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REPORT
UNDER THE AGREEMENT ON SCIENTIFIC COOPERATION
WITH AIRES HUMAN GENOME RESEARCH FOUNDATION

Subject: Study of high-frequency electromagnetic radiation impact and Aires resonators influence on behavior, genetic and epigenetic processes in cells of central and peripheral organs (models organisms: rat (*Rattus norvegicus*) and honey bee (*Apis mellifera* L.)

SECOND STAGE: Study of electromagnetic radiation, emitted by the router, and influence of Aires resonators - converters on stability of genetic apparatus in bone marrow cells of rat lines with different excitability of the nervous system.

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UNDER CONTRACT ON SCIENTIFIC COOPERATION BETWEEN FSBSI PAVLOV INSTITUTE OF PHYSIOLOGY OF THE RUSSIAN ACADEMY OF SCIENCE AND AIRES HUMAN GENOME RESEARCH FOUNDATION “Study of exposure to high frequency electromagnetic radiation and Aires resonators influence on behavior, genetic and epigenetic processes in cells of midline and peripheral organs (*Rattus norvegicus* and *Apis mellifera* L. models)”

THE SECOND STAGE (October 2016 – April 2017): study of exposure to electromagnetic radiation of a router and Aires resonators influence on stability of genetic apparatus in bone marrow cells of rats, originating from lines with different nervous system excitability levels.

Technical progress, increased power generation, communication systems development involve wide practical application of electromagnetic radiations of different frequencies, which affect living organisms. It is known that EMR enhancement, caused by common use of household appliances, mobile phones and wireless routers, affects genetic apparatus of cells in different organs of humans and animals. At the moment, accumulated evidence indicates that electromagnetic fields of different ranges, including mobile phones and WiFi ranges, can induce a wide spectrum of genetic damages, modify genes expression, affect structural and functional properties of cell nuclei (Kryukov, 2000; Deynekina, 2002; Boyko et al., 2010, Gapeev, 2006, Garaj-Vrhovac et al., 1992, Tice et al., 1999,2002, et al.) Ways of EMR influence on genetic apparatus of cells and organism on the whole are still not quite clear (Gapeev et al., 2000, 2001, Gapeev, 2006). Individual peculiarities of response to electromagnetic fields exposure, role of the nervous system’s functional state in mechanisms of responsiveness/ resistance to effect of such factors are hardly known.

The relevant task is to create systems, protecting from destructive EMR influence, and to study mechanisms of EMR influence on genetic processes in the cells of midline and peripheral organs of model objects subject to inherent properties of nervous processes, in particular, nervous system excitability. At present, Aires Company has created devices, which, based on fractal-matrix nanotechnologies of EMR transformation, efficiently redistribute EMR and have therapeutical value (<http://www.aires.spb.ru/nano.html>, <http://airestech.ru>). However, no studies on mechanisms of protective influence of the indicated devices on cells’ chromosome apparatus, providing for peculiarities of genetically determined nervous system excitation level, are available.

Purpose and objectives of the second stage:

1) to carry out the second replication of the experiment on assessment of EMR-caused impairment upon 6 h/day 4 day operation of a standard WiFi router, and protective influence of

Aires Defender fractal-matrix resonators on genetic apparatus of cycling bone marrow cells in male Vistar line rats.

2) comparative study of EMR influence on state of genetic apparatus of cycling bone marrow cells in male rates of VP and NP lines, differing in the level of the nervous system excitation, upon 6 h/day 4 day operation of a standard WiFi router.

3) comparative assessment of Aires Defender fractal-matrix resonators on state of genetic apparatus of cycling bone marrow cells upon EMR router damaging action in male rates of VP and NP lines, differing in the level of the nervous system excitation.

MATERIAL AND METHODS.

Subject if the study was rats of two lines, selected subject to nervous system excitability threshold – VP and NP (high and low excitability threshold correspondingly) (Vaido, 2000), and of initial Vistar line (body weight 300 – 350 g). VP and NP line rats were bred in the standard conditions of the animal facility in the higher nervous activity genetics laboratory of Pavlov Institute of Physiology of the Russian Academy of Science. The rats had a free access to water and food. Vistar line rats came from the central animal facility of FSBSI Pavlov Institute of Physiology of the Russian Academy of Science. After arrival the animals spent at least two weeks in conditions of the laboratory animal facility to ensure adjustment thereof. The males were kept in groups of six in standard cages with standard diet.

