



Brief communication

In vitro testing of cellular response to ultra high frequency electromagnetic field radiation

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ABSTRACT

The aim of this study was to evaluate whether low-level, ultra high frequency (UHF) irradiation of 935 MHz influences the cell structure and growth of V79 cells. UHF field was generated inside a Gigahertz Transversal Electromagnetic Mode cell (GTEM-cell) with a Hewlett-Packard signal generator. The electric field strength was 8.2 ± 0.3 V/cm and the average specific absorption rate (SAR) was calculated to be 0.12 W/kg. Cell samples were cultivated in a humidified atmosphere at 37 °C with 5% CO₂. Prepared cell samples were exposed to a 935 MHz continuous wave frequency field for 1, 2, and 3 h. The structure of microtubule proteins has been determined using the immunocytochemical method. Cell growth was determined by cell counts for each hour of exposure during five post-exposure days. Negative- and positive-cell controls were included into the experimental procedure. In comparison with control cells, the microtubule structure clearly altered after 3 h of irradiation ($p < 0.05$). Significantly decreased growth was noted in cells exposed for 3 h three days after irradiation ($p < 0.05$). It seems that the 935 MHz, low-level UHF radiation affects microtubule proteins, which consequently may obstruct cell growth.

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1. Introduction

Common concern about biological effects of ultra high frequency (UHF) electromagnetic (EM) fields in the range from 300 MHz to 3 GHz grows with development of telecommunications systems. So far, many reported biological effects of UHF are considered inconclusive in terms of their significance to living beings (Elwood, 2003). Different *in vitro* and *in vivo* effects on cell proliferation and cell membrane structure have been reported (Grundler and Keilmann, 1978; Phelan et al., 1992). There is also a possibility that UHF could interact with different apoptosis pathways in brain cells (Peinnequin et al., 2000; Panagopoulos et al., 2007; Zhao et al., 2007). No other effects have been reported, such as the effects on the cell cycle, enzyme activity, gene expression, DNA, and chromosome damage (Dutta et al., 1992; Cleary et al., 1996; Fritze et al., 1997; Malyapa et al., 1997; Penafiel et al., 1997; Goswami et al., 1999; Bisht et al., 2002; Zeni et al., 2003). An *in vivo* study showed that rat blood-forming system could be affected by low-level UHF fields (Trosic et al., 2002, 2004a, 2004b). There have been reported that long-term intermittent exposure to RF fields can enhance the probability that mice carrying a lymphomagenic oncogene will develop lymphomas (Repacholi et al., 1997). Also, UHF radiation has been shown to cause disturbance in erythrocytes maturation and proliferation, along with micronu-

cleus formation, as well as binucleation and multinucleation of alveolar macrophages in rats (Trosic, 2001; Busljeta et al., 2004).

Since studies of irradiation effects in mobile phone frequency ranges often present inconsistent results, it seems that the biological potency of UHF radiation could be elucidated using biological markers of exposure at the cellular macromolecular level.

In this study, we decided to use the V79 cell strain, because the status of this established line is well-known and commonly used in the cytotoxicological studies. No deviation has been expected during the course of regular cultivation (Bianchi, 1995). At the macromolecular level, we followed an extremely dynamic structure i.e., microtubule proteins, which functions are based on constant exchange of free charged tubulin subunits. In addition, process is continuously occurring in the proliferating cells. Consequently, the cell proliferation depends on proper dynamic instability rate of microtubule arrangement. We supposed that such biological scheme might be sensitive substrate in basic bioelectromagnetic research. Therefore, the aim of this study was to evaluate whether low-level 935 MHz frequency radiation affects microtubule structure and the growth of V79 cells 1, 2, and 3 h of exposure.

2. Material and methods

For this study, we used a continuous line of V79 cells, that is, lung fibroblasts of the Chinese hamster cultivated in a RPMI 1640 nutrient medium (SIGMA Chemical CO, St. Louis, USA) and supplemented with penicillin (20 units/ml), streptomycin (20 mg/

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ml), and 10% (vol/vol) heat-inactivated fetal calf serum (SIGMA Chemical CO, St. Louis, USA). The cells were kept in 25 cm² flasks (TPP, Switzerland) at 37 °C and humidified atmosphere with 5% CO₂. For the assay, cells were detached using 0.25% Trypsin/EDTA solution (SIGMA Chemical CO, St. Louis, USA), re-suspended, seeded, and pre-incubated for 24 h (Freshney, 2000a).

