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OVERPRODUCTION OF FREE RADICAL SPECIES IN EMBRYONAL CELLS EXPOSED TO LOW INTENSITY RADIOFREQUENCY RADIATION

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***Aim*: Long-term exposure of humans to low intensity radiofrequency electromagnetic radiation (RF-EMR) leads to a statistically significant increase in tumor incidence. Mechanisms of such the effects are unclear, but features of oxidative stress in living cells under RF-EMR exposure were previously reported. Our study aims to assess a production of initial free radical species, which lead to oxidative stress in the cell. *Materials and Methods:* Embryos of Japanese quails were exposed *in ovo* to extremely low intensity RF-EMR of GSM 900 MHz (0.25 μW/cm²) during 158–360 h discontinuously (48 c — ON, 12 c — OFF) before and in the initial stages of development. The levels of superoxide ($O_2^{\cdot-}$), nitrogen oxide (NO·), thiobarbituric acid reactive substances (TBARS), 8-oxo-2'-deoxyguanosine (8-oxo-dG) and antioxidant enzymes' activities were assessed in cells/tissues of 38-h, 5- and 10-day RF-EMR exposed and unexposed embryos. *Results*: The exposure resulted in a significant persistent overproduction of superoxide and nitrogen oxide in embryo cells during all period of analyses. As a result, significantly increased levels of TBARS and 8-oxo-dG followed by significantly decreased levels of superoxide dismutase and catalase activities were developed in the exposed embryo cells. *Conclusion*: Exposure of developing quail embryos to extremely low intensity RF-EMR of GSM 900 MHz during at least one hundred and fifty-eight hours leads to a significant overproduction of free radicals/reactive oxygen species and oxidative damage of DNA in embryo cells. These oxidative changes may lead to pathologies up to oncogenic transformation of cells.**

***Key Words*: non-ionizing radiation, reactive oxygen species, superoxide, nitrogen oxide, 8-oxo-2'-deoxyguanosine, carcinogenesis.**

Worldwide spread of wireless technologies increased exposure of humans to radiofrequency electromagnetic radiation (RF-EMR) thousands times during the last decades [1]. And continuing intensive development of the wireless technologies at the moment leads us to the inevitable perspective of "wireless future", where the background level of RF-EMR on the planet will only increase. The question is how much radiofrequency radiation human beings could accept without adverse effects in the long-term perspective. Unfortunately, today we have the first sound signals on the human health problems from the long-term radiofrequency exposure by wireless devices. Firstly, during the last years a group of epidemiological studies that indicate a statistically significant increase in different kinds of tumors among long-term or "heavy" users of cellular phones was published. It was reported on increased risk in brain tumors [2–4], acoustic neuroma [5, 6], tumors of parotid glands [7], seminomas [8], melanomas [9] and lymphomas [10] in these cohorts of people. Secondly, it was reported on a significant increase in tumor incidence among people living nearby cellular base transmitting stations [11, 12]. And third, today a bulk of experimental studies reveals significant metabolic changes in living cells under the exposure to low intensity RF-EMR (see, for example) [13, 14]. Especially important in the last group of studies is the findings on a significant activation of oxidative processes in the exposed biological models [15–19].

Taking in mind a huge pathogenic potential of reactive oxygen species (ROS), we have checked the effects of low intensity RF-EMR on the production of ROS in embryo cells in a model of Japanese quail embryo *in ovo*. We used a commercial model of a cellular phone of the GSM 900 MHz standard as a realistic source of low intensity RF-EMR. Then we used Japanese quail embryos as one of sensitive biological models in risk assessment studies [20]. The important advantage of this model is immune immaturity of embryonic cells in the early stages of embryogenesis [21]. We aimed to assess the initial form of ROS, superoxide ($O_2^{\cdot-}$), and nitrogen oxide (NO·) in the exposed embryo cells. As well we assessed a possible initiation of lipid peroxidation and oxidative damage of DNA in the exposed cells.