International principles of the Declaration of Helsinki on humane treatment of animals were observed upon work with animals.

Description of VP and NP line rats.

To study genetically determined connections between functional state of nervous system (excitability), brain functioning and a wide range of behavioral characteristics, breeding program was started in the seventies of the last century. The purpose of the program was to breed rat lines with different levels of nervous system excitability (Vaido, Sitdikov, 1979). Vistar line rats (Rappolovo) served as a starting material. Vistar rats are outbred line of albino rats (*Rattus norvegicus* species), they were bred in 1906. Currently, this is a widely spread line, used in medical and biological studies.

Direct selection was based on threshold of neuromuscular excitability upon electric current stimulation (rectangular electrical 2 ms pulses) of tibial nerve (n. Tibialis). 4 lines of rats with different excitability thresholds were bred: VP1, NP1, VP2, NP2 (high and low thresholds, 1 – 2: selection program number) (Vaido, Sitdikov, 1979). At present, two lines, which show the most different excitability thresholds, are maintained: VP1(VP) and NP2(NP). The indicated

lines have a history of over 70 selection generations. Unidirectional changes of peripheral and central nervous system divisions excitation (mostly, in subcortical system) are detected in these rats. Influence of nervous system excitability, and corresponding differences in manifestation of instinctive reactions and behavior characteristics, emotionality, aggressiveness, altruism, peculiarities of behavior strategy, ability to develop conditioned reflexes are found. Different elements of hormonal control systems, transmitters metabolism, ion channels functioning, structural and functional characteristics of nerve cell membranes change in rats of the indicated lines. Molecular and genetic studies show divergence of lines by the level of some genes expression as well. The lines have different stress responsiveness in deprivation of paradoxical phase, immobilization, short-term and long-term emotional and noxious stress tests. Long-term post-stress behavioral changes, persisting for 6 months, are demonstrated for the first time upon exposure of VP and NP rats to long-term emotional and noxious stress sensu K. Geht. Disorders of higher nervous activity are present in lines with different nervous system excitation level, however, each line presents specific disorders: VP line with low excitability – development of depression-like condition, increase of excitability, aggression, disruption of plastic processes; NP line with high excitability – appearance and preservation of compulsive movements, thus enabling to use these lines as a model to study post-stress pathology (depressed mood, post-trauma stress disorder (PTSD), compulsive disorder (CD)). Long-term stress effects are based on neuron structural changes in different brain structures, differential post-stress modifications of chromosomes in neurons and cells of peripheral organs, which is shown in characteristics studies of heterochromatin (condensed chromatin) and different epigenetic modifications of DNA and histone proteins in neurons (Vaido et al., 2009). At present, these lines are used to study genetic and epigenetic mechanisms of adaptive and pathological brain state in extreme conditions, and to search for nervous system excitability correlates (Dyuzhikova et al., 2015).

In the work presented here a standard WiFi router (LinkSys E1200-EE/RU wireless router) was used. The specifications thereof are as follows: wireless coupling frequency: 2.4GHz, number and type of antennae: 2 internal antennae, standard antenna(e) gain factor, dBi: 4 dBi.

To study EMR router influence, “home” cage with the animals was placed into the Faraday cage (Fig. 2), the router was placed under the upper lid of the cage on the removable shelf, in the center of the cage. Experimental groups were under the router influence during 4 days, 6 hours each day (8 am – 2 pm). Controls included groups of rats, who were placed into the Faraday cage without the router for the same period of time, and intact animals.

Aires Defender fractal-matrix resonators – transformers (special annular diffraction grids) were also used in the experiment (Zhabrev et al., 2005). Interaction of electromagnetic field with

Aires Defender results in structural transformation, partial suppression of the former, which does not hinder distance data exchange between message devices (the stage report of Gedeminas Vilnius Technical University).

To assess resonators influence on damaging action of router EMR, 6 resonators-transformers were used. They were placed in the center of each face of the Faraday cage (Fig. 2). One of the experimental groups of each tested line was exposed to the router effect in the Faraday cage with resonators during 4 days, 6 h per day. Moreover, one of the experimental groups of Vistar line was used to assess the effect of resonators as such (without the router action) on the studied parameters of the bone marrow cells.

