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Confirmation Studies of Soviet Research on Immunological Effects of Microwaves: Russian Immunology Results

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This paper presents the results of a replication study performed to investigate earlier Soviet studies conducted between 1974 and 1991 that showed immunological and reproductive effects of long-term low-level exposure of rats to radiofrequency (RF) electromagnetic fields. The early studies were used, in part, for developing exposure standards for the USSR population and thus it was necessary to confirm the Russian findings. In the present study, the conditions of RF exposure were made as similar as possible to those in the earlier experiments: Wistar rats were exposed in the far field to 2450 MHz continuous wave RF fields with an incident power density in the cages of 5 W/m^2 for 7 h/day, 5 days/week for a total of 30 days, resulting in a whole-body SAR of 0.16 W/kg. Effects of the exposure on immunological parameters in the brain and liver of rats were evaluated using the complement fixation test (CFT), as in the original studies, and an additional test, the more modern ELISA test. Our results, using CFT and ELISA, partly confirmed the findings of the early studies and indicated possible effects from non-thermal RF exposure on autoimmune processes. The RF exposure resulted in minor increases in formation of antibodies in brain tissue extract and the exposure did not appear to be pathological. In addition, a study was conducted to replicate a previous Soviet study on effects from the injection of blood serum from RF-exposed rats on pregnancy and foetal and offspring development of rats, using a similar animal model and protocol. Our results showed the same general trends as the earlier study, suggesting possible adverse effects of the blood serum from exposed rats on pregnancy and foetal development of intact rats, however, application of these results in developing exposure standards is limited. Bioelectromagnetics 31:589–602, 2010.

Key words: non-thermal RF exposure; rats; complement fixation test; ELISA; autoimmunity; brain extract; stress reaction; pregnancy

INTRODUCTION

A series of Soviet studies published between 1974 and 1991 dealt mainly with effects on the immune system of rats exposed to radiofrequency (RF) electromagnetic fields (EMF) at 2375 MHz (0.1–10 W/m^2). The results of these studies have served, in part, as the basis for the public RF standards of the former USSR and the current Russian standard. Because the results of these studies were taken into account in these important standards, the World Health Organization's (WHO) International EMF Project considered it essential that Russian scientists and an independent laboratory replicate the results of these studies.

Studies by Soviet (Russian and Ukrainian) scientists reported that RF irradiation of rats disrupted the antigenic structure of brain tissue [Vinogradov and Dumansky, 1974, 1975; Shandala and Vinogradov, 1982; Shandala et al., 1983; Vinogradov and Naumenko, 1986]. Their results suggested that semichronic exposure at 5 W/m^2 evoked a pronounced

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autoimmune response compared to Sham-exposed animals. Shandala et al. [1983], Vinogradov and Naumenko [1986] and Vinogradov et al. [1991] reported differences in immune responses between Sham-exposed and RF-exposed rats immediately after the exposure by assessing basophile degranulation, plaque-forming activity and content of complement-binding antibodies in blood serum. In the second phase of their experiments, intact rats were immunized by an intraperitoneal injection of saline brain extracts from control and exposed animals. The autoimmune response was assessed 2 and 3 weeks later by cytological and morphological alterations in brain, spleen, bone marrow, and blood cells. Brain extract from rats exposed at 5 W/m^2 evoked pronounced autoimmune responses both 2 and 3 weeks after immunization: the basophile degranulation index, antibody levels and percentage of plaque-forming cells rose in immunized animals compared to controls. In addition, this treatment increased the content of plasmocytes in the spleen and decreased the number of small lymphocytes in bone marrow. Vinogradov and Dumansky [1974, 1975] studied the content of complement-binding antibodies in blood serum in exposed and unexposed rats over time following termination of the RF exposure. They showed that titres of antibodies against brain antigens peaked approximately 2–3 weeks after the exposure in both groups, but a further decline in the level of antibodies was more pronounced in the Sham-exposed group. A study on pre- and postnatal development was performed by Shandala and Vinogradov [1982] to investigate the effect of auto-antibodies from exposed rats on foetal and offspring development. They found that after the injection of blood serum from exposed rats (7 h/day for 30 days at 5 W/m^2) into intact female rats on the 10th day of pregnancy, embryo mortality was high in the exposed group (28%) and absent in control groups (biological controls and pregnant rats injected with blood serum from unexposed rats). Significantly higher offspring mortality was found at the end of the first postnatal month in the exposed group (31%) compared to control (10%) and unexposed (17%) groups.

Since these effects were found at rather low incident power densities, replication and extension of these studies by two independent laboratories (the former SRC Institute of Biophysics, Moscow, Russia, now the Federal Medical Biophysical Centre FMBA, and the IMS Laboratory, University of Bordeaux, Pessac, France) were conducted using similar scientific methods, but a modern exposure system and biological methods [Statement of Work, 2006]. Replication of these studies was part of the WHO RF Research Agenda [WHO, 2006]. Thus, the objectives of the present study were twofold: (1) to confirm the results of previous Soviet studies indicating effects of semi-chronic non-thermal exposure to RF on immunological parameters in the brain of exposed rats and (2) to replicate the adverse influence of blood serum from exposed rats on prenatal and early postnatal development of offspring.

MATERIALS AND METHODS

General Scheme of the Present Study

The study of immunological and reproductive effects of long-term low-level microwave exposure was conducted on Wistar rats in a blind manner. The general scheme of the study is shown in Figure 1. Three groups of rats were formed, each consisting of 16 males: (1) RF-exposed group included rats that were exposed to low-intensity RF in an anechoic chamber, (2) Sham-exposed group included rats that were treated in the same way as (1) but were not RF-exposed, and (3) cage control group included rats that were kept in the animal room. Rats from each group were donors of blood serum and tissues on the 7th and 14th day after termination of the exposure. The immunology study was performed on blood serum and brain and liver extracts taken at both time points. In the study on pre- and early postnatal development of offspring, blood taken on the 14th day after the exposure from Sham-exposed and RF-exposed rats was injected into intact pregnant rats on the 10th day of pregnancy.

Animals

Forty-eight male outbred Wistar (WI) rats were obtained from the Scientific Centre of Biomedical Technologies of the Russian Academy of Medical Science (SCBT RAMS, Moscow, Russia) in August 2006. The rats were 4- to 5-weeks old and weighed 120–135 g at the time of purchase. They were left for a 1-week quarantine period and then were adapted in the exposure chamber for another week. Mature rats (90 females and 30 males) for the study on pre- and early postnatal development of offspring were obtained from the SCBT RAMS in October 2006 and weighed between 150 and 160 g at the time of purchase. These animals were quarantined for 2 weeks. All rats were investigated for bacterial infections (salmonella, *E. coli*), leptospirosis, dermamycosis (trichophytosis and microspores), ectoparasites (ticks and lice), and endoparasites (intestinal worms). All animals were in good health.

