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ORIGINAL RESEARCH

Monochromatic red light of LED protects embryonic cells from oxidative stress caused by radiofrequency radiation

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ABSTRACT

Objective: Oxidative mechanisms of the mutagenic and carcinogenic potential of radiofrequency radiation (RFR) have been demonstrated recently. This opens the need for antioxidative approach for protection of living cells from harmful effects of RFR. In this study, we aimed to assess the antioxidant potential of monochromatic red light of light-emitting diodes (LED) in RFR-exposed embryonic cells.
Methods: One group of Japanese quail embryos was exposed in ovo to GSM 900 MHz RFR (I = 1 14 μW/cm2; SAR = 0.17 mW/kg; t = 158 h; discontinuously) before and during the first hours of incubation. The second group of embryos was exposed to RFR in the same regimen and additionally to LED red light (λmax = 630-650 nm; I = 0.1 mW/cm2; t = 180 c; discontinuously). The third group of embryos were served as control. The rate of somitogenesis, level of lipid peroxidation, activity of superoxide dismutase (SOD) and catalase in tissues of 38-h embryos were assessed.
Results: Red light of LED exposure resulted in statistically significant reversion of the rate of somitogenesis decreased under RFR exposure; as well as in reversion of significantly increased level of lipid peroxidation and decreased catalase activity in tissues of RFR exposed embryos. In vitro significant suppression of SOD and catalase activities by short-term RFR exposure were partially reversed by LED red light treatment.
Conclusion: Red light of LED can protect embryonic cells from oxidative stress caused by low intensity RFR exposure. This is of particularly importance in terms of potential mutagenicity and carcinogenicity of low intensity RFR, which in turn depends on the oxidative potential of RFR.

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INTRODUCTION

Classification of radiofrequency radiation (RFR) as "possibly carcinogenic to humans" (group 2B) by the International Agency for Research on Cancer (IARC) / the World Health Organization (WHO) in 2011 [1] was a milestone on the way to awareness of global risk for human biology from expansion of wireless technologies all over the world. There exists a number of epidemiological studies which demonstrate carcinogenic effects of low intensity RFR emitted by cell phones [2-5] and base transceiver stations [6, 7]. To that, there is a set of experimental data showing that low intensity RFR results in cancer promotion in animal models [8-11]. In addition, during the last decades dozens of experimental studies on expressive mutagenic effects of low intensity RFR were published [12].

During the recent years persuasive experimental data was published on oxidative effects of low intensity RFR shed light with regard to possible molecular mechanisms as mutagenic and carcinogenic effects. Our recent meta-analysis of available peer-reviewed experimental studies on oxidative effects of low intensity RFR revealed, that among a hundred of studies more than 90% demonstrated significant oxidative effects of RFR exposure [13, 14]. It is experimentally proven that low intensity RFR, despite its non-ionizing nature, can activate key reactive oxygen species (ROS) generating systems, *i.e.* non-phagocytic NADH oxidase and mitochondrial pathway [15-17]. Likewise, a marker of oxidative damage of DNA, 8-hydroxy-2'-deoxyguanosine (8-OH-dG) was detected in many studies on risk assessment of low intensity RFR [16-20]. For example, on the model of quail embryo, we demonstrated a significant overproduction of superoxide radicals, nitrogen oxide, lipid peroxides and oxygen damage of DNA in embryonic cells under extremely low intensity RFR exposure [16].

While oxidative effects of low intensity RFR became a well proven phenomenon, antioxidative approaches on prevention of hazardous effects caused by RFR exposure emerge. Thus, the effectiveness of some antioxidants for mitigation of RFR exposure effects was demonstrated. Such protective effects have been reported for melatonin [21-25], vitamin E and C [26, 27], selenium and L-carnitine [28].

Earlier we ascertained that monochromatic red light of helium-neon (He-Ne) laser and light-emitting diodes (LED) is a modulator of oxidative status of living organisms [29, 30]. Particular regimens of monochromatic red light demonstrated pronounced antioxidant effects in bird embryos under stress conditions [31]. In the present study, we demonstrate that oxidative stress developed in quail embryonic cells due to 900 MHz RFR exposure can be mitigated by LED red light treatment. Moreover, for the first time, we demonstrate direct suppression of key antioxidant enzymes, superoxide dismutase (SOD) and catalase, under RFR exposure in *in vitro* models; and confirmed a potential of monochromatic red light to reactivate these enzymes.