Special preliminary measurements of WiFi router radiation energy were made in four points on the floor of the cage without and with the resonators. Kharchenko antenna was used for measurements (Fig 1).

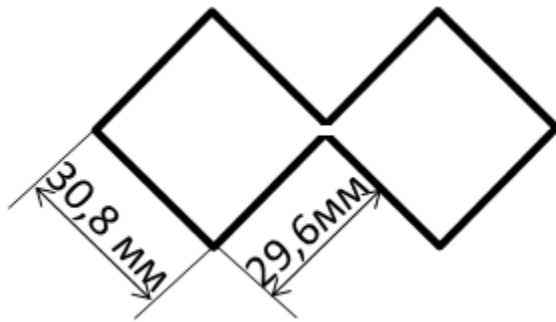
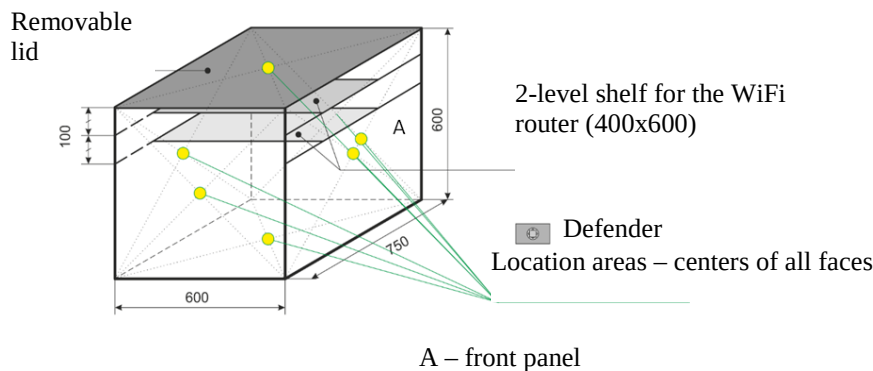


Fig. 1. Kharchenko antenna

Fig 2 shows the faraday cage and WiFi router and antenna layout therein.

Faraday cage for studies



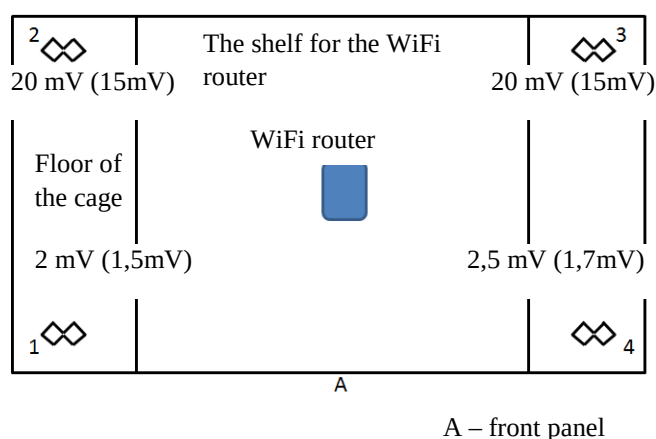


Fig. 2. Faraday cage and layout of the WiFi router and Kharchenko antenna therein. Points of the antenna location (1-4) and signal value (mV) at the antenna output without and with (in the brackets) resonators are shown.

The signal, received by the antenna, in layout points (1, 2, 3, 4, Fig. 2) is as follows, Table 1.

Table 1.

Layout point	1	2	3	4
Without resonators	2mV	20mV	20mV	2.5 mV
With resonators	1.5mV	15mV	15mV	1.7 mV

Bone marrow cells sample preparation. Upon extraction from femoral bones, 24 hours after the end of exposure, bone marrow was fixed in the fresh Clarke's solution (1 part of glacial acetic acid: 3 parts of ethanol) and stored at +4°C. Bone marrow samples were tinted with 2% solution of aceto-orcein and squash preparations were made according to the standard method (Makarov, Safronov, 1978; Dev et al., 2009).

Bone marrow samples analysis. Squash preparations of bone marrow cells were analyzed, using Mikromed-3 microscope at 640-1600x magnification. Chromosome aberrations at the anaphase – telophase stages (standard ana-telophase method) were recorded subject to additional recommendations (Daev et al., 2009). Min 200 cells of each animal were analyzed. Record was made of the number of normal and aberrant anaphases – telophases with the following deviations: single rearrangements (fragment, bridge, laggard), multiple rearrangements (two and more abnormalities of any type per cell).

