

Microtubular structure impairment after GSM-modulated RF radiation exposure

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The objective of the study was to investigate whether low-level 915 MHz GSM-modulated radiofrequency (RF) radiation impairs microtubular structure and affects normal cell growth. V79 cells were exposed to a GSM-modulated field in a Gigahertz Transversal Electromagnetic Mode cell (GTEM cell) for 1, 2, and 3 h. Signal generator combined with power and chip modulator generated the electromagnetic field (EMF). The electric field strength was adjusted to 10, 20, and 30 V/m, and the average specific absorption rate (SAR) was calculated to be 0.23, 0.8, and 1.6 W/kg. The structure of microtubule proteins was assessed by indirect immunocytochemistry, and cell growth was determined based on cell counts taken every day over six post-exposure days. Three-hour radiation exposure significantly altered microtubule structure regardless of the electric field strength. Moreover, on the third post-exposure day, three-hour radiation significantly reduced cell growth, regardless of field strength. The same was observed with two-hour exposure at 20 and 30 V/m. In conclusion, 915 MHz GSM-modulated RF radiation affects microtubular proteins in a time-dependent manner, which, in turn, affects cell proliferation. Our future research will focus on microtubule structure throughout the cell cycle and RF radiation effects on mitotic spindle.

KEY WORDS: cell growth; cytoskeleton; *in vitro*; 915 MHz; mobile phone radiation

Numerous biological effects reported to be caused by radiofrequency (RF) radiation are open to doubt in terms of their significance for living beings (1). So far, several *in vitro* and *in vivo* studies (2–5) were questioned and dismissed based on their lack of consistency and reproducibility. Various investigations have suggested that RF fields may promote cancer, but evidence to these effects was suggestive at best, not substantive (1, 6). However, RF radiation was found to interact with different apoptosis pathways in living cells (7–9). A number of studies have also investigated its genotoxic and other effects on the cell cycle, enzyme activity, gene expression, DNA, oxidative stress, and chromosomes (10–19).

For RF energy to impair physiological function or trigger a disease in humans or animals there must be a mechanism by which physical forces exerted by electric and magnetic fields or charged particles alter molecules, chemical reactions, cell membrane, or biological structure (20–22). This mechanism could be connected to microtubule dynamics. It is known that microtubule assembly and disassembly takes place at specific time in the cellular cycle, and that dynamic exchange of charged tubulin subunits on and off microtubule fibres influences the movement of cytoplasmic vesicles, granules, and organelles like mitochondria or chromosomes during mitosis (23).

Dynamic instability of microtubular arrangement controls most of cell proliferation, so it stands to reason that it might be an appropriate substrate for research of bioelectromagnetic effects. Furthermore, as this bipolar system could be sensitive to external RF fields, we thought it logical to examine their effects at the cellular macromolecular level. Our investigation aimed at evaluating whether GSM-modulated 915 MHz RF radiation and corresponding specific absorption rates could really affect microtubular structure and cell growth.

MATERIALS AND METHODS

For the experiment we used the Chinese hamster fibroblast cell line (V79) because of its well-known properties and common use in cytotoxic studies (24). Cells were maintained in a strict line with Freshney's instructions (25), and cultivation procedure was the same as described elsewhere in detail (26).

Electromagnetic field was created inside a 5402 Gigahertz Transversal Electromagnetic Mode Cell (GTEM, ETS-Lindgren, Cedar Park, TX, USA). 915 MHz field was generated with a tracking generator and an Anritsu 2721B spectrum analyser combined with an RF 3146 power amplifier module (RF Micro Devices, Greensboro, NC, USA) and a Polaris chipset RF 2722 signal modulator (RF Micro Devices). Quality and uniformity of the produced signal was measured by an antenna connected to the spectrum analyser. RF signal was modulated to the 900-

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MHz GSM band in line with mobile network systems common in Europe, Asia, Africa, and mainly South America. This signal is designed for wide-area cellular operations with maximum output powers permissible for mobile operations. Although 2G is being decommissioned around the world, the selected frequency is also used in the 3G and 4G networks.

Each exposure protocol was run on three independent cell samples. The field strength of the GSM-modulated electromagnetic field (EMF) was 10, 20, and 30 V/m. The cells were exposed in parallel for one, two, and three hours.

The strength and uniformity of the electric field created inside the GTEM cell was determined with an HI 468 measuring instrument (ETS Lindgren, Cedar Park, TX, USA) and an HI 6005 test probe (ETS Lindgren). The field in reference was controlled with the Anritsu 2721B spectrum analyser (Atsugi, Kanagawa, Japan). The strength of the field created inside the GTEM cell was proven to be homogenous, with only slight variations of ± 1 V/m.

Sham-exposed (negative control) cells were treated in the same manner as the exposed ones but were not irradiated at any point, that is, the radiofrequency generator was switched off during simulation.

In addition to negative control, radiation exposed cells treated with 0.1 mmol/L colchicine (Sigma-Aldrich, St. Louis, MO, USA) were used as a positive control. Colchicine is an antimetabolic agent able to attach to free tubulin subunits and suppress polymerisation and destruction of microtubules.