Rats were kept in an animal room (except during RF and Sham exposure, at which time they were placed in separate anechoic chambers) having a temperature of 21–23 °C, humidity of 40–60%, 12 h light (7:00 am to 7:00 pm) and 12 h dark cycles, and air flow of 100 m^3/h.

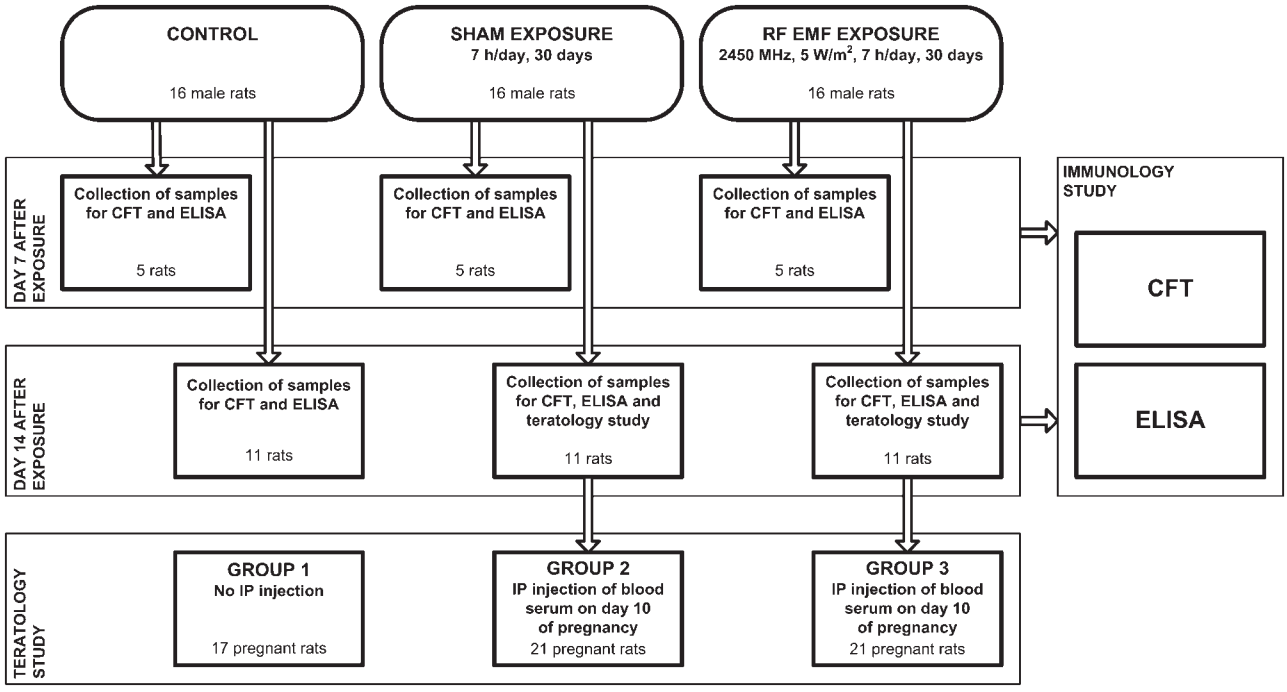

Fig. 1. General scheme of the study illustrating all stages, sizes of animal groups and collection of biological samples for immunology and teratology studies.

In the immunology study, four rats were placed in clear plastic cages 0.28 m × 0.42 m × 0.15 m. In the study on pre- and early postnatal development of offspring, one pregnant rat and her offspring were kept in a separate cage of the same size. Cage bedding was changed twice a week.

The rats were provided a standard food diet containing wheat, barley, maize, soybean, wheat bran, soluble fish protein concentrate, dried milk, sprout meal, calcium carbonate, vitamins A, B1, B2–6, B12, D3, E, H, K3 and Co, Cu, Fe, I, Mn, Se, Zn. Lactating rats were additionally fed with fresh vegetables (carrots, cabbage) and stale white bread. Beginning on postnatal day 20, offspring consumed dry forage, white bread and vegetables according to a recommended ration [Loskutova, 1980]. Food and water were not placed in the cages during RF exposure. At the same time that RF exposure was conducted, food and water were removed from cages of Sham-exposed rats. The control rats had free access to food and water at all times.

All animals were managed according to the guidance for laboratory practice [Ministry of Health, 2003]. The local ethics committee approved the study.

RF Exposure Conditions

The exposure system and conditions were made as similar as possible to those in the original studies [Vinogradov and Dumansky, 1974, 1975; Shandala and Vinogradov, 1982; Vinogradov and Naumenko, 1986]. Rats were exposed in the far field to an elliptically polarized 2450 MHz continuous wave RF field from above the ring at an incident power density of 5 W/m^2 at the cage location for 7 h/day, 5 days/week for a total of 30 days of exposure.

Actual and Sham RF exposure was carried out in two shielded anechoic chambers. The walls, floor and ceiling of the chambers were covered by RF-absorbing material made of ferrite-based pyramidal elements 0.05 m high. The absorbing frequency range of the material was 300 MHz–15 GHz and the reflection factor was minus 15–20 dB over the frequency range. Outer walls of the chambers were covered with welded steel sheets. The chambers had combined extraction and input ventilation. Chamber 1 for the Sham-exposed animals was 10 m × 3 m × 3.5 m, and chamber 2 for RF-exposed animals was 6 m × 3 m × 3.5 m. To make the chambers visually the same size, a black opaque curtain was put in chamber 1. Since there was no daylight in the chambers, they were illuminated with six high-efficiency lamps (26 W) with colour temperature of 4200 K (daylight). Prior to exposing the rats, the air temperature, relative humidity, air exchange rate, lighting, electric field strength and magnetic flux density in the frequency range 5 Hz–30 kHz, and gamma-dose rate were measured in both chambers. The environmental conditions in both chambers were

very similar. During the study, air temperature and relative humidity in the chambers were recorded immediately before and after each exposure. The air temperature and relative humidity in chamber 1 were in the range of 20–21 °C and 38–62%, respectively; in chamber 2 these parameters were in the range of 20–22 °C and 36–62%, respectively.

The Sham and RF-exposed animals were placed in special cages arranged in a ring in each chamber (Fig. 2). The cages (Atelier Déco Volume, Limoges, France) were made of dielectric materials, Plexiglas and PVC, with holes for ventilation. Each ring consisted of 16 cages with one rat per cage. Rats were free to move and cages were covered with transparent lids. Each ring was placed above the floor on eight styrofoam stands 0.18 m high.