Statistical analysis. Frequency of detected mitosis distortions was calculated in percents. Data was checked for homogeneity and normality of tested values distribution (Glotov et al., 1982).

Based on the check individual data were combined in groups. To determine significance of differences between variants the following was used: ANOVA test, Student t-test (upon normal distribution of data within groups), or Man-Whitney U test (upon abnormal distribution of data within groups); software applied - Statgraphics Centurion XV11 and Statistica 6.0.

FINDINGS

Repeated experiments on assessment of the router's electromagnetic radiation effect and influence of Aires resonators on stability of genetic apparatus in marrow bone cells of Vistar line rats enabled to confirm that router's action during 4 days, 6 h a day, causes disruption of genetic apparatus of cycling bone marrow cells: total frequency of mitotic disorders increases fourfold in comparison with control 2 (Faraday cage, 4 days x 6 hours) and with intact control 1 (Fig. 3). Resonators application resulted in decrease of chromosome disorders by 2.8 times against the proper router's action (Fig. 3). However, it is to be noted that in the repeated experiments combined effect of the router and resonators did not result in complete restoration of chromosome aberration level to the control level. Frequency of mitosis disorders at the background of significant decrease in comparison with the router's effect statistically exceeded values of control 1 and 2. Animals exposure in the Faraday cage with resonators only did not cause alteration of chromosome aberrations frequency in comparison with both control groups, however it was lower than in the "router + resonators" group.

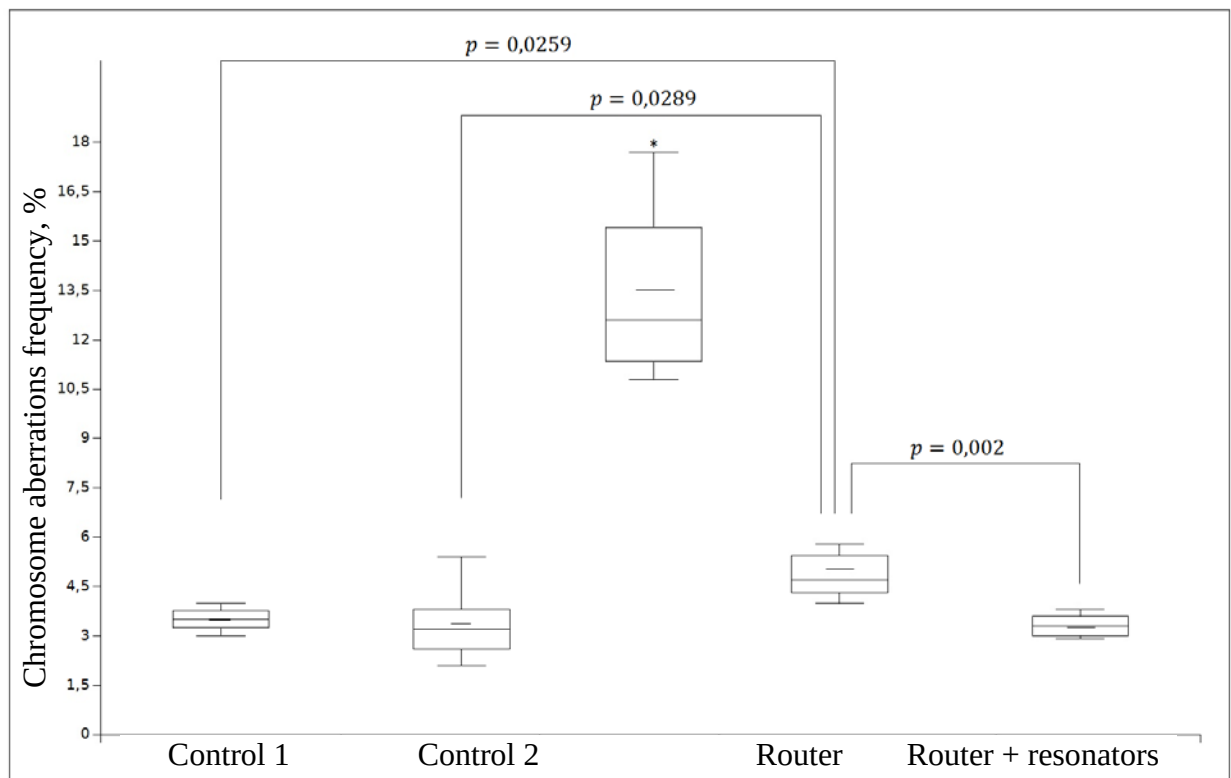


Fig.3. Frequency of chromosome aberrations in bone marrow cells of Vistar line male rats after exposure to electromagnetic radiation of the router and resonators. Median values, medium, boundaries of the first and third quartiles, min and max values of the sampling are presented.

