

In vitro non-thermal oxidative stress response after 1800 MHz radiofrequency radiation

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Abstract. In this study possible connection between radiofrequency exposure (RF) and development of oxidative stress was investigated by measuring impairment in cellular oxidation-reduction balance immediately after RF exposure. Fibroblast cells V79 were exposed for 10, 30 and 60 minutes to 1800 MHz RF radiation. Electric field strength was 30 V/m and specific absorption rate (SAR) was calculated to be 1.6 W/kg. Electromagnetic field was generated within Gigahertz Transversal Electromagnetic Mode cell (GTEM) equipped by signal generator, amplifier and modulator. Cell viability was determined by CCK-8 colorimetric assay and level of reactive oxygen species (ROS) was detected by dihydroethidium staining. Reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) were used to assess cell antioxidant activity while lipid oxidative damage was evaluated measuring concentration of malondialdehyde. Viability of V79 cells remained within normal physiological values regardless of exposure time. Increased level of superoxide radicals was detected after 60-min exposure. Significantly higher GSH level was observed immediately after 10-min exposure with higher but insignificant activity of GSH-Px. Lipid oxidative damage in exposed cell samples was not observed. Short-term RF exposure revealed transient oxidation-reduction imbalance in fibroblast cells following adaptation to applied experimental conditions.

Key words: Fibroblast cells — Viability — Glutathione — Malondialdehyde — Oxidation-reduction balance

Abbreviations: CAT, catalase; CCK-8, Cell Counting kit-8; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GSH, glutathione; GSH-Px, glutathione peroxidase; GTEM, Gigahertz Transversal Electromagnetic Mode cell; MBCl, monochlorobimane; MDA, malondialdehyde; PBS, phosphate buffer saline; PUFAAs, polyunsaturated fatty acids; RF, radiofrequency; ROS, reactive oxygen species; SAR, specific absorption rate; SOD, superoxide dismutase; TBA, thiobarbituric acid; V79, Chinese hamster lung fibroblast cells.

Introduction

Remarkable progress in the field of telecommunications resulted in great public concern for possible adverse effects

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induced by radiofrequency (RF) electromagnetic radiation emitted from mobile phones. Although biological interactions and potential health impacts of RF exposure have been extensively studied, only those effects that produce measurable tissue heating are well understood. At the same time there are many uncertainties and questions regarding biological effects that occur in the absence of heating, at the non-thermal level. One of the important mechanisms that should be considered in risk evaluation of non-thermal

radiation exposure is connected to reactive oxygen species (ROS), alteration in radical scavengers and development of oxidative stress (Blank 2008; Georgiou 2010; Marjanović et al. 2012; Trošić et al. 2012).

Unlike low concentrations which are known to regulate key cellular physiological functions, high level of ROS has the ability to induce oxidative damage to cellular components. Damage to lipids, proteins and nucleic acids severely compromise cell health and viability or induce a variety of cellular responses through generation of secondary reactive species (Dalle-Donne et al. 2006). So far several studies have shown RF capacity to enhance effects of stress inducing chemicals at cellular level (Höytö et al. 2008; Luukkonen et al. 2009). Moreover, RF radiation alone was found to increase generation of ROS and oxidative damage in different exposure systems. Effects were observed in human spermatozoa (De Iuliis et al. 2009), stable continuous cell lines (Friedman et al. 2007; Yao et al. 2008) as well as in primary cell cultures (Campisi et al. 2010; Xu et al. 2010). On the other hand most of conducted *in vitro* studies were not able to find connection between free radical production and RF radiation exposure (Lantow et al. 2006; Zeni et al. 2007; Brescia et al. 2009; Hong et al. 2012).