RF was generated by a diathermy unit, SMV-150-1 "Luch-11" magnetron (Electronic Medical Apparatuses (EMA), Moscow, Russia), with a standard helical antenna having an external diameter of 90 mm. The generator produced continuous RF at 2450 ± 50 MHz and was connected to the antenna using a feeder about 8.5 m long, made of RK50-11-21 coaxial cable (Kazenergokabel, Pavlodar, Kazakhstan) with Teflon insulation. The antenna was fixed 2.35 m above the floor in chamber 2, and was mounted on a bracket made of plastic and wood (Fig. 2). The output of the "Luch-11" was set to 71.0 ± 7.3 W antenna input power.

Measurements of equivalent plane wave power density were made using a Narda EMR-20 broadband meter (Pfullingen, Germany), connected to a personal computer through a fiber-optic link. The meter was equipped with an isotropic E-field sensor. The accuracy of measurements was ± 2 dB. Background measurements of the power density in the chambers did not exceed 0.0017 W/m^2 at all measurement points. Measurements of the power density were performed at points corresponding to the geometric centre of each cage at 0.22 m above the floor in the absence of the animals and cages.

The equivalent plane wave power density values at the reference point were recorded after turning the generator on and just before turning it off. Periodic (two times a week) monitoring of generator output

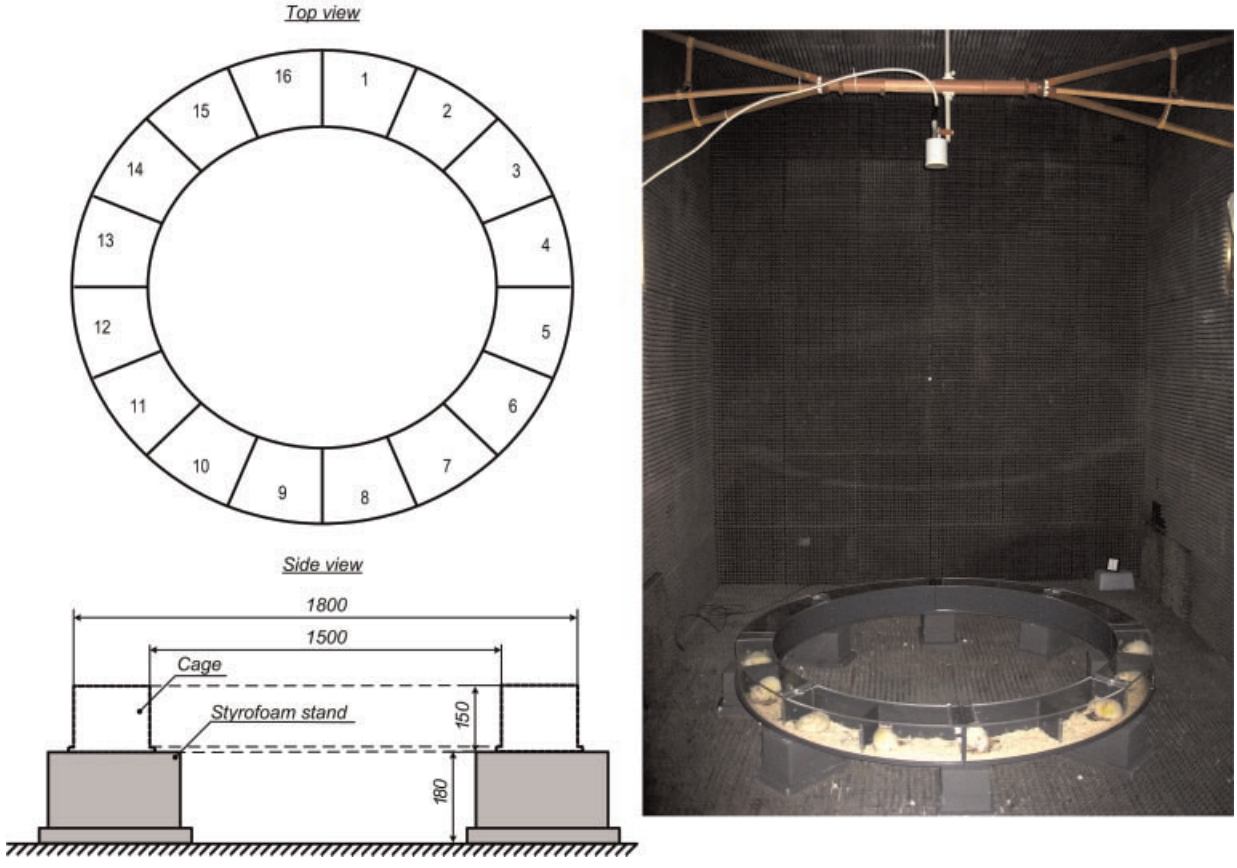

Fig. 2. General scheme of the RF exposure setup, illustrating the ring containing the cages for the animals (sketch) and the fixed antenna above the ring. [The color figure for this article is available online at wileyonlinelibrary.com.]

power was carried out by measuring the power density at the reference point for 7 h. The output power was found to be reasonably stable (less than ±0.5 dB).

During exposure, the behaviour of the rats was monitored with a web camera connected to a computer. The web camera was located well away from the animals so as not to interfere with the RF exposure. Rats were calm and slept most of time. To conduct the experiment in a blind manner the two groups of rats were marked with different colours by a small label of 10–15 mm painted on the rat's back. Investigators who evaluated the animal studies were not informed which rat colour belonged to the RF- or Sham-exposed group. After termination of the exposure, rat tissues were sampled for the two studies.

Immunological study: Complement Fixation Test

The complement fixation test (CFT) was conducted at low temperature (4 °C) [Shubik, 1987] to evaluate the ability of antibodies (mainly IgM subclass) in blood to react with antigens in brain and liver extracts [Sinaya and Birger, 1949; Birger, 1982]. The CFT was implemented in the same manner as the original Soviet studies. Blood serum, brain and liver were taken from five rats from each group on the 7th day after 30-day RF exposure and from 11 rats from each group on the 14th day after 30-day RF exposure.

Methods of blood sampling and preparation of tissue homogenates from brain and liver were the same as in the original Soviet studies [Vinogradov and Dumansky, 1974, 1975; Vinogradov and Naumenko, 1986]. Each rat was anaesthetized by intraperitoneal injection of 0.75 ml of 5% hexenal solution. Anaesthetized rats were held on an operating table and the chest surface was sterilized with a 5% solution of iodine in ethanol. The thorax was opened and the lungs and heart gently cut. The blood in the chest cavity was collected with a sterile Pasteur pipette and placed in sterile test tubes and sealed. Collected blood was placed in an incubator and maintained at 37 °C for 30 min. Then, using the Pasteur pipette, clots of fibrin were removed from the walls of the test tube. For the best collection of serum, the test tube was placed for 30–60 min in a refrigerator (4 °C). The serum was collected using a sterile Pasteur pipette and centrifuged at 2000*g* for 5 min. Transparent serum was used in serological reactions (1 ml). Extracted brain and liver were cut with scissors into small samples, separated from connecting and fatty tissues, and washed in physiological saline solution so that they were free of blood. Then, one part by weight of crushed brain/liver was added to four parts of physiological saline solution, made homogeneous using a glass tissue homogenizer, and centrifuged at 2000*g* for 20 min. The fluid above the brain/liver mix was placed in ampoules, sealed and kept frozen at −20 °C.