Legend: Control 1 – intact rats, control 2 – rats, placed in the Faraday cage, * - differences against each group of “control 1”, “control 2”, “router+resonators” and “resonators” are statistically significant ($p < 0.0005$), brackets denote significantly different values, p- significance point.

During the second stage of studies under the contract it was shown that router's high frequency electromagnetic radiation upon exposure within 4 days, 6 h a day, caused disruption of genetic apparatus of cycling bone marrow cells in male rats of both lines under study - VP and NP: total frequency of mitotic disorders in low excitation line animals increases by 1.6 times in comparison with control 2 (Faraday cage, 4 days x 6 hours) and by 1.8 times in comparison with intact control 1 (Fig. 4). In rats of highly excitable NP line in the same conditions aberration frequency increases by 2.6 times in comparison with control 2 (Faraday cage, 4 days x 6 hours) and by 3.4 times in comparison with intact control 1 (Fig. 4), the indicated results are characterized by high confidence according to all applied statistical criteria. Even though no statistically significant interstrain differences in chromosome disorders after exposure to the router's EMR were detected in the used samples, however degree of bone marrow cells genome instability is higher in rats of NP line against VP line (Fig. 4), which may be indicative of their higher sensitivity to effect of EMR of the range under study.

It is important to note that after the animals exposure in the Faraday cage (control 2), a higher level of chromosome disorders was detected in VP line rats in comparison with the alternative NP line rats, however, no differences with relevant intact control groups were revealed.

Aires Defender fractal-matrix resonators' protective effect was assessed in conditions of the router action during 4 days, 6 h a day, which, as previously shown (First stage report), produce the most damaging effect on the chromosome apparatus of bone marrow cells of Vistar line rats. Resonators application during the router's action results in lower frequency of mitosis damage in cycling bone marrow cells in comparison with the router's action without protection: VP line rats – by 1.9 times, NP line rats – by 3.8 times. The indicated level is comparable to control 2 (faraday cage, 4 days, 6 h a day) and intact control 1 (Fig. 4). It is to be noted that in spite of absence of statistically significant interstrain differences with regard to frequency of chromosomes damage upon joint router and resonators action, based on rate of decrease of mitosis disorders, caused by the router's EMR, efficiency of the resonators' protective effect is higher in NP line rats with high excitability against NP line rats with low excitability. Conspicuous is the tendency of the test items values decrease upon exposition with resonators in both lines rats against control groups.

