as increased incidence of lymphomas/leukemias in males.

Conclusions These results call for a re-evaluation of the safety of non-ionizing radiation.

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S-50Hz MF carcinogenicity; cancer promotion; Sprague-Dawley rats; life-span bioassays; breast cancer; hemolymphopoietic neoplasias; heart malignant schwannomas

Introduction

From 1979 until now the results of numerous epidemiological research projects carried out on children living in houses near electricity power lines as well as on occupationally-exposed workers have suggested that there is a potential carcinogenic risk from electricity-generated magnetic fields. An initial association between exposure and leukemia in children was proposed in 1979 (Wertheimer & Leeper 1979). Subsequently Milham (1982) reported a correlation between leukemia and extremely low-frequency magnetic fields (ELFMF) in adults. An increased relative risk of breast cancer was observed in women, particularly in those under 55 years of age (Wertheimer & Leeper 1979). An excess of male breast cancers associated with ELFMF exposure was reported later (Matanoski and Breysse 1989; Matanoski et al. 1991; Demers et al. 1991; Tynes et al. 1992). In 2000, a pooled analysis identified a significant increased risk of childhood leukemia at exposures in excess of 0.4 μT (Ahlbom et al. 2000). The epidemiological evidence led the International Agency for Research on Cancer to classify ELFMF as a possible carcinogen (World Health Organization [WHO]-IARC 2002). Further case control studies (Draper et al. 2005; Kroll et al. 2010; Sermage-Faure et al. 2013) and a pooled analysis based on primary data (Kheifets et al. 2010) confirmed the reliability of an approximately two-fold increased risk of childhood leukemia at magnetic fields levels above 0.3–0.4 μT.

However, although during the past three decades several epidemiological studies have taken into consideration various exposure situations using different approaches, consistent evidence of an association was observed only with leukemia in children (Kheifets et al. 2010) and chance or confounding factors, including selection bias, might have contributed to uncertainty in correlating exposure to ELFMF and other tumor sites, particularly the ones related to occupational exposure (Grellier et al. 2014).

Indeed, despite the epidemiological evidence of an association, experimental studies in which ELFMF were administered alone have failed to provide conclusive confirmation. Experimentally, it has not been possible to identify a carcinogenic effect from magnetic fields and no accepted mechanism by which they might cause cancer has been described. Up to now, five long-term carcinogenicity bioassays on ELFMF administered alone, four conducted on rats (Margonato et al. 1995; Mandeville et al. 1997; Yasui et al. 1997; Boorman et al. 1999b) and one on mice (McCormick et al. 1999) have failed to show convincing evidence of any carcinogenic effect. The results of the NTP study showed equivocal evidence for the carcinogenic activity of 60-Hz magnetic fields in Fischer 344 rats on the basis of the increased incidence of thyroid gland C-cell neoplasms in males exposed to 2 or 200 μT. There was no evidence of carcinogenicity in male rats exposed at 1000 μT or again in female or male mice (Boorman et al. 1999b).

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Several studies have been performed to evaluate the carcinogenic effects of combined exposure to ELFMF and to well-known chemical carcinogenic agents. Up to now, the results of these studies have shown either weak or equivocal evidence of the ELFMF capacity to enhance the carcinogenic effects of initiating agents with particular reference to mammary cancer (Beniashvili et al. 1991; Boorman et al. 1999a; Fedrowitz & Loscher 2008), lymphoma and leukemias (Boorman et al. 1999b; McCormick et al. 1999; Babbitt et al. 2000) and skin cancer (WHO-IARC 2002).

In conclusion, studies to date have typically lacked the size to identify rare events and have not lasted long enough to track diseases in later life. Critically, they have also not taken into account *in utero* exposure, apart from the study conducted by Mandeville et al. (1997) on small groups of Fisher 344 rats in which exposure began from day 20 of gestation.

This background motivated the Ramazzini Institute to embark on a project of life-span experimental studies on ELFMF designed to evaluate the carcinogenic potentiality of ELFMF alone and also in association with other known carcinogenic agents.

The experiments were planned as an integrated experimental project in which the exposure of the experimental animals to ELFMF started from prenatal life and lasted until natural death. The aim of the studies was to assess the qualitative-quantitative carcinogenic effects of sinusoidal-50 Hz MF (S-50Hz MF), trying to simulate possible human exposure situations. Moreover, large experimental groups were used in order to increase the statistical power and thus improve the evaluation of possible low-magnitude oncogenic effects. For this purpose the project includes studies to assess: (1) the qualitative-quantitative potential carcinogenic effects of S-50Hz MF alone with reference to intensity and continuity-discontinuity of electric current; (2) the carcinogenic effects of S-50Hz MF combined with acute exposure to ionizing radiation; (3) the carcinogenic effects of S-50Hz MF combined with exposure to carcinogenic chemical agents such as formaldehyde or aflatoxin B1; and (4) the possible pathogenic mechanisms at the basis of potential carcinogenic effects, as revealed by molecular profiling investigations.

The plan of the project, encompassed four experiments using 7133 rats in all. The four experiments started concurrently and the experimental animals were those born during the breeding of 2100 breeders. Table 1 presents the experimental design of two experiments in which animals were exposed only to S-50Hz MF or S-50Hz MF plus a single acute dose of 0.1 Gy of gamma radiation. Experiment No. 1 includes one control group that is common to experiment 2.

Carcinogenic effects on Sprague-Dawley rats exposed to ELFMF from prenatal life until natural death and to 0.1 Gy γ radiation delivered in a single acute exposure at 6 weeks of age

This paper deals with the results of the experiment in which four groups of male and female Sprague-Dawley rats were exposed to 0, 20 or 1,000 μT of S-50Hz MF from prenatal life until natural death as well as to 0.1 Gy of γ radiation delivered in a single acute exposure at 6 weeks of age. This particular experiment encompassed 1658 male and female Sprague-Dawley rats.

The highest S-50Hz MF dose level was selected on the basis of data available in the literature. The lowest dose was chosen as representative of possible human exposure scenarios, particularly in the workplace (Portier & Wolfe 1998). The 0.1 Gy dose was chosen as being very close to radiation exposures during some medical investigations and because it was the lowest dose tested by us in an experiment evaluating the overall dose-response carcinogenic effects of γ radiation, (Soffritti et al. 2015).

Materials and methods

To expose the animals to S-50Hz MF and γ radiation, specific radiation exposure conditions and facilities were designed and constructed.

S-50 Hz MF exposure conditions and facilities

In order to give all the experimental groups the same environmental conditions (i.e., a temperature of 22 ± 3 °C, a relative humidity of 40–60% and 12 h/day homogeneous diffusion of light) the rats were located in a room of 60 × 15 × 4 m, in all more than 900 m^2.

The MF exposure system was constructed so as to satisfy a number of technical conditions, namely: (1) the magnetic field was linearly polarized; (2) the field lines were horizontal and parallel to the ground; (3) the field uniformity was better than ± 10%; (4) the current supply had a maximum harmonic distortion of 3%; (5) the field rise time at power-up was at least 10 periods (for 50 Hz, 200 ms); (6) the current generator was noiseless; (7) the joule effect on windings did not alter the environmental temperature, a maximum variation of 2 °C being tolerated near coils; (8) coil noise and vibration were absent; and (9) the natural field level was no more than 0.1 μT and all mutual interaction of the system was avoided, while in any case the control group stayed in the same room.