Electromagnetic field was generated within the certified Gigahertz Transversal Electromagnetic Mode Cell (GTEM-cell) manufactured by ETS Lindgren (Mod. 5402, ETS Lindgren, USA). Signal generator was used to produce electromagnetic field frequency of 935 MHz (Hewlett Packard HP8657, Palo Alto, USA) (ESCO Company EMCO, 1999).

Cell samples were exposed in triplicate for 1, 2, and 3 h to the UHF EM radiation. Negative control cells were kept in the same experimental conditions, but were not exposed to UHF EM radiation. Positive control cells used in the study were treated with colchicine (Colchicine, SIGMA Chemical CO, St. Louis, USA). Average specific absorption rate (SAR) for a single cell was 0.12 W/kg. SAR was calculated by averaging the individual parameters of the cell components in accordance with their volume fraction in live cell according to Steffensen's mathematical model (WHO 1993; Steffensen et al. 1995). Temperature inside the GTEM-cell was measured at 10 min intervals in order to preserve steady temperature of 37 °C throughout irradiation.

Indirect immunocytochemical method was used to determine microtubule proteins in irradiated and negative- and positive-control cells (Bell Jr. et al., 1995). According to Freshney's instructions from 2000, cell culture samples in the total volume of 5 ml were initially seeded on Permax Lab-Tek Chamber Slides (Nunc, Roskilde, Denmark) at a concentration of 2.5×10^4 cells/ml. After irradiation, the samples were washed and permeabilized with 0.5% triton X-100 (SIGMA Chemical CO, St. Louis, USA) in Dulbecco's Phosphate Buffered Saline (PBS, Imunološki zavod, Zagreb, Croatia). Subsequently, the cells were fixed in 4% paraformaldehyde. Microtubule proteins were marked by primary antibody (IgG anti- β -tubulin, SIGMA Chemical CO, St. Louis, USA). Secondary antibody was a conjugate of anti-mouse IgG and fluorescein isothiocyanate (FITC, Imunološki zavod, Zagreb, Croatia). The smears of cells were mounted in fluorescent mounting medium. To determine cell proliferation, V79 cells were plated in the concentration of 1×10^4 cells/ml on 24-well plates (TPP, Switzerland). For each hour

of exposure, cell proliferation was determined by cell count on post-exposure hours 24, 48, 72, 96, and 120. Proliferation was analyzed using a light microscope with 400 $\times$ magnification (Freshney, 2000b). The results are presented in the semi logarithmic scale. Each data point represents the mean value obtained from six separate cell samples. For assessing statistical difference between groups the data were analyzed using non-parametric Mann–Whitney *U* test. Statistical analyses were performed with software package Statistica, Version 7.0 (StatSoft, USA).

3. Results

In relation to the negative and positive controls, V79 cells showed increased and time-dependent susceptibility to UHF irradiation (Fig. 1a and b). Irregularities were observed in both cell morphology and microtubule protein structure. Frequent cytoplasm membrane knobs and bubbles are considered phenotypic changes caused by irradiation. For 1 and 2 h of exposure, nearly all microtubules were normally developed, and did not differ from the negative-control microtubules. We did not observe any damage of the mitotic spindle or irregularities in the distribution of microtubule proteins (Fig. 2a and b). Structural differences from negative controls were observed in cells exposed to UHF irradiation for 3 h ($p < 0.05$). Changes identified as grainy fluorescent clusters were comparable to those observed in positive control cells (see Fig. 2c). This grainy structure suggests that microtubule fibers are highly dissipated. Figs. 2–4 show the growth curves of V79 cell cultures after 1, 2, and 3 h of 935 MHz microwave irradiation and of the negative controls. In comparison with the negative-control cells, a significant difference was found in cultures three days after microwave exposure that lasted 3 h ($p < 0.05$) (Fig. 4). The observed decline in the exposed cell count on the fifth post-exposure day seems to be the consequence of cell culture confluence and depletion of nutrient in the medium. A similar decrease in control cell count on the same day confirms this assumption.

