SECTION 8

Evidence for Effects on the Immune System Supplement 2012

Immune System and EMF RF

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I. INTRODUCTION

Population exposure to electromagnetic fields (EMF) from mobile phones is continuous and long-term. Unfortunately this is still not taken into account in international standards. Thus it is important to consider immunological studies that relate to chronic and long-term exposure to EMF since the immune system was considered as a critical system in studies conducted in the former USSR. The results of these studies were important for developing standards in the former USSR and the current Russian exposure limits.

Both national and international scientists have studied the immune system as a possible critical system from short exposure to radiofrequency (RF) fields of low intensity (Fiskeko et al. 1999a; Novoselova et al. 1999; Kolomeitcheva et al. 2002; Cleary et al. 1990; Czerska et al.1992; Moszczynski et al. 1999; Stankiewicz et al. 2006; Nasta at al. 2006, Prisco et al. 2008; Johansson 2009; Pinto et al. 2010; Sambucci et al. 2010; Ait-Aissa et al. 2012 and others). These studies were performed under different conditions of EMF exposure as well as different methods and end-points. Analysis of these study results still does not allow criteria for standards development. However, there are only a few studies that are important and were performed in the 1970-1990s by scientists at the Kiev Institute of Public Hygiene headed by Academician Mikhail Shandala (Dronov and Kuritseva 1971; Vinogradov and Dumanski, 1974, 1975; Shandala and Vinogradov, 1982; Vinogradov et al. 1985; Shandala, et al.1983, 1985**;** Vinogradov and Naumenko, 1986; Vinogradov et al.1987; Vinogradov et al, 1991).

It should be emphasized that these studies were conducted many years ago using methodological recommendations published by the Ukrainian Ministry of Health in 1981 on evaluation of biological actions of microwave radiation of low intensity necessary for development of hygienic regulations (Ukrainian Ministry of Health 1981). Using these recommendations all studies were conducted under the same conditions and so subsequent studies can be considered as a replication of the previous studies that was important for the validity of the final results.

In the first pilot studies conducted in the beginning of the 1970s it was shown that exposure to RF with power density of 15 $\mu W/cm^2$ resulted in disruption of the antigen structure of brain tissue leading to the formation of sensitized lymphocytes and the development of autoimmune reactions.

These studies have been described and translated by Repacholi et al (2012) and part of

the translation from this paper has been incorporated here.

Dronov and Kiritseva (1971) exposed 15 rabbits to 50 μW/cm^2 and 5 rabbits to 10 μW/cm^2 UHF (no frequency given) fields for 4h/day for 4 months. The 15 animals exposed to 50 μW/cm^2 were divided into 3 groups of 5 animals each; the 1st group was sensitized (injected with an antigen) during exposure, the 2nd group sensitized before exposure, and the 3rd group sensitized after exposure. The 10 μW/cm^2 group was sensitized during exposure. Immunological changes were assessed using the agglutination reaction, the reaction to indirect hemagglutination, and differential determination of macro- and micro-globulin antibodies with a sedimentation constant of 19S (IgM) and 7S (IgG), respectively. The authors reported that 50 μW/cm^2 caused a decreased antibody response only when exposure occurred prior to or during sensitization and no effect was produced from the 10 μW/cm^2 exposure.

Vinogradov and Dumanski (1974) exposed white rats EMF 2450MHz at 50 μW/cm^2 for 5 h/day for 14 days. The authors reported alterations to the structure and/or expression of tissue antigens using the method of anaphylaxis with desensitization. In this study 25 white rats were included, of which 20 were UHF exposed (PD of 50 μW/cm^2). Sera from these and 5 control animals were investigated for the content of antibodies against normal and exposed animals, using the complement binding reaction in the cold. The reaction was started immediately after exposure and weekly afterwards for one month. The results of theses experiments are shown in Table 1.

Table 1. Complement binding reaction in white rats after UHF exposure (M ± m)
(Vinogradov and Dumansky 1974 modified from Repacholi et al. 2012)

Antigen from brain tissue of	Background		Immediately after radiation		After 1 week		After 2 weeks		After 3 weeks		After 4 weeks	
	No. of positive reactions	Log_{10} antigen titre	No. of positive reactions	Log_{10} antigen titre	No. of positive reactions	Log_{10} antigen titre	No. of positive reactions	Log_{10} antigen titre	No. of positive reactions	Log_{10} antigen titre	No. of positive reactions	Log_{10} antigen titre
Exposed rats	0	0	7	1.60±0.19	17	2.1±0.11*	18	2.46±0.2**	18	2.51±0.06**	5	1.54±0.31
Normal rats	0	0	6	1.50±0.14	18	1.80±0.13	16	1.95±0.06	4	1.45±0.18	0	0

* $p < 0,05$
** $p < 0,01$

The authors concluded RF exposure could induce expression of antigens not normally expressed in brain tissues and/or alter antigen structure of normally expressed antigens.

