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Original Research

Oxidative effect of low-intensity microwave radiation in the model of developing quail embryos

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ABSTRACT

Objective: Exposure of humans to low-intensity microwave (MW) radiation under some circumstances leads to several medical conditions, including headache, chronic fatigue, and even cancer. Mechanisms of these effects in many cases may depend on oxidative stress caused by MW exposure. Our study aims to assess oxidative stress features in embryonic cells under low-intensity MW exposure in the first stage of embryogenesis. **Methods:** Embryos of Japanese quails were exposed *in ovo* to low-intensity MW of global system for mobile communication (GSM) 900 MHz (0.25 μW/cm^2) during 158-360 h discontinuously (48 c - ON, 12 c - OFF) before and in the initial stages of development. The levels of superoxide ($O_2^{\bullet -}$), nitrogen oxide (NO$^\bullet$), and 8-oxo-2′-deoxyguanosine (8-oxo-dG) were assessed in cells of 38-h, 5-, and 10-day exposed embryos and compared to the control group. Lucigenin-enhanced chemiluminescence was used for assessment of GSM modulation role in MW-induced oxidative effects. **Results:** A significant persistent overproduction of superoxide, nitrogen oxide, and 8-oxo-dG in GSM MW-exposed embryonic cells during all periods of analyses was detected. **Conclusion:** Exposure of developing quail embryos to low-intensity MW of GSM 900 MHz during the first stages of embryogenesis resulted in a significant overproduction of superoxide and nitrogen oxide and oxidative damages of DNA in embryonic cells. These effects were interpreted to be depended on the GSM modulation of MW.

KEY WORDS: 8-oxo-2′-deoxyguanosine, embryogenesis, microwave radiation, nitrogen oxide, superoxide

INTRODUCTION

In 2015, the total number of active SIM cards for mobile phones became equal to earth population. Mobile telephony is the very first technology when a source of microwave (MW) radiation, a cell phone, is situated just near the human brain. Unfortunately, today we have some sound signals on the human health problems caused by long-term MW exposure from wireless devices. During the last years, epidemiological studies indicated on the significantly increased risk of different types of tumors among heavy users of a cell phone, including brain tumors [1-3], acoustic neuroma [4,5], tumors of parotid glands [6], seminomas [7], melanomas [8], and lymphomas [9]. Furthermore, it was reported on a significant increase in tumor incidence among people living nearby cellular base transmitting stations [10,11]. And finally, a bulk of experimental studies revealed a significant metabolic changes in living cells under the exposure to low-intensity MW [12,13]. Especially important are the findings on a significant activation of oxidative processes in exposed biological models [14,15].

Taking in mind pathogenic potential of reactive oxygen species (ROS), we have tested the effects of low-intensity MW on the production of ROS in embryonic cells. We used a commercial model of a cell phone of the global system for mobile communication (GSM) 900 MHz standard as a realistic source of low-intensity MW. We aimed to assess the initial forms of ROS, superoxide ($O_2^{\bullet -}$), and nitrogen oxide (NO$^\bullet$) in the exposed embryonic cells. Furthermore, we assessed a possible initiation of oxidative damages of DNA in the exposed cells.

Our data indicate that exposure of quail embryos to low-intensity MW of GSM 900 MHz, far below the international safety limits, leads to a significant overproduction of superoxide and nitrogen oxide and oxidative damages of DNA in embryonic cells. Here, we firstly demonstrate a significance of GSM modulation for oxidative effects of MW.

MATERIALS AND METHODS

Embryos

Japanese quail embryos from fresh hatching eggs were used for experiments. Two groups - analogs of hatching eggs were formed for each experiment (n = 10). One group was the control and the other was exposed to MW. Brooding of the embryos *in ovo* was carried out in a foam plastic incubator designed for the experiments, free of metal covers. Thus, MW was neither shielded nor reflected on the incubator structures. Quail hatching eggs were incubated in optimal conditions (temperature 38-38.5°C, relative humidity 60%), long axis horizontally, and turned over manually triple a day.

MW Radiation Exposure

A commercial cell phone of the GSM 900 MHz standard (Nokia 3120; Nokia Corporation, Espoo, Finland) assigned to a local mobile connection provider was used as a source of modulated MW. The GSM standard is still the most common standard for mobile communication. This radiation is frequency modulated, with channel rotation frequency of 217 Hz; hence, it belongs to the pulsed mode radiation [16]. 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 MW (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. MW intensities were assessed by the radio frequency (RF) field strength meter (Extech; Nashua, NH, USA).

