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

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Running title: Cellular stress response after RF 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 minute-exposure. Significantly higher GSH level was observed immediately after 10 minute-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.

Keywords: fibroblast cells; viability; glutathione; malondialdehyde; oxidation-reduction balance

List of abbreviations

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

Introduction

Remarkable progress in the field of telecommunications resulted in great public concern for possible adverse effects 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 at 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 (Yao et al. 2008; Friedman et al. 2007) 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; Hong et al. 2012; Brescia et al. 2009).

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 (Valko et al. 2004; De Zwart et al. 1999).

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 37ºC in a humidified atmosphere with 5% CO_2. Cells were routinely grown in 25 cm^2 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 $5x10^4$ cells/ml and incubated overnight at 37°C and 5% CO_2. 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 2h and absorbance of yellow colored product was measured at 450 nm by means of plate reader device (1420 Multilabel Counter Victor^3, 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 ($O_2^{\cdot -}$). Cells were seeded 24 h before radiation exposure in 96-well tissue culture plates at concentration of $1x10^5$ cells/ml. After irradiation cells were washed with PBS and incubated with 20 μM DHE for 30 min at 37°C. Fluorescence intensity was quantified by means of plate reader device (1420 Multilabel Counter Victor3, Perkin Elmer, Walthman, MA, USA) 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 minutes 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 $1x10^5$ 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 Victor™ 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 $1x10^5$ cells/ml. The next day, after radiation exposure cell samples were washed, detached and centrifuged (1000 g, 4°C, 10 min). Cell pellet was resuspended in 1ml PBS, sonicated and centrifuged 15 min at 10 000 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 $2x10^5$ 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 phosphate buffer saline (PBS, Sigma). Cell samples were briefly centrifuged (5000 rpm for 5 min at 4ºC) 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 90º 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 Victor™ 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 ± 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 irradiated cells remained within normal physiological values and did not differ significantly compared to negative control group. Furthermore, short-term 10 and 30 minutes radiation did not cause any significant effect on the level of superoxide radicals. On the other hand, after 60 minutes 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 μM H_2O_2 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 minute 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 H_2O_2 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-minute exposure. During continued 30 and 60 minute 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).

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 (Pavicic and Trosic 2008; Trošić and Pavičić 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 radiofrequency 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 minute 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 minute-exposure. Moreover, after the same exposure time we observed the highest activity of 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 1h 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, 1h/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 her 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 (Esmekaya et al. 2011; Bilgici et al. 2013; Ozgur et al. 2013; Dasdag et al. 2008). Significant changes in oxidative stress parameters were also seen in human blood samples after acute 1 h, 2 h, 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 1h radiation exposure (872 MHz, 5W/kg).

In the cell considerable amount of H_2O_2 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 minutes 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**.**

Figure legends

Figure 1 Cell viability of V79 cells exposed to 1800 MHz radiation for 10, 30 and 60 minutes

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 H_2O_2 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 minutes.

* Statistically significant difference compared to control cell samples ($P < 0.05$)

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

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

References:

1. Abel S., Gelderblom W.C. (1998): Oxidative damage and fumonisin B_1-induced toxicity in primary rat hepatocytes and rat liver *in vivo*. Toxicology 131, 121-131
2. Aly A.A., Cheema M.I., Tambawala M., Laterza R., Zhou E., Rathnabharathi K., Barnes F.S. (2008): Effects of 900-MHz radio frequencies on the chemotaxis of human neutrophils *in vitro.* IEEE Trans Biomed Eng. 55, 795–797
3. Ballardin M., Tusa I., Fontana N., Monorchio A., Pelletti C., Rogovich A., Barale R., Scarpato R. (2011): Non-thermal effects of 2.45 GHz microwaves on spindle assembly, mitotic cells and viability of Chinese hamster V-79 cells. Mutat Res. 716, 1-9
4. Belesten J., Wright A. (1995): European Community: FLAIR common assay for whole-blood glutathione peroxidase (GSH-Px); results of an inter-laboratory trial. Eur J Clin Nutr. 49, 921–927
5. Bilgici B., Akar A., Avci B., Tuncel O.K. (2013): Effect of 900 MHz radiofrequency radiation on oxidative stress in rat brain and serum. Electromagn Biol Med. 32, 20–29
6. Blank M. (2008): Protein and DNA reactions stimulated by electromagnetic fields. Electromagn Biol Med. 27, 3-23
7. Bradford M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72, 248–254
8. Brescia F., Sarti M., Massa R., Calabrese M.L., Sannino A., Scarfi M.R. (2009): Reactive oxygen species formation is not enhanced by exposure to UMTS 1950 MHz radiation and co-exposure to ferrous ions in Jurkat cells. Bioelectromagnetics 30, 525–535