The data obtained indicate that exposure of quail embryos to extremely low intensity RF-EMR of GSM 900 MHz, far below the ICNIRP safety limits, leads to a significant overproduction of superoxide and nitrogen oxide in embryo cells and resulted in the significant features of oxidative stress, including oxidative damage of DNA. Undoubtedly pronounced oxidative stress in the embryo cells under the exposure to low intensity RF-EMR can serve as the basis for the subsequent adverse metabolic changes in living cells up to its oncogenic transformation under some specific conditions.

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Abbreviations used: 8-oxo-dG – 8-oxo-2'-deoxyguanosine; ELF – extremely low frequency; EPR –electron paramagnetic resonance; ETC – electron transport chain; GSM – Global System for Mobile communication; HIF-2 – hypoxia-inducible factor-2; ICNIRP – International Commission on Non-Ionizing Radiation Protection; NBT – nitro blue tetrazolium; NO· – nitrogen oxide; $O_2^{\cdot-}$ – superoxide; OH· – hydroxyl radical; RF-EMR – radiofrequency electromagnetic radiation; ROS – reactive oxygen species; SAR – specific absorption rate; SOD – superoxide dismutase; TBARS – thiobarbituric acid reactive substances.

MATERIALS AND METHODS

Biological model. Embryos of Japanese quail *in ovo* were used for the experiments. Fresh hatching eggs were purchased at Bila Tserkva Poultry Farm (Ukraine). Two similar groups of eggs matched to the incubation standards were formed for each experiment (n=10). One group was the control, and the other was exposed to RF-EMR.

Brooding of the embryos *in ovo* was carried out in a foam plastic incubator designed especially for the experiments, free of metal covers. So we have neither shielded nor reflected RF-EMR on the incubator surface. The quail hatching eggs were incubated in close to the optimal conditions (temperature 38.0–38.5 °C, relative humidity 60%), long axes horizontally, and turned over manually triple per day.

RF-EMR exposure. A commercial model of a cellular phone of the GSM 900 MHz standard (Nokia 3120) assigned to a local mobile connection provider (Kyivstar, Ukraine) was used as a source of modulated RF–EMR. The muted and silenced cell phone was activated due to auto-redial computer program Autoringup (Russia), which guaranteed a discontinuous activation of the cell phone as a source of RF–EMR (48 c — ON, 12 c — OFF). The cell phone was placed on a plastic setup 3 cm over the surface of hatching eggs of the exposed group. Assessment of the RF-EMR exposure and control of the background level of non-ionizing radiation were carried out by the RF Field Strength Meter (USA).

In order to maximize the time of RF-EMR exposure we started irradiation of quail embryos of the exposed groups *in ovo* 120 h (5 days) before the incubation. This procedure was performed in the laboratory at room temperature. Then the exposure of embryos *in ovo* was continued inside the incubator during the actual brooding of the embryos. Depend on the term of analysis the exposure lasted 38 h, 120 h (5 days) or 240 h (10 days) during the incubation. Thus, the total exposures of the quail embryos were 158 h, 240 h or 360 h depending on the term of the analysis.

The embryos of control groups were subjected to the same procedures as the exposed groups' embryos except for the RF–EMR exposure. During the irradiation of the exposed embryos, the control embryos were kept in the same conditions 10 cm from the exposed group, shielded a few layers of an aluminum foil (total thickness of 0.2 mm).

The average intensity of RF-EMR on the surface of hatching eggs of the exposed groups was 0.25±0.008 μW/cm^2. A calculated specific absorption rate (SAR) value for quail embryos in our experiments was about 3 μW/kg [20]. The RF–EMR background level in the laboratory during the experiments was 0.001 μW/cm^2 (in range of 100–3000 MHz). The level of RF-EMR radiation in a zone of the control group, while the cellular phone was under the operation in a zone of the exposed embryos, was 0.002 μW/cm^2.

Assessment of free radical species production and oxidative stress in embryo cells. For the analysis of possible pro-oxidative effects of RF-EMR exposure we assessed the production of superoxide, nitrogen oxide, thiobarbituric acid — reactive substances (TBARS) and 8-oxo-2'-deoxyguanosine (8-oxo-dG) in cells/tissues of 38-h, 5-day and 10-day embryos. As well the activity of key antioxidant enzymes, superoxide dismutase (SOD) and catalase, were assessed in embryo tissues in these terms of analysis.