Therefore these early studies established that exposure to RF at power density (PD) of 50 μW/cm^2 could result in changes in antigenic structure of tissue and blood proteins. These changes were characterized by the appearance of new nonspecific antigenic qualities and partial elimination of normal antigens, i.e. the exposure resulted in changes of antigenic structure of tissues. However, this conclusion required confirmation and further exploration. As a result a few subsequent studies were performed at longer long-term RF exposures.

Vinogradov and Dumanski (1975) reported that exposure to 2450 MHz fields 7h/day for 30 days at 50 μW/cm² induced autoantibodies reacting with brain tissue antigens in Guinea pigs, white Wistar rats and rabbits. Autoimmune reactions were identified using the complement binding reaction (CBR) and plaque forming cell techniques that revealed the presence of antigen-specific antibodies and antigen-specific antibody-producing cells, respectively. Moreover, leukocytes from UHF-exposed Guinea pigs showed a reduced serum-mediated phagocyte activity.

To obtain the antigen from exposed brain tissue, brains from donor animals, housed under the same conditions as experimental ones, were sacrificed immediately at the end of the exposure cycle. Blood to conduct the CBR was collected according to the following schedule: background, immediately after exposure, and then after 2, 4, 6, and 8 weeks after exposure. The results are shown in Table 2. The study showed that RF exposure of animals (guinea pigs and rats) at 50 μW/cm² resulted in the alteration of protein structure in brain tissues and production of circulating brain antigens.

Sampling time	Guinea pigs			White rats		
	No. of reactions	No. of positive reactions	Log_{10} of antibody titres (M±m)	No. of reactions	No. of positive reactions	Log_{10} of antibody titres (M±m)
Background	24	0	-	20	0	-
Immediately after exposure	24	19	1.95 ± 0.06	20	7	1.60 ± 0.19
2 weeks after exposure	24	20	2.77 ± 0.04	20	18	2.46 ± 0.2
4 weeks after exposure	24	20	2.56 ± 0.05	20	18	2.51 ± 0.06
6 weeks after exposure	24	18	2.05 ± 0.07	20	19	2.10 ± 0.11
8 weeks after exposure	24	13	1.71 ± 0.05	20	5	1.54 ± 0.31

Table 2. Dynamics of titres of antigens against brain in Guinea pigs and white rats after UHF exposure at 50 μW/cm² ,Vinogradov and Dumansky 1975 (From Repacholi et al. 2012)

The results shown in Table 2 indicate a time-dependence in the formation of circulating antibodies against the brain. The antibody titre in Guinea pigs increased in time after the exposure and reached a maximum 2 weeks after exposure ($\log_{10}$ of the titre was 2.77 ± 0.04). The authors concluded that chronic exposure to RF at a PD of 50 μW/cm^2 resulted in the formation of brain antigens in the animals. This process was observed using brain tissue from both exposed and non-exposed animals. The highest titres of compliment binding were observed 10-14 days after exposure.

The results of the subsequent study, published in the same paper (Vinogradov and Dumansky 1975), indicated a similar time-dependent trend suggesting that the action was consistent. The authors investigated the cellular auto-immune reaction by determining the number of spot forming cells, synthesising antibodies against its own erythrocytes in the blood. The study was conducted on Guinea pigs and white rats that were exposed for one month to UHF fields at a PD of 50 μW/cm². The Jerne reaction in blood was performed before exposure, immediately after the end of exposure, and then after 2 and 4 weeks. Results of the study are shown in Table 3.

Animal species	No. of animals	Background	Immediately after exposure	2 weeks after exposure	4 weeks after exposure
Guinea pigs	10	2.1 ± 0.21	2.8 ± 0.4	14.7 ± 1.1	9.01 ± 0.6
P-value			> 0.05	< 0.001	< 0.001
White rats	7	1.5 ± 0.15	1.57 ± 0.20	10.4 ± 1.0	6.7 ± 0.8
P-value			> 0.05	< 0.001	< 0.001

Table 3. Percentage of spot forming cells from Guinea pigs and white rats after UHF monthly exposure at a PD of 50 μW/cm² (M±m),
Vinogradov and Dumansky 1975 (Modified from Repacholi et al. 2012)

As seen from Table 3, a statistically significant increase in the percentage of spot forming cells was observed during the second week after exposure and was quite stable. Four weeks after the exposure the % still remained high.