The reaction of complement fixation was conducted on six different blood serum dilutions in physiological saline solution (1:5, 1:10, 1:20, 1:40, 1:80 and 1:160) with respective brain/liver homogenates, and the outcome of the reaction was judged by a group of three experts for visual assessment of the amount of precipitate and liquid colour. The outcome of the reaction was assigned "−" if there was no reaction between antibodies and antigens, and a rank ranging from "+" to "++++" was assigned based on the intensity of the reaction between antibodies and antigens. Serum dilutions at which "++" positive reactions were registered were used to study the difference in the amount of antibodies to brain/liver antigens between the three groups of rats. For this purpose, the Mann–Whitney *U*-test was used and differences at $P < 0.05$ were considered to be statistically significant. For comparison with the results of the original Soviet studies, logarithms of serum dilutions at which "++" positive reactions were registered were calculated in the following manner. A numerical value was assigned corresponding to a serum dilution at which "++" positive reactions were registered, and the logarithm was calculated. For example, if the positive reaction occurred at 1:5 dilution, a value of 5 was assigned; if the positive reaction occurred at 1:10 dilution, a value of 10 was assigned, and so on. Logarithms of the assigned values were calculated. If there was no positive reaction at any dilution, the logarithm was assigned a 0 value.

ELISA

In addition to the CFT, the ELISA test was used to evaluate immunological responses induced by RF exposure via analysis of the level of antibodies reacting with selected antigens [Semballa et al., 2004; Nasta et al., 2006; Mangas et al., 2008]. This test was not used in the original Soviet studies. ELISA was performed using the blood serum samples collected for the CFT on days 7 and 14 after the exposure. Circulating antibodies (IgA, M and G isotypes) were evaluated for 16 antigens, shown in Table 1, selected by our French collaborators based on the results of the earlier Soviet studies suggesting autoimmune and degenerative processes. ELISA tests were conducted in a blind manner. The coded sera were diluted at 1/500 in buffer and diluted circulating antibodies were pipetted into the wells of the ELISA plates, provided by the IMS Laboratory of the University of Bordeaux. The intensity of the reaction was measured twice, as an optical density (OD) or absorbance for each blood serum at 492 nm (maximum colour absorption) and 620 nm

TABLE 1. Characteristics of the Chemical Substances Tested Using the ELISA Test Method

Abbreviations	Tested antigens	Pathological significance
3OH Kyn	3 Hydroxykynurenin	Tryptophan metabolites: **neurotoxic** (3OH Kyn, 3OH Ant, Quina) or **immunomodulators** (Ant/Xant, KynA)
Ant/Xant/3OH Ant	Anthranilic and xanthurenic and 3OH anthranilic acids	
KynA	Kynurenic acid	
Quina	Quinolinic acid	
Aze	Azelaic acid	Products of oxidative processes and hydrolysis of unsaturated fatty acids: **autoimmunity**
MDA + 4HNE	Malondialdehyde residue + 4-hydroxynonenal	Final products of **lipoperoxydation**
C6-C8-C10-C12	Fatty acids with small chains	Non-accessible to the immune system; recognized following membrane alteration or after abnormal interaction with other constituents of the self: **autoimmunity**
C6-C8-C10-C12 OH	Hydroxylated fatty acids	
Pi	Phosphatidyl inositol	
Pal/Myr/Ole	Palmitic/myristic/oleic acids	
NO2Tyr	NO2-tyrosine	Neoantigenes resulting from an **hyperproduction of NO** and **peroxynitrite**
NOArg	NO-arginine	
NOCys + NOBSA	NO-cystein + NO-bovine serum albumin	
NOMet + NOAsn + NOHis	NO-methionine + NO-asparagine + NO-histidin	
Now + NOTyr	No-tryptophan + NO-tyrosin	
CAT	Cystenyl-catecholamines: dopamine + L-DOPA + HVA + epinephrine + nor-epinephrine	Oxidation of catecholamines and nucleophilic attack by SH of thiol groups: **neurotoxicity**

Bold font indicates the main processes associated with the tested antigens. (Courtesy of Dr. M. Geffard, University of Bordeaux, France)

(optical dispersion). Blank wells were measured at the same time and readings were subtracted. If measurements were negative, they were set to 0. Since the blank measurement was subtracted, only ODs above 0.1 were considered as measurements above noise. To validate the quality of the plates, positive controls were obtained using known antibodies against some tested chemical substances and the test was shown to be appropriate. The results of the ELISA test on 16 antigens were analyzed using the *Z*-approximation of the Mann–Whitney *U*-test; a *P*-level <0.05 was considered to be significant. To study crucial biological changes, OD measurements were pooled in groups characterizing the reaction of an antibody (IgA, IgM or IgG) with chemically similar antigens; the first group comprised products of the interaction of amino acids with nitric oxide (NO) or its derivatives and the second group comprised fatty acids.

Selection of Pregnant Rats and Formation of Groups for Study of Exposed Blood Serum on Reproductive Endpoints

The animal model was similar to the one used in an earlier study conducted by Shandala and Vinogradov [1982]. Overnight mating was performed with one male and three females. Vaginal smears were taken the next morning. Mating was considered successful if sperm and/or a vaginal plug were found. The day of finding sperm was considered to be day 0 postconception (p.c.).

For the study, 59 sperm-positive rats were selected following mating and divided into three groups (Table 2, Fig. 1). The first group (group 1) comprised 17 sperm-positive female rats that served as controls. The second group (group 2) consisted of 21 female rats to which 1 ml of blood serum from Sham-exposed rats, taken on day 14 after the exposure, was injected IP on day 10 p.c. The third group (group 3) included 21 female rats to which 1 ml of blood serum from RF-exposed rats, taken on day 14 after the exposure, was injected IP on day 10 p.c.