9. Campisi A., Gulino M., Acquaviva R., Bellia P., Raciti G., Grasso R., Musumeci F., Vanella A., Triglia A. (2010): Reactive oxygen species levels and DNA fragmentation on astrocytes in primary culture after acute exposure to low intensity microwave electromagnetic field. Neurosci Lett. 473, 52–55
10. Crouzier D., Perrin A., Torres G., Dabouis V., Debouzy J.C. (2009): Pulsed electromagnetic field at 9.71 GHz increase free radical production in yeast (Saccharomyces cerevisiae). Pathol Biol. 57, 245–251
11. Czyz J., Guan K., Zeng Q., Nikolova T., Meister A., Schönborn F., Schuderer J., Kuster N., Wobus A.M. (2004): High frequency electromagnetic fields (GSM signals) affect gene expression levels in tumor suppressor p53-deficient embryonic stem cells. Bioelectromagnetics 25, 296–307
12. Dalle-Donne I., Rossi R., Colombo R., Giustarini D., Milzani A. (2006): Biomarkers of oxidative damage in human disease. Clin Chem. 52, 601–623
13. Dasdag S., Bilgin H.M., Akdag M.Z., Celik H., Aksen F. (2008): Effects of long term mobile phone exposure on oxidative-antioxidative processes and nitric oxide in rats. Biotechnol Biotechnol Equip. 22, 992–997
14. De Iuliis G.N., Newey R.J., King B.V., Aitken R.J. (2009): Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. PLoS One. 4, e6446
15. Del Vecchio G., Giuliani A., Fernandez M., Mesirca P., Bersani F., Pinto R., Ardoino L., Lovisolo G.A., Giardino L., Calzà L. (2009): Effect of radiofrequency electromagnetic field exposure on in vitro models of neurodegenerative disease. Bioelectromagnetics 30, 564–672

16. De Zwart L.L., Meerman J.H., Commandeur J.N., Vermeulen N.P. (1999): Biomarkers of free radical damage applications in experimental animals and in humans. Free Radic Biol Med. 26, 202–226
17. Esmekaya M.A., Ozer C., Seyhan N. (2011): 900 MHz pulse-modulated radiofrequency radiation induces oxidative stress on heart, lung, testis and liver tissues. Gen Physiol Biophys. 30, 84–89
18. Ferreira A.R., Bonatto F., de Bittencourt Pasquali MA, Polydoro M., Dal-Pizzol F., Fernández C., de Salles A.A., Moreira J.C. (2006): Oxidative stress effects on the central nervous system of rats after acute exposure to ultra high frequency electromagnetic fields. Bioelectromagnetics 27, 487–493
19. Freshney R.I. (2000): Cell lines, Protocol 12.2. In: Culture of Animal Cells: A Manual of Basic Techniques. (Ed. R.I. Freshney), pp. 184–188, Viley-Liss Inc, New York
20. Friedman J., Kraus S., Hauptman Y., Schiff Y., Seger R. (2007): Mechanism of short-term ERK activation by electromagnetic fields at mobile phone frequencies. Biochem J. 405, 559–568
21. Georgiou C.D. (2010): Oxidative stress-induced biological damage by low-level EMFs: mechanism of free radical pair electron spin-polarization and biochemical amplification. In: Non-thermal effects and mechanisms of interacttion between electromagnetic fields and living matter. (Eds. L. Giuliani and M. Soffritti), pp. 63–113, Bologna, Firenza
22. Halliwell B., Gutteridge J.M.C. (2007): Free radicals in biology and medicine. 4th ed. Oxford University Press Inc, New York
23. Hong M.N., Kim B.C., Ko Y.G., Lee Y.S., Hong S.C., Kim T., Pack J.K., Choi H.D., Kim N., Lee J.S. (2012): Effects of 837 and 1950 MHz radiofrequency

radiation exposure alone or combined on oxidative stress in MCF10A cells. Bioelectromagnetics 33, 604–611