MATERIALS AND METHODS

Biological Model

Embryos of Japanese quail at the stage of gastrula *in ovo* were used in the experiments. Three similar groups of fresh hatching eggs matched to incubation standards were formed for each experiment (n = 8-10). The embryos of first group were exposed to 900 MHz RFR, and those of second group were exposed to RFR in the same regimen and additionally treated by LED red light. The third group of embryos was an unexposed control. Incubation of the embryos *in ovo* was carried out in a foam plastic incubator, free of metal covers, under proper conditions (temperature 38.0-38.5°C, relative humidity 60%), long axes of eggs horizontally, and turned over manually three times per day. All experiments have been approved by the Bioethics committee of Bila Tserkva National Agrarian University and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

RFR Exposure and LED Treatment

Combine system of a commercial 3G USB-modem Huawei E173 and commercial model of a cellular phone of the GSM 900 MHz standard Nokia 3120 was used as a realistic source of modulated RFR. The system was activated due to auto-redial computer program Autoringup, which guaranteed a discontinuous activation of the system as a source of RFR (48 c on / 12 c off). The modem was placed 3 cm over the hatching eggs surface and the cell phone was placed 3 cm under a plastic setup for hatching eggs of the exposed groups. Assessment of the RFR intensity was carried out by the RF field strength meter (Alfalab Inc, Salt Lake City, UT, USA).

The embryos of the first and second groups were irradiated *in ovo* for 120 h (5 days) before the incubation. This procedure was performed at room temperature. Then the exposure of embryos was continued inside the incubator during the first 38 h of incubation. Thus, the total exposure period of the quail embryos was 158 h discontinuously. The average intensity of RFR on the surface of hatching eggs of the exposed groups was 14 μW/cm^2. A calculated specific absorption rate (SAR) value for quail embryos in these experiments was 0.17 mW/kg. The calculation was performed according to [32].

The embryos of the second group, additionally to RFR exposure, were treated by red light of LED. The setup of 20 single LED L7113PDC/H (λ_{max} = 630-650 nm) was used for treatment. The red light exposure was carried out 180 sec discontinuously (60 c each time on the second, 8th and 24th h of incubation) in the dark. The intensity of red light on the hatching egg surface was 0.1 mW/cm^2. Such regimen of LED monochromatic red light application was demonstrated by us earlier to be effective for stimulation of quail embryogenesis [33]. The intensity of light was assessed by a Radiant Flux Meter (Laser Technologies, Kharkiv, Ukraine).

The embryos of control groups were subjected to the same procedures as the embryos of exposed groups except for the RFR and LED light treatment. The control and exposed groups of hatching eggs were shielded and shaded from each other by a few layers of aluminum foil.

Analysis of Somitogenesis

A number of pairs of differentiated somites in a bird embryo is well known objective integral index of early embryonic development. Analysis of somites was carried out as described earlier [32]. Briefly, after 38 h of incubation, embryonic development was stopped by cooling the hatching eggs in cold water (10°C). Embryos were taken off the surface of yolk using filter paper rings and washed carefully in cold saline solution. Counting the numbers of differentiated somite pairs and visual analysis of possible embryo abnormalities were carried out under a light microscope with 24x magnification. Unfertilized eggs were excluded from the subsequent statistical analysis.

Assessment of Oxidative Stress in Embryo Cells

For the express assessment of oxidative/antioxidative effects of RFR exposure and LED red light treatment, the production of thiobarbituric acid reactive substances (TBARS) and activity of key antioxidant enzymes, SOD and catalase, in 38-h embryo tissues were carried out. Fresh homogenates of the embryo tissues were prepared on ice (0°C) and dissolved in saline solution (1:10 strictly).

Thiobarbituric acid reactive substances

For the assessment of lipid peroxidation in the embryo tissues, the reaction of lipid peroxides with TBA in presence of Fe^{2+} ions was used [34, 35]. Briefly, 1.5 ml of 1% orthophosphoric acid was added to 0.15 ml of the diluted homogenate 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 reaction mixtures were centrifuged at 3,000 rpm for 10 min. The level of TBARS was measured spectrophotometrically at λ = 532 nm.

Superoxide dismutase

The activity of SOD was determined using an assay based on the competition of SOD and nitro blue tetrazolium (NBT) for superoxide [36]. Superoxide was produced 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 presence of SOD in the sample was detected spectrophotometrically at λ = 540 nm.