Fresh homogenates of the embryo tissues were prepared in the cold (0 °C) and dissolved in saline solution (1:10 strictly). The whole 38-h and 5-day embryos washed in cold saline solution and the separate tissues of liver, heart and brain of 10-day embryos were used for preparing of homogenates.

Superoxide assay. The production of superoxide in embryonic cells was assessed by electron paramagnetic resonance (EPR) spin-trapping technique using radiospectrometer RE-1307 (Russia) at a room temperature [22, 23]. A specific spin trap 1-hydroxy-4-dimethylamino-2,2,6,6-tetramethyl-piperidin dihydrochloride (Novosibirsk Institute of Organic Chemistry, Russia) was used for trapping of superoxide and transforming it into the stable nitroxyl radical (g=2.005). The spin trap concentration in the samples was 0.5 mM. The EPR signal of nitroxyl radical was recorded in each sample triple with 2 min intervals. The rate of superoxide generation in the samples was measured through the dynamic of the nitroxyl radical signal and expressed in nmole per gram of wet tissue per min (nmol g^{-1} min^{-1}).

Nitrogen oxide assay. The nitrogen oxide production in embryo cells was assessed by the EPR method with using of specific spin trap sodium diethyldithiocarbamate (Sigma–Aldrich, Germany) [23, 24]. The EPR signal of stable iron nitrosyl complexes with g=2.03 was measured after 5 min incubation of the samples with the spin trap. The EPR signal was measured triple, every 2 min, in each sample using the radiospectrometer RE-1307 at liquid nitrogen temperature (T=77 K). The rate of nitrogen oxide production in the embryo cells was measured through the dynamic of the EPR signal with g=2.03 and expressed in nmole per gram of wet tissue per min (nmol g^{-1} min^{-1}).

TBARS assay. For the assessment of lipid peroxidation in the embryo tissues a reaction of lipids with TBA in a presence of Fe^{2+} ions was used [25, 26]. Briefly, to 0.15 ml of the diluted homogenate 1.5 ml of 1% orthophosphoric acid was added followed by addition of 0.5 ml 0.75% of TBA, and $FeSO_4 \cdot 7H_2O$ to 0.5 μM. The reaction was carried out for 30 min in test tubes placed in boiling water and stopped in cold water. Then the test tubes were centrifuged at 3,000 rpm for 10 min. The level of TBARS was measured in supernatants by spectrophotometer Specoll 11 (Germany) at λ=532 nm.

The 8-oxo-dG assay. The level of 8-oxo-dG, marker of oxidative damage of DNA in the cell, was determined by solid phase extraction from the tissues of 38-h and 5-day embryos. The assessment of 8-oxo-dG concentration in the samples was carried out spectrophotometrically at λ=260 nm [27].

Superoxide dismutase activity was assessed using the assay based on a competition of SOD and nitro

blue tetrazolium (NBT) for superoxide [28]. Superoxide was produced in the reaction medium in a reaction of NADH with phenazine methosulfate in the presence of oxygen. A decrease of hydrazine tetrazolium level (which formed in a reaction of superoxide with NBT) due to a presence of SOD of the sample was detected spectrophotometrically at λ=540 nm.

Catalase activity assessment in the embryo tissues was made using a reaction of decomposition of hydrogen peroxide (H_2O_2; 0.03% solution) added into the samples. The determination of the hydrogen peroxide residual in the sample was carried out using its reaction with molibdate ammonium (4% solution) [29]. Molibdate ammonium produces with H_2O_2 a color complex, which level was assessed spectrophotometrically (λ=410 nm).

Statistical analysis. The data were expressed as the mean ± standard error of the mean (M±m). Student's t-test was used for the statistical analysis, with a significance levels * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ as compared with the matched controls.

RESULTS

Superoxide radical production in the exposed to RF-EMR embryo cells increased significantly. In cells of the exposed 38-h embryos the rate of superoxide production was on 57.5% ($p<0.01$) higher as compared to the unexposed control embryos. The production of superoxide in the tissues of livers of 10-day exposed embryos was on 78.6% ($p<0.05$) higher than in the control. The overproduction of superoxide in the tissues of hearts of 10-day exposed embrios was 51.5% as compared with the control (Fig. 1).