Subsequently the same authors (Vinogradov and Dumansky, 1975) performed a study to investigate adverse properties of blood serum after UHF exposure based on the determination of changes in the phagocytic capacity of the cells. Fifteen Guinea pigs were included in the study, which were exposed to UHF at a PD of 50 μW/cm^2 for 1 month. Phagocytosis was determined three times – before exposure and 2 and 4 weeks after the exposure. Table 4 shows the results of phagocytosis in three stages of the study. These data indicate that serum from the exposed animals has a pronounced suppressive effect both on phagocyte number and the phagocyte index. This effect was pronounced in blood serum collected 2 weeks after exposure and remained for another 2 weeks.

Guinea pig serum before exposure		Guinea pig serum 2 weeks after exposure		Guinea pig serum 4 weeks after exposure	
Phagocyte no.	Phagocyte index	Phagocyte no.	Phagocyte index	Phagocyte no.	Phagocyte index
63.4 ± 3.2	6.28 ± 0.5	29.6 ± 2.4 $P < 0.001^{*}$	3.61 ± 0.56 $P < 0.01^{**}$	22.9 ± 3.0 $P < 0.001^{*}$	4.10 ± 0.6 $P < 0.05^{**}$

* compared to the phagocyte number in Guinea pig before exposure
** compared to the phagocyte index in Guinea pig before exposure

Table 4. Suppression of the phagocyte reaction under the influence of sera from exposed animals, Vinogradov and Dumansky 1975(From Repacholi et al. 2012)

Considering the results of these three studies it can be concluded that long-term RF exposure at low intensity (50 μW/cm^2) results in auto-allergic reactions.

Shandala et al. (1983) exposed CBA mice and Wistar rats to 2375 MHz (7 h/day). When mice were exposed to 0.1 or 10 mW/cm^2 it increased spontaneous and mitogen-stimulated (PHA) cell proliferation, which persisted for 30 days after the last exposure. When rats were exposed for 3 months to 1 or 5 μW/cm^2 or for 1 month at 10, 50, 500 μW/cm^2, there was a decrease in proliferative response to PHA, still evident 3 months post exposure. No effects were observed with 10 and 50 μW/cm^2 in rats. The authors concluded that RF exposure induced important changes in T-cell immunity.

Vinogradov et al. (1985) exposed white Wistar rats for 30 days to 10, 50, 500 μW/cm^2 (2375 MHz) and a sham-exposed group used as controls. Induction of autoantibodies toward brain tissue antigens (brain extracts) was evaluated with the complement binding/fixation assay and pathological effects assessed by injecting auto-antibody-containing sera into pregnant animals. Electrophoresis patterns of sera immunoglobulin were also evaluated. Exposure to 50 and 500 μW/cm² induced autoantibodies to brain tissue antigens as revealed by indirect degranulation of basophiles and complement fixation assays. No effects were induced from exposure to 10 μW/cm^2. Exposure to 50 and 500 μW/cm^2 also decreased cell proliferation (blast formation). Sera from exposed (or sham-exposed) rats were injected into pregnant rats to verify whether the presence of the autoantibodies was pathological. Sera from rats exposed to 500 μW/cm^2 increased post-implantation loss and decreased the number, body weight and length of the newborns. Analyses of soft tissues from the fetuses revealed the presence of hemorrhage in subcutaneous tissues, peritoneal cavity, liver and brain. The authors also reported that exposure to 500 μW/cm^2 (but not 10 μW/cm^2 or 50 μW/cm^2) led to alterations in immunoglobulin electrophoresis, with the appearance of a new peak similar to that of class A antibodies, and concluded that it caused strong changes in physico-chemical and

immunological properties of serum humoral factors. The authors concluded that such changes might render proteins naturally produced in the body as immunologically "foreign" and stimulate auto-immune responses.

To repeat the results of Shandala et al. (1985) and Vinogradov and Naumenko (1986) exposed Wistar rats to 2375 MHz fields at 50 or 500 μW/cm² for 30 days for 7 h/day and confirmed that exposure to 500 μW/cm² induced anti-brain antibodies using complement binding and basophiles degranulation assays, and increased plaque-forming cells, suggesting RF exposure altered brain tissues rendering them immunogenic. When rats were injected with extracts from animals exposed to 500 μW/cm² the authors also reported an increased number of reticulo-endothelial and plasma cells in bone marrow and spleen and a decreased number of small lymphocytes in bone marrow.