To maximize the time of MW exposure, we started irradiation of quail embryos of the exposed groups *in ovo* 120 h (5 days) before the incubation. This procedure was performed at room temperature. Then, the exposure of embryos *in ovo* was continued inside the incubator during the 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 analysis.

The embryos of control groups were subjected to the same procedures as the exposed ones except for the MW exposure. The exposed and control embryos were kept and incubated in the same conditions 10 cm from each other, shielded a few layers of aluminum foil.

The average intensity of MW on a surface of hatching eggs of exposed groups was 0.25 μW/cm^2. A calculated specific absorption rate (SAR) value for quail embryos in our experiments was about 3 μW/kg [17]. The intensity of MW in the zone of control hatching eggs was 0.003 μW/cm^2. The RF background in the laboratory during the experiments was 0.001-0.002 μW/cm^2 (in range of 100-3000 MHz).

Superoxide Assay

The rate of superoxide generation in embryonic cells was assessed by electron paramagnetic resonance (EPR) spin-trapping technique using radiospectrometer RE-1307 (EPSI; Chernogolovka, Russia) at a room temperature [18,19]. 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 nanomoles per gram of wet tissue per minute (nmol/g/min).

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) [19,20]. 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 radio spectrometer RE-1307 at liquid nitrogen temperature (T = 77 K = –196.15°C). The rate of nitrogen oxide production in embryonic cells was measured through the dynamic of EPR signal with g = 2.03 and expressed in nmol/g/min.

The 8-oxo-dG Assay

The level of 8-oxo-dG, marker of oxidative damages 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 made spectrophotometrically at λ = 260 nm [21].

Lucigenin-enhanced Chemiluminescence

A 12-day embryo brain homogenates was used to compare effect of modulation of MW on superoxide generation [22]. Three sets of samples of native embryo brain homogenates (n = 10) were used in this experiment. One set of samples was exposed before the analysis to GSM 900 MHz MW (0.21 μBτ/cm^2) during 20 min. The second set of samples was exposed to non-modulated MW (850-950 MHz, 7.72 μBτ/cm^2) during 20 min. The third set of samples was unexposed control. As a source of non-modulated MW, we used generator SJM-4357. Chemiluminescence analysis was carried out immediately after the exposure by chemiluminometer SmartLum-5773 (Interoptika-S; Moscow, Russia). For

activation of chemiluminescence in the samples, 100 μM solution of lucigenin (Sigma-Aldrich; Taufkirchen, Germany) was used.

Statistical Analysis

The data were expressed as the mean ± standard error of the mean. 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 to the matched controls. Statistica 6.0 software was used for the analysis.

RESULTS

Superoxide radical generation in cells of MW-exposed 38-h embryos had statistically significantly higher rate (57.5%, $P = 0.0029$) as compared to the unexposed control embryos. The rate of generation of superoxide in cells of livers of 10-day exposed embryos was also significantly (78.6%, $P = 0.0208$) higher than in the control. The overproduction of superoxide in cells of brains of 10-day exposed embryos, however, was statistically insignificantly (51.5%, $P = 0.3343$) higher as compared to the control [Figure 1].

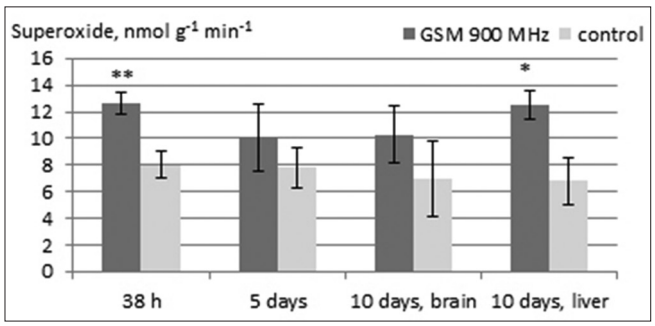

Figure 1: Rate of superoxide generation in cells of quail embryos after the exposure to low-intensity microwave of global system for mobile communication 900 MHz (0.25 μW/cm², 158-360 h, discontinuously): $n = 5$-7, mean ± standard error of the mean, nmol/g/min, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