4. Discussion

Trosic et al. (2004b) found that UHF EM whole-body irradiation led to greater proliferation and quicker maturation of the rat haematopoietic cells. Although proposed a plausible mechanism of

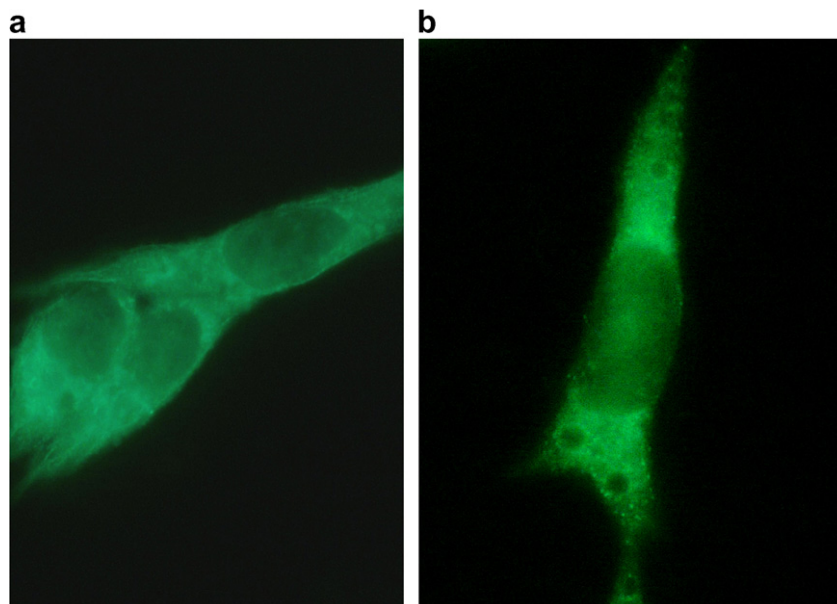


Fig. 1. Microtubule structure of unexposed control V79 cells (a) and positive control (b) cells.