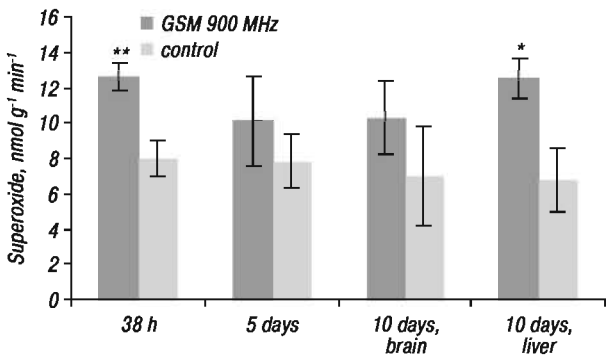

Fig. 1. The rate of superoxide generation in cells of quail embryos after the exposure to low intensity RF-EMR of GSM 900 MHz (0.25 μW/cm²; 158–360 h; discontinuously): n=5–7; M±m; nmol g^{-1} min^{-1}

Nitrogen oxide production in the cells of RF-EMR exposed embryos increased dramatically during the all period of analysis. In the tissues of 38-h exposed embryos the rate of NO· generation increased on 80% ($p<0.001$) and in 5-day embryos — on 56.8% ($p<0.001$) compared with the matched controls (Fig. 2). As well a statistically significant increase in NO· production was detected in the tissues of 10-day RF-EMR exposed embryos. In liver of the exposed 10-day embryos the level of NO· production was on 38% ($p<0.05$) and in brain it was on 64.5% ($p<0.05$) higher as compared with the unexposed control.

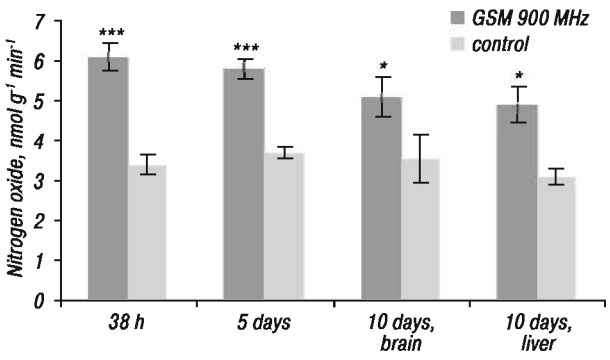

Fig. 2. The rate of nitrogen oxide generation in cells of quail embryos after the exposure to low intensity RF-EMR of GSM 900 MHz (0.25 μW/cm²; 158–360 h; discontinuously): n=5–7; M±m; nmol g^{-1} min^{-1}

Level of TBARS in the RF-EMR exposed embryo tissues as well had a tendency to increase as compared with the control (Fig. 3). Although the difference between the exposed and control embryos was not as pronounced as for superoxide and nitrogen oxide. A significant difference between the groups in the level of TBARS was detected firstly in the tissues of 5-day embryos (14.5%; $p<0.01$). In the 10-day exposed embryos the significantly increased level of TBARS was detected in the tissues of heart, on 32.1% ($p<0.01$) as compared with the control.

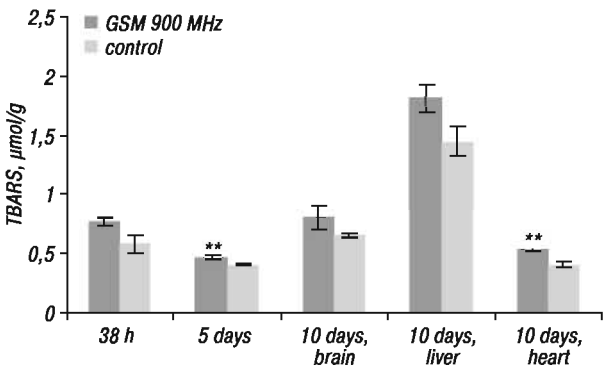

Fig. 3. The level of thiobarbituric acid reactive substances in homogenates of quail embryo tissues after the exposure to low intensity RF-EMR of GSM 900 MHz (0.25 μW/cm²; 158–360 h; discontinuously): n=5–7; M±m; μmol/g