The primary site for transduction of extracellular chemical and physical signals is considered to be the cell membrane which is also likely to be affected by RF radiation exposure. Effect was seen on cell membrane receptors (Stankiewicz et al. 2006), membrane motility and fluidity (Aly et al. 2008; Crouzier et al. 2009; De Iuliis et al. 2009), as well as synaptic activity and number of synapses in cultured rat neurons (Xu et al. 2006). High content of polyunsaturated fatty acids (PUFAs) as well as solubility of oxygen and free radicals in the fluid lipid bilayer contributes to membrane susceptibility toward oxidative damage. Increased ROS production and/or decreased antioxidant activity enables lipid peroxidation, radical chain reactions and formation of malondialdehyde (MDA), reliable biomarker of oxidative stress (De Zwart et al. 1999; Valko et al. 2004).

The aim of this study was to investigate possible connection between short-term 1800 MHz RF exposure and cell membrane oxidative damage by measuring cell viability, ROS level, cellular antioxidant defense and concentration of MDA immediately after RF exposure.

Materials and Methods

Cell culture and radiofrequency exposure protocol

Stable continuous cell line of Chinese hamster lung fibroblast cells (V79) was grown in a RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma). Cell cultures were maintained at

37°C in a humidified atmosphere with 5% CO₂. Cells were routinely grown in 25 cm² cell culture flasks until the exponential phase of growth and growth medium was refreshed every four days during sub-culturing. Before radiation exposure, cells were detached from culture flasks by brief trypsinization using 0.25% Trypsin/EDTA solution (Sigma), resuspended, seeded and pre-incubated for 24 h (Freshney 2000). Cell samples were exposed to RF radiation within Gigahertz Transversal Electromagnetic Mode (GTEM) cell (Mod. 5402, ETS Lindgren, USA) for 10, 30 and 60 minutes. Electromagnetic radiation frequency 1800 MHz was generated by spectral analyzer (Anritsu MS2711B, USA), amplified (RF 3146 Power Amp Module, RF Micro Devices, Greensboro, USA) and modulated (RF 2722 Polaris chip, RF Micro Devices, Greensboro, USA). Electric field strength was 30 V/m and an average specific absorption rate (SAR) for a single cell was calculated to be 1.6 W/kg. SAR value was determined by averaging the individual parameters of the cell components with their volume fraction in the live cell according to Steffensen's mathematical model (Steffensen et al. 1995; McIntosh et al. 2003). Control cell samples were kept in the same experimental conditions, except they were not exposed to RF radiation. All experiments were carried out in triplicates. Temperature inside the GTEM-cell was monitored entire time during the experimental procedure and was kept at 37°C by means of specially constructed water bath system.

Cellular viability assay

The viability of V79 cells was evaluated using a sensitive colorimetric assay Cell Counting kit-8 (CCK-8, Sigma). The cells were seeded in 96-well tissue culture plates at concentration of 5×10^4 cells/ml and incubated overnight at 37°C and 5% CO₂. The next day cell samples were irradiated for 10, 30 and 60 minutes. Positive control (treated with 5% DMSO) and negative control cell samples (not exposed to radiation) were also included. Immediately after radiation exposure cell culture medium was replaced and medium containing 10% CCK-8 solution was added to the well. Cell plates were incubated at 37°C for 2 h and absorbance of yellow colored product was measured at 450 nm by means of plate reader device (1420 Multilabel Counter Victor³, Perkin Elmer, Walthman, MA, USA). For every exposure time, corresponding positive and negative control group there were eight independent samples included. Data were expressed as percentage of absorbance compared to relevant negative control.