Catalase

Assessment of catalase activity was carried out in the embryo tissues using a reaction of decomposition of hydrogen peroxide (H_2O_2; 0.03% water solution) added

into the samples. An assessment of hydrogen peroxide residuals in the samples was carried out using its reaction with molibdate ammonium (4% water solution) [37]. Molibdate ammonium produces with H_2O_2 a colored complex, which is assessed spectrophotometrically (λ = 410 nm).

In Vitro Experiments

During the SOD and catalase activity assessment, as described above, the exposure to RFR or RFR/LED light were applied to the reaction mixtures. For SOD activity assay, 10 μM saline solution of SOD from bovine erythrocytes (Sigma-Aldrich, Taufkirchen, Germany) was prepared. For catalase activity assay, a saline solution (1:10) of fresh homogenate of native 38-h embryo tissues was used. One set of reaction mixtures for each enzyme was exposed to GSM 900 MHz RFR with 0.25 μW/cm^2 intensity from activated muted cell phone for 10 min during the assay procedure. The second set of reaction mixtures was exposed to the same regimen of RFR and simultaneously to LED red light with 0.1 mW/cm^2 intensity for 10 min. The control sets of mixtures were not exposed.

Statistical Analysis

The data were expressed as the mean ± standard error of the mean (SEM). Student's t-test was used for the statistical analysis, with a significance level of $P < 0.05$.

RESULTS

The embryos of control and exposed groups were developed normally without visual malformations on the 38th h of incubation (Figure 1). Meanwhile, somitogenesis of quail embryos exposed to GSM 900 MHz RFR was slightly but significantly depressed as compared to the control embryos. The number of differentiated somite pairs in RFR exposed embryos was 11.2% ($P < 0.05$) lower as compared to the unexposed control, in full accordance with our previous results [32]. Monochromatic red light treatment of the RFR-exposed embryos resulted in a restoration of somitogenesis rate up to the control level (Figures 1 and 2).

Level of TBARS in tissues of RFR exposed embryos was 37.5% higher ($P < 0.05$) than in the control group (Table 1). This confirms our previous data on expressive pro-oxidative effect of GSM 900 MHz RFR exposure of quail embryos [16]. An additional LED red light treatment of the embryos resulted in a significant decrease of TBARS level in embryo tissues at a rate of 30.4% ($P < 0.001$) as compared to the RFR-only exposed embryos.

The activity of SOD was slightly decreased in tissues of RFR exposed embryos, 17.3% lower as compared to the control (Table 1), but the difference was not statistically significant. The LED red light application did not induce significant changes in SOD activity in 38-h quail embryo tissues. On the other hand, activity of catalase was found to be significantly decreased (78.6%, $P < 0.01$) in tissues of 38-h RFR exposed embryos as compared to the control (Table 1). Importantly, the