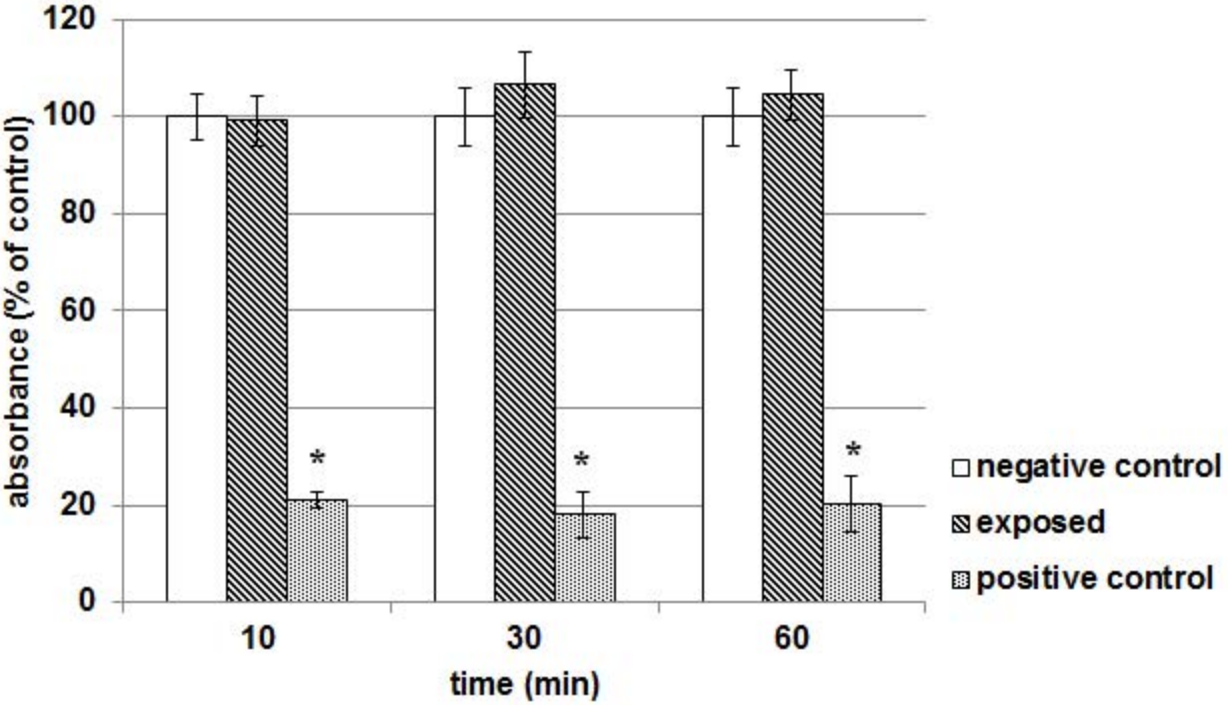
24. Höytö A., Luukkonen J., Juutilainen J., Naarala J. (2008): Proliferation, oxidative stress and cell death in cells exposed to 872 MHz radiofrequency radiation and oxidants. Radiat Res. 170, 235–243
25. Huang T.Q., Lee M.S., Oh E., Zhang B.T., Seo J.S., Park W.Y. (2008): Molecular responses of Jurkat-T cells to1763 MHz radiofrequency radiation. Int J Radiat Biol. 84, 734-741
26. Hulbert A.J., Pamplona R., Buffenstein R., Buttemer W.A. (2007): Life and death: metabolic rate, membrane composition, and life span of animals. Physiol Rev. 87, 1175–1213
27. Ilhan A., Gurel A., Armutcu F., Kamisli S., Iraz M., Akyol O., Ozen S. (2004): Ginkgo biloba prevents mobile phone-induced oxidative stress in rat brain. Clin Chim Acta. 340, 153–162
28. Irmak M.K., Fadillioğlu E., Güleç M., Erdoğan H., Yağmurca M., Akyol O. (2002): Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits. Cell Biochem Funct. 20, 279-283
29. Lantow M., Schuderer J., Hartwig C., Simkó M. (2006): Free radical release and HSP70 expression in two human immune-relevant cell lines after exposure to 1800 MHz radiofrequency radiation. Radiat Res. 165, 88–94
30. Lee S., Johnson D., Dunbar K., Dong H., Ge X., Kim Y.C., Wing C., Jayathilaka N., Emmanuel N., Zhou C.Q., Gerber H.L., Tseng C.C., Wang S.M. (2005): 2.45 GHz radiofrequency fields alter gene expression in cultured human cells. FEBS Lett. 579, 4829–4836

31. Leszczynski D., Nylund R., Joenväärä S., Reivinen J. (2004): Applicability of discovery science approach to determine biological effects of mobile phone radiation. Proteomics 4, 426–431
32. Luukkonen J., Hakulinen P., Mäki-Paakkanen J., Juutilainen J, Naarala J. (2009): Enhancement of chemically induced reactive oxygen species production and DNA damage in human SH-SY5Y neuroblastoma cells by 872 MHz radiofrequency radiation. Mutat Res. 662, 54–58
33. Marjanović A.M, Pavičić I., Trošić I. (2012): Biological indicators in response to radiofrequency/microwave exposure. Arh Hig Rada Toksikol. 63, 407–416
34. Marjanovic A.M., Pavicic I., Trosic I. (2015): Cell oxidation–reduction imbalance after modulated radiofrequency radiation. Electromagn Biol Med. 34, 381-386
35. McIntosh R.L., McKenzie R.J., Iskra S., Carratell A., Standaerz P. (2003): Computation of SAR in cell culture fasks exposed to 900 MHz GSM type signals in a modified TEM cell. Appl Comput Electromagn Soc J. 18, 107–113
36. Moustafa Y.M., Moustafa R.M., Belacy A., Abou-El-Ela S.H., Ali F.M. (2001): Effects of acute exposure to the radiofrequency fields of cellular phones on plasma lipid peroxide and antioxidase activities in human erythrocytes. J Pharm Biomed Anal. 26, 605–608
37. Ni S., Yu Y., Zhang Y., Wu W., Lai K., Yao K. (2013): Study of oxidative stress in human lens epithelial cells exposed to 1.8 GHz radiofrequency fields. PLoS One 8, e72370
38. Nylund R., Leszczynski D. (2004): Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation. Proteomics 4, 1359–1365