In utero development and newborns. On day 15 of pregnancy, 5–6 pregnant female rats from each group were sacrificed to evaluate embryo mortality. Also, the number of implants, corpora lutea of pregnancy, live embryos, resorbed embryos, as well as the mass of the embryos and placentas were recorded in each group of rats. Embryo development and placental formation was assessed by weight. On day 20 of pregnancy, four female rats from groups 2 and 3 were sacrificed to evaluate total in utero mortality and the fertility index; the number of implants and live embryos were also

TABLE 2. Characteristics of the Three Groups of Pregnant Female Rats Selected for the Study

Group	Characteristics	Number of pregnant female rats	Time of injection (days)	Number of rats at different times of observation		
				Sacrificed on day 15 of pregnancy	Sacrificed on day 20 of pregnancy	Left for offspring study
1	Biological control	17	—	6	—	11
2	Rats injected with blood serum from Sham-exposed rats	21	10	6	4	10[a]
3	Rats injected with blood serum from RF-exposed rats	21	10	5	4	12

[a]One rat was found not to be pregnant.

recorded for these rats. In each group, 11–12 pregnant female rats were kept alive until delivery to study offspring development and survival. At delivery, the number of newborns in a litter, body mass of newborns, number of stillborns and apparent birth defects were registered. During pregnancy, daily monitoring of rats was performed including the behaviour and eating activity, and the condition of the hair cover and mucous layers of the nose, auricles, eyes and tail skin. Rats who had spermatozoa in their vaginal smear but did not develop offspring at supposed times were sacrificed to establish the presence or absence of pregnancy.

Postimplantation loss was assessed for each group using the following equation:

$$\text{Embryo mortality } (M_{\text{u}}) = \frac{C}{A + C} 100\% \qquad (1)$$

where A is the total number of live embryos and C is the total number of resorbed embryos in the respective group.

Total in utero embryo mortality was assessed for each group using the following equation:

$$\text{Total in utero mortality } (M_{\text{tu}}) = \frac{B\text{-}A}{B} 100\% \qquad (2)$$

where A is the total number of live embryos and B is the total number of corpora lutea in the respective group.

Postnatal offspring mortality after postnatal day 30 was assessed for each group with the following equation:

$$\text{Postnatal mortality } (M_{\text{p}}) = \frac{N\text{-}D}{N} 100\% \qquad (3)$$

where N is the total number of newborns and D is the number of live offspring after postnatal day 30 in the respective group.

Study on the effects on postnatal development of the offspring. Postnatal offspring development was studied for the first 30 postnatal days using generally accepted integral and specific parameters. The integral parameters were body mass and survival. Changes in body mass were determined over the first postnatal month by weekly measurements. The specific parameters were appearance of hair cover, detachment of auricles, opening of eyes, eruption of incisors and onset of independent eating.

A one-way ANOVA was used for comparison of grouped means if normality of distributions and homogeneity of variances were confirmed; in case the results were significant, a multiple comparison test (Tukey test) was used to test pairwise differences between the studied groups. If a single-factor ANOVA was not applicable, the Kruskal–Wallis and corresponding nonparametric multiple comparison tests were used.

RESULTS

Body Mass

Changes in the average body mass for the three groups of male rats (RF-exposed, Sham-exposed and control rats) during quarantine, 6-week exposure, and 1 week after the exposure were found to comply with the average mass of rats in normal experimental studies [Darenskaya et al., 2004]. Table 3 shows that the gain in body mass was similar in the three groups. There were no statistically significant differences in average body mass between the RF- and Sham-exposed groups. However, after the exposure they were statistically lower than in the control group, by 18 and 27 g, respectively ($P < 0.01$). This can be explained by the fact that the control rats had food and water all the time while Sham- and RF-exposed rats were restricted in food and water during exposure. Body mass of pregnant and lactating rats in the study on pre- and early postnatal development of offspring was not monitored.

Dosimetry

Simulations performed by Dr. Philippe Lévêque, the contracted dosimetrist for our study, showed that the exposure setup provided power density values in the

TABLE 3. Changes in the Average Body Mass (g) of Rats From the Three Studied Groups Before Exposure (Week 0), Over the Exposure Period (Weeks 1–6) and After Exposure (Week 7)

	Average body mass (g) of rats at weeks of the study							
	Before exposure	Exposure						After exposure
Groups	0	1	2	3	4	5	6	7
RF-exposed	146.6 ± 2.3	157.5 ± 4.5	200.6 ± 5.0	215.3 ± 6.0	244.1 ± 7.1	269.4 ± 6.9	276.6 ± 8.0	285.6 ± 7.1[a]
Sham-exposed	137.2 ± 1.3	157.5 ± 4.3	189.1 ± 7.3	198.4 ± 9.1	235.3 ± 8.1	253.8 ± 8.4	263.8 ± 6.9	276.6 ± 6.2[a]
Cage control	142.8 ± 2.5	170.9 ± 4.4	204.1 ± 7.2	236.9 ± 7.5	262.8 ± 8.6	276.3 ± 9.7	283.4 ± 9.2	303.7 ± 9.5

[a]Statistically significant difference compared to cage control group ($P < 0.01$).

cages of about 5 W/m^2; however, the RF was slightly perturbed by the ring of cages. The power density at the geometric centre of the cages varied from 4.29 (2.70 to 6.78) to 5.93 (3.74 to 9.37) W/m^2 with an average value of 4.95 (3.12 to 7.82) W/m^2. SAR was evaluated for these exposure conditions using the finite-difference time-domain (FDTD) method. For the calculations a rat model with 36 different tissues and a resolution of 0.75 mm (Brooks Air Force Base, San Antonio, TX) was used. To estimate the SAR in the rat model, only a part of the entire ring was considered (1/4 of the structure). The power density distributions were similar to those when the entire system was considered. Four rat phantoms were placed in cages in one-fourth of the ring. The SAR levels were evaluated in the whole-body and different tissues/organs for each rat model. The mean and standard deviation values were estimated in the real exposure conditions for each tissue/organ. The whole-body SAR was 0.16 ± 0.04 W/kg at 5 W/m^2. The averaged SAR in the brain was about 0.16 W/kg. A maximum peak SAR value of 9.9 W/kg was calculated in the tail skin; maximum peak SAR value for the brain was 1.0 W/kg. The influence of the distance between two rats placed in their cages was studied and showed a very weak coupling between animals under our exposure conditions. Whole-body SAR variation caused by RF polarisation was about 15%. Different rat positions inside cages changed the whole-body SAR value by less than 5%.