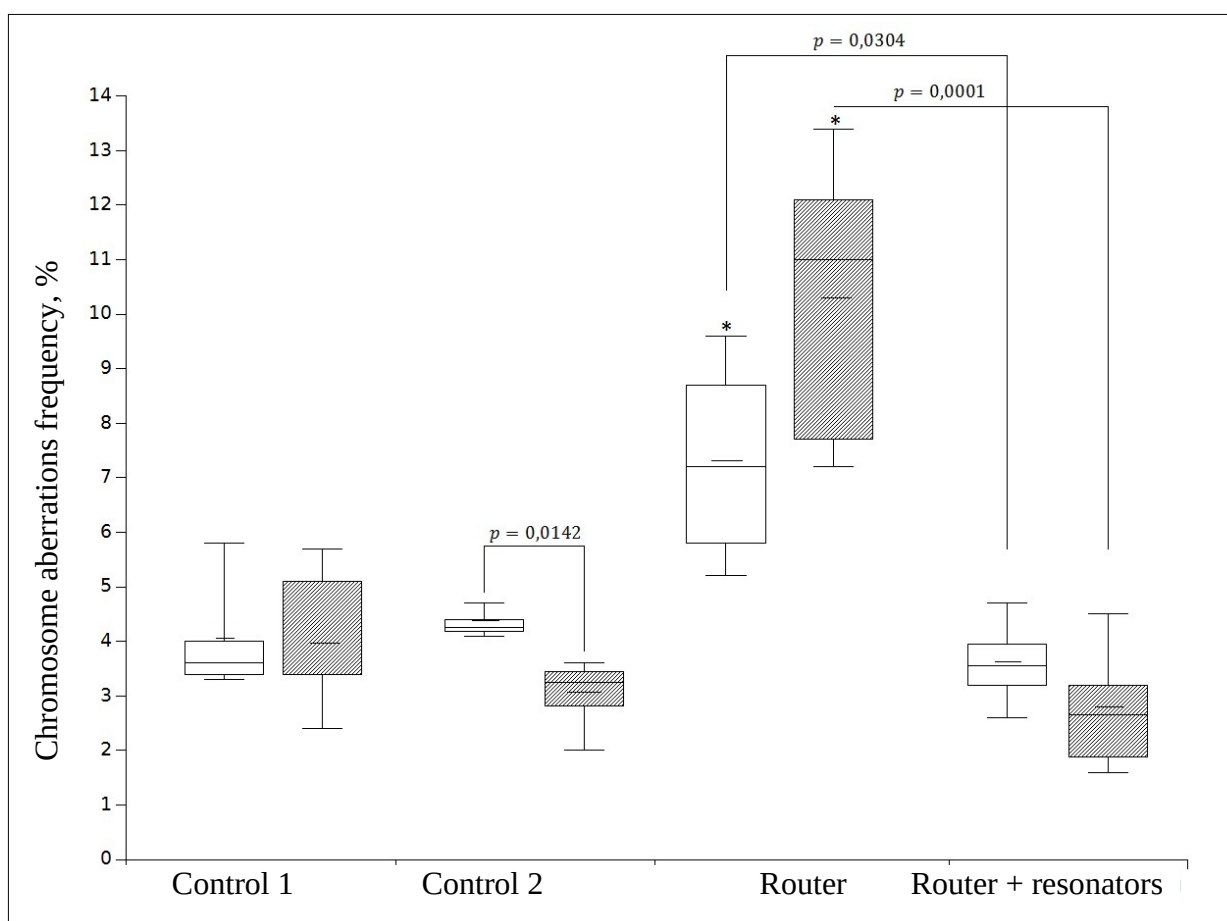


Fig.4. Frequency of chromosome aberrations in bone marrow cells of VP and NP lines male rats after exposure to electromagnetic radiation of the router and resonators. Median values, medium, boundaries of the first and third quartiles, min and max values of the sampling are presented

Legend: no hatching – VP line, hatching – NP line. Control 1 – intact rats, control 2 – rats, placed into the Faraday cage. * - differences with the group of control 1 and control 2 of the relevant line are statistically significant ($p < 0,05$), brackets denote significantly different values, p- significance point.

DISCUSSION

Findings of the repeated assessment of chromosome aberrations level in bone marrow cells of Vistar line male rats conformed that the router's electromagnetic radiation, upon exposure thereto during 4 days, 6 h a day, has a cytogenetic activity and induces chromosome aberrations. We have shown for the first time (First stage report) and it was confirmed by findings of the second research stage, that Aires Defender fractal matrix resonators – transformers have a protective effect on genetic apparatus of cycling bone marrow cells in Vistar line male rats against the router's EMR action. However, efficiency thereof was different in the first and the

second stages of the experiment, which could be caused by initially higher frequency of chromosome disorders in control groups of rats, used in the first experiment, and/or other seasonal and weather factors. It is to be highlighted that the resonators as such do not influence the level of chromosome aberrations in bone marrow cells of Vistar line rats in comparison with control groups.

Findings of evaluation of chromosome aberration level in bone marrow cells of VP and NP lines male rats with contrasting excitability of nervous system have shown that the router's electromagnetic radiation during 4 days, 6 h a day, induces mitotic disorders in animals of both lines. No statistically significant interstrain differences in chromosome disorders frequency were revealed, however, rate of bone marrow cells genome instability increase is larger in NP line rats against VP rats, which demonstrates dependency of sensitivity thereof to EMR exposure under study on functional state of nervous system. Sensitivity of bone marrow cells in was higher in Vistar rats than in VP line rats by all applied statistical criteria ($t=4,36$, $p=0,0018$; $F=19,04$, $p=0,0018$, $W=-15$, $p=0,008$), however it was not different from and was comparable with NO line. Therefore, character of EMR damaging effect on chromosome apparatus of bone marrow cells depends on genotype and is connected with excitability level of animals' nervous system.