The average specific absorption rate (SAR) established on a single-cell level was 0.23, 0.8, and 1.6 W/kg at 10, 20, and 30 V/m, respectively. SAR was calculated by averaging the individual parameters of cell components in accordance with their volume fraction in a living cell (27, 28). Following the method described by Buttiglione et al. (29) dosimetry results in our study were obtained in a homogeneous cell culture arranged in the form of plastic chamber slides filled with 5 mL of culture medium. In order to calculate SAR values, we used the medium dielectric constant (ϵ_r) of 75, its effective electric conductivity (σ) of 1.8 S/m (numerical calculation), and aqueous sample density of 1 kg/L as input parameters (26, 30). To provide a steady temperature throughout irradiation the temperature of the nutrient medium (contained in plastic chamber slides and placed inside the GTEM cell) was continuously measured using a temperature sensor (NTA 880, Oregon Scientific, Tualatin, OR, USA). Temperature did not rise during irradiation and kept at 36.3 ± 0.3 °C, which corresponds to physiological cell temperature.

Microtubular proteins in irradiated, negative, and positive control cell samples were determined with indirect immunocytochemical analysis (31). In brief, cells (in the total volume of 5 mL) were initially seeded on Permanox Lab-Tek Chamber Slides (Nunc, Roskilde, Denmark) at the concentration of 2.5×10^4 cells/mL. After 24 hours, cells were irradiated for 1, 2, and 3 h. After irradiation, the samples

were washed, permeabilised, and fixed in paraformaldehyde. Microtubular proteins were marked with a complex of primary IgG anti- β -tubulin antibody produced in mouse (Sigma-Aldrich, St. Louis, MO, USA) and secondary antibody representing a conjugate of anti-mouse IgG and fluorescein isothiocyanate (FITC, Sigma-Aldrich, St. Louis, MO, USA). Once mounted in a fluorescent medium, 1,000 cells per slide were analysed with a fluorescent light microscope (400x magnification, Olympus BX61, Tokyo, Japan). Microtubule damage was evaluated by determining structural differences in irradiated cells. Changes identified as grainy fluorescent clusters were compared with those observed in positive control cells. This grainy structure suggests that microtubule fibres are highly dissipated and therefore damaged.

In order to measure cell proliferation rate, 1×10^4 cells/mL from each group were seeded on 24-well plates and counted for six post-exposure days under a light microscope at 400x magnification using improved Neubauer counting chamber (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

Statistical differences between the subsets were analysed with non-parametric Mann-Whitney *U* test. All data were presented as means \pm standard deviations of three independent samples per subset. Statistical analyses were run on the software package Statistica, version 13.2 (StatSoft, Tulsa, OK, USA).

RESULTS

Figure 1 shows the percentage of damaged V79 cells after 1, 2, and 3 h of exposure to GSM-modulated 915 MHz radiation. Although the cells were exposed to different electric field strengths, they showed the same pattern of time-dependent microtubular impairment. Three-hour irradiation at all electric field strengths caused significant microtubular damage. At shorter irradiations, microtubules developed normally and did not differ from negative controls (Figure 2).

Three days after the three-hour exposure, cells counts were significantly lower than in negative control ($P < 0.05$) (Figure 3A, 3B, and 3C). Two-hour exposure to 20 and 30 V/m fields (corresponding to 0.8 and 1.6 W/kg SAR, respectively), also resulted in significantly lower proliferation rate (Figures 3B and 3C) on day 3 post-exposure. However, on day 4 post-exposure, cells counts returned to normal in every exposed group. The decline in the cell count on the fifth post-exposure day is most likely a result of cell culture confluence and depletion of nutrient in the medium, considering that parallel decrease was observed in control cells (there was no difference in proliferation rate between exposed and negative control cells).

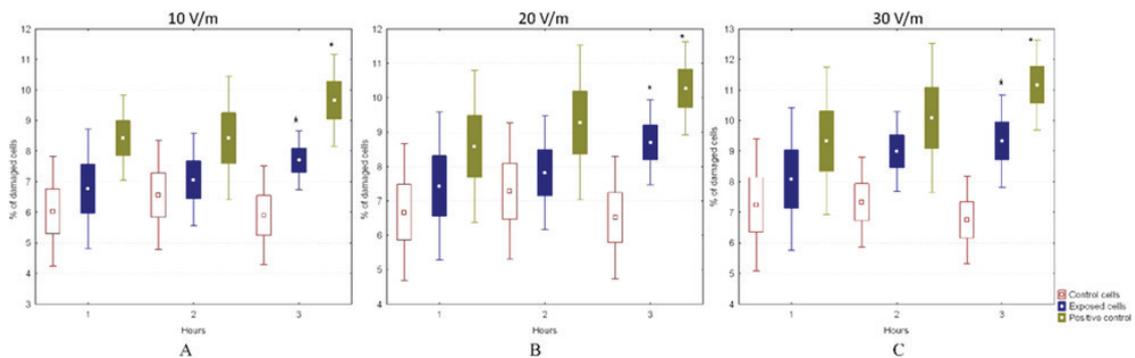


Figure 1 Microtubular structure impairment in V79 cells after 1, 2, and 3 h of exposure to GSM-modulated 915 MHz field at the electric field strength of 10 V/m corresponding to SAR of 0.23 W/kg (A), 20 V/m at the SAR 0.8 W/kg (B) and 30 V/m corresponding to SAR of 1.6 W/kg (C). Control cells – unexposed to radiation (sham exposure); Positive control – cells treated with colchicine. *P<0.05