Vinogradov et al. (1991) exposed female Fisher rats to 2375 MHz (500 μW/cm², 7 h/day for 15 days). Exposure effects were assessed by injecting lymph node cells from exposed or sham-exposed animals into normal recipient rats. This was to determine if it was possible to transfer the "conditions of autoimmunity caused by the exposure" into recipient animals. Analyses were then performed on both donor and recipient rats and, consistent with previous reports, the authors found exposure reduced mitogen-stimulated cell proliferation (PHA and Con A) and induced auto-antibodies toward brain tissue antigens as shown by basophiles degranulation and plaque forming cell assays. Moreover, cells injected from exposed animals (but not from sham-exposed rats) "led to analogous conditions" in normal recipient rats.

Shandala and Vinogradov (1982) exposed 11 pregnant white Wistar rats to UHF (500 $\mu W/cm^2$, 7 h/day for 30 days) and reported an increased response to fetal liver antigens in terms of both frequency of antibody-producing lymphocytes in blood and auto-antibodies in serum, compared to 11 unexposed controls. Lymphocytes from exposed pregnant rats also showed a reduced mitogen-stimulated cell proliferation compared with controls. When sera were injected into pregnant rats (10 exposed and 10 controls) "to evaluate the pathological meaning of the auto-antibodies", sera from exposed rats increased embryo lethality during pregnancy and higher offspring mortality at around 1 month of age.

Shandala et al. (1985) exposed female Wistar rats to UHF fields (2375 MHz) at 50 and 500 $\mu W/cm^2$ for 7 h/day for 30 days. They investigated induction of autoantibodies and found these exposures induced the formation of autoantibodies to brain tissue extract using the basophiles degranulation technique. The authors then investigated the immunogenicity of brain extracts from exposed animals by injecting these extracts into normal animals. Their hypothesis was that normal tissue should not induce antibodies to brain tissue since recipient animals should recognize them as their own tissues. If exposure to UHF induced alterations in antigen expression and/or structure, the

tissue extract should become immunogenic and therefore able to raise an antibody response. The authors reported that brain tissue extracts from animals exposed to 50 and 500 μW/cm^2 induced antibodies in injected animals, but basophiles degranulation was seen only in animals injected with extracts from animals exposed to 500 μW/cm^2. To assess the pathological significance of the autoantibodies they injected sera from animals exposed to 500 μW/cm^2 into pregnant rats and this increased post-implantation loss. No effects were induced by the injection of sera from animals exposed to 50 μW/cm^2. The authors concluded that only exposure to 500 μW/cm² was capable of inducing anti-brain antibodies, leading to an adverse effect.

When Vinogradov et al. (1987) reviewed the results of these immunological studies they concluded that exposure to UHF at a power density of 500 μW/cm^2 irreversibly damages organisms while 50 μW/cm^2 induces some effects often non pathogenic, and 10 μW/cm^2 does not affect any immunological parameters. This early assessment seems to have been given much credence by all subsequent standards committees.

When the public health standards committees analyzed all studies they agreed with Vinogradov et al. (1987):

- 100-500 μW/cm^2 chronic daily exposure can induce persisting pathological biological reactions (based on the immunology studies above), the most striking effect being offspring death after injection of foreign serum.
- ~ 50 μW/cm^2 is the threshold exposure for unfavorable biological effects (based on the immunology studies above). These effects were not pathological since the organism could compensate for the exposure but continual compensation could lead to long-term adverse effects and thus should be protected against.
- ≤10-20 μW/cm^2 chronic exposure does not induce any noticeable biological changes in small laboratory animals.

Therefore, specialists from the Kiev Institute in 1970-1980s showed that there was a clear dose-dependence in biological effects of RF on the immune system. Chronic RF exposure at 500 μW/cm^2 in the frequency range 1750-2750 MHz resulted in significant changes in the immune status of immunocompetent globulin fractions, and changes in antigenic structure of tissue and blood proteins resulted in the development of autoimmune processes. Chronic exposure at 1-20 μW/cm^2 did not result in changes to immunological status. These results, as well as studies of other systems of the animal chronically exposed to RF fields at the same PDs were used for establishing the first standards in the former USSR.