Level of 8-oxo-dG in the RF-EMR exposed embryo cells increased significantly, 2–3-fold as compared with the control. So, the level of 8-oxo-dG in cells of the exposed to low intensity RF-EMR 38-h embryos was on 128% ($p<0.001$), and in cells of the exposed 5-day embryos — on 229% ($p<0.001$) higher as compared with the matched controls (Fig. 4)

Activities of SOD and catalase in the RF-EMR exposed embryo cells. The exposure of the quail embryos to low intensity RF-EMR of GSM 900 MHz resulted in significant changes in the antioxidant enzymes' activities. So, the activity of SOD, an enzyme, which dismutase superoxide radicals into hydrogen peroxide, was decreased significantly in the tissues of the 5-day exposed embryos (on 34.4%; $p<0.05$) and in the tissues of heart in the 10-day exposed embryos (on 48.3%; $p < 0.05$) as compared with the control (Fig. 5).

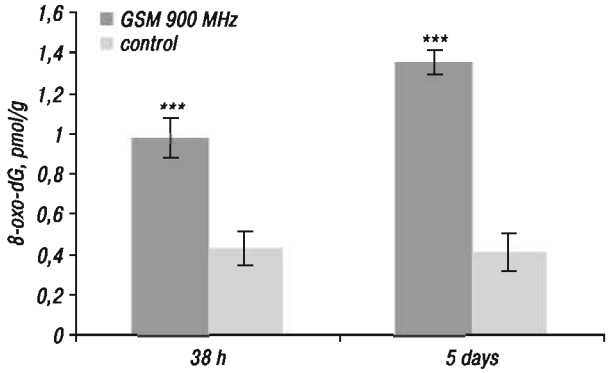

Fig. 4. The level of 8-oxo-2'-deoxyguanosine in cells of quail embryos after the exposure to low intensity RF-EMR of GSM 900 MHz (0.25 μW/cm²; 158–360 h; discontinuously): n=5–7; M±m; pmol/g

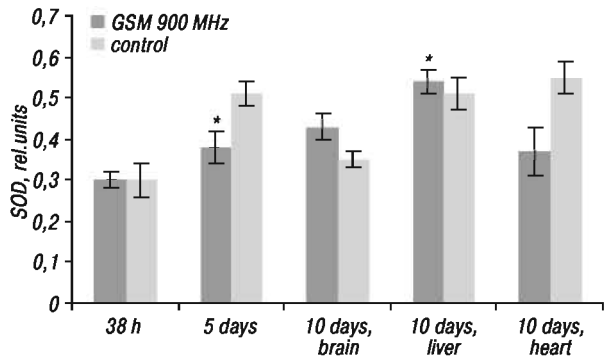

Fig. 5. The level of superoxide dismutase activity in homogenates of quail embryo tissues after the exposure to low intensity RF-EMR of GSM 900 MHz (0.25 μW/cm²; 158–360 h; discontinuously): n=5–7; M±m; rel. units

As well the activity of catalase, an enzyme, which protects cells from hydrogen peroxide, was decreased in the tissues of exposed embryos in some periods of the analysis. So, in the tissues of 38-h exposed embryos and in the tissue of liver of the 10-day exposed embryos the levels of catalase activity were, respectively, on 20% ($p<0.05$) and 21% ($p<0.05$) lower as compared with the matched controls (Fig. 6). On the other hand, in the tissues of 5-day exposed embryos the level of catalase activity was significantly, on 60.4% ($p<0.05$) higher than in the control.

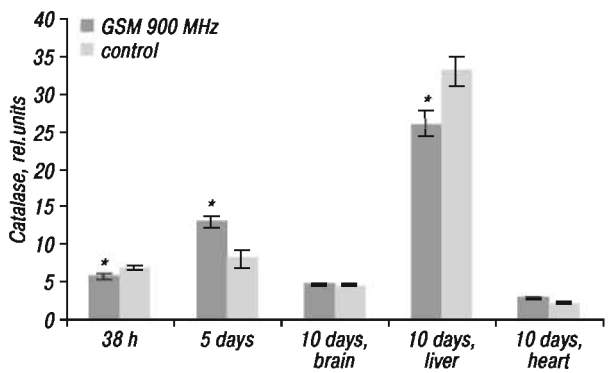

Fig. 6. The level of catalase activity in homogenates of quail embryo tissues after the exposure to low intensity RF-EMR of GSM 900 MHz (0.25 μW/cm²; 158–360 h; discontinuously): n=5–7; M±m; rel. units