39. Oktem F., Ozguner F., Mollaoglu H., Koyu A., Uz E. (2005): Oxidative damage in the kidney induced by 900-MHz-emitted mobile phone: protection by melatonin. Arch Med Res. 36, 350–355
40. Ozguner F., Altinbas A., Ozaydin M., Dogan A., Vural H., Kisioglu A.N., Cesur G., Yildirim N.G. (2005): Mobile phone-induced myocardial oxidative stress: protection by a novel antioxidant agent caffeic acid phenethyl ester. Toxicol Ind Health. 21, 223–230
41. Ozgur E., Kismali G., Guler G., Akcay A., Ozkurt G., Sel T., Seyhan N. (2013): Effects of prenatal and postnatal exposure to GSM-like radiofrequency on blood chemistry and oxidative stress in infant rabbits, an experimental study. Cell Biochem Biophys. 67, 743–751
42. Pavicic I., Trosic I. (2008): In vitro testing of cellular response to ultra high frequency electromagnetic field radiation. Toxicol In Vitro. 22, 1344–1348
43. Stander B.A., Marais S., Huyser C., Fourie Z., Leszczynski D., Joubert A.M. (2011): Effects of non-thermal mobile phone radiation on breast adenocarcinoma cells. S Afr J Sci. 107, 1–9
44. Stankiewicz W., Dabrowski M.P., Kubacki R., Sobiczewska E., Szmigielski S. (2006): Immunotropic influence of 900 MHz microwave GSM signal on human blood immune cells activated in vitro. Electromagn Biol Med. 25, 45–51
45. Steffensen K., Raskmark P., Pedersen G. (1995): FDTD calculations of the EM-field distribution in a microtiter suspension well. In: Proceedings of the COST 244: Biomedical Effects of Electromagnetic Fields, pp. 80–87, Kuopio
46. Trošić I., Pavičić I. (2009): Disturbance of cell proliferation in response to mobile phone frequency radiation. Arh Hig Rada Toksikol. 60, 109–115

47. Trošić I., Pavičić I., Marjanović A.M., Bušljeta I. (2012): Non-thermal biomarkers of exposure to radiofrequency/microwave radiation. Arh Hig Rada Toksikol 63 (Suppl.), 67–73
48. Valko M., Izakovic M., Mazur M., Rhodes C.J., Telser J. (2004): Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Bochemistry. 266, 37–56
49. Valko M., Leibfritz D., Moncol J., Cronin M.T., Mazur M., Telser J. (2007): Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 39, 44–84
50. Xu S., Ning W., Xu Z., Zhou S., Chiang H., Luo J. (2006): Chronic exposure to GSM 1800-MHz microwaves reduces excitatory synaptic activity in cultured hippocampal neurons. Neurosci Lett. 398, 253–257
51. Xu S., Zhou Z., Zhang L., Yu Z., Zhang W., Wang Y., Wang X., Li M., Chen Y, Chen C., He M., Zhang G., Zhong M. (2010): Exposure to 1800 MHz radiofrequency radiation induces oxidative damage to mitochondrial DNA in primary cultured neurons. Brain Res. 1311, 189–196
52. Yao K., Wu W., Wang K., Ni S., Ye P., Yu Y., Ye J., Sun L. (2008): Electromagnetic noise inhibits radiofrequency radiation-induced DNA damage and reactive oxygen species increase in human lens epithelial cells. Mol Vis. 14, 964–969
53. Zeni O., Di Pietro R., d'Ambrosio G., Massa R., Capri M, Naarala J, Juutilainen J., Scarfi M.R. (2007): Formation of reactive oxygen species in L929 Cells after exposure to 900 MHz radiation with and without co-exposure to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone. Radiat Res. 167, 306–311