Russian-French study performed under WHO EMF project (2006-2009)

Considering the importance of the results obtained in 1970-1980s (described above) for harmonization of standards (performed in a special program on development of a scientific basis for setting standards for RF EMF) the International Advisory Committee of the World Health Organization's (WHO) Program "EMF and health" included in 2006 research agenda to perform studies to attempt to replicate the results of the earlier immunological studies.

With the purpose to replicate and confirm the results of the earlier Soviet studies we selected two major immunological and teratological studies described above; these were Vinogradov and Dumansky 1974 and Shandala and Vinogradov 1982.

In our replication study the original scientific methods were used, but a modern exposure system, dosimetric and biological methods were used. The study was conducted in a blind manner; in addition to the CBR, the ELISA test was used to evaluate immunological responses induced by RF exposure.

Preparatory work for the replication study began in 2006: a program and detailed protocol of the study were developed and were subsequently discussed and agreed with WHO and approved by an independent International Advisory Committee (IAC), who included scientists from Germany (J. Bushmann), Italy (C. Pioli) and USA (R. Sypnewski). The Committee was chaired by the head of WHO EMF project Dr. Mike Repacholi.

With agreement with WHO, the former SRC Institute of Biophysics (now the Federal Medical Biophysical Centre of FMBA, Moscow, Russia) was chosen to implement the study. Animal exposure and dosimetric evaluations were jointly performed by specialists from the Centre for Electromagnetic Safety (Moscow, Russia) and the IMS laboratory (University of Bordeaux, France). The RF exposure conditions were jointly agreed by the scientific group and the IAC. The exposure geometry resulted in relatively uniform exposure of animals in the study as confirmed by dosimetric evaluations.

Scientists in the key specialties were invited to perform the replication study. During the quarantine period (14 days) and exposure period (30 days) the animals were handled in a blind manner by scientists from the radiobiological laboratory of the Institute of Biophysics (supervised by Prof. N.G. Darenskaya).

The replication study began in October 2006. The International Advisory Committee monitored all steps of the study, including the final results and conclusions. The final scientific report and conclusions of the replication study were reviewed by IAC. The main results of the study were published in English in "Bioelectromagnetics" journal (Grigoriev et al. 2010a) and as a series of papers

in Russian in the "Radiation Biology. Radioecology" journal (Grigoriev et al. 2010, Lyaginskaya et al. 2010). English translation of these papers was published in "Biophysics" journal (Grigoriev et al. 2010b-e, Lyaginskaya et al. 2010).

The following section briefly describes this replication study (Grigoriev et al 2010a-e).

The study of immunological and reproductive effects of long-term low-level microwave exposure was conducted on Wistar (WI) rats in a blind manner. There were three groups of rats, each consisting of 16 males: (1) the RF-exposed group included rats that were exposed to low-intensity RF in an anechoic chamber, (2) the sham-exposed group included rats that were treated in the same way as (1) but were not RF-exposed, and (3) the cage control group included rats 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 pregnant rats on the 10th day of pregnancy. For the latter study mature rats (90 females and 30 males) were used.

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 Sham and RF-exposed animals were placed in special cages arranged in a ring in each chamber (Fig. 1). The cages (Atelier Deco 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.

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. 1). The output of the ''Luch-11'' was set to 71.0 ± 7.3 W antenna input power.