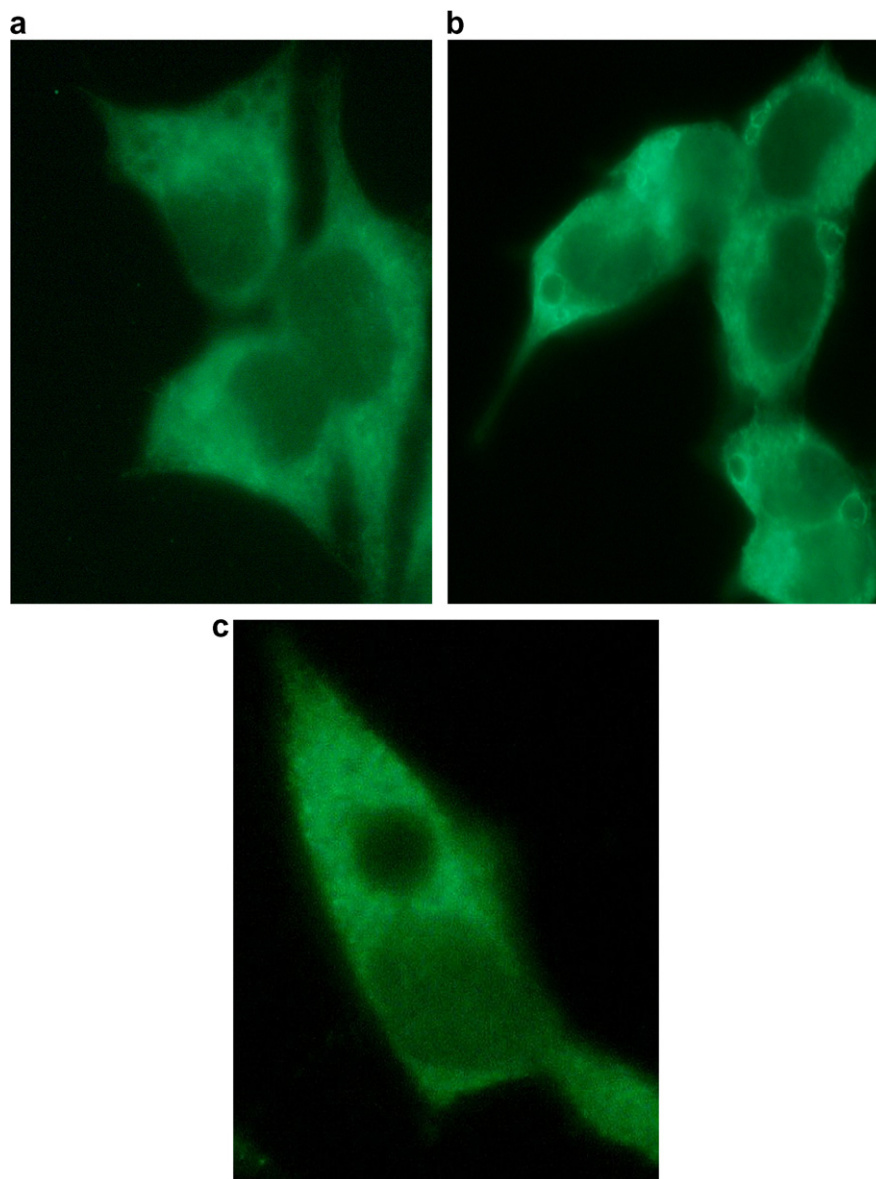


Fig. 2. Microtubule structure of V79 cells after 1 h (a), 2 h (b), and 3 h (c) of 935 MHz electromagnetic field radiation.

UHF EM action *in vivo*, we believe that *in vitro* approach would give a more accurate insight into this mechanism, particularly when it comes to the polar protein structures. Microtubule fibers represent extremely dynamic structure which functioning dependent on dynamical instability, that is, continuously binding and releasing of free tubule proteins. It was reasonably to assume that the external UHF EM radiation might interact with polar cytoskeletal structure. This structure greatly contributes to the balance between internal physiological electrostatic forces. Depending on the dipole moment, an external electric field could, in theoretically, disturb the equilibrium (Ortner et al., 1983) and increase the number of free cytoplasmatic tubulin proteins, identified by immunocytochemical staining and fluorescence microscopy as fluorescent grains. In our experiment, V79 cells showed increased time-dependent susceptibility of microtubular structure to UHF radiation (Fig. 1a and b). Visible grains denote impairment of microtubular fibers. Italian authors studied the influence of modulated UHF radiation on fibroblast cell morphology after 1-h exposure to electromagnetic field frequency of 902.4 MHz and SAR-a of 0.6 W/kg. They reported morphological changes caused by increased expres-

sion of genes coded for structural proteins (Pacini et al., 2002). French et al. investigated the effects of 835 MHz on human astrocytoma cells at power density of 40 and 8.1 mW/cm². The alteration of actin protein structure was noticed at field power density of 40 mW/cm², whereas changes in cell proliferation were observed at 8.1 mW/cm² (French et al., 1997). Our results are in concordance with their findings, even though the experimental conditions were different (Figs. 3–5). This suggests that various mammalian cells respond to external UHF radiation in a similar way. Thus, mobile phone frequencies may be capable of affecting fundamental biological processes even if there is no heating. In contrast, Stagg's and Higashikubo's group did not observe changes in the transit of cells through G1, G2 or S phase and in cell division immediately after exposure to modulated 835.62 MHz frequency wave up to 100 h, and SAR of 0.6 W/kg (Stagg et al., 1997; Higashikubo et al., 2001). Nevertheless, they have demonstrated the capability of low-power UHF radiation to induce reversible alteration in the cell protein structure. Microwaves affect the kinetics of conformational changes of the protein β -lactoglobulin. UHF irradiation accelerates these changes in the direction towards the equilibrium

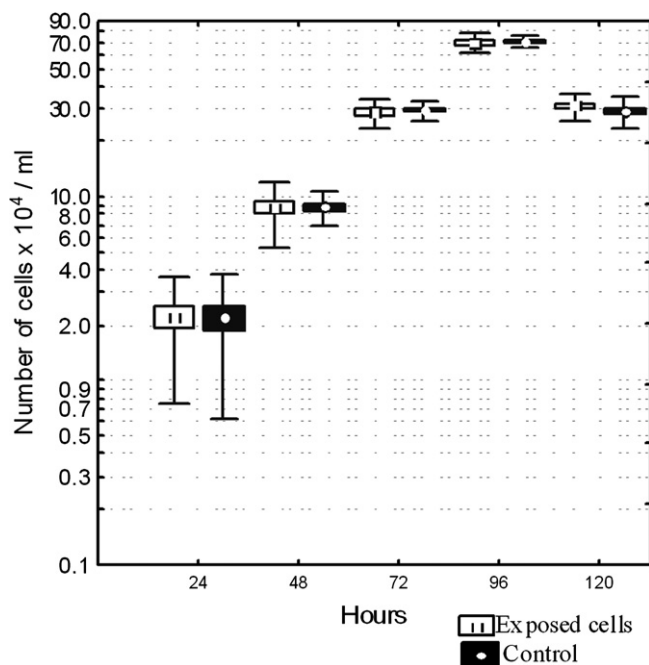


Fig. 3. Growth curve of unexposed V79 cells and cells exposed to 935 MHz electromagnetic field radiation for 1 h.