DISCUSSION

The features of significant oxidative stress were detected in the quail embryos exposed to low intensity RF-EMR of GSM 900 MHz. It is of note, that the intensity of RF-EMR applied in our study was three orders of magnitude lower than ICNIRP safety limits [30]. Moreover it was order of magnitude lower than the strictest national safety limits on RF-EMR (*e.g.* in Ukraine or Switzerland) [13]. As we know, it's the lowest intensity of RF-EMR ever effectively used to demonstrate the overproduction of free radical species in the cells/tissues under non-ionizing radiation exposure. Although some relevant studies without indication of exact levels of RF-EMR exposure possibly used close low intensities [31, 32].

Previously the oxidative stress features in the living cells under RF-EMR exposure were detected in *in vivo* and *in vitro* models [15, 17, 33, 34], including human cells and fluids [35, 36]. As well there exist many studies demonstrated significant mutagenic effects in the living cells under the exposure to low intensity RF-EMR (see review) [37]. But the present study is the first, where so low intensity of RF-EMR as 0.25 μW/cm² and SAR value in 3 μW/kg led to a significant overproduction of free radicals / reactive oxygen species and oxidative damage of DNA in the cells. For comparison, the effective intensities of RF-EMR used in the other studies on ROS overproduction effects were about 200 μW/cm² [15] and SAR value of 0.4 W/kg and higher [16]. We could suppose that comparatively long-term exposure of the embryos (158 h and more) as well as modulated and pulsed character of the radiation applied in our experiments were crucial for pronounced effects revealed. Particularly, a few studies reported on more biological effectiveness of modulated RF-EMR as compared with non-modulated one [38, 39]. It is of note, earlier we demonstrated that this regime of RF-EMR exposure applied to developing quail embryos resulted in a significantly increased level of single- and double-strand breaks of DNA and depression of somitogenesis, while shorter exposure (38-h) led to the opposite effects [20].

It is important that both superoxide and nitrogen oxide, which overproductions were detected in our experiments, are the free radical species. That is why we could state on the free radicals overproduction in living cells as the first step response of the cell on RF-EMR exposure. On the other hand, it is not clear yet the mechanisms of the free radicals' overproduction in the cell under RF-EMR exposure [15, 16]. Previously both mitochondrial and NADH oxidase pathways of superoxide generation were experimentally supported to be activated under low intensity RF-EMR exposure. As we used the spin trap for the EPR detection of superoxide specifically in mitochondria, our data support a mitochondrial pathway of superoxide overproduction. But it is still unclear the site of interaction of modulated RF-EMR with mitochondria structures/electron transport chain (ETC) complexes of mitochondria. At least three sites of superoxide generation in ETC

are known at the moment: complex I [40], complex II [41], and complex III [42].

As for the stable significant overproduction of nitrogen oxide in the exposed embryo cells revealed in our experiments, the question remains is it an additional expression of NO-synthases under the RF-EMR exposure or a direct activation of the NO-synthase molecules presented in the cell at the moment of irradiation. It is important that, for example, direct interaction of RF-EMR with NADH oxidase was demonstrated previously [15]. But additional research should be done on the mechanism of NO· overproduction in the cell under low intensity RF-EMR exposure. On the other hand, a significant overproduction of NO· may itself lead to disturbing in ETC and increase a generation of superoxide in the cell [43].

The increased levels of these free radical species (superoxide and nitrogen oxide) in the embryo cells due to RF-EMR exposure resulted in a significant activation of peroxidation processes and depression of key antioxidant enzymes, SOD and catalase, activities. And the dramatic consequence of the increased levels of $O_2^{\cdot-}$ and NO· in the exposed embryo cells was a pronounced oxidative damage of DNA, which was manifested in the 2–3-fold ($p<0.001$) increased level of 8-oxo-dG.