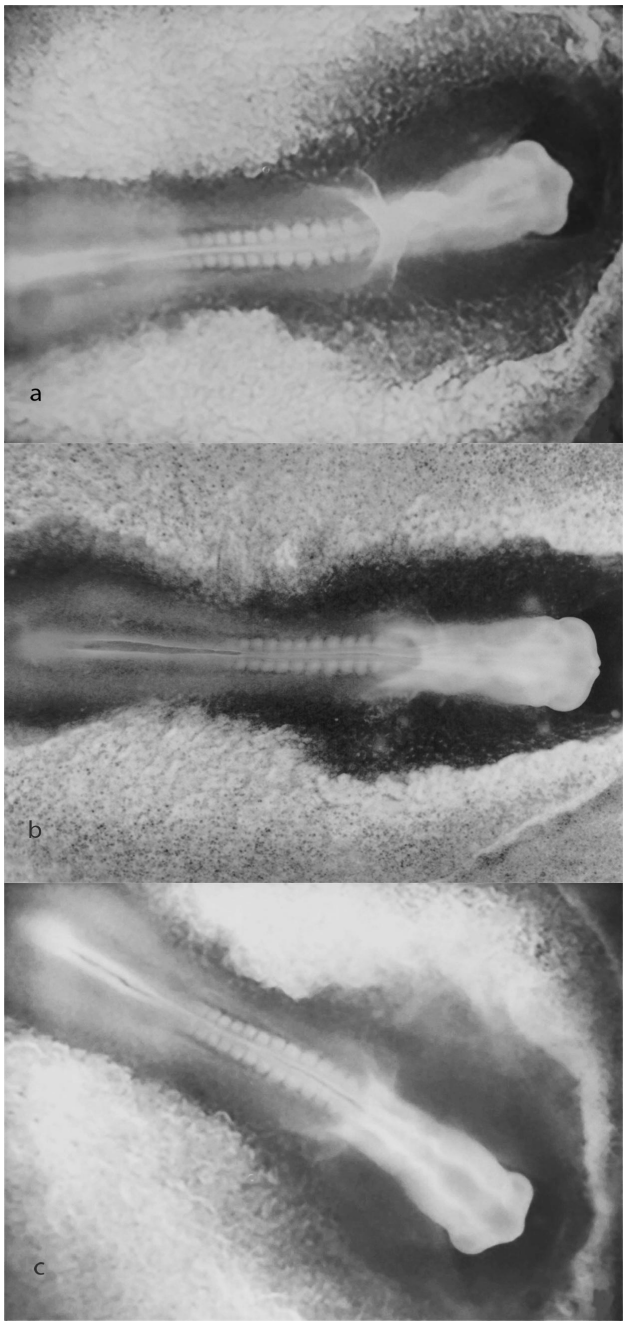

Figure 1. Microscopic pictures (x 24) of 38-h quail embryos (ventral): ***(a)*** control; ***(b)*** after GSM 900 MHz RFR exposure (14 μW/cm^2; 0.17 mW/kg; discontinuously 120 h before and 38 h during the incubation) *in ovo*; ***(c)*** after GSM 900 MHz RFR exposure and LED red light treatment (λ_{max} = 630-650 nm, 0.1 mW/cm^2; 180 c discontinuously during the first hours of incubation) *in ovo*.

LED monochromatic red light treatment resulted in a significant reactivation of the enzyme. The activity of catalase in 38-h embryo tissues after red light exposure increased by 99.2% ($P < 0.01$) as compared to the RFR-only exposed embryos.

To elucidate if GSM 900 MHz RFR can directly affect antioxidant enzymes or a change in catalase activity was only a feedback on significant ROS overproduction in RFR exposed cells, *in vitro* experiments were carried out. RFR exposure of 10 μM SOD solution for 10 min during the assay reaction demonstrated statistically significant

Table 1. Levels of thiobarbituric acid reactive substances (TBARS), and superoxide dismutase (SOD) and catalase activities in 38-h quail embryo tissues after *in ovo* exposure to GSM 900 MHz RFR (14 μW/cm²; 0.17 mW/kg; discontinuously 120 h before and 38 h during the incubation), and LED red light treatment (λ_{max} = 630-650 nm, 0.1 mW/cm²; 180 c discontinuously during the first hours of incubation)

Groups	Exposure dose (mJ/cm²)	TBARS (μmol/g)	SOD activity (rel. units)	Catalase activity (ncat/g)
Control		0.797 ± 0.099	0.163 ± 0.026	7.921 ± 0.855
RFR	6370	1.096 ± 0.025*	0.139 ± 0.012	4.434 ± 0.517**
RFR + LED	6370+18	0.763 ± 0.054+++	0.112 ± 0.021	8.834 ± 1.086++

Values are presented as mean ± SEM; n = 7-8; *P < 0.05 and **P < 0.01 compared to control, ++P < 0.01 and +++P < 0.001 compared to RFR group.

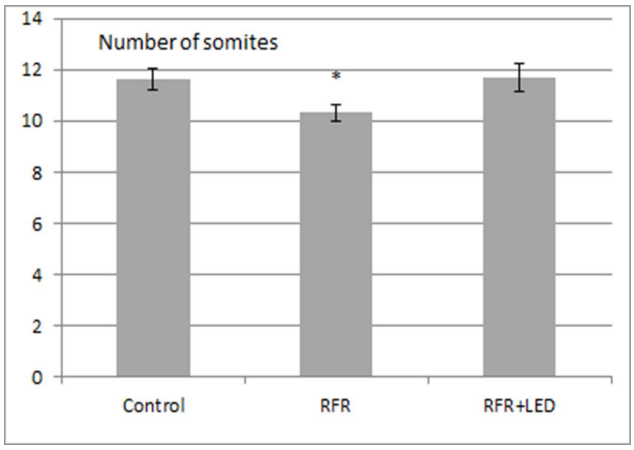

Figure 2. Effects of GSM 900 MHz RFR (14 μW/cm²; 0.17 mW/kg; discontinuously 120 h before and 38 h during the incubation) and LED red light treatment (λ_{max} = 630-650 nm, 0.1 mW/cm²; 180 c discontinuously during the first hours of incubation) on the rate of somitogenesis in 38-h quail embryos (n = 7-8; mean ± SEM; *P < 0.05 compared to control).