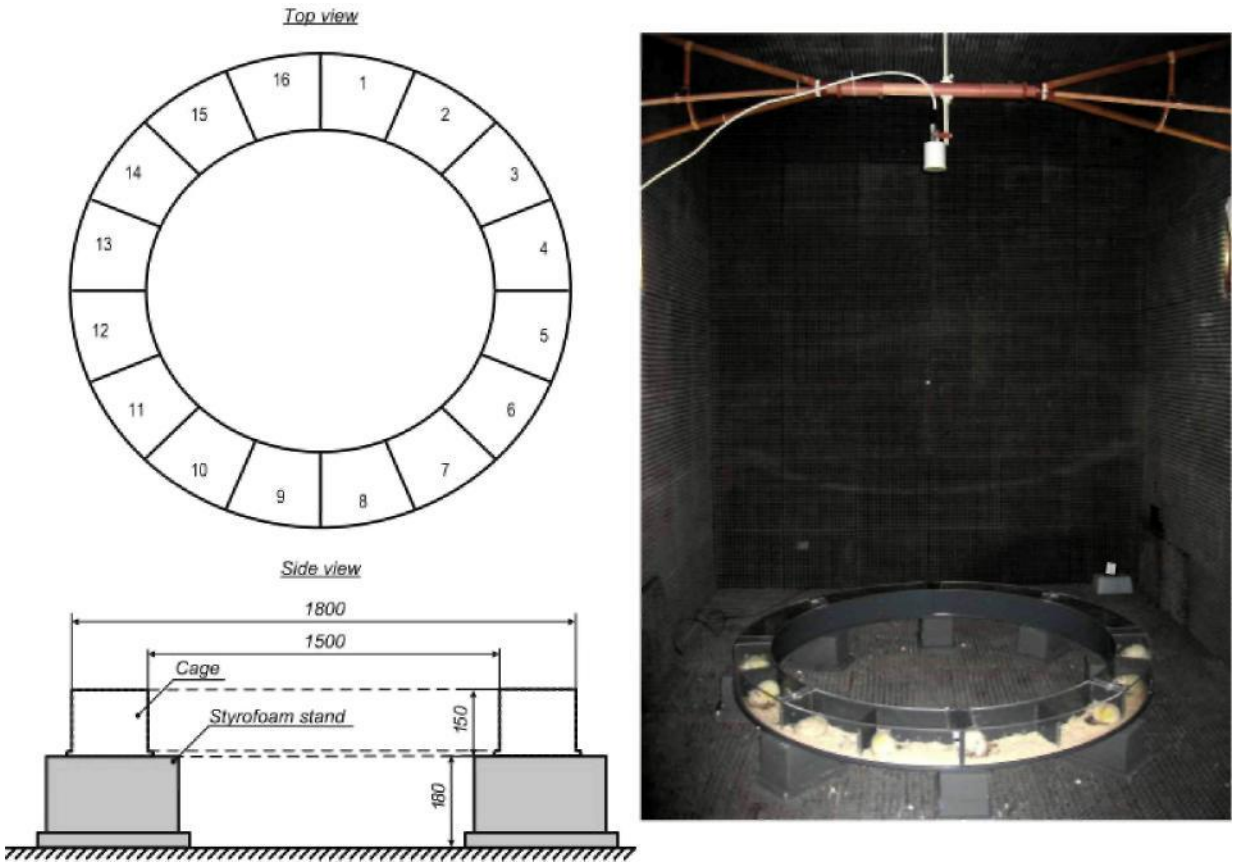

Fig. 1. General scheme of the RF exposure setup, illustrating the ring containing the cages for the animals (sketch) and the fixed antenna above the ring (from Grigoriev et al. 2010a)

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. A detailed description of the exposure conditions and dosimetric measurements is provided in Grigoriev et al. 2010a. Dosimetric calculations were performed by Dr. Philippe Leveque, the contracted dosimetrist for our study. They showed that the whole-body SAR evaluated for the exposure conditions was 0.16 ± 0.04 W/kg. 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. After termination of the exposure, rat tissues were sampled for the two studies (immunological and teratological).

Study of the effects on the immune system

The immunological study was performed using the Complement Fixation Test (or Complement Binding Reaction) at low temperature (Shubik, 1987) and the modern ELISA test.

The Complement Fixation Test (CFT) was used 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.

The 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). They are described in detail in Grigoriev et al. 2010a.

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 color.

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, selected by our French collaborators based on the results of the earlier Soviet studies suggesting autoimmune and degenerative processes (Grigoriev et al 2010a).

The results of our CFT showed that there were no statistically significant differences in the levels of antibodies against brain (or liver) antigens between the three groups on day 7 after termination of RF exposure (Grigoriev et al 2010a). 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 the increase was not statistically significant; in the Sham-exposed group the 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 ($P < 0.01$). However, our CFT results showed that the difference between the Sham-exposed and control groups was almost significant, which could be explained by stress and other factors. The appearance of antibodies against liver antigens was smaller than against brain antigens (Grigoriev et al 2010a). The results of our CFT are shown in Fig. 2 in units used in the original studies.