It is known that superoxide itself doesn't affect DNA. The most aggressive form of ROS, which does affect the DNA molecule, is hydroxyl radical (OH·). Hydroxyl radicals are generated in the cell in Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$) and in Haber-Weiss reaction ($O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH\cdot + OH^-$) [44]. Taking in mind that superoxide is transformed by SOD into H_2O_2, both the aforementioned reactions depend on superoxide concentration and activity of antioxidant enzymes in the cell and thus could be activated due to RF-EMR exposure. On the other hand, a presence in the RF-EMR exposed cells increased concentration of NO· in addition to superoxide will lead to formation of peroxynitrite ($ONOO^-$), the other aggressive form of ROS, which can cause DNA damage [44].

We should underline, that the model of bird embryo is extremely sensitive to physical and chemical exposures and is one of the most valuable risk assessment models. We could not expect such the expressive and fast effects of low intensity RF-EMR exposure in all other biological models including human cells/tissues. Nevertheless, these findings are in line with previously published data on increased levels of ROS and NO· in living cells after low intensity RF-EMR exposure, although under much higher intensities [17, 33, 46, 47]. The great pathogenic potential of ROS in the cell, including its role in carcinogenesis [44, 48], allows us suppose that overproduction of free radical species, namely superoxide and nitrogen oxide, in RF-EMR exposed living cells is one of the plausible key mechanisms for the next pathological transformation of cells. Reactive oxygen species, and particularly hydroxyl radical, can directly damage the DNA molecule. The persistent oxidative damage of DNA could be a first step of mutagenic and carcinogenic processes [44]. Thus oxidative damage of DNA resulted in alters of transcription rate, replication errors and genomic instability [49]. In turn these processes are associated with carcinogenesis. And in different cancer tissues an increased level of oxidative damage of DNA were reported [44].

The important feature of the phenomena of ROS overproduction under the low intensity RF-EMR exposure is its non-thermal nature. Obviously, so low intensity RF-EMR as used in our experiments and some other studies could not produce any significant thermal effects in the cells/tissues (at least in classical meaning). On the other hand, much more intensive RF-EMR (with 4 and 5 W/kg SAR value) which undoubtedly did produce thermal effects in biological tissues, did not induce pro-oxidative effects [50, 51].

According to the data obtained we hypothesize that low intensity RF-EMR exposure leads to dysfunction of mitochondria, which results in superoxide overproduction, cell hypoxia and ROS-induced mutagenesis. As well matrix metalloproteinase are activated in these conditions [15]. These events could be a trigger for oncogenic transformation of cells stabilized due to activation of hypoxia-inducible factor-2 (HIF-2) [52, 53].

We could reveal the experimental evidences of cancer development/promotion in rodents caused by long-term low intensity RF-EMR exposure [54–57]. Moreover, in 2011 the World Health Organization / the International Agency for Research on Cancer recognized RF-EMR as a possible carcinogen for humans due to a significantly increased level of gliomas in "heavy" users of cell phones [58].

It is significant that some medical experts propose to restrict the intensity of RF-EMR for chronic exposure of humans even stricter as compared to the intensity demonstrated to be effective in this study. For example, recently the Austrian Medical Association released a Guideline on EMR-related health problems and illnesses, where it was recommended safety limit on RF-EMR chronic exposure (4 h and more per day) in 0.0001 $\mu W/cm^2$ [59]. We could discuss a technical possibility of such a restriction in modern society, but from the biological safety point of view, it seems absolutely reasonable.

It is of note, that a commercial cellular phone as a source of RF-EMR inevitably produces additionally to RF-EMR as well extremely low frequency (ELF) magnetic field due to electric currents in a battery circuit. Reportedly, ELF magnetic field can vary from a few μT up to a few tens μT of magnitude nearby a cell phone [60]. And strictly speaking we could not exclude some modulating effects of magnetic field in our experiments.

In conclusion, the exposure of developing quail embryos *in ovo* to extremely low intensity RF-EMR of GSM 900 MHz during at least one hundred and fifty-eight hours discontinuously leads to the significantly increased rates of superoxide and nitrogen oxide generation in embryo cells. This was accompanied by a significantly increased level of lipid peroxidation, a depression of key antioxidant enzymes activity, and significantly, 2–3-fold, increased level of oxidative damage of DNA in embryo cells.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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