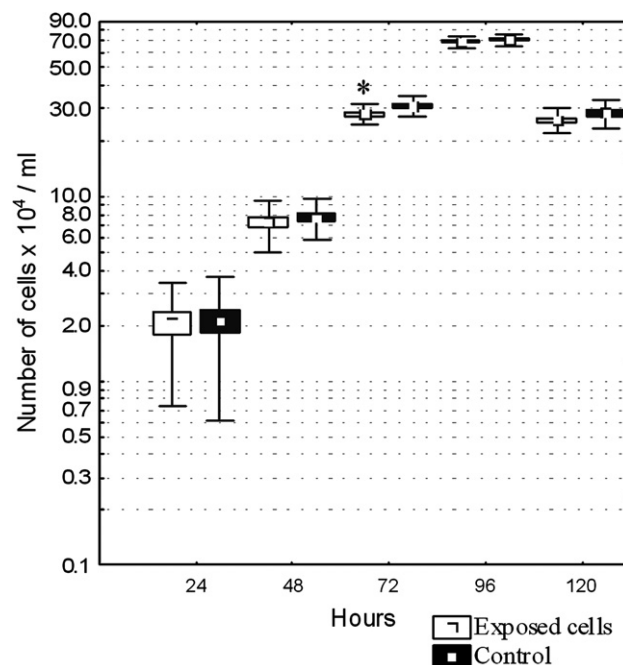


Fig. 5. Growth curve of unexposed V79 cells and cells exposed to 935 MHz UHF electromagnetic field radiation for 3 h (* significance $p < 0.05$).

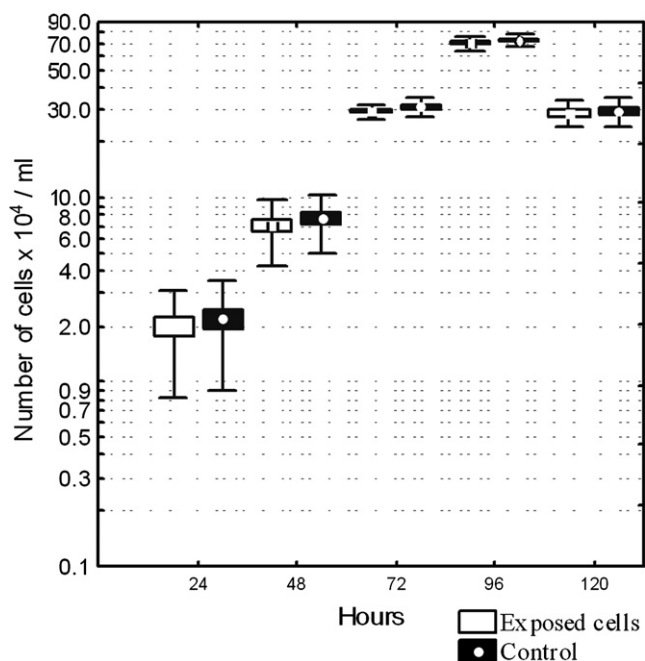


Fig. 4. Growth curve of unexposed V79 cells and cells exposed to 935 MHz electromagnetic field radiation for 2 h.

state by excitation of collective intrinsic modes in the protein. These non-thermal effects of 2.45 MHz microwaves were consistently reproducible in repeated experiments (Bohr and Bohr, 2000a, 2000b). Dipole momentum of microtubule structures fulfils basic condition for vibration excitation and creation of internal oscillated electric field (Pokorný et al., 1999). Oscillations in cellular electric field correspond to those of the external EM field, which may result in considerable biological effect.

The results obtained under the applied laboratory conditions indicate that low-intensity 935 MHz radiation possesses the capacity to affect microtubule proteins, which may consequently obstruct the growth of V79 cells.