54. Zhao R., Zhang S., Xu Z., Ju L., Lu D., Yao G. (2007): Studying gene expression profile of rat neuron exposed to 1800 MHz radiofrequency electromagnetic fields with cDNA microassay. Toxicology 235, 167–175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20
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absorbance (% of control)
*
*
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10
30
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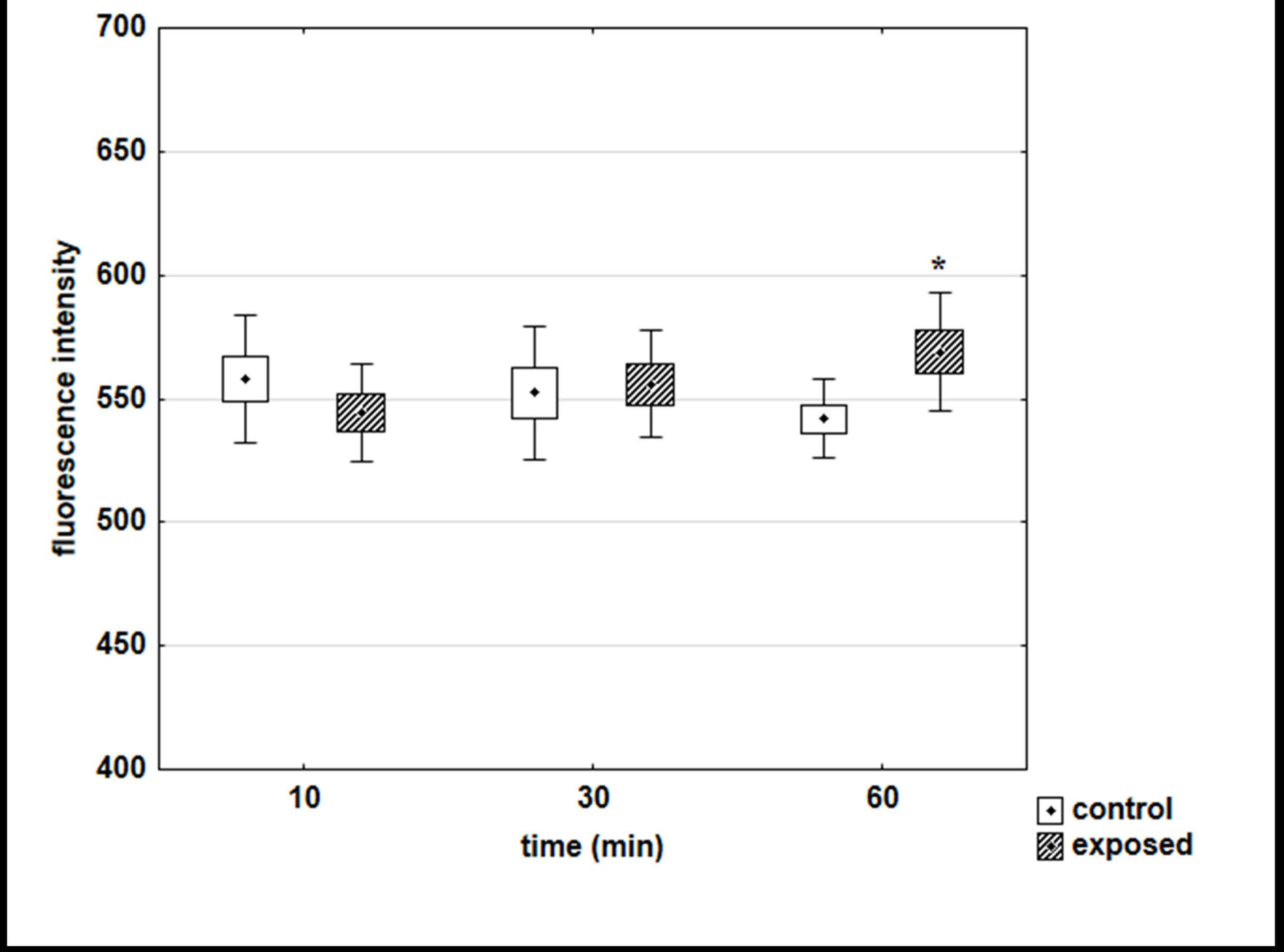
time (min)
negative control
exposed
positive control