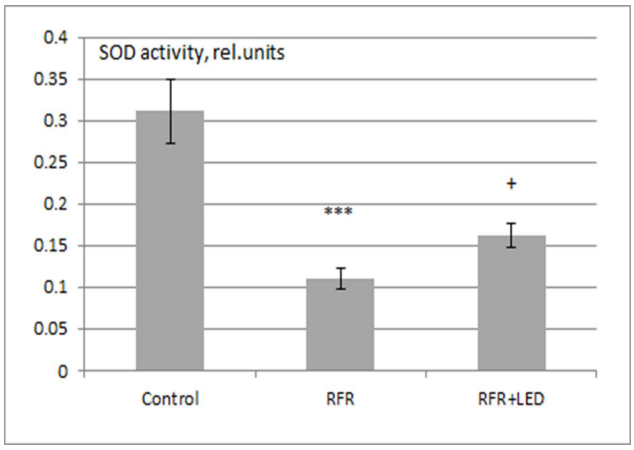

Figure 3. Levels of SOD activity in 10 μM SOD from bovine erythrocytes saline solution after *in vitro* exposure to GSM 900 MHz RFR (0.25 μW/cm², 10 min continuously during the assay procedure) and LED red light treatment (λ_{max} = 630-650 nm, 0.1 mW/cm² for 10 min continuously simultaneously with RFR exposure) (n = 7; mean ± SEM; ***P < 0.001 compared to control, +P < 0.05 compared to the RFR-exposed group).

suppression of the enzyme activity (64.3%, P < 0.001) as compared to the control unexposed reaction mixtures (Figure 3). LED red light applied to the reaction mixtures along with RFR exposure has led to a partial, but statistically significant restoration of SOD activity (46%, P < 0.05) as compared with RFR-only exposed solution.

Similarly, GSM 900 MHz RFR exposure of native 38-h embryo tissue homogenate for 10 min during the assay on catalase activity revealed a significant suppression of this enzyme activity (23.4%, P < 0.001) as compared to unexposed control homogenate (Figure 4). The application of LED red light simultaneously with RFR exposure of homogenate led to a statistically significant reactivation of catalase (21.4%, P < 0.05) as compared to RFR-only exposed homogenates.

DISCUSSION

It appears that the majority of recent epidemiological and experimental studies support public concern on potential risk of low intensity RFR from modern wireless devices for human health. Also, extremely high sensitivity of living cells to low intensity RFR was demonstrated in some studies. For example, as low as 0.1 μW/cm² intensity of RFR and SAR of 0.3 μW/kg were found to be effective in inducing significant oxidative stress in living cells [16, 38]. This is particularly important as the modern international safety limits on RFR exposure are based solely on the thermal effects of this type of radiation and restrict RFR intensity to 450-1000 μW/cm² and SAR to 2 W/kg [39]. Despite the decision of the IARC/WHO to classify RFR as possible carcinogen to humans, an ordinary human being will hardly be able to restrict his or herself in excessive implementation of such kind of wireless technologies as mobile telephony or wireless Internet. In this situation, it is important to propose evidence-based approaches for mitigation of hazardous effects along with strong limitation of excessive exposure. A tremendous progress has been achieved during the recent years in understanding of oxidative potential of this factor through activation of key ROS generating systems in living cells [15-17]. Thus, this was experimentally proven in hundreds of studies that oxidative stress is an intrinsic feature of interaction of low intensity RFR with living cells [13, 14]. Moreover, in some research the effectiveness of classical antioxidants against oxidative effects of low intensity RFR has been demonstrated [21-28, 40, 41].

Monochromatic red light of low intensity lasers and LED was used as effective therapeutic agents for years [42-44]. Also, strong antioxidant effects of some monochromatic red light regimens were demonstrated in different models [30]. Protective effect of monochromatic red light of He-Ne laser or LED against ionizing radiation [45] and dioxin toxicity [46] was demonstrated in a model of bird embryos. In addition, earlier we demonstrated that monochromatic light of LED (red and blue, but not