Chromosome aberrations (bridges and fragments), detected in anaphase and telophase of mitosis are caused by incorrect repair of DNA damages at the interphase stage and/or replication errors (Aguilera, Garsia-Muse, 2013). Average cell cycle duration makes 23 – 24 h for bone marrow cells of a rat and a mouse (Mejer et al., 2013). Apparently, damaging effect of EMR factors and DNA disorders formation occur long before its implementation at the chromosome level, at initial stages of EMR action.

Change of stability of cycling bone marrow cells genetic apparatus when exposed to the router's EMR, may be caused by cell oxidative stress. Mutagenic activity thereof is based on genotoxic action of endopathic causes of humoral nature and free-radical products of peroxidation (Achudume et al., 2010). It is known that key neuroendocrine factors of DNA stress damage induction are corticosterone and catecholamines: adrenaline and noradrenaline (Flint et al., 2007, Hara et al., 2011).

Revealed chromosome disorders may affect functioning of immune system components, related to bone marrow processes, cause immune system suppression, immunopoiesis and hematopoiesis arrest (Flach et al., 2014), which will have an effect on the organism state and vital capacity thereof.

At present, ways of EMR influence on somatic cells genome remain under-explored. The following hypothesis have been advanced and partially sustained: effects of EMR influence on the cell within EHF are related to change of intracellular signal systems activity, effect on

secondary messengers, DNA, ferments (Gapeev, Chemeris, 2000; Gapeev et al., 2001). Problems of physical and chemical mechanisms of EMR reception are still mostly unsettled. It is suggested that EHF EMR influences weak electrostatic bonds, participating in maintenance of biomolecules and submolecular structures conformation (Gapeev, 2006). It is also suggested that neuroendocrine system is involved in EMR reception, mediating effect thereof on functioning of individual organs and the organism on the whole (Gapeev, 2006).

Studies of individual peculiarities of sensibility/ resistance to EMR effect and means of protection against damaging effect of EMR on genetic apparatus are also of considerable interest. This aspect is directly related to development of EMR-caused disorders prevention and correction methods based on individual approach. It is well known that genotype influences degree of chromosomes damage, caused by mutagenes, genotoxicants, stress factors of different nature (e.g. Rosenfeld et al., 2001, Semenov et al., 2010, Kovaleva, 2008). However, little is known of genotype-specificity of EMR influence (Irons et al., 2012, Vereschako, 2015). This paper shows dependence of chromosome aberrations frequency increase under the influence of wireless router's EMR on the line (Vistar, NP, VP). Given that, chromosome apparatus of NP line highly-excitability animals is more prone to EMR damaging effect against VP line. Previously, we have shown that NP line highly-excitability rats are more sensitive to cyclophosphan mutagen and to psychoemotional stress than VP line rats, it is also evidenced by increase of cytogenetic disorders in bone marrow cells (Bykovskaya et al., 1994, Dyuzhikova et al., 1996).

During the second stage of the work we have shown that efficiency of resonators' protective effect may depend on functional state of animals' nervous system, on genotype of the rats under study. Thus, high excitability of nervous system determines a more prominent decrease of mitotic disorders under resonators' influence, when the router is used. It is worth noting that there is a tendency of chromosome anomalies decrease upon both lines rats exposure with resonators in comparison with the control.

Mechanism of resonators protective effect is being actively studied currently. It is known that interaction of electromagnetic field with Aires Defender results in structural transformation of the former (Zhabrev et al., 2005). Electromagnetic field voltage is concentrated along the count directions, while considerably decreasing along other directions, which, on the whole, results in attenuation of total electromagnetic exposure, exceeding organism's threshold sensitivity to electromagnetic radiation. Thus, resonators' protective features are, apparently, based on restructurization (transformation) of incident EMR, which decreases damaging effect hereof on cycling cells.

On the whole, findings of the performed study enable to conclude that it is more promising to use lines with already known characteristics of nervous system to reveal EMR mechanisms and study ways of attenuation of damaging action thereof.

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