700
650
600
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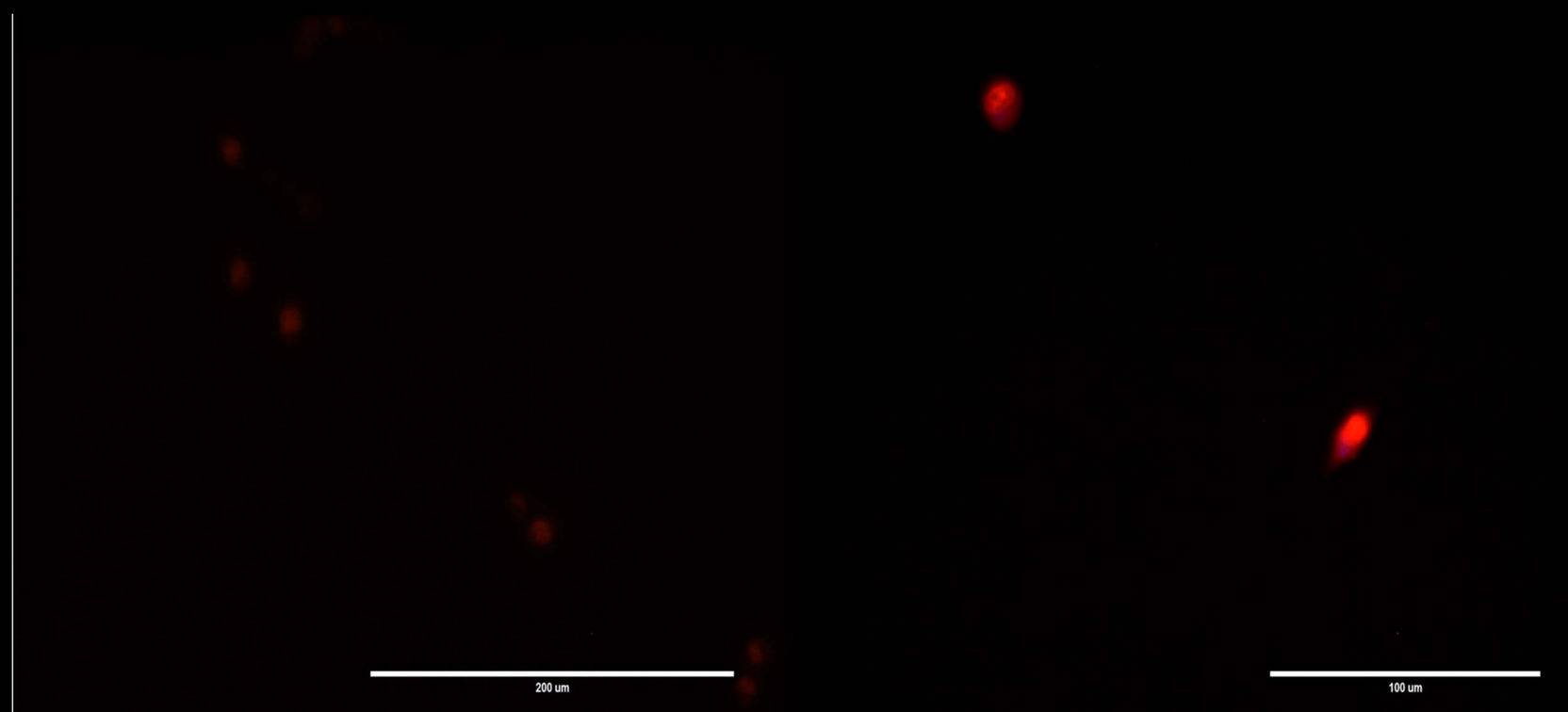
500
450
400
fluorescence intensity
*
10
30
60
time (min)
control
exposed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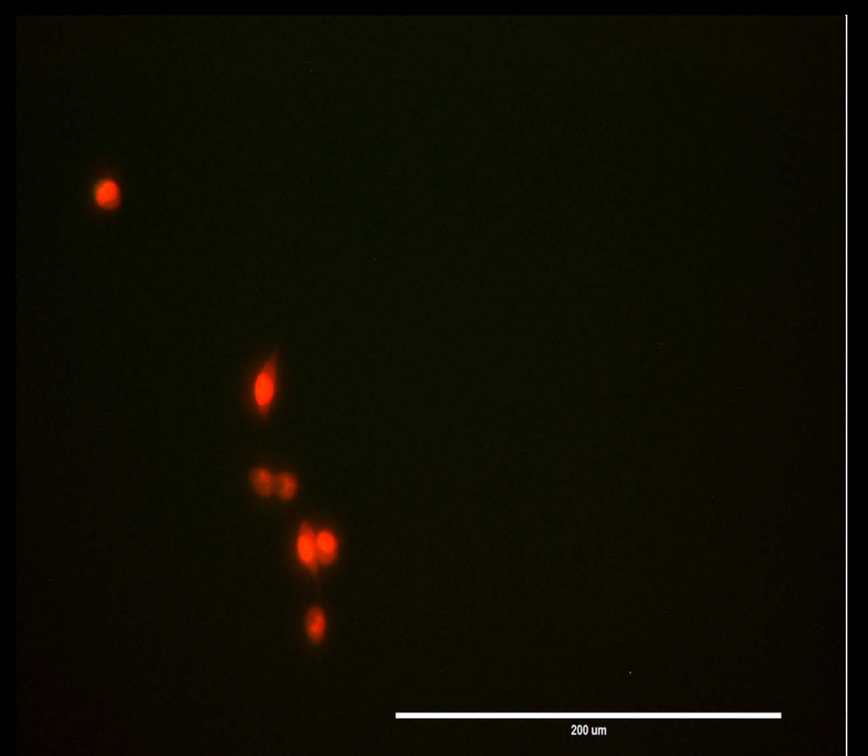