Effects on the Immune System

The results of our CFT, indicating levels of antibodies reacting with brain and liver homogenates in the three studied groups of rats on days 7 and 14 after exposure, are presented in Table 4. The table comprises median serum dilutions at which a ++ positive reaction was registered. Also in Table 4, the average of the logarithms of titres at which a ++ positive reaction was

TABLE 4. Results on the Formation of Antibodies in Brain and Liver of Rats From Long-Term Low-Level RF Exposure

				Statistical characteristics	
Antigen	Day after exposure	Group of rats	Number of rats	Median	Logarithm (log) of titre (M ± SE)[a]
Brain	7	Cage control	5	<1:5	0.34 ± 0.21
		Sham-exposed	5	1:5	0.68 ± 0.21
		RF-exposed	5	1:5	0.68 ± 0.18
	14	Cage control	11	1:5	0.69 ± 0.08
		Sham-exposed	11	1:10[b]	0.89 ± 0.05[d]
		RF-exposed	11	1:20[c]	1.19 ± 0.07[e]
Liver	7	Cage control	5	< 1:5	0.28 ± 0.17
		Sham-exposed	5	1:10	0.66 ± 0.28
		RF-exposed	5	1:5	0.54 ± 0.23
	14	Cage control	11	< 1:5	0.06 ± 0.06
		Sham-exposed	11	1:5	0.38 ± 0.11
		RF-exposed	11	1:5	0.44 ± 0.13

[a]M ± SE is arithmetic mean of the logarithm of antigen titre and SE is the standard error.
[b]Different from cage control group at P-level ≥0.05 (using the Mann–Whitney U-test).
[c]Different from Sham-exposed group at P-level <0.01 (using the Mann–Whitney U-test).
[d]Different from cage control group at P-level <0.05 (using the t-test).
[e]Different from Sham-exposed group at P-level <0.01 (using the t-test).

observed are included to compare with the results of the earlier Soviet studies. It is seen from Table 4 that on day 7 for cage control rats the median serum dilution was below 1:5, that is, in most rats the reference positive reaction was not observed when minimum diluted serum was used in the reaction with brain and liver homogenates, thus indicating a small amount of antibodies in blood serum. In Sham- and RF-exposed rats the median serum dilution was similar in the reaction with brain homogenates and was 1:5, but was different in the reaction with liver homogenate, being 1:10 and 1:5, respectively. No statistically significant differences in the levels of antibodies against brain (or liver) antigens were seen between the three groups on day 7 after termination of RF exposure. On day 14 after RF exposure, an increase in the median serum dilution was seen in the reaction with brain homogenates in the three studied groups compared to the median levels registered on day 7. Only in the control group was the increase not statistically significant; in the Sham-exposed group median serum dilution increased from 1:5 to 1:10, and in the RF-exposed group the increase was more pronounced, from 1:5 to 1:20. The levels of antibodies against liver antigens did not change significantly. On day 14 after termination of the exposure, the difference in levels of antibodies against brain antigens between RF- and Sham-exposed groups became statistically significant ($P < 0.01$). Nevertheless, the results show that the difference between the Sham-exposed and control groups was almost significant ($P \approx 0.06$), which could be explained by stress and other factors (as discussed below). The appearance of antibodies against liver antigens was smaller than against brain antigens.

Circulating antibodies directed against 16 antigens (indicated in Table 1) were evaluated in coded serum from each group of rats. Table 5 gives the median OD for IgA, IgG and IgM interacting with antigens resulting from a hyperproduction of NO and peroxynitrite. Table 6 gives the median ODs for IgA, IgG and IgM interacting with fatty acids and products of oxidative processes. It is seen from Tables 5 and 6 that there was an increased amount of compounds resulting from interaction of amino acids with NO or its derivatives (NO_2-tyrosine, NO-arginine, NO-cysteine + NO-bovine serum albumin, NO-methionine + NO-asparagine + NO-histidine, NO-tryptophan + NO-tyrosin), as well as fatty acids with short chains (C6-C8-C10-C12; C6-C8-C10-C12; Pal/Myr/Ole) in blood serum from RF-exposed rats. Antibodies to Aze (product of oxidation of fatty acids) were determined only in the IgM fraction on day 7 after the exposure, and median ODs were equal to 0.31, 0.20 and 0.21 in RF-exposed, Sham-exposed and control groups, respectively. The difference between the RF- and Sham-exposed groups was statistically significant ($P < 0.05$). The response was weak to Ant/Xant/3OH Ant and was absent for the remaining antigens (3OH Kyn, CAT, MDA + 4HNE, Pi, Quina). As a rule, antibodies to conjugated antigens were seen for IgM, rarely seen for IgG, and were completely absent for IgA. The levels of antibodies were higher on day 7 after exposure compared to those on day 14 after exposure and the differences were not statistically significant between the control and Sham-exposed groups. However, in the RF-exposed group the difference in the levels of antibodies on days 7 and 14 was statistically significant (Tables 5 and 6).

Effects on Pre- and Postnatal Development of Offspring

A response to injection of blood serum was observed in one rat from the Sham-exposed group and three rats from RF-exposed group. These rats were sluggish, slow-moving, refused food and water, and lay rolled up in a ball most of the time. Such response

TABLE 5. Content of Antibodies (IgA, IgM and IgG subclasses) to Products of Interaction of Amino Acids With Nitric Oxide (NO) or Its Derivatives (NO_2-Tyrosine, NO-Arginine, NO-Cysteine + NO-Bovine Serum Albumin, NO-Methionine + NO-Asparagine + NO-Histidine, NO-tryptophan + NO-Tyrosin) in Blood of Rats (Median Optical Densities)

			Immunoglobulin (Ig)		
Group of rats	Day after exposure	Number of reactions	A	G	M
Cage control	7	25	<0.1	0.14	0.13
Sham-exposed		25	<0.1	0.13	0.14
RF-exposed		25	<0.1	0.18[a]	0.21[b]
Cage control	14	55	<0.1	0.11	0.12
Sham-exposed		55	<0.1	0.14	0.11
RF-exposed		55	<0.1	0.15	0.17[c]

[a]Different from Sham-exposed group at P-level = 0.02.
[b]Different from Sham-exposed group at P-level <0.001.
[c]Different from Sham-exposed group at P-level = 0.05.

TABLE 6. Content of Antibodies (IgA, IgM and IgG Subclasses) to Fatty Acids With Small Chains, Hydroxylated Fatty Acids, and Palmitic/Myristic/Oleic Acids (C6-C8-C10-C12; C6-C8-C-C12OH; PAL/MYR/OLE) in Blood of Rats (Median Optical Densities)

			Immunoglobulin (Ig)		
Group of rats	Day after exposure	Number of reactions	A	G	M
Cage control	7	15	<0.1	0.13	0.24
Sham-exposed		15	<0.1	0.23	0.26
RF-exposed		15	<0.1	0.25	0.36[a]
Cage control	14	33	<0.1	0.14	0.25
Sham-exposed		33	<0.1	0.15	0.28
RF-exposed		33	<0.1	0.17	0.28

[a]Different from Sham-exposed group at P-level = 0.02.

continued for up to 1 h. Three of the four pregnant rats later delivered normal offspring and one rat from the RF-exposed group had all embryos resorbed.