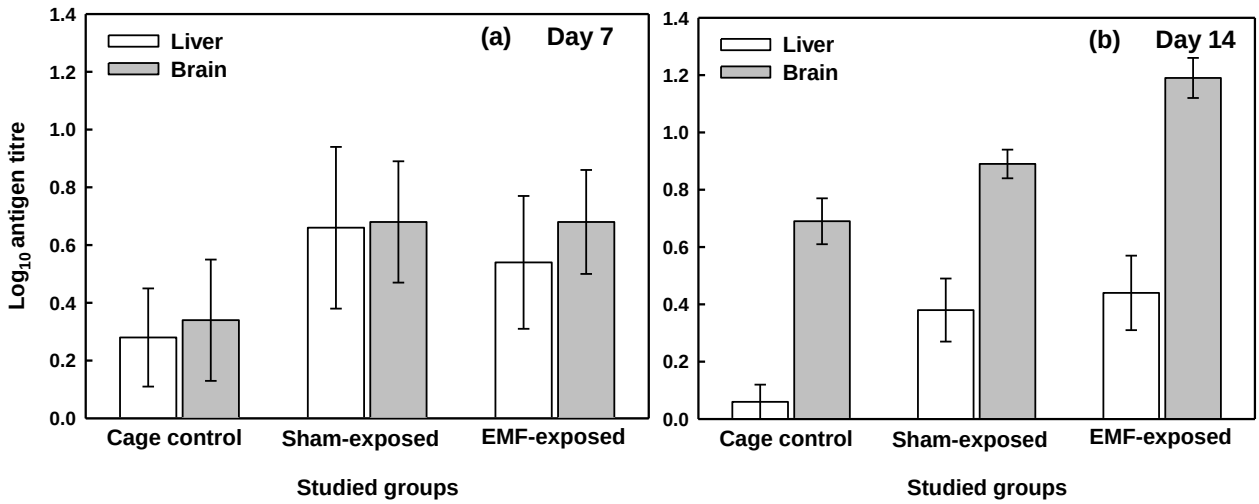

Fig.2. Average $\log_{10}$ antigen titre in the three groups of rats on day 7 (a) and day 14 (b) after the termination of the exposure shown for liver (white boxes) and brain (grey boxes) antigens. Vertical bars represent standard errors. The results are shown in units used in the original studies.

In our opinion, a notable increase in the level of antibodies against brain antigens seen in the Sham- and RF-exposed groups of rats on day 14 after termination of the 30-day RF exposure 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. Comparison of our results with the results of earlier Soviet studies showed that the formation of antibodies against brain antigens was less pronounced in our study but the general trend was similar. 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.

Results of the evaluation of circulating antibodies directed against 16 antigens using the ELISA test showed that there was an increased number 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. Fig. 3 shows content of antibodies (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) on days 7 (a) and 14 (b) after the termination of the exposure. Levels of antibodies of IgA subclass were below

detection limit.

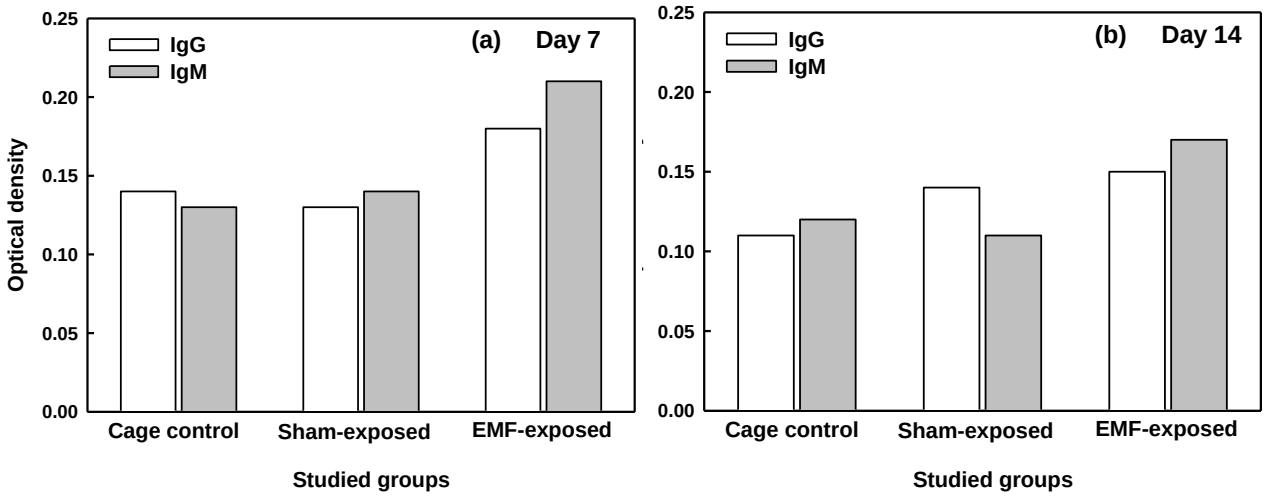

Fig. 3. Content of antibodies (IgM and IgG subclasses) to products of interaction of amino acids with nitric oxide (NO) or its derivatives in blood of rats from the three studied groups on days 7 (a) and 14 (b) after the termination of the exposure (median optical densities)

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 ($P < 0.05$). 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 (Grigoriev et al 2010a). 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 (Grigoriev et al 2010a).