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References

- Bell Jr., P.B., Safiejko-Mroccka, B., Bohn, W., Albrecht, R.M., Malecki, M., Burdett, I.D., 1995. Improved methods for preserving macromolecular structures and visualizing them by fluorescence and scanning electron microscopy. *Scanning Microscopy* 9, 843–860.
- Bianchi, V., 1995. V 79 Cytotoxicity test for membrane damage. In: O'Hare, S., Atterwill, C.K. (Eds.), *In Vitro Toxicity Testing Protocols* (Methods in Molecular Biology). Humana Press Inc., Totowa, NJ, pp. 151–161 (Chapter 17).
- Bisht, K.S., Moros, E.G., Straube, W.L., Baty, J.D., Roti Roti, J.L., 2002. The effect of 835.62 MHz FDMA or 847.74 MHz CDMA modulated radiofrequency radiation on the induction of micronuclei in C3H 10T1/2 cells. *Radiation Research* 157, 506–515.
- Bohr, H., Bohr, J., 2000a. Microwave-enhanced folding and denaturation of globular proteins. *Physical Review E-Statistical Physics, Plasmas, Fluids, and Related Interdisciplinary Topics* 61, 4310–4314.
- Bohr, H., Bohr, J., 2000b. Microwave enhanced kinetics observed in ORD studies of a protein. *Bioelectromagnetics* 21, 68–72.
- Busljeta, I., Trosic, I., Milkovic-Kraus, S., 2004. Erythropoietic changes in rats after 2.45 GHz nonthermal irradiation. *International Journal of Hygiene and Environmental Health* 207, 549–554.
- Cleary, S.F., Cao, G., Liu, L.M., 1996. Effects of isothermal 2.45 GHz microwave radiation on the mammalian cell cycle: comparison with effects of isothermal 27 MHz radiofrequency radiation exposure. *Bioelectrochemistry and Bioenergetics* 39, 167–173.
- Dutta, S.K., Das, K., Ghosh, B., Blackman, C.F., 1992. Dose dependence of acetylcholinesterase activity in neuroblastoma cells exposed to modulated radio-frequency electromagnetic radiation. *Bioelectromagnetics* 13, 317–322.
- Elwood, J.M., 2003. Epidemiological studies of radio frequency exposures and human cancer. *Bioelectromagnetics*, 24.
- ESCO Company EMCO, 1999. Operating manual Gigazertz Transverse Electromagnetic cell (GTEM). Model 5400 Series, ETS test systems. Austin, USA.
- French, P.W., Donnellan, M., McKenzie, D.R., 1997. Electromagnetic radiation at 835 MHz changes the morphology and inhibits proliferation of a human astrocytoma cell line. *Bioelectrochemistry and Bioenergetics* 43, 13–18.
- Freshney, R.I., 2000a. Cell lines, Protocol 12.2. In: Freshney, R.I. (Ed.), *Culture of Animal Cells: A Manual of Basic Techniques*. Wiley-Liss Inc., New York, pp. 184–188.
- Freshney, R.I., 2000b. Quantitation, Protocol 20.7. In: Freshney, R.I. (Ed.), *Culture of Animal Cells: A Manual of Basic Techniques*. Wiley-Liss Inc., New York, pp. 319–321.