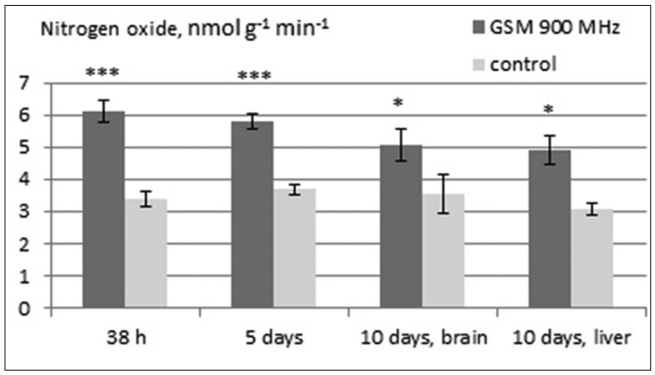

Figure 2: Rate of nitrogen oxide generation in cells of quail embryos after the exposure to low-intensity microwave of global system for mobile communication 900 MHz (0.25 μW/cm², 158-360 h, discontinuously): $n = 5$-7, mean ± standard error of the mean, nmol/g/min, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

Nitrogen oxide generation rate in MW-exposed embryonic cells increased dramatically during all periods of analysis. In cells of 38-h exposed embryos, the rate of $NO^{\bullet}$ generation increased significantly (80%, $P = 0.0001$) and in 5-day embryo's cells (56.8%, $P = 0.0001$) as compared to the matched controls [Figure 2]. Furthermore, a statistically significant increase in $NO^{\bullet}$ generation was detected in cells of 10-day MW-exposed embryos. In liver of 10-day exposed embryos, the level of $NO^{\bullet}$ production was significantly (38%, $P = 0.0229$) higher than in the control. In brain, it was significantly (64.5%, $P = 0.0145$) higher as compared to the unexposed control, too.

Level of 8-oxo-dG in MW-exposed embryonic cells increased significantly in 38-h embryos (128%, $P = 0.0007$) and in 5-day embryos (229%, $P = 0.0001$) as compared to the matched controls [Figure 3].

Lucigenin-enhanced chemiluminescence of 12-day native embryo brain homogenates demonstrated significant overproduction of superoxide in samples exposed to GSM modulated MW, while non-modulated MW did not induce any change in superoxide production in the samples as compared to the control. Thus, 20 min of GSM-modulated MW exposure

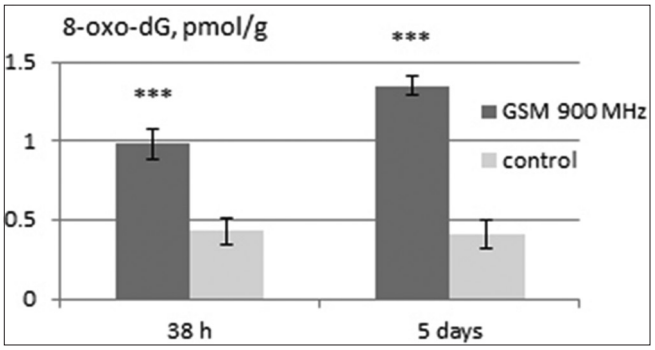

Figure 3: Level of 8-oxo-2'-deoxyguanosine in cells of quail embryos after the exposure to low-intensity microwave of global system for mobile communication 900 MHz (0.25 μW/cm², 158-360 h, discontinuously): $n = 5$-7, mean ± standard error of the mean, pmol/g, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

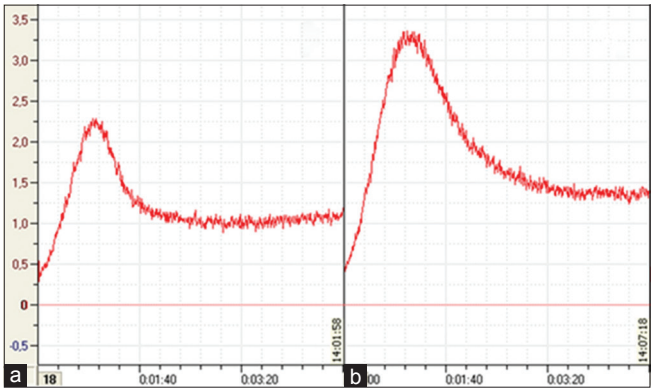

Figure 4: Lucigenin-enhanced chemiluminescence of 12-day quail embryo brain's homogenates: (a) Control; (b) exposed to global system for mobile communication 900 MHz microwave (0.21 μW/cm², 20 min, discontinuously) before the analysis; X-axis, time of analysis, min; Y-axis, intensity of chemiluminescence signal, relative units

increased significantly lucigenin-enhanced chemiluminescence signal in samples (36.1%, $P = 0.0046$) as compared to the control samples [Figure 4], while non-modulated MW exposure did not affect the intensity of chemiluminescence in the samples ($P = 0.8948$, graphics are not shown).