Intracellular ROS measurement

Cellular ROS production was measured by fluorescence probe dihydroethidium (DHE) which allows intracellular

detection of superoxide radicals (O_2^-). Cells were seeded 24 h before radiation exposure in 96-well tissue culture plates at concentration of 1×10^5 cells/ml. After irradiation cells were washed with phosphate buffer saline (PBS, Sigma) and incubated with 20 μM DHE for 30 min at 37°C. Fluorescence intensity was quantified by means of plate reader device at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The image analysis of intracellular ROS was carried out by seeding the cells on Permanox Lab-Tek Chamber Slides (Nunc Naperville, USA) at concentration of 2.5×10^4 cells/ml. The next day, after radiation exposure, cell culture medium was removed and samples were washed with PBS. Non-irradiated and positive control cell samples exposed to H_2O_2 for 15 min were also included. After the treatment cells were incubated with 20 μM DHE (Sigma) for 30 min in the dark. At the end of incubation period fluorescent dye was removed and cells were washed four times with PBS. Fluorescence of the cells was examined by EVOS FL Auto Cell Imaging System (ThermoFisher Scientific, Waltham, USA) using 585 nm excitation and 624 nm emission filter.

Quantification of reduced glutathione (GSH)

Concentration of GSH in V79 cells was quantified using a fluorogenic bimane probe, monochlorobimane (MBCl, Sigma). Cells were seeded at concentration of 1×10^5 cells/ml in 96-well plates. The next day, after radiation exposure, cells were washed with PBS and incubated with 20 μM MBCl for 20 min at 37°C. Fluorescence intensity was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm by VictorTM multilabel reader (Perkin Elmer, MA). Negative (non-irradiated) and positive (treated with 500 μM H_2O_2) controls were included for every radiation exposure time.

Glutathione peroxidase (GSH-Px) activity

V79 cells were grown overnight in 12-well tissue culture plate at concentration of 1×10^5 cells/ml. The next day, after radiation exposure cell samples were washed, detached and centrifuged (1000 $\times g$, 4°C, 10 min). Cell pellet was resuspended in 1 ml PBS, sonicated and centrifuged 15 min at 10 000 $\times g$. Supernatant was collected and stored at -80°C. The GSH-Px activity in V79 cells was measured by the European standardized method (Belesten and Wright 1995). In order to increase assay sensitivity for the measurement of GSH-Px samples were pre-diluted for 60 times instead of a recommended 110-fold dilution used for blood samples. The amount of glutathione oxidized by t-butyl hydroperoxide was determined by following the decrease in the β -NADPH concentration, and the decrease in absorbance at 340 nm was measured by spectrophotometry (Cary 50 UV-Vis, Varian Inc. CA, USA). One unit of GSH-Px is the

number of micromoles of β -NADPH oxidized *per minute*. Activity of GSH-Px was expressed *per gram* of total protein (U/g protein).

Lipid peroxidation

Concentration of MDA in the cell was determined in reaction with thiobarbituric acid (TBA) according to a modified method of Abel and Gelderblom (1998). Cells were seeded at concentration of 2×10^5 cells/ml. The next day cells were exposed to RF radiation, washed, detached and collected by centrifugation. Supernatant was removed and cell pellets were sonicated on ice in PBS. Cell samples were briefly centrifuged (5000 rpm for 5 min at 4°C) and supernatant was collected for total protein and MDA measurement. Subsequently 200 μl of cell lysate was mixed with equal volume of solution containing 1% trichloracetic acid (Kemika, Zagreb, Croatia) and 0.67% TBA (Sigma) and heated for 20 min at 90°C. The cell samples were placed on ice and then centrifuged at 1000 rpm for 10 min. Supernatant was transferred to 96 well plates in duplicates and absorbance was detected at 530 nm by VictorTM multilabel reader (Perkin Elmer, MA). Concentration of MDA in the cell samples was calculated from MDA standard curve obtained from different concentrations of MDA standard (1,1,3,3-tetramethoxypropane; Sigma). Results were expressed as nmol/mg protein.

Protein determination

Protein concentration of cell lysates was measured by Bradford method (1976) using Total protein kit micro (Sigma). Samples, standards, and blanks were done in triplicate and absorbance was measured in 96-well plates at 570 nm (1420 Multilabel Counter Victor3, Perkin Elmer).

Statistics

Statistical analyses were performed with Statistica 13.0 (StatSoft, Inc., USA) software package. Data obtained for control and exposed group of samples were compared by non-parametric Mann-Whitney U-test. Values of $p < 0.05$ were considered statistically significant. The data used for statistical analysis were expressed as mean \pm standard deviation of the mean.