Study of pregnancy. On day 15 of pregnancy, that is, 5 days after injection of blood serum, the number of live embryos per animal did not differ significantly among the studied groups and was equal to 7.5 ± 0.4, 8.3 ± 0.2 and 7.4 ± 0.4 in groups 1, 2 and 3, respectively. The average mass of embryos of rats from groups 2 and 3 was similar (190.4 ± 5.4 and 185.4 ± 4.7 mg, respectively) and was higher than in the control group (151.1 ± 1.6 mg). The mass of placentas of rats from group 2 statistically differed from control rats (182.0 ± 5.9 and 172.7 ± 2.2 mg, respectively) but was not statistically different from group 3 (175.9 ± 4.2 mg). The ratios of placenta-to-embryo mass (so-called ''placental coefficient'') were 1.14 ± 0.16, 0.96 ± 0.03 and 0.95 ± 0.04 in groups 1, 2 and 3, respectively, and did not differ significantly between each other.

Data on embryo mortality evaluated on day 15 of pregnancy are shown in Table 7. It is seen that embryo mortality was higher in rats from group 3, however, this was not significantly different compared to the other groups.

On day 20 of pregnancy, that is, 10 days after injection of blood serum, the number of live foetuses per animal did not differ significantly between groups 2 and 3 and was equal to 8.3 ± 0.7 and 7.5 ± 0.8, respectively. The average foetal mass in rats also did not differ significantly between these groups and was equal to 3.8 ± 0.1 and 3.7 ± 0.1 g, respectively. In utero foetal mortality on day 20 of pregnancy increased compared to that on day 15, and did not differ significantly between the rats from groups 2 and 3, being $19.5 \pm 6.3\%$ and $23.1 \pm 6.8\%$, respectively.

All rats from groups 1 and 2 delivered offspring on day 22 of pregnancy; in group 3, two rats delivered offspring on day 22 of pregnancy and another two on day 23. Of the total number of pregnant rats left for delivery, offspring were delivered in 100% of rats in the control group (11 rats from 11 animals); 90% of rats from group 2 (9 rats from 10 animals) and 33.3% of rats from group 3 (4 rats from 12 animals). From the group of rats injected with blood serum from the Sham-exposed animals (group 2) two rats that did not deliver offspring were sacrificed, one was found not to be pregnant, and another had all embryos resorbed. Eight rats from the group of rats injected with blood serum from RF-exposed animals (group 3) that did not deliver offspring were also sacrificed and all were found to have their embryos resorbed. Because the body mass of rats was not measured during pregnancy, it was not known when the resorption of embryos occurred.

Total in utero foetal mortality was evaluated using the data on foetal mortality on days 15 and 20 of pregnancy and foetal resorption in rats that were pregnant but did not deliver offspring. Table 8 shows that total

TABLE 7. Embryo Mortality in Rats on the 15th Day of Pregnancy

	Number of				
Group of rats	Pregnant rats	Implants	Live embryos	Resorptions	Embryo mortality, $M_u \pm m$, %[a]
1	6	47	45	2	4.3 ± 2.9
2	6	53	50	3	5.7 ± 3.2
3	5	42	37	5	11.9 ± 4.6

[a]m is CI half-width.

TABLE 8. Total In Utero Mortality

	Group of rats					
	1		2		3	
Time of observations	Number of implants	Number of resorptions	Number of implants	Number of resorptions	Number of implants	Number of resorptions
Day 15 of pregnancy	47	2	53	3	42	5
Day 20 of pregnancy			41	8	39	9
Rats without offspring	—	—	—	—	70	70
Total number	47	2	94	11	151	84
Total, $M_{tu} \pm m$, %	4.3 ± 2.0	11.7 ± 3.3	55.6 ± 4.0			

Different from group 1 at P-level <0.05.
Different from group 2 at P-level <0.001.

in utero mortality among rats from group 3 was significantly higher compared to rats from groups 1 and 2 ($55.6 \pm 4.0\%$, $4.3 \pm 3.0\%$ and $11.7 \pm 3.3\%$, respectively).

Influence on prenatal development was assessed from the number of live foetuses on day 20 of pregnancy and the number of live newborns at delivery. Table 9 shows that in rats from group 3, the number of live foetuses and newborns per pregnant rat was significantly lower than in groups 1 and 2 (3.8 ± 1.1, 8.1 ± 1.1 and 8.7 ± 0.8, respectively). However, the number of live foetuses and newborns in rats that had live offspring did not differ significantly between the groups and was equal to 8.1 ± 1.1, 10.2 ± 0.9 and 8.7 ± 1.3 in groups 1, 2 and 3, respectively.

Offspring mortality and development. High postnatal mortality during the first 30 days of life was observed in our study in the control group (34%). This result does not correspond to the normal outcomes for these rats and our data for postnatal period cannot be used in the analysis. For this reason, the results of our study of offspring development are not included in this paper.

DISCUSSION

A CFT was used in our replication study to evaluate the presence of antibodies (mainly IgM) reacting with brain or liver homogenates. A notable increase in the level of antibodies against brain antigens was seen in the Sham- and RF-exposed groups of rats on day 14 after termination of the 30-day RF exposure. Such an increase could be explained by long-term hypokinesia (reduced movement during the whole experiment) and stress reactions of the animals. It is known that hypokinesia in space [Ivanov and Shvets, 1978] or in laboratory animals [Portugalov et al., 1976] results in an increase in autoantibodies in blood serum available for complement fixation. However, on the 14th day after the 30-day exposure, the increase in antibodies against brain antigens in the RF-exposed group was statistically different from the Sham-exposed group, even noting their state of hypokinesia. Formation of antibodies against brain antigens is less pronounced in our study compared with the results of earlier Soviet studies [Vinogradov and Dumansky, 1974, 1975; Vinogradov et al., 1991] but the general trend is similar. Vinogradov et al. [1991] showed average logarithms of titres against brain antigens equal to 1.2 ± 0.1 in the Sham-exposed group and 2.8 ± 0.2 in the RF-exposed group after termination of the RF exposure. Vinogradov and Dumansky [1974, 1975] reported no statistically significant differences between average logarithms of titres against brain antigens in Sham-exposed and RF-exposed groups on day 7 after the termination of exposure (1.8 ± 0.1 and 2.1 ± 0.1, respectively), however, on day 14 after the RF exposure, maximum levels of antibodies against brain antigens were seen in these groups and differences between the Sham- and RF-exposed groups became significantly different (1.95 ± 0.06 and 2.5 ± 0.2, respectively). According to our results, shown in Table 4, on the 7th day after RF exposure, antibodies against brain tissue were similar between the Sham and RF-exposed groups; on the 14th day after exposure, antibodies against brain tissue increased in the Sham-exposed group from 0.68 ± 0.21 to 0.89 ± 0.05, and in the RF-exposed group from 0.68 ± 0.18 to 1.19 ± 0.07 and the differences between the two groups became statistically significant. It should be noted that the earlier studies evaluated characteristics of immunity using different parameters that allowed a more reliable estimate of the expression of autoimmune processes due to chronic non-thermal RF exposure. However, assessment and analysis of these parameters was not included in our replication study.