On the whole, 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 unfavorable influence remained.

Study of the effects on pre- and postnatal development of offspring

The animal model in the teratology study on investigation of the exposed blood serum on reproductive endpoints was similar to the one used in an earlier study conducted by Shandala and Vinogradov (1982). Three groups of rats were in this study. 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 1ml 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 were studied using the following scheme (Grigoriev et al 2010a). 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 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. Study on the effects on postnatal development of the offspring. Offspring development was studied for the first 30 postnatal days using generally accepted integral and specific parameters. 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 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 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.

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 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 showed 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±0.1g, 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 ± 6.3% and 23.1 ± 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 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. Fig.4 shows that total in utero mortality among rats from group 3 was significantly higher compared to rats from groups 1 and 2 (55.6 ±4.0%, 4.3 ±3.0% and 11.7 ±3.3%, respectively).

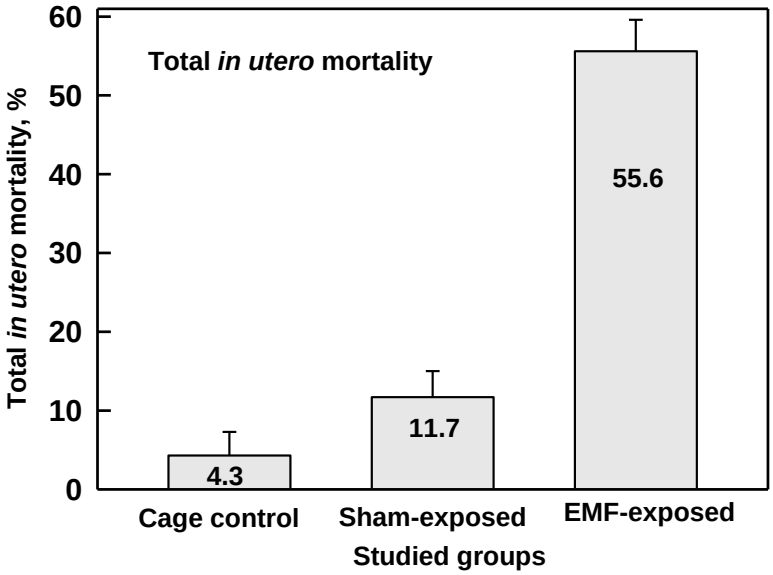

Fig. 4. Total *in utero* mortality in the three groups of rats

The 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. It was shown in our study that in rats from group 3, the number of live foetuses and newborns per pregnant rat (3.8 ± 1.1) was significantly lower than in groups 1 and 2 (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 (Grigoriev et al 2010a).

High postnatal mortality was observed during the first 30 days of life in our study of offspring mortality and development 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.

High *in utero* mortality in rats injected with blood serum from RF-exposed animals (55.6 ± 4.0%) than in female rats injected with serum from Sham-exposed animals (11.7 ± 3.3%) shown in our study suggests 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. 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 considered the experiment on pre- and early postnatal development of offspring as a pilot study that argues for the necessity of carrying out a larger and more powerful study.

The main conclusions from our study were as follows (Grigoriev et al. 2010a):

- 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 for7 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.

Analysis of the results of our study on RF effects on immune system allowed conclusion that data used in 1976 for development of RF standards in the USSR that are still in action in Russia were reasonable.

In an analogous study performed by our French colleagues using a similar protocol (except that CFT reaction was not implemented) (University of Bordeaux, IMS laboratory) no changes in immune status of animals were registered (Poulletier et al. 2009). However, in our opinion there were a few reasons that could influence the final results of this study. First of all, differences in the status of the experimental animals in these two studies. For example, the average body mass of rats at the end of our study was 275 g, and 400 g in the French study. More detailed discussion of these and other differences between the studies was provided in our comment (Grigoriev 2011).

Analogous results were obtained by our Ukrainian colleagues in a replication study (Tomashevskaya et al 2004). Unfortunately, these results were published as a brief summary in Ukrainian language. This study was conducted in the following conditions: chronic exposure of white outbred rats at 450 MHz for 2 h/day for 4 months. There were three experimental groups of rats exposed at different PDs: 250, 500 and 1000 mW/cm^2 and a sham-exposed group.

II. CONCLUSION

Available data allow the conclusion that the immune system is a critical system for evaluation of the effect of RF at low intensity and should be taken into consideration for development of standards.

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