- Fritze, K., Wiessner, C., Kuster, N., Sommer, C., Gass, P., Hermann, D.M., Kiessling, M., Hossmann, K.A., 1997. Effect of global system for mobile communication microwave exposure on the genomic response of the rat brain. *Neuroscience* 81, 627–639.
- Goswami, P.C., Albee, L.D., Parsian, A.J., Baty, J.D., Moros, E.G., Pickard, W.F., Roti Roti, J.L., Hunt, C.R., 1999. Proto-oncogene mRNA levels and activities of multiple transcription factors in C3H 10T 1/2 murine embryonic fibroblasts exposed to 835.62 and 847.74 MHz cellular phone communication frequency radiation. *Radiation Research* 151, 300–309.
- Grundler, W., Keilmann, F., 1978. Nonthermal effects of millimeter microwaves on yeast growth. *Zeitschrift fur Naturforschung Section C Biosciences* 33, 15–22.
- Higashikubo, R., Ragouzis, M., Moros, E.G., Straube, W.L., Roti Roti, J.L., 2001. Radiofrequency electromagnetic fields do not alter the cell cycle progression of C3H 10T1/2 and U87MG cells. *Radiation Research* 156, 786–795.
- Malyapa, R.S., Ahern, E.W., Straube, W.L., Moros, E.G., Pickard, W.F., Roti Roti, J.L., 1997. Measurement of DNA damage after exposure to electromagnetic radiation in the cellular phone communication frequency band (835.62 and 847.74 MHz). *Radiation Research* 148, 618–627.
- Ortner, M.J., Galvin, M.J., Irwin, R.D., 1983. The effect of 2450-MHz microwave radiation during microtubular polymerization in vitro. *Radiation Research* 93, 353–363.
- Pacini, S., Ruggiero, M., Sardi, I., Aterini, S., Gulisano, F., Gulisano, M., 2002. Exposure to Global System for Mobile Communication (GSM) cellular phone radiofrequency alters gene expression, proliferation, and morphology of human skin fibroblasts. *Oncology Research* 13, 19–24.
- Panagopoulos, D.J., Chavdoula, E.D., Nezis, I.P., Margaritis, L.H., 2007. Cell death induced by GSM 900-MHz and DCS 1800-MHz mobile telephony radiation. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 626, 69–78.
- Peinnequin, A., Piriou, A., Mathieu, J., Dabouis, V., Sebbah, C., Malabiau, R., Debouzy, J.C., 2000. Non-thermal effects of continuous 2.45 GHz microwaves on Fas-induced apoptosis in human Jurkat T-cell line. *Bioelectrochemistry and Bioenergetics* 51, 157–161.
- Penafiel, L.M., Litovitz, T., Krause, D., Desta, A., Mullins, J.M., 1997. Role of Modulation on the effect of microwaves on ornithine decarboxylase activity in L929 cells. *Bioelectromagnetics* 18, 132–141.
- Phelan, A.M., Lange, D.G., Kues, H.A., Luttj, G.A., 1992. Modification of membrane fluidity in melanin-containing cells by low-level microwave radiation. *Bioelectromagnetics* 13, 131–146.
- Pokorný, J., Jelínek, F., Trkal, V., Šrobár, F., 1999. Vibration in microtubules. Paper presented at: Electricity and Magnetism in Biology and Medicine, Kluwer Academic/Plenum Publishers, Bologna, Italy, pp. 967–970.
- Repacholi, M.H., Basten, A., Gebiski, V., Noonan, D., Finnie, J., Harris, A.W., 1997. Lymphomas in E-Pim1 transgenic mice exposed to pulsed 900 MHz electromagnetic fields. *Radiation Research* 147, 631–640.
- Stagg, R.B., Thomas, W.J., Jones, R.A., Adey, W.R., 1997. DNA synthesis and cell proliferation in C6 glioma and primary glial cells exposed to a 836.55 MHz modulated radiofrequency field. *Bioelectromagnetics* 18, 230–236.
- Steffensen, K.V., Raskmark, P., Pedersen, G.F., 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.
- Trosic, I., 2001. Multinucleated giant cell appearance after whole body microwave irradiation of rats. *International Journal of Hygiene and Environmental Health* 204, 133–138.
- Trosic, I., Busljeta, I., Kasuba, V., Rozgaj, R., 2002. Micronucleus induction after whole-body microwave irradiation of rats. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 521, 73–79.
- Trosic, I., Busljeta, I., Modlic, B., 2004a. Investigation of the genotoxic effect of microwave irradiation in rat bone marrow cells: in vivo exposure. *Mutagenesis* 19, 361–364.
- Trosic, I., Busljeta, I., Pavicic, I., 2004b. Blood-forming system in rats after whole-body microwave exposure; reference to the lymphocytes. *Toxicology Letters* 154, 125–132.
- WHO, 1993. *Environmental Health Criteria 137: Electromagnetic Fields (300 Hz to 300 GHz)*. World Health Organization, Geneva.
- Zeni, O., Chiavoni, A.S., Sannino, A., Antolini, A., Forigo, D., Bersani, F., Scarfi, M.R., 2003. Lack of genotoxic effects (micronucleus induction) in human lymphocytes exposed in vitro to 900 MHz electromagnetic fields. *Radiation Research* 160, 152–158.
- Zhao, T.Y., Zou, S.P., Knapp, P.E., 2007. Exposure to cell phone radiation up-regulates apoptosis genes in primary cultures of neurons and astrocytes. *Neuroscience Letters* 412, 34–38.