Levels of intracellular forms of active oxygen and NO were evaluated. Normally, they are necessary for

TABLE 9. Rat Fertility in the Three Studied Groups

Group of rats	Total number of pregnant rats	Sacrificed on day 20 of pregnancy			Deliveries			Total number of newborns and live foetuses on day 20 of pregnancy	Fertility		
		Total number	With live foetuses	Total number of live foetuses	Left for delivery	Gave birth			% of rats with live offspring	Number of live foetuses and newborns per rat that gave birth	Number of live foetuses and newborns per pregnant rat
						Number	%				
1	11	—	Not assessed	Not assessed	11	11	100	90	100	8.1 ± 1.1[a]	8.1 ± 1.1[a]
2	14	4	3	33	10	9	90	122	85.7	10.2 ± 0.9	8.7 ± 0.8
3	16	4	3	30	12	4	33.3[b]	61	43.8[b]	8.7 ± 1.3	3.8 ± 1.1

[a]CI half-width.
[b]Different from group 2 at P-level <0.01.

tissues and organisms to function since they work as signalling species for regulation of the cellular network, and determine the response to a perturbing exposure [Mikhaylov et al., 2003]. It was shown in our study (Tables 5 and 6) that RF exposure resulted in an increase in the content of amino acids bound with NO compounds, as well as lipids, including non-saturated fatty acids with short chains and their oxidation products (Aze). Enhanced production of these compounds that activate the peroxidation of lipids, the decreased production of antioxidants and the failure of DNA and protein-repair processes result in cellular oxidative stress. In our study, development of oxidative stress was weak and short-term. The maximum content of antigen-specific bound antibodies was seen on day 7 after termination of the RF exposure and subsequently decreased on day 14 (Tables 5 and 6).

A study was conducted to replicate the study of Shandala and Vinogradov [1982] on the influence of blood serum from exposed rats with an increased amount of antibodies on pre- and early postnatal development of offspring. The study showed high in utero mortality in rats injected with blood serum from RF-exposed animals ($55.6 \pm 4.0\%$) than in female rats injected with serum from Sham-exposed animals ($11.7 \pm 3.3\%$). These data suggest a more pronounced embryotoxic effect from RF-exposed serum compared to Sham-exposed serum. The in utero mortality in our study was higher than in the study of Shandala and Vinogradov [1982] in all groups of rats (Table 10). It is seen from Table 10 that the number of animals that delivered live offspring and the number of foetuses per rat were similar in both studies. However, we cannot guarantee that the effects depend only on the influence of RF exposure since there was high variability in the following parameters: offspring mortality, mass of embryos, placental coefficient and unusually high mortality in offspring at later ages.

In our opinion, Shandala and Vinogradov [1982] chose a rather complex model that can be subject to variable results and is not an appropriate model for assessing the impact on human health from RF exposure. There are stress responses in the rats, participation of a number of very complex functional systems, and pregnancy itself changes the functional condition of all rat systems. These could all contribute to the wide data scatter seen in our results. It should be noted that our experiment was carried out 25 years after the original study. Unfortunately, a lot of information required to replicate this study was lacking in the original publications, making comparisons with our results more difficult. Because of these problems, we consider the experiment on pre- and early postnatal development of offspring as a pilot study that argues for

TABLE 10. Comparison of the Test Results of Teratology Experiments with Findings of Shandala and Vinogradov [1982]

Parameters	Study	Groups 1	2	3
In utero mortality, %	Our study	4.3 ± 2.0	11.7 ± 3.3[a]	55.6 ± 4.0[b]
	Shandala and Vinogradov [1982]	0	0	28
Animals with live offspring, %	Our study	100	85.7 ± 8.0	43.8 ± 12[c]
	Shandala and Vinogradov [1982]	100	100	50
Number of live foetuses per rat	Our study	8.1 ± 1.1	10.2 ± 0.9	8.7 ± 1.3
Number of live foetuses per pregnant rat	Our study	8.1 ± 1.1	8.7 ± 1.3	3.8 ± 1.1[c]
	Shandala and Vinogradov [1982]	7.9 ± 0.6	9.0 ± 0.78	2.6 ± 1.04

[a]Different from group 1 at P-level <0.05.
[b]Different from group 2 at P-level <0.001.
[c]Different from group 2 at P-level <0.01.

the necessity of carrying out a larger and more powerful study.

Our CFT study showed the same tendency of RF exposure to influence the formation of antibodies to brain tissue homogenates as the results of the earlier Soviet-era studies. However, our study showed that quantitative interpretation of the CFT outcomes was rather complex and could be influenced by assumptions accepted in the study. The ELISA test supported our views on the occurrence of intracellular oxidative stress reactions from RF exposure, showing possible development of pathological processes if an unfavourable influence remains. Our study on the influence of blood serum from RF-exposed rats injected into pregnant rats showed the same tendency as earlier Soviet studies on adverse effects on pre- and early postnatal development of offspring. Statistically significant results were obtained using four parameters (Table 10). These parameters of the RF-exposed group differed statistically from Sham-exposed and control animals.

CONCLUSIONS

This study was conducted using the methodology of the original experiments conducted in the USSR [Vinogradov and Dumansky, 1974, 1975; Shandala and Vinogradov, 1982] and the agreed protocol of the Bordeaux–Moscow Project on Confirmation studies of the Russian data on immunological effects of microwaves [Statement of Work, 2006]. Autoimmunity was evaluated using the original methodology developed in the USSR [Vinogradov and Dumansky, 1974, 1975; Shandala and Vinogradov, 1982]. The original methodology was a CFT, however, our study was expanded to include the ELISA test. The Russian study was conducted in accordance with WHO recommendations on RF biological research and Good Laboratory Practice (GLP) principles.

The results of our immunology study using the CFT and ELISA tests partly confirmed the results of the Soviet research groups on the possible induction of autoimmune responses (formation of antibodies to brain tissues) and stress reactions from RF exposure (30-day exposure for 7 h/day for 5 days/week at a power density of 5 W/m^2, i.e., long-term non-thermal RF exposure).

The results of our study on prenatal development of offspring suggested possible adverse effects of the blood serum from exposed rats (30-day exposure for 7 h/day for 5 days/week at a power density of 5 W/m^2) on pregnancy and embryo–foetal development in rats, in agreement with the earlier results of Shandala and Vinogradov [1982], although the model used by Shandala and Vinogradov [1982], which was intentionally replicated here, is not considered an appropriate one for assessing human health effects from RF exposure.

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