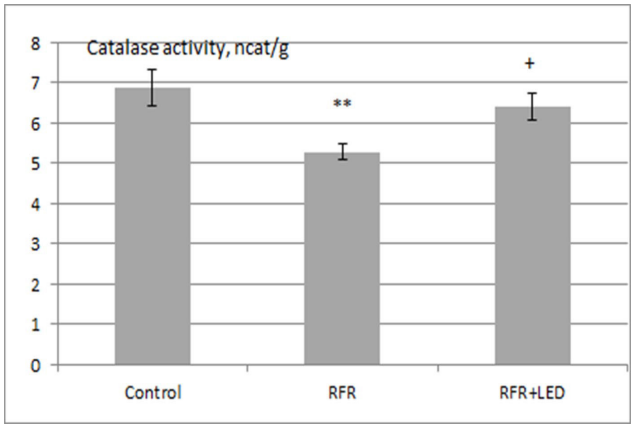

Figure 4. Levels of catalase activity in a saline solution (1:10) of fresh homogenate of 38-h native quail embryo tissues after *in vitro* exposure to GSM 900 MHz RFR (0.25 μW/cm^2, 10 min continuously during the assay procedure) and LED red light (λ_{max} = 630-650 nm, 0.1 mW/cm^2 for 10 min continuously, simultaneously with RFR exposure) (n = 7; mean ± SEM; **P < 0.01 compared to control, **P < 0.05 compared to the RFR-exposed group).

green) was effective for stimulation of somitogenesis in suppressed quail embryos [33]. That is why the use of monochromatic red light of LED for mitigation of oxidative stress in embryo cells caused by GSM 900 MHz RFR exposure seemed to be promising.

Indeed, the present study demonstrates a significant protective effect of low intensity monochromatic red light (λ_{max} = 630-650 nm) against both suppression of somitogenesis rate and oxidative stress in embryonic cells caused by RFR exposure of quail embryos. It has been shown for the first time that a particular regimen of LED red light significantly eliminates the hazardous effects of GSM 900 MHz RFR exposure in living cells. It was demonstrated that antioxidant potential of the applied regimen of LED red light was sufficient to normalize oxidative disturbances in embryonic cells. Both a significant TBARS overproduction and a decreased level of catalase activity caused by RFR exposure were normalized due to monochromatic red light application. Obviously, the mechanism of such favorable effect is based on biological activity of monochromatic red light, which includes activation of key enzymes of energetic metabolism [42, 47] and antioxidant defense system [30, 43].

In this study, we also demonstrate for the first time direct suppression of activity of key antioxidant enzymes, SOD and catalase, due to short-term GSM 900 MHz RFR exposure *in vitro*. The mechanism of such effect is unclear, but the possibility of low intensity RFR to change enzymes' activity was already demonstrated *in vitro* for NADH oxidase [15]. Also, conformational changes in protein macromolecules were detected under low intensity RFR exposure. Thus, low intensity RFR accelerated conformational changes in β-lactoglobulin through excitation of so called collective intrinsic modes in the protein [48, 49]. Similarly, a frequency dependent effect on intrinsic flexibility in insulin protein structure due to applied oscillating electric field was demonstrated [50]. Moreover, macromolecular structure of cytoskeleton was significantly altered in fibroblasts of Chinese hamster after the exposure to low intensity modulated RFR [51]. Therefore, induction of some critical conformation changes in particular antioxidant enzymes caused by modulated RFR exposure, which can lead to partial deactivation of the enzymes, seems plausible.

On the other hand, reactivation of SOD and catalase by monochromatic red light demonstrated in our experiments has a long-term experimental background. For both enzymes, a possibility of reactivation by monochromatic red light was revealed previously, and plausible biophysical mechanisms were proposed [52, 53]. It is essential that both these enzymes absorb in a red part of the light spectrum.

It is important to note that persistent oxidative stress induces oxidative damage of DNA and can cause malignant transformation of living cells [54, 55]. It is known that, in addition to mutagenic effects, ROS play an important role as a second messenger for intracellular signaling cascades which can also induce oncogenic transformation [56]. Moreover, in recent years evidences on the relationship between oxidative stress condition, epigenetic changes and carcinogenesis were obtained [57-59]. That is why the results on protective effect of red light of LED from oxidative stress caused by low intensity RFR exposure could be very useful in elaboration of new protective approaches against the potential hazards from excessive RFR exposure caused by modern wireless technologies.

In conclusion, this study demonstrates a high antioxidant potential of LED monochromatic red light (λ_{max} = 630-650 nm) and its effectiveness in treatment of oxidative stress developed in embryonic cells due to low intensity GSM 900 MHz RFR exposure. The results are of particular importance with the aspect of potential mutagenicity and carcinogenicity of low intensity RFR, which in turn is closely connected with oxidative potential of this type of radiation.

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