Results

Figure 1 shows the viability of fibroblast cells in non-irradiated (negative control), radiation-exposed and DMSO-treated (positive control) cell samples. During the course of radiation exposure, the viability of irradia-

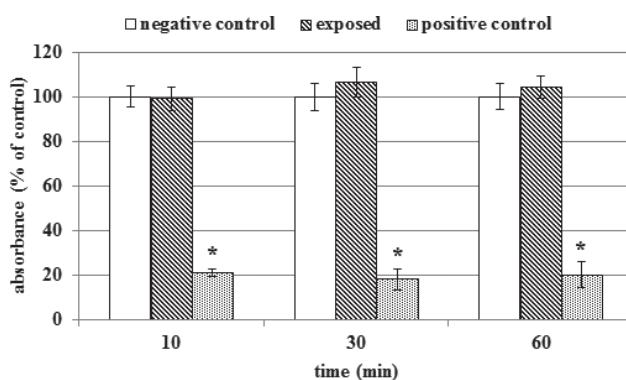


Figure 1. Cell viability of V79 cells exposed to 1800 MHz radiation for 10, 30 and 60 minutes. * $p < 0.05$ vs. negative control cell.

ated cells remained within normal physiological values and did not differ significantly compared to negative control group. Furthermore, short-term 10- and 30-min radiation did not cause any significant effect on the level of superoxide radicals. On the other hand, after 60-min exposure superoxide radicals in the cell increased and were significantly higher ($p < 0.05$) compared to corresponding

control group (Figure 2A). Observed difference was further confirmed and visualized by fluorescence microscopy as shown in Figure 2B. Concentration of GSH in radiation-exposed cells, negative control group and positive control treated with 500 μ M H₂O₂ was used to assess the non-enzymatic antioxidant activity of fibroblast cells (Figure 3). Significantly higher GSH level ($p < 0.05$) was observed immediately after 10-min exposure. During prolonged radiation exposure, difference between negative control and exposed cell samples decreased and was considered to be insignificant. In positive control group, GSH decrease was connected to duration of hydrogen peroxide treatment; longer H₂O₂ exposure reduced concentration of GSH in the cell. Figure 4 shows the enzymatic antioxidant activity of V79 cells determined by measuring activity of GSH-Px. Although insignificant the highest activity of GSH-Px in exposed cell samples was detected after 10-min exposure. During continued 30 and 60 min, RF exposure activity of an enzyme in the cell decreased. Lipid oxidative damage in the cell was evaluated by measuring concentrations of MDA. Even though the concentration of MDA in the cell continuously increased, the observed changes were not considered to be statistically significant when compared to corresponding control group (Figure 5).