The exposure system was based on independent devices. Each simple exposure device served at least 500 rats, leaving enough space to isolate ill/moribund rats.

In order to satisfy stray field requirements, a good solution was obtained by using a toroidal-shaped device. Figure 1 shows the device's magnetic structure. All the devices needed were identical and the different intensity of MF was obtained by properly tuning the power supplies which were of the current-controlled type. The toroidal shaped device guarantees the absence of interference between the structures. The fact is that, about 1 m away from the external torus boundary producing 1 milli Tesla, the field level is approximately 0.1 μTesla (Montanari 2003).

The toroid was designed with 24 coils made of three turns of insulated copper cable, mounted on a superstructure of aluminum composed of two insulated parts in order to avoid a closed loop subject to total field. The total copper cross section was 11 × 28 mm^2, and the total current used for 1 mT level was 359.6 A. The electric power was supplied by low

Table 1. The integrated project on S-50Hz MF.

Experiments *N.*	No. of animals	Treatment[a] (μTesla)	Other treatment	Duration	Type of study
Experiment 1[b]	5,029	0; 2; 20; 100; 1,000 C/D[c]	–	Life-span	Full study
Experiment 3	657	0; 20; 1,000	γ radiation, 0.1 Gy single dose at 6 weeks of age	Life-span	Full study

[a]The treatment with ELFMF started from fetal life and lasted until spontaneous death.
[b]The first experiment control group of over 500 males and 500 females was in common with experiment 3.
[c]C/D: Continuity/Discontinuity of electric current.

Figure 1. Apparatus for non-ionizing radiation S-50Hz MF exposure.

density current and the large amount of a good thermal-conducting insulation prevented heating, leaving the device at room temperature. Vibrations and noise were proved to be absent.

Mounted inside the toroidal magnet was a wooden support structure for rat cages. One of the toroids to be used was mounted and treated in order to verify the correctness of the computed parameters pertaining to the experiment. All results were in agreement with computed values.

A magnetic field probe was placed at a representative animal location to monitor the fields. An information system continuously stored the exposure data throughout the experiment.

The details of the exposure system have been described by Montanari (2003). The apparatus was also positively evaluated by a representative of the USA National Institute of Standards and Technology.

Gamma radiation exposure conditions and facility

The animal facility for irradiation is located on the ground floor of the laboratory (in a different location from the S-50Hz MF facility), inside a properly shielded irradiation room (bunker), 5 × 4m and 3 m high, communicating with the animal housing premises through a 5 × 4 m room, where the control board and exposure monitor facilities are set up. This room houses all the equipment needed to prepare animals for irradiation.

The radiation source was a therapy unit supplying Co_{60} with an activity of about 56 TBq (1500 Ci). The apparatus made it possible to radiate up to 10 animals at the same time, with an absorbed dose rate of about 0.21 Gy/min. Dose measurement was made using a Nuclear Enterprise dosimeter type 2571 A, with a 0.6 cc graphite ionization chamber, calibrated in terms of dose absorbed to water with 4% uncertainty.

Treatment at the required acute dose of 0.1 Gy was divided into two equal irradiations, performed on the ventral and dorsal side of the animals respectively. In this way the rats were treated by two opposite irradiation fields, with an almost homogeneous dose distribution. More information on ionizing radiation apparatus is reported by Soffritti et al. (2015).

Diet

Ordinary feed was delivered in pellets *ad libitum* and provided by "Laboratorio Dottori Piccioni" (Milan, Italy) as has been the practice at the Cesare Maltoni Cancer Research Center of the Ramazzini Institute (CMCRC/RI) for more than 40 years. All the animals received tap water *at libitum*.

To avoid significant alterations, every 6 months biological and chemical examinations of the animal feed and tap water were performed. The results were recorded in 'Feed data analysis' and 'Water data analysis' reports and properly stored in the CMCRC/RI experimental data archives.

Experimental animals

The animals used for the experiment were Sprague-Dawley rats from the same colony used for more than 40 years at the CMCRC/RI. The basic expected tumorigram and its fluctuations are based upon data derived from more than 18,000 historical controls.

The generation of experimental animals was performed in the following way: (a) inbred breeders were randomized by body weight in four groups in such a way as to have no more than one brother and sister per group; (b) the size of breeder groups was dictated by the number of offspring required; (c) mating of the breeders that generated the offspring for the experiments was strictly outbred (made possible by the pedigree identification number of each animal); it was synchronized among groups and lasted 5 days; and (d) all the offspring of each litter from these breeders were assigned to the respective experimental groups.

All the male and female breeders were euthanized by CO_2 over-exposure respectively 3 weeks after birth and 1 week after weaning offspring.

The experimental animals were weaned at 5 weeks of age, identified by ear punch (Jackson Laboratory method) and distributed by sex, litter by litter, until the planned number for each group was reached. After weaning, animals received ordinary feed and tap water *ad libitum*. They were housed 5 per cage, in polycarbonate cages (41 × 25 × 15) with covers made of non-magnetic material and a shallow layer of white wood shaving as bedding. All the animals were kept in a

temperature-controlled environment at 23 ± 2 °C and 50–60% of relative humidity, with 12 h/day light/dark alternation. The experiments were conducted according to the Italian law regulating the use and humane treatment of animals for scientific purposes (Decreto Legislativo 1992).

Treatment

Treatment with S-50Hz MF began during fetal life exposing the female breeders from the 12th day of pregnancy. The daily exposure to S-50Hz MF for both breeders and offspring was 19 h and for the offspring lasted until natural death. The animals of groups III and IV were also treated with an acute dose of 0.1 Gy of γ radiation at 6 weeks of age. The animals of group II were exposed only to γ radiation.

Conduct of the experiment

Housing

All animals were kept in highly standardized environmental and diet conditions, the same as used for more than 40 years in our laboratories.

Duration of the experiments

All the animals were monitored until their spontaneous death (life-span experiment).

Feed and water consumption

The daily feed and water consumptions were measured in a sample of 100 animals (50 males and 50 females) from each group starting from 6 weeks of age, every 2 weeks, for the first 8 weeks, and then at 4 week intervals, until 110 weeks of age.

Body weight

Body weight was recorded from the age of 6 weeks, every 2 weeks for the first 8 weeks, every 4 weeks until 110 weeks of age, and then every 8 weeks until the end of the experiment.

Health control

Animal health and behavior were checked 3 times daily throughout the entire experiment.

Clinical control

Checking for pathological lesions, including mammary tumors, was performed every 2 weeks for the first 8 weeks and every 4 weeks until the end of the experiment.

Necropsy and fixation

All dead rats were submitted to necropsy and the following organs and tissues were taken: Skin, subcutaneous tissue, mammary gland, brain, pituitary gland, Zymbal gland, salivary glands, Harderian glands, cranium, tongue, thyroid and parathyroid, pharynx, larynx, thymus, trachea, lung, heart, diaphragm, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach (fore and glandular), intestine (four levels), bladder, prostate, uterus, ovaries, testes, interscapular fat pad and subcutaneous, mediastinal and mesenteric lymph nodes. The organs and tissues collected were preserved in a 70% solution of Solvanol (a mixture of ethyl and isopropyl alcohol respectively, approx. 60% and 40%, obtained from Vital srl, Bologna, Italy), and 30% distilled water, apart from bone tissues which were preserved in 10% formalin and then decalcified.

Trimming

All lesions were trimmed so as to include a portion of adjacent normal tissue. As far as normal tissues and organs are concerned, trimming was performed according to standard laboratory procedures.

Histopathology

The trimmed specimens were processed and embedded in paraffin blocks according to standard operating procedures (SOP) of the laboratory. Then 3–5 μm sections were cut and routinely stained with Hematoxylin-Eosin. A histopathology evaluation was performed by the same group of pathologists. The supervisor reviewed all lesions of oncological interest as well as any open to dubious interpretation. In the pathological diagnosis, all the pathologists used the same evaluation criteria and the same classification described in the specific SOP and long adopted at the CMCRC/RI. The diagnoses are reported in the experimental registries.

Statistical analysis

Statistical evaluation of the various malignant tumors was based on the Cox proportional hazard regression model (Cox 1972) which was adjusted for possible differential survival. The *p*-values are reported in the tables. For those endpoints for which some dose groups had no cases, a simple Mantel-Haenszel model was used since there was no difference in survival between the exposed groups.

Results

The experiment proceeded smoothly without any noticeable unexpected alteration of the clinical status of the animals in the various groups. No differences were observed in body weight among control vs. treated male and female groups (Figure 2). The survival among males and females of the various groups is presented in Figure 3. The data showed that no change in survival for either sex was observed among the groups. Significant oncological results related to site-specific tumors are reported in Tables 2–6.

Mammary gland tumors

The mammary lumps of males and females were carefully monitored during the experiment. From the age of

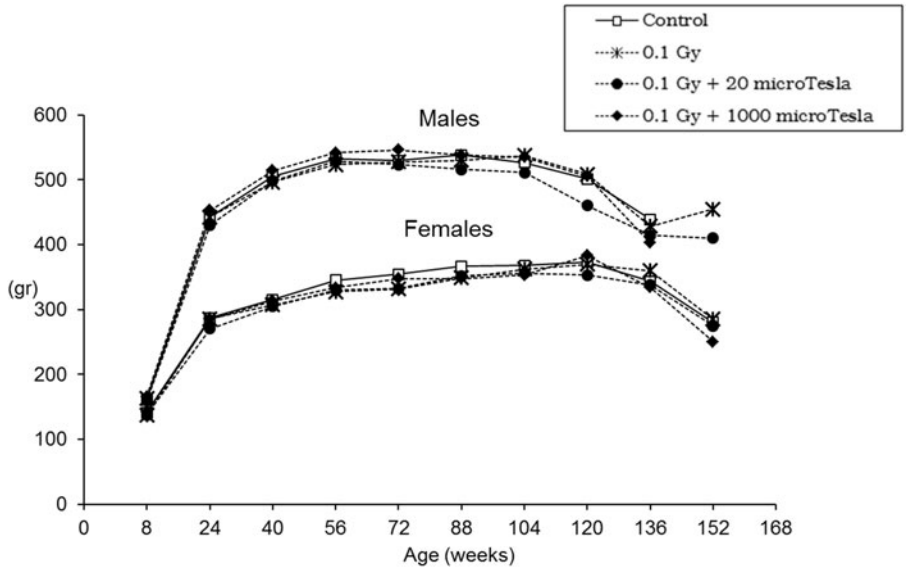

Figure 2. Mean body weight in males and females.

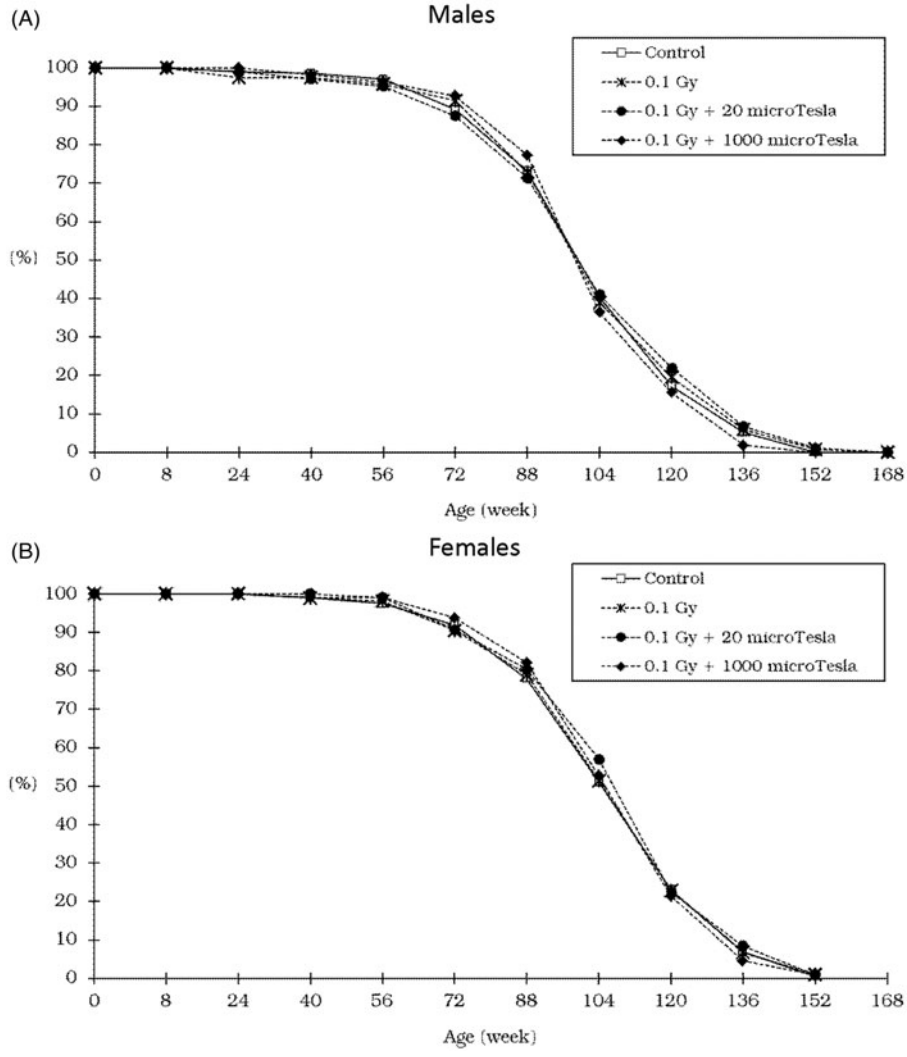

Figure 3. Survivals in males and females.

72 weeks until death a sharp increased incidence of mammary lumps was clinically observed in females exposed to S-50Hz MF plus 0.1 Gy compared to the other groups.

Comparison with untreated control group

The incidences of males and females bearing benign and malignant tumors of mammary glands are reported in Table 2. No difference in the incidence of mammary

Table 2. Incidence of mammary glands tumors in male (M) and female (F) Sprague-Dawley rats exposed to S-50Hz MF and/or γ radiation[a,b] compared to untreated controls.

			Tumor-bearing animals[c]					
	Animals		Fibroadenomas		Adenocarcinomas		Total tumors	
Group No. (μT/Gy)	Sex	No.	No.	%	No.	%	No.	Per 100 animals
I	M	500	12(13)	2.4	1	0.2##	14	2.8
(0/0)	F	501	206(269)	41.1	32	6.4##	301	60.1
	M + F	1001	218	21.8	33	3.3	315	31.5
II	M	118	4	3.4	0	-	4	3.4
(0/0.1)	F	105	44(56)	41.9	8	7.6	64	61.0
	M + F	223	48	21.5	8	3.6	68	30.5
III	M	105	5	4.8	3	2.9**	8	7.6
(20/0.1)	F	107	53(66)	49.5	8(9)	7.5	75	70.1
	M + F	212	58	27.4	11	5.2	83	39.2
IV	M	110	9(11)	8.2◆◆	1	0.9	12	10.9
(1000/0.1)	F	112	51(77)	45.5	18(19)	16.1**	96	85.7
	M + F	222	60	27.0	19	8.6**	108	48.6

[a]The treatment with S-50 Hz MF for 19 h/day, in continuous way (C), started on the 12th day of pregnancy and lasted until natural death.
[b]γ radiation delivered as a single acute exposure at 6 weeks of age.
[c]Between brackets the number of tumors (one animal can bear more than one tumor).
**Statistically significant ($p \leq 0.01$) using Cox Proportional Hazard model. For M + F a stratified analysis was used.
◆◆Statistically significant ($p \leq 0.01$) using the Mantel-Haenszel Model for the analysis (used for incidental lesions).
##Near the control incidence is the *p*-value ($p \leq 0.01$) using Cox Regression Model for the analysis of trend.

Table 3. Incidence of animals bearing atypical precursors or adenocarcinomas of mammary gland in male (M) and female (F) Sprague-Dawley rats exposed to S-50Hz MF and/or γ radiation[a,b] compared to untreated controls.

			Animals bearing atypical precursors[c]		Animals bearing Adenocarcinomas		Total number of animals bearing adenocarcinomas or atypical precursors[d]	
	Animals							
Group No. (μT/Gy)	Sex	No.	No.	%	No.	%	No.	%
I	M	500	0	0.0◇	1	0.2##	1	0.2◇◇
(0/0)	F	501	15	3.0◇◇	32	6.4##	47	9.4◇◇
	M + F	1001	15	1.5	33	3.3	48	4.8
II	M	118	1	0.8	0	-	1	0.8
(0/0.1)	F	105	4	3.8	8	7.6	12	11.4
	M + F	223	5	2.2	8	3.6	13	5.8
III	M	105	1	1.0	3	2.9**	4	3.8◆
(20/0.1)	F	107	14	13.1◆◆	8	7.5	22	20.6◆◆
	M + F	212	15	7.1◆◆	11	5.2	26	12.3◆◆
IV	M	110	2	1.8◆	1	0.9	3	2.7◆
(1000/0.1)	F	112	13	11.6◆	18	16.1**	31	27.7◆◆
	M + F	222	15	6.8◆◆	19	8.6**	34	15.3◆◆

[a]The treatment with S-50 Hz MF for 19 h/day, in continuous way (C), started on the 12th day of pregnancy and lasted until natural death.
[b]γ radiation delivered as a single acute exposure at 6 weeks of age.
[c]Mammary gland atypical precursors include: animals bearing atypical hyperplasia in single mammary gland or in fibroadenoma; they are counted only once according to the most severe lesion.
[d]Animals bearing more than one type of lesion are plotted only once according to the most severe lesion.
**Statistically significant ($p \leq 0.01$) using Cox Proportional Hazard model. For M + F a stratified analysis was used.
◆Statistically significant ($p \leq 0.05$) or
◆◆($p \leq 0.01$) using the Mantel-Haenszel Model for the analysis (used for incidental lesions). For M + F a stratified analysis was used.
##Near the control incidence is the *p*-value ($p \leq 0.01$) (excluding the 0.1 Gy group) using Cox Regression Model for the analysis of trend.
◇Near the control incidence is the *p*-value ◇($p \leq 0.05$) or ◇◇($p \leq 0.01$) (excluding the 0.1 Gy group) using the Mantel Haenszel Model for incidental lesions for trend analysis.

fibroadenomas or adenocarcinomas was observed between male and female untreated rats compared to those treated with 0.1 Gy.

Comparing the untreated controls with groups treated with 20 or 1000 μT plus 0.1 Gy, the data show: (1) a significant dose-related increased incidence of mammary carcinomas in males ($p \leq 0.01$) and females ($p \leq 0.01$); (2) a significant increased incidence in males exposed to 20 μT plus 0.1 Gy ($p \leq 0.01$) and in females exposed to 1000 μT plus 0.1 Gy ($p \leq 0.01$). In females a sharp difference in cumulative hazard may be observed among the group treated with 1000 μT plus 0.1 Gy compared to untreated controls and the group exposed only to 1000 μT (Figure 4). Moreover, in females the total number *per* 100 animals of breast tumors (benign and malignant aggregated) was 60.1 in untreated controls and 61.0 in females exposed to 0.1 Gy compared to 70.1 and 85.7 among the groups exposed to 20 μT or 1000 μT plus 0.1 Gy, respectively.

The incidences of males and females bearing atypical precursors (namely atypical hyperplasia in the glands or in fibroadenomas) or adenocarcinomas of the mammary gland are reported in Table 3. Compared to the untreated control group, a significant dose-related increased incidence of animals bearing atypical precursors occurred in males ($p \leq 0.05$) and females ($p \leq 0.01$), in particular in females exposed to both levels of S-50Hz MF plus γ radiation ($p \leq 0.01$) and in

Table 4. Incidence of animals bearing mammary adenocarcinomas, or atypical precursors aggregated with mammary adenocarcinomas in male (M) and female (F) Sprague-Dawley rats exposed to S-50Hz MF and γ radiation[a,b] compared to 0.1 Gy treated group.

	Animals		Animals bearing mammary adenocarcinomas		Animals bearing mammary adenocarcinomas or atypical precursors[c]	
Group No. (μT/Gy)	Sex	No.	No.	%	No.	%
I	M	118	0	–	1	0.8◇◇
(0/0.1)	F	105	8	7.6	12	11.4◇◇
	M + F	223	8	3.6	13	5.8
II	M	105	3	2.9	4	3.8◆◆
(20/0.1)	F	107	8	7.5	22	20.6◆◆
	M + F	212	11	5.2	26	12.3
III	M	110	1	0.9	3	2.7◆
(1000/0.1)	F	112	18	16.1*	31	27.7◆◆
	M + F	222	19	8.6	34	15.3

[a]The treatment with S-50 Hz MF for 19 h/day, in continuous way (C), started on the 12th day of pregnancy and lasted until natural death.
[b]γ radiation delivered as a single acute exposure at 6 weeks of age.
[c]Mammary gland atypical precursors include: animals bearing atypical hyperplasia in single mammary gland or in fibroadenoma; they are counted only once according to the most severe lesion.
*Statistically significant compare to 0.1 Gy group ($p \leq 0.05$) using Cox Proportional Hazard model.
◆Statistically significant compare to 0.1 Gy group ($p \leq 0.05$) or
◆◆($p \leq 0.01$) using the Mantel-Haenszel Model for the analysis (used for incidental lesions).
Near the 0.1 Gy group (positive control) incidence is the *p*-value ◇◇($p \leq 0.01$) using the Mantel-Haenszel Model for incidental lesions for trend analysis.

Table 5. Incidence of heart malignant schwannomas and hemolymphoreticular neoplasias in male (M) and female (F) Sprague-Dawley rats exposed to S-50Hz MF and/or γ radiation[a,b] compared to untreated controls.

	Animals		Animals bearing heart malignant schwannomas		Animals bearing hemolymphoreticular neoplasias	
Group No. (μT/Gy)	Sex	No.	No.	%	No.	%
I	M	500	1	0.2##	83	16.6
(0/0)	F	501	0	-	68	13.6
	M + F	1001	1	0.1	151	15.1
II	M	118	0	-	18	15.3
(0/0.1)	F	105	1	1.0	13	12.4
	M + F	223	1	0.4	31	13.9
III	M	105	2	1.9*	19	18.1
(20/0.1)	F	107	1	0.9	21	19.6
	M + F	212	3	1.4	40	18.9
IV	M	110	3	2.7**	28	25.5*
(1000/0.1)	F	112	0	-	16	14.3
	M + F	222	3	1.3	44	19.8

[a]The treatment with S-50 Hz MF for 19 h/day, in continuous way (C), started on the 12th day of pregnancy and lasted until natural death.
[b]γ radiation were delivered as a single acute exposure at 6 weeks of age.
*Statistically significant ($p \leq 0.05$) or
**$p \leq 0.01$) using Cox Proportional Hazard model.
##Near the control incidence is the *p*-value ($p \leq 0.01$) (excluding the 0.1 Gy group) using Cox Regression Model for the analysis of trend.

Table 6. Incidence of animals bearing hemolymphoreticular neoplasias in male (M) and females (F) Sprague-Dawley rats exposed to S-50Hz MF and/or γ radiation[a,b] compared to 0.1 Gy treated group.

	Animals		Animals bearing hemolymphoreticular neoplasias	
Group No. (μT/Gy)	Sex	No.	No.	%
I	M	118	18	15.3
(0/0.1)	F	105	13	12.4
	M + F	223	31	13.9
II	M	105	19	18.1
(20/0.1)	F	107	21	19.6
	M + F	212	40	18.9
III	M	110	28	25.5*
(1000/0.1)	F	112	16	14.3
	M + F	222	44	19.8

[a]The treatment with S-50 Hz MF for 19 h/day, in continuous way (C), started on the 12th day of pregnancy and lasted until natural death.
[b]γ radiation delivered as a single acute exposure at 6 weeks of age.
*Statistically significant compare to 0.1 Gy group ($p \leq 0.05$) using Cox Proportional Hazard model.

males exposed to 1000 μT plus 0.1 Gy ($p \leq 0.05$). Moreover the data show that when the males and females bearing atypical precursors are respectively aggregated to males and females bearing adenocarcinomas, a significant dose-related increased incidence occurred in males ($p \leq 0.01$) and females ($p \leq 0.01$), in particular in males and females exposed to 20 μT plus γ radiation ($p \leq 0.01$, respectively) and in males and females exposed to 1000 μT plus 0.1 Gy ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Aggregation of animals bearing benign tumors or atypical mammary lesions with animals bearing adenocarcinomas is justified to gain more insight into the evidence of the carcinogenicity of a given agent (McConnell et al. 1986). Specifically, progression from benign to malignant neoplasms has been suggested for mammary gland neoplasms in rats (van Zwieten et al. 1984; Russo 2015) to show the potential carcinogenic risk. Moreover, cytologically, atypical mammary hyperplasias are characterized by cells that have some of the neoplastic characteristics of low-grade ductal carcinoma *in situ* (Fabian et al. 2000; Singletary 2003; WHO-OMS-IARC 2003). As reported by the Cancer Committee of the College of American Pathologists (Fitzgibbons et al. 1998), and documented also by other less recent studies (Dupont et al. 1993), the relative risk of developing a mammary cancer among patients with atypical ductal hyperplasia is 4–5 times higher.

Histologically the adenocarcinomas in both male and female rats showed papillary and solid lobular structures with an increased nuclear/cytoplasm ratio and prominent nucleoli of the epithelial cells. In some cases, metastasis occurred in the lung.

In our historical controls mammary cancer in male rats is a very rare tumor. Indeed, out of 2415 males the overall

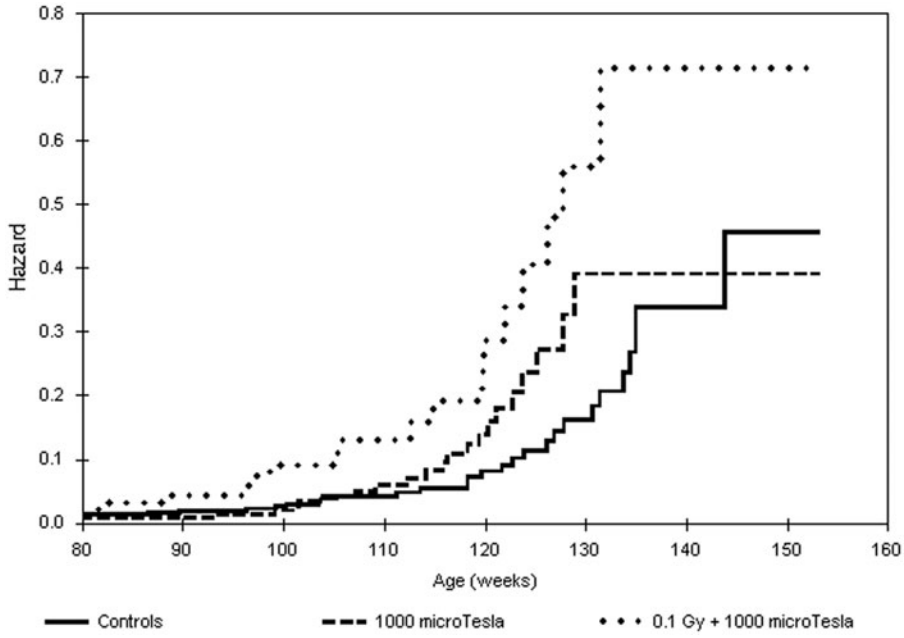

Figure 4. Hazard for mammary gland adenocarcinomas in females compared to untreated controls.

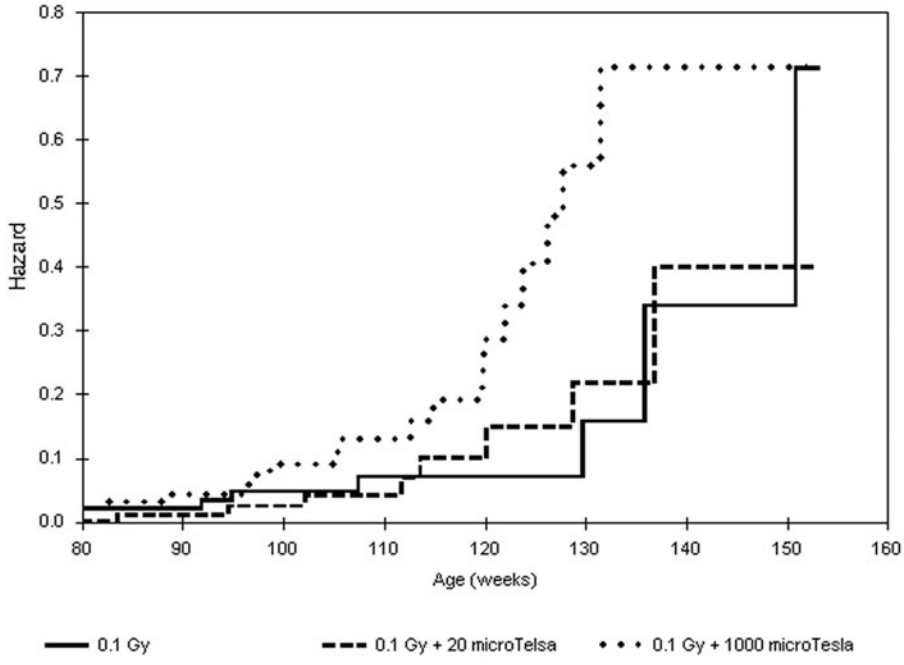

Figure 5. Hazard for mammary gland adenocarcinoma in females compared to 0.1 Gy treated group.

incidence is 0.5% (0–1.3%). In females, out of 2424 rats the overall incidence of mammary cancers is 8.9% with a range of 2.9–14.2%.

Comparison with the 0.1 Gy treated control group

Table 4 reports the incidences of mammary tumors in rats exposed to 20 or 1000 μT plus 0.1 Gy compared to rats exposed only to 0.1 Gy. The data show a still significant increased incidence of mammary adenocarcinoma in females exposed to 1000 μT plus 0.1 Gy ($p \leq 0.05$). The evidence of an increased cumulative hazard for adenocarcinomas in females induced by exposure to 1000 μT plus 0.1 Gy compared to the groups exposed to 20 μT plus 0.1 Gy or 0.1 Gy is shown in Figure 5 in which a sharp difference may be observed starting after 120 weeks of age. When the animals bearing adenocarcinomas are aggregated to animals bearing atypical precursors (including atypical cellular hyperplasia in a single mammary gland or areas of cellular atypia in fibroadenomas), a significant dose-related increased incidence occurred in males ($p \leq 0.01$) and females ($p \leq 0.01$), as well as in males exposed to 20 μT or 1000 μT plus 0.1 Gy ($p \leq 0.01$ and $p \leq 0.05$, respectively) and in females exposed to 20 μT or 1000 μT plus 0.1 Gy ($p \leq 0.01$ in both cases).

Malignant schwannomas of the heart and hemolymphoreticular neoplasias (HLRN)

The incidences of animals bearing malignant schwannomas of the heart and HLRN are reported in Table 5.

Compared to untreated controls, a significant dose-related increased incidence ($p \leq 0.01$) of heart malignant schwannomas occurred in males treated with S-50Hz MF and 0.1 Gy. Concerning males exposed to 20 μT plus 0.1 Gy ($p \leq 0.05$) or to 1000 μT plus 0.1 Gy, a significant increased incidence of malignant schwannomas was observed in both groups ($p \leq 0.01$, respectively) compared to untreated controls. When compared to the group exposed to 0.1 Gy alone, the differences in the incidences were no longer significant.

Heart malignant schwannoma is not a frequent tumor among male Sprague-Dawley rats from our colony. Out of 2415 males, the overall incidence of heart malignant schwannomas is 0.7% (range 0–2%). Microscopically, malignant schwannomas involved the left ventricle with extension inside the cavity, infiltration of the sub-endocardium and involvement of the right ventricle and the aortic valve. Neoplastic cells arranged in palisading structures, pleomorphic cells, giant nuclei, and mitotic figures are observed in neoplastic tissues. Immunohistochemical characterization was positive for S-100 protein stain.

The incidence of HLRN in males shows a significant increase at the exposure to 1000 μT plus 0.1 Gy as compared to negative controls ($p \leq 0.05$). The increased incidence is still significant ($p \leq 0.05$) when the males exposed to 1000 μT plus 0.1 Gy are compared to males exposed to 0.1 Gy (Table 6). The evidence of the increased cumulative hazard of hematopoietic tumors induced by 1000 μT plus 0.1 Gy in males compared to exposure to 0.1 Gy or 20 μT plus 0.1 Gy is shown in Figure 6.

Lymphoma and leukemia are neoplasias arising from the hemolymphoreticular tissues and aggregation of them is allowed because solid and circulating phases are common in many hematopoietic neoplasms and a distinction would be artificial (Harris et al. 2001). In our historical untreated male controls the overall incidence of hematopoietic neoplasias is 20.5% (range 8.0–30.9) out of 2415 male rats.

Compared to the higher range of historical controls, the incidence of HLRN in males exposed at the highest dose level of S-50Hz MF and γ radiation is slightly lower, but significantly higher than the concurrent 0.1 Gy and untreated control group.

Discussion

The aim of this study was to evaluate the carcinogen-promoting effects of S-50Hz MF life-span exposure using a γ radiation initiation/promotion protocol in Sprague-Dawley rats.

The results of the study show, for the first time, that exposure of Sprague-Dawley rats to S-50Hz MF from prenatal life until natural death plus acute low-dose γ radiation delivered at 6 weeks of age, compared to untreated controls, significantly enhances the incidence of several tumors in males and females, namely: (a) a significant dose-related increased incidence of mammary adenocarcinomas in males and females in particular in males exposed to 20 μT plus 0.1 Gy and in females exposed to 1000 μT plus 0.1 Gy; (b) in males a significant dose-related increased incidence of heart malignant schwannomas with a significant increase among males exposed to 20 μT plus 0.1 Gy ($p \leq 0.05$) and to 1000 μT plus 0.1 Gy; and (c) a significant increased incidence of hematopoietic neoplasias in males treated at 1000 μT plus 0.1 Gy. Concerning our S-50Hz MF alone study arm, no effects were shown on these cancer endpoints.

When rats exposed to MF and 0.1 Gy are compared with the group exposed to 0.1 Gy alone, a significant increased incidence was observed in mammary adenocarcinomas among females exposed to 1000 μT plus 0.1 Gy, as well as in

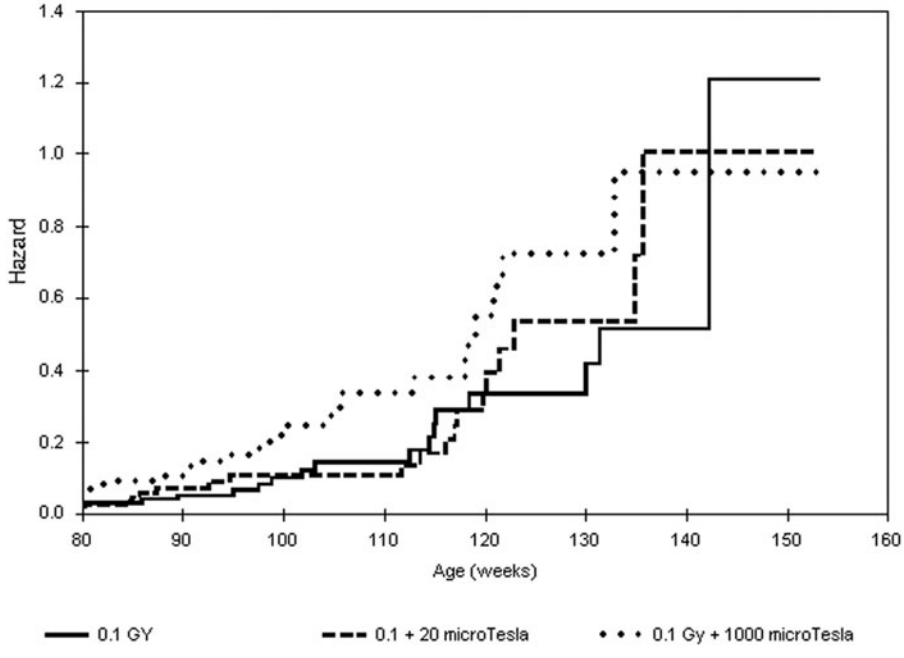

Figure 6. Hazard for hemolymphoreticular neoplasias in males compared to 0.1 Gy treated group.

hematopoietic neoplasms among males exposed to 1000 μT plus 0.1 Gy.

The long-term bioassays on rodents hitherto published to test the carcinogenic potential of 50/60 Hz MF alone have failed to show clear evidence of carcinogenic effects. However, the large diffusion of ELFMF in the general and occupational environment has motivated widespread interest in testing the interaction of ELFMF with certain known chemical and physical carcinogens.

Up to now, several sub-chronic studies have been conducted to test the carcinogen-promoting activity of ELFMF using initiation-promotion animal models and trying to induce various types of tumor, mainly mammary cancers in female rats or lymphoma/leukemia in mice and rats.

Mammary cancers

Concerning studies on initiation-promotion of mammary tumors in female rats, the first results were reported by Beniashvili et al. (1991). They showed that treatment with N-Methyl-N-Nitrosurea (MNU) as the initiator followed by exposure to 50 Hz MF for 2 years enhanced the induction of mammary cancers. These results were confirmed later in a second experiment conducted by the same group (Anisimov et al. 1996). However, as reported by Boorman et al. (2000), their inadequate reporting of experimental methods and lack of engineering details rule out any assessment of the significance of the results. These are the only studies in which MNU has been used.

More studies have been conducted on magnetic fields using a 7,12-dimethyl-benz[a]anthracene (DMBA) rat mammary tumor model. In a series of German studies it was reported that DMBA-initiated female Sprague-Dawley rats exposed to ELFMF in experiments lasting 13 weeks had an earlier onset, larger size and increased incidence of mammary tumors (Mevissen et al. 1993; 1996, 1998; Loscher & Mevissen 1994). In another study, in which the exposure to ELFMF lasted 27 weeks after treatment with DMBA, the results were similar (Thun-Battersby et al. 1999). In a study conducted by Ekstrom et al. (1998) in which Sprague-Dawley rats were treated with an initiating dose of DMBA followed by exposure to ELFMF for 25 weeks, no difference was observed in mammary tumor incidence compared to animals exposed only to DMBA.

Other studies, conducted in the framework of the U.S. National Toxicology Program (NTP) by Boorman et al. (1999a) and Anderson et al. (1999), both using the DMBA model, failed to find a promoting effect at either 50 or 60 Hz MF. The differences in the results observed between the NTP and the German studies supported the hypothesis that genetic differences between substrains of Sprague-Dawley rats used in the different laboratories may be involved (Anderson et al. 2000).

In a careful review of the published literature on magnetic fields and mammary cancer in rodents (12 studies), Boorman et al. (2000) wrote that 'when considered in total, the results of these studies demonstrated either negative or inconsistent positive results across five endpoints (tumor incidence, number of tumor-bearing rats, time to tumor, total tumors and tumor size)' and he concluded with a citation of the U.S. National Institute of Environmental Health Science that 'the collection of studies provides strong evidence of no effect of magnetic fields on the promotional development of mammary cancer' (NIEHS 1999).

However further studies conducted by the German laboratory in early 2000 showed that 50 Hz MF produced an enhanced proliferation of mammary epithelium in female Sprague-Dawley rats (Fedrowitz et al. 2002).

In a subsequent experiment the same authors showed that the genetic background plays a pivotal role in effects of magnetic exposure (Fedrowitz et al. 2004). Based on this assumption, Fedrowitz and Loscher (2005) tested the effects of MF in inbred Fisher 344 rats. They showed that exposure of mammary glands to MF, in females, increased the number of terminal end buds in breast tissues, which are the site of origin of mammary carcinomas as reported by Russo and Russo (1996). Furthermore a 26-week study conducted on Fisher 344 rats exposed to DMBA and MF showed a significant increased incidence of mammary adenocarcinomas in treated females compared to sham controls (Fedrowitz & Loscher 2008).

Later, Fedrowitz and Loscher (2012) published the results of a mammary gland gene expression study performed on MF-sensitive female Fisher 344 and MF-insensitive Lewis rats aimed to elucidate candidate genes involved in differences of MF response in mammary glands. After 2 weeks of sham- or 50 Hz MF-exposure, the Fisher 344 breast tissue showed alterations in gene expression which were not observed in Lewis rats and could be considered correlated to the MF-susceptibility of F344.

In summary, our results with S-50Hz MF prenatal life-span exposure combined with acute exposure to 0.1 Gy γ radiation support the hypothesis that 50Hz MF enhances the risk of mammary cancer in females, the evidence being the time of appearance of mammary adenocarcinomas (Figure 7), significant increased incidence of cancers, and increased numbers of mammary tumors per 100 rats. Moreover the fact that the cumulative hazard of mammary cancer observed in females exposed to 1000 μT alone for the life-span proved fairly similar to the concurrent negative controls, as shown in Figure 4, reinforces the conclusion that the significant increased incidence of mammary cancers is not accidental but related to exposure to 1000 μT S-50Hz MF plus 0.1 Gy γ radiation. Significant carcinogenic effects in the form of breast cancer were also shown for the first time in male rats. In order to identify new molecular targets of S-50Hz MF exposure, a gene expression analysis in the mammary gland tissue of the male and female Sprague-Dawley rats of this study will be performed inside the molecular biology laboratory at the CMCRC/RI.

Malignant schwannomas of the heart

Concerning malignant schwannomas of the heart, this is a rare tumor in rodents, as it is in humans. Indeed, primary malignant schwannoma of the heart in humans comprises only 0.75% of all primary cardiac tumors and thus is very rare

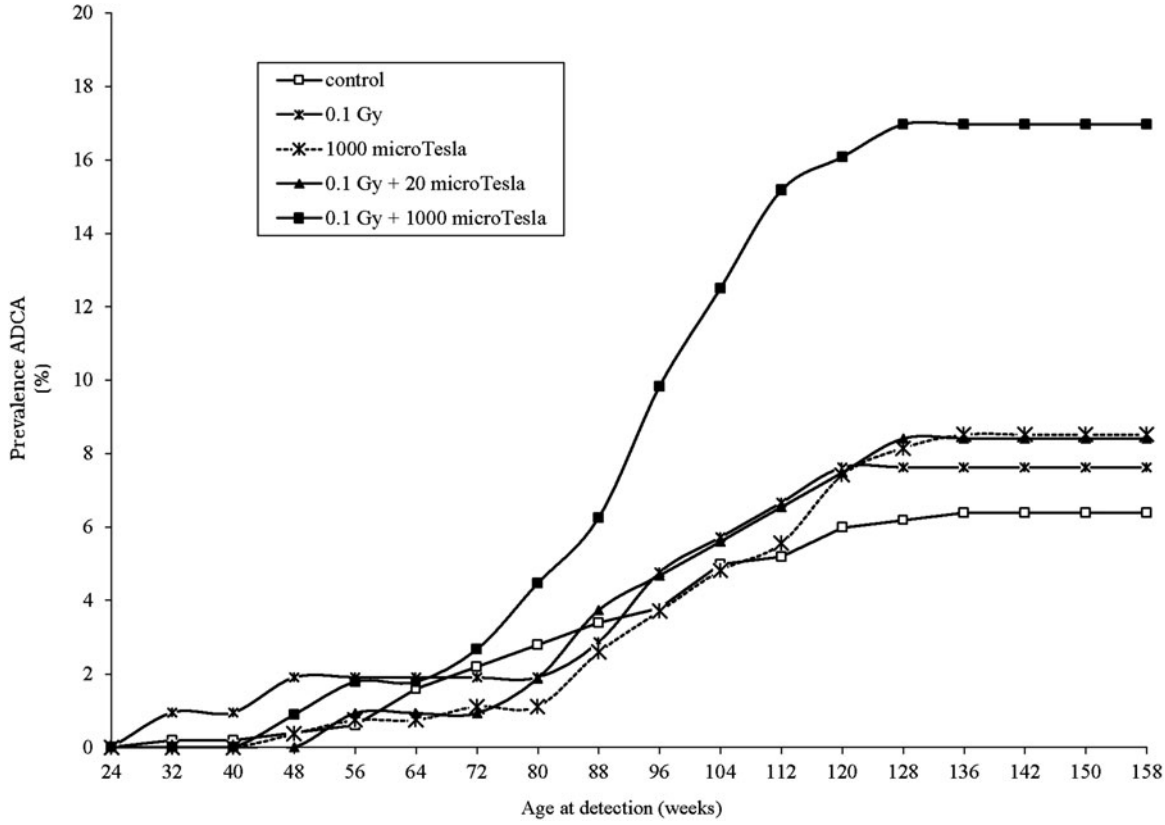

Figure 7. Cumulative prevalence of glandular mammary adenocarcinomas clinically observed in female rats at the age of the onset and histopathologically evaluated.

(Morishita et al. 1988). In experiment No. 1 of our project in which male rats were exposed only to 1000 μT, out of 253 males exposed, two animals bearing malignant schwannomas of the heart were observed (0.8%). Overall, in the same experiment out of 2004 males exposed to five dose levels of S-50Hz MF from prenatal life until death, we observed eight animals (0.04%) bearing malignant schwannomas (personal communication of data not yet published). These data may support a correlation with the exposure to S-50Hz MF plus 0.1 Gy γ radiation.

Hemolymphoreticular neoplasias (HLRN)

As reported by Boorman et al. (2000b), several animal studies in rats and mice have been conducted to evaluate the potential leukemogenic effects of 50/60 Hz MF. Of course, due to the limited statistical power of long-term bioassays to detect significant increased incidence of leukemia in early age, it is very difficult to reproduce in these studies the leukemogenic effects detected in children exposed to ELFMF. However the four already reported long-term bioassays published up to now failed to show any leukemogenic effects of ELFMF in experimental test conditions.

In a large long-term bioassay in which, after exposure to ionizing radiation, groups of female mice were exposed to 60 Hz MF for 120 weeks, no effects on HLRN were observed (Babbitt et al. 1999). In another initiation/promotion study, groups of mice were exposed to DMBA by way of initiation and afterwards exposed to 50Hz–1000 μT for 16 weeks. The overall incidence of lymphoma was similar among the groups (Shen et al. 1997). Other studies conducted using various transgenic models or transplanted tumor models did not show any leukemogenic effect. All in all, the animal studies reported do not provide evidence that 50 or 60 Hz MF induces HLRN.

Conclusions

The results of this study have demonstrated for the first time that exposure to S-50Hz MF from prenatal life until natural death enhances the carcinogenic effects of γ radiations in male and female Sprague-Dawley rats.

The results of our study cannot be compared to those of the studies conducted in the past because of our different experimental design, large number of animals per group, starting exposure from prenatal life and duration of observation until natural death, our complete histopathological evaluation of all organs and tissues, as well as the possibility we had of comparing the incidence of various tumors in rats treated with 0.1 Gy and among negative controls as well as with concurrent males and females exposed to 1000 μT MF alone.

The type and level of 0.1 Gy exposure planned for this study cannot be considered unusual in the human working place or in general life. For instance, during computed tomography (CT) investigations, the organ being studied typically receives a radiation dose from 15 mGy (in adults) to 30 mGy (in children) with an average of 2–3 scans per study (which corresponds at least to 120 mGy, which is >0.1 Gy) (Brenner & Hall 2007). Radiation exposure from CT scans and increased cancer risk in adults (Sodickson et al. 2009) and in children (Pearce et al. 2012) has been reported. Moreover S-50Hz MF

may enhance progression of a number of lesions from benign to malignant. Indeed, our results on mammary cancer as well as on leukemia and malignant schwannomas of the heart should call attention to situations in which exposure to MF may be associated with exposure to low doses of well-known carcinogenic agents such as ionizing radiation or other chemical carcinogens.

In conclusion, in our opinion these results call for a re-evaluation of the safety of non-ionizing radiation particularly at this time when the pressure to move from conventional fuels-based mobility to electric mobility deserves high priority in the EU and US and other industrialized countries.

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Disclosure statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper. They also declare that their funding sources had no direct role in the study design, data collection, analysis and interpretation of the data, in the writing of the manuscript, or in the decision to publish the work.

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