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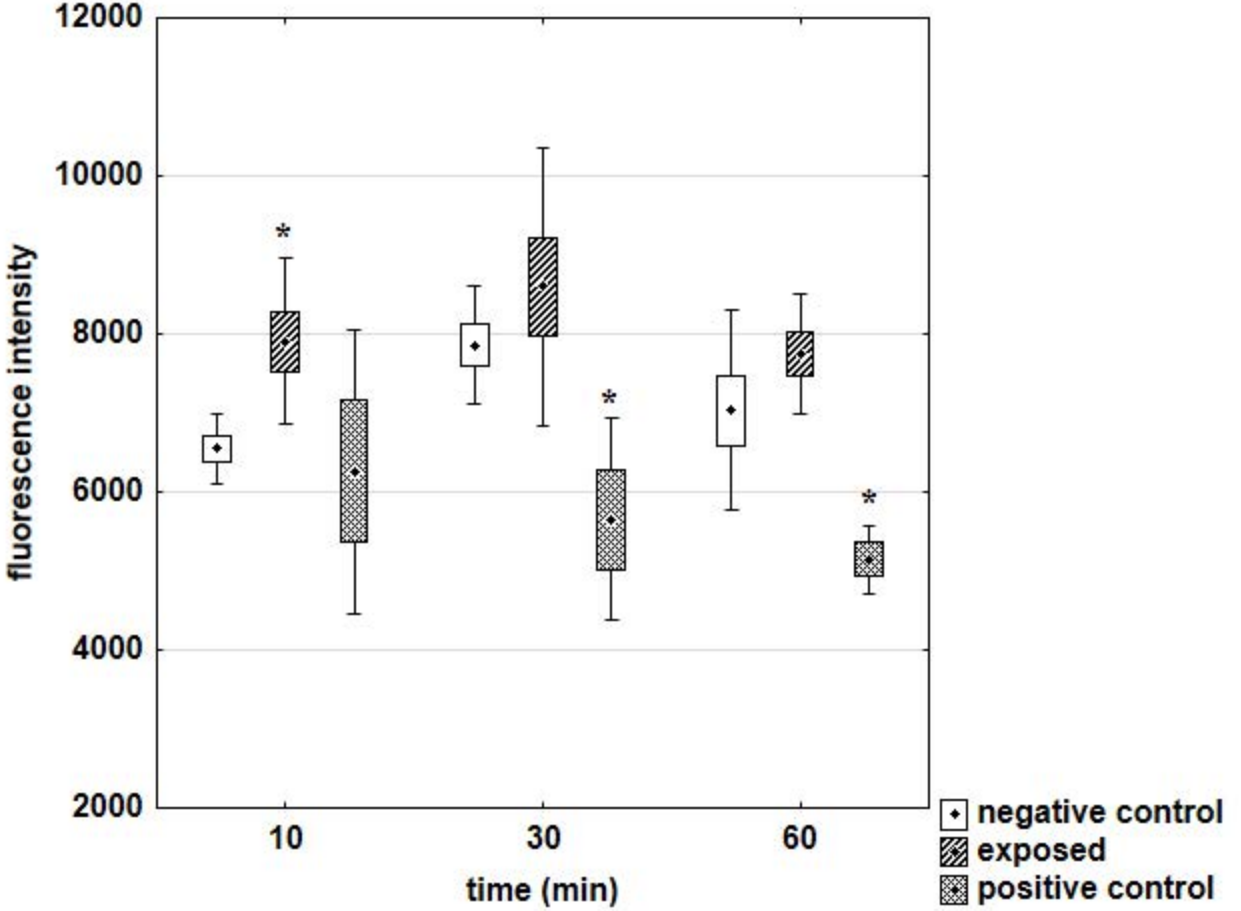
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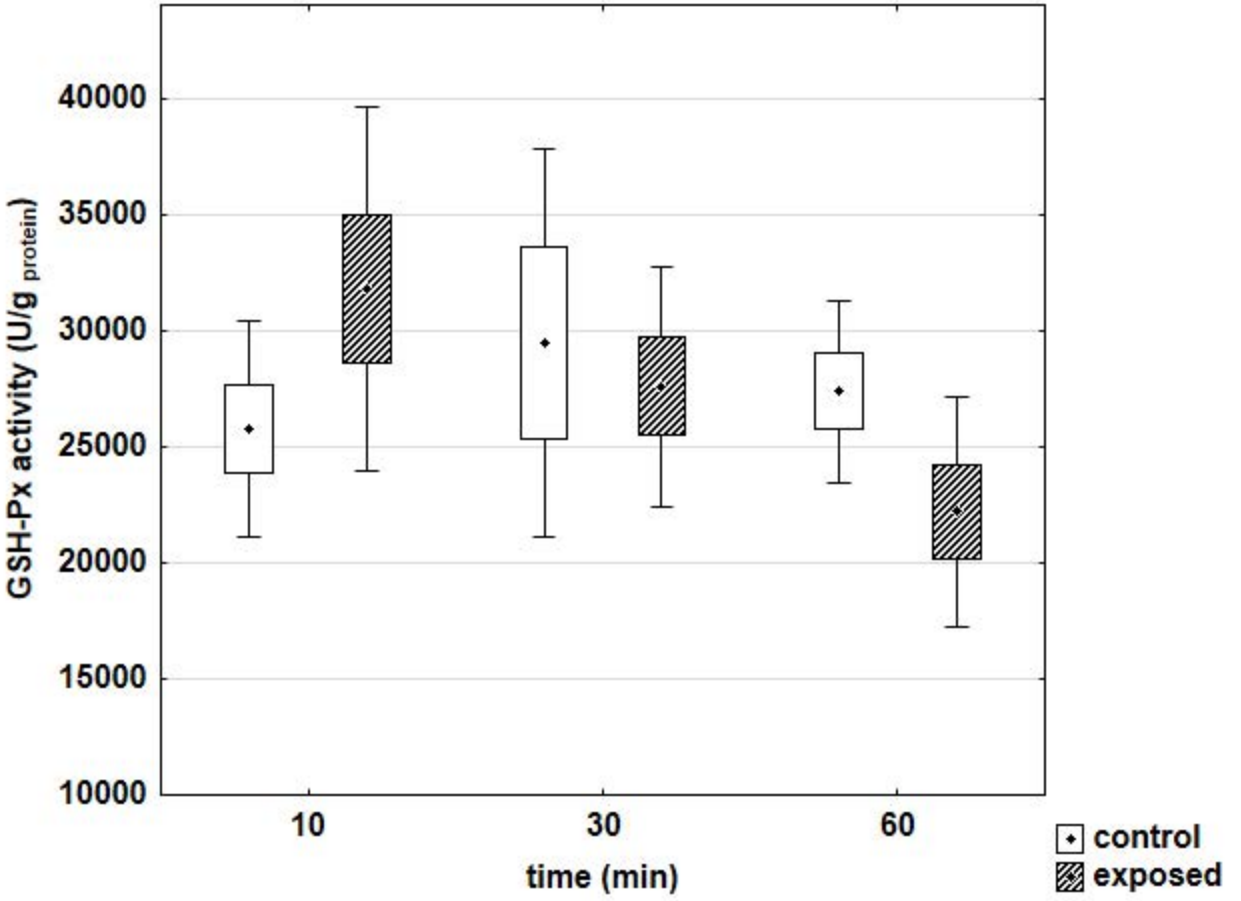
10000
8000
6000
4000
2000
fluorescence intensity
10
30
60
time (min)
negative control
exposed