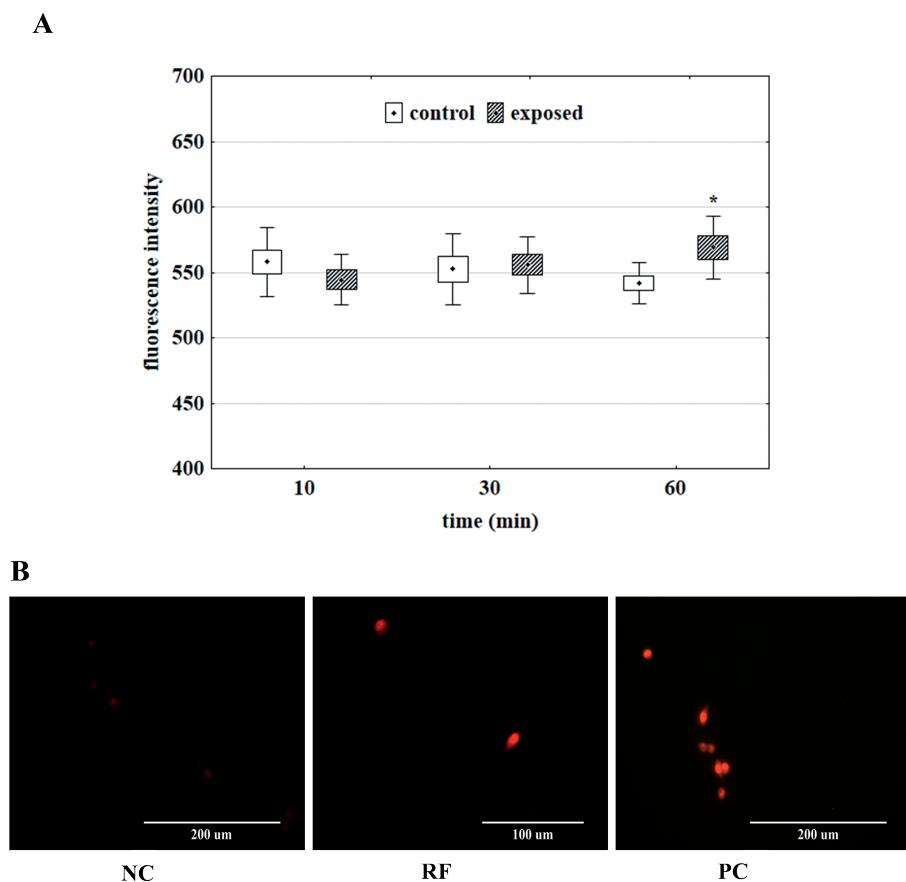


Figure 2. Analysis of superoxide radicals in V79 cells using dihydroethidium (DHE) as a probe assay. **A.** Quantification of DHE fluorescence intensity in fibroblast cells exposed to 1800 MHz RF radiation for 10, 30 and 60 minutes. * Statistically significant difference compared to control cell samples ($p < 0.05$). **B.** Images of DHE staining in fibroblast cells not exposed to radiation (NC-negative control), exposed to 60-minute RF exposure (RF-exposed) and exposed to H₂O₂ for 15 min (PC-positive control).

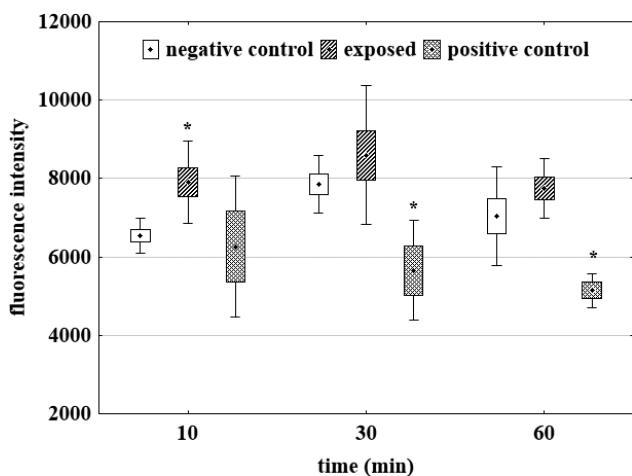


Figure 3. Level of GSH in V79 cells exposed to 1800 MHz RF radiation for 10, 30 and 60 min. * $p < 0.05$ vs. negative control cell.

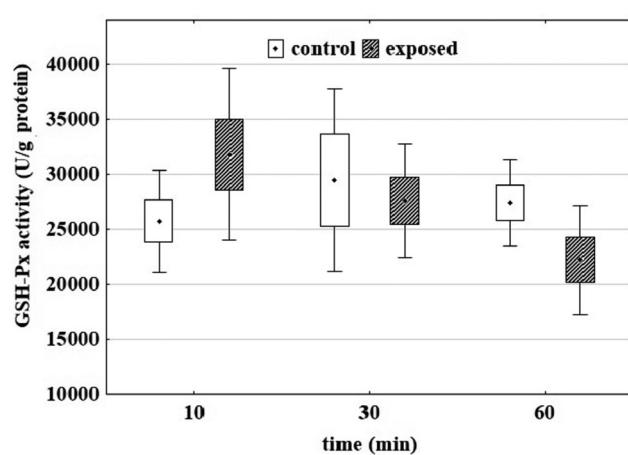


Figure 4. Activity of GSH-Px in V79 cells exposed to 1800 MHz RF radiation for 10, 30 and 60 min.

Discussion

Studies have shown that short-term RF exposure causes multiple gene expression alterations associated with cytoskeleton, signal transduction pathway and metabolism in different cell culture lines (Nylund and Leszczynski 2004; Leszczynski et al. 2004; Lee et al. 2005; Zhao et al. 2007). Lee et al. (2005) found that 2 h and 6 h exposure to RF fields at 2.45 GHz affects gene expression level in cultured human HL-60 cells. Apoptosis-related genes were found to be upregulated while expression level of genes involved in cell cycle was downregulated. In addition, ability of RF radiation to directly interact with polar structures of cell cytoskeleton fibers was also detected (Pavlicic and Trosic 2008; Trošić and Pavićić 2009). Study of Ballardin et al. (2011) showed that short-term 2.45 GHz radiation exposure induce alteration of mitotic apparatus and cell apoptosis of fibroblast cells (V79), as well as moderate reduction in the rate of cell division. On the contrary, in our study viability of exposed cell samples remained within normal physiological values and did not differ significantly compared to control. Cell samples were exposed to lower RF radiation at 1800 MHz and results are in an agreement with studies conducted on *in vitro* models of neurodegenerative disease (Del Vecchio et al. 2009) adenocarcinoma (Stander et al. 2011) immune (Huang et al. 2008) or stem cells (Czyz et al. 2004). Since the EM field was generated in a high-quality GTEM chamber which ensures a constant and uniform field and stable physiological temperature throughout irradiation, any changes observed in the exposed cells were considered to be consequence of non-thermal RF exposure.

The results of our previous study on fibroblast cells revealed possible impairment in cellular oxidation-reduction

balance after 1800 MHz RF exposure (Marjanovic et al. 2015). Increased ROS production, mainly hydrogen peroxide, was observed only after short-term 10-min irradiation, while with prolonged RF exposure, level of ROS decreased indicating possible antioxidant activation. As a follow up we further investigated stress response of fibroblast cells focusing on antioxidant defense mechanisms and lipid oxidative damage. It is known that GSH-Px activity is dependent upon GSH availability and that its biochemical function is involved in reduction of free hydrogen peroxide and lipid hydroperoxides. In our study significantly higher level of GSH was detected immediately after 10-min exposure. Moreover, after the same exposure time we observed the highest activity of

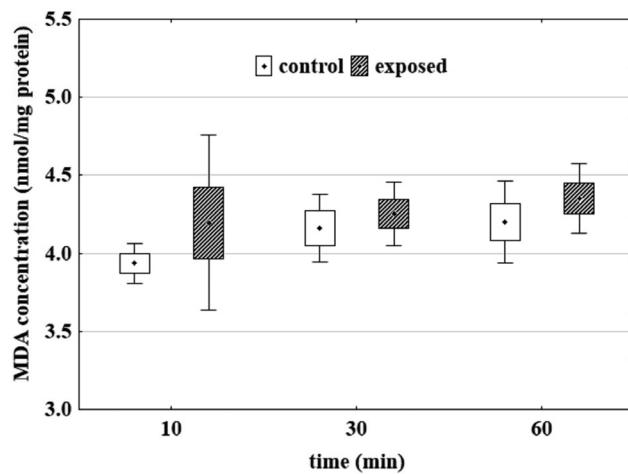


Figure 5. Concentration of MDA in V79 cells exposed to 1800 MHz RF radiation for 10, 30 and 60 min.

GSH-Px. This increased activity of cellular non-enzymatic and enzymatic defense points to a rapid cell response to applied RF radiation. Within prolonged RF exposure we did not observe any impairment in cellular antioxidant defense, indicating that these changes were only transient. Unlike radiation-exposed samples, GSH level in samples treated with hydrogen peroxide decreased with longer exposure time, which is a result of greater GSH consumption in the presence of radicals and more severe oxidative stress effect. In a study of Ni et al. (2013) more pronounced stress effect of 1.8 GHz radiation was observed in human lens epithelial cells (HLE B3). They observed significant decrease in expression level of catalase (CAT), superoxide dismutase (SOD) and GSH-Px after 1 h exposure. Furthermore, increased level of ROS was detected after shorter (0.5, 1 and 1.5 h) and MDA after longer (6, 12 and 24 h) RF exposure. *In vivo* decreased antioxidant activity was observed in rats after shorter (7 days, 1 h/day) (Ilhan et al. 2004) or longer, (10 days 30 min/day) 900 MHz RF exposure (Oktem et al 2005; Ozguner et al. 2005).

The physicochemical properties as well as chemical reactivity of cell membrane make it particularly sensitive to oxidative damage (Hulbert et al. 2007). Oxidative stress-induced peroxidation of membrane lipids can result in a loss of membrane fluidity, inactivation of membrane-bound receptors or enzymes which in turn impair normal cellular function and their permeability (Halliwell and Gutteridge 2007). So far several *in vivo* studies showed connection between long-term RF exposure, increased lipid peroxidation and development of oxidative stress (Dasdag et al. 2008; Esmekaya et al. 2011; Bilgici et al. 2013; Ozgur et al. 2013). Significant changes in oxidative stress parameters were also seen in human blood samples after acute 1, 2 and 4 h mobile phone radiation exposure (Moustafa et al. 2001). On the other hand Irmak et al. (2002) were not able to find lipid damage in rabbit serum or brain after 900 MHz radiation, even though other oxidative parameters were significantly altered. Similar results were found in rat's central nervous system after acute exposure to ultra high electromagnetic fields (UHF-EMF; 800–1800 MHz). There were no observable changes in the level of lipid and protein damage as well as in non-enzymatic antioxidant defense (Ferreira et al. 2006). In our *in vitro* study there were no statistically significant changes in the level of lipid oxidative damage. Given that higher MDA concentrations in previously mentioned studies were only detected after longer RF exposure or in a more sensitive cell culture lines these effects were expected. Results are in an agreement with study of Höytö et al. (2008) where no RF radiation related differences in lipid peroxidation level of fibroblast cells (L929) were observed after 1 h radiation exposure (872 MHz, 5 W/kg).

In the cell considerable amount of H₂O₂ is produced through dismutation reactions from superoxide radicals.

These radicals are mainly generated in the mitochondria but also from membrane NADH oxidase (Valko et al. 2007). Friedman et al. (2007) showed that short-term RF exposure causes activation of NADH oxidase in the plasma membrane and generation of ROS which is involved in initiation of extracellular signal-regulated kinase (ERK). The effect was observed after 5 min exposure and maximum activation was seen after 10 and 15 min irradiation. Furthermore, Xu et al. (2010) found that after 24 h exposure to 1800 MHz radiation increased ROS level caused significant oxidative damage to mitochondrial DNA. These results indicate possible role of superoxide radicals in oxidation-reduction balance and development of macromolecular oxidative damage. Although in our study shorter radiation exposure did not cause considerable effect on the level of superoxide radicals, significantly higher level was observed after 60 min irradiation. The results of our study on fibroblast cells (V79) indicate transient oxidation-reduction imbalance and activation of cellular adaptive mechanisms in response to short-term 1800 MHz RF exposure. Future studies should give special consideration to the role of superoxide radicals and mitochondria as possible target of RF radiation exposure.

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Conflict of interest. The Authors declare that there are no conflicts of interest.

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