DISCUSSION

Previously oxidative effects in living cells under low-intensity MW exposure were detected in *in vivo* and *in vitro* models [23-26], including human cells and fluids [27,28]. As well there exist many studies demonstrated significant mutagenic effects in living cells under the exposure to low-intensity MW [29]. However, the present study is one of the few whereas low intensity of MW as 0.25 μW/cm^2 and SAR value in 3 μW/kg revealed a significant overproduction of ROS and oxidative damages of DNA in living cells. For comparison, according to the International Commission on Non-ionizing Radiation Protection (ICNIRP) recommendations, safety limits for MW exposure are about 450-1000 μW/cm^2 of intensity and 2 W/kg of SAR value [30]. We could suppose that comparatively long-term discontinuous 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. It is of note, earlier we demonstrated that this regime of MW exposure applied to 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 [17]. Furthermore, here, we firstly demonstrate that GSM modulation is critical for superoxide overproduction in the MW-exposed embryonic cells. Previously, the important role of modulation of MW for calcium-dependent effects in neurons was demonstrated [31].

It is important that both superoxide and nitrogen oxide, which overproductions were detected in our experiments, are free radical species. Thus, we could state on the free radicals' overproduction in living cells as the first step response of the cell on GSM-modulated MW exposure. On the other hand, it is not clear yet the mechanisms of the free radicals' overproduction in the cell under MW exposure. Previously, both mitochondrial and NADH oxidase pathways of superoxide generation were experimentally supported to be activated under low-intensity MW exposure [26,32]. As we used the spin trap for the EPR detection of superoxide specifically in mitochondria, our data support a mitochondrial pathway of superoxide overproduction. It is still unclear the site of interaction of modulated MW with mitochondria structures or electron transport chain (ETC) complexes of mitochondria. At least three sites of superoxide generation in ETC are known at the moment: Complex I [33], Complex II [34], and Complex III [35].

As for the stable statistically significant overproduction of nitrogen oxide in the exposed embryonic cells revealed in our experiments, the question remains if it is an additional expression of NO-synthases under the MW exposure or a direct activation of the NO-synthase molecules presented in the cell at the moment of irradiation. For example, direct interaction of MW with NADH oxidase was demonstrated previously [26]. On the other hand, a significant overproduction of NO$^\bullet$ may itself lead to disturbing in ETC and increase a generation of superoxide in the cell [36].

The increased levels of superoxide and nitrogen oxide in embryonic cells due to MW exposure can result in a significant activation of peroxidation processes and depression of key antioxidant enzymes [37]. Moreover, the dramatic consequence of the increased levels of $O_2^{\bullet-}$ and NO$^\bullet$ in the exposed embryonic cells was a pronounced oxidative damages of DNA, for example, 2-3-fold increased level of 8-oxo-dG.

It is known that superoxide itself does not affect the DNA. The most aggressive form of ROS, which does affect the DNA molecule, is hydroxyl radical ($^\bullet$OH). Hydroxyl radicals are generated in the cell in Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^\bullet OH + OH^-$) and in Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^\bullet OH + OH^-$) [38]. Taking in mind that superoxide is transformed by superoxide dismutase 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 MW exposure. On the other hand, a presence in the MW-exposed cells increased concentrations of NO$^\bullet$ and superoxide will lead to formation of other aggressive form of ROS, which can cause DNA damages, peroxynitrite ($ONOO^-$) [38].

Our findings are in line with previously published data on increased levels of ROS and NO$^\bullet$ in living cells after low-intensity MW exposure [14,15] although we used much lower intensity of MW. The huge pathogenic potential of ROS in the cell, including its role in carcinogenesis [38,39], allows us to hypothesize that overproduction of free radical species, namely, superoxide and nitrogen oxide, in MW-exposed living cells is one of the key mechanisms for the next pathological transformation of cells. The persistent oxidative damages of DNA could be a first step of mutagenic and carcinogenic processes [38]. Thus, oxidative damages of DNA resulted in alters of transcription rate, replication errors, and genomic instability [40]. In turn, these processes are associated with carcinogenesis. Moreover, in different cancer tissues, an increased level of oxidative damages of DNA was reported [38].

Recently, the Austrian Medical Association released a Guideline on EMR-related health problems and illnesses, where it was recommended safety limit on MW chronic exposure (4 h and more per day) in 0.0001 μW/cm^2 [41]. We could discuss a technical possibility of such a restriction in modern world, but from biological safety point of view, it seems absolutely reasonable.

CONCLUSION

We demonstrated that exposure of developing quail embryos to low-intensity MW of GSM 900 MHz, three orders of magnitude lower than the ICNIRP safety limits, during the first stages of embryogenesis resulted in a statistically significant overproduction of superoxide and nitrogen oxide, and oxidative

damages of DNA in embryonic cells. The effects depended on GSM modulation of MW.

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