positive control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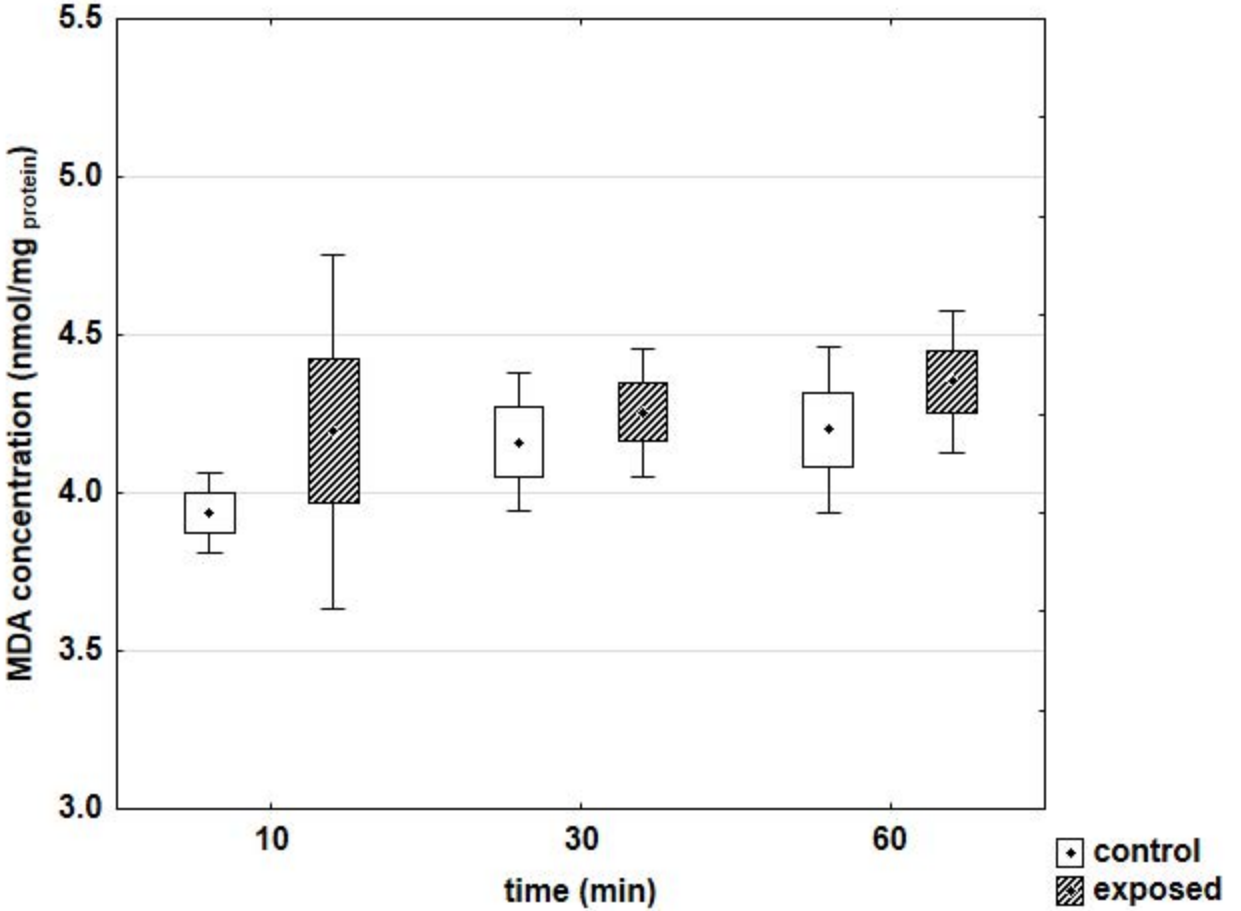
35000
30000
25000
20000
15000
10000
GSH-Px activity (U/g protein)
10
30
60
time (min)
control
exposed


5.5
5.0
4.5
4.0
3.5
3.0
MDA concentration (nmol/mg protein)
10
30
60
time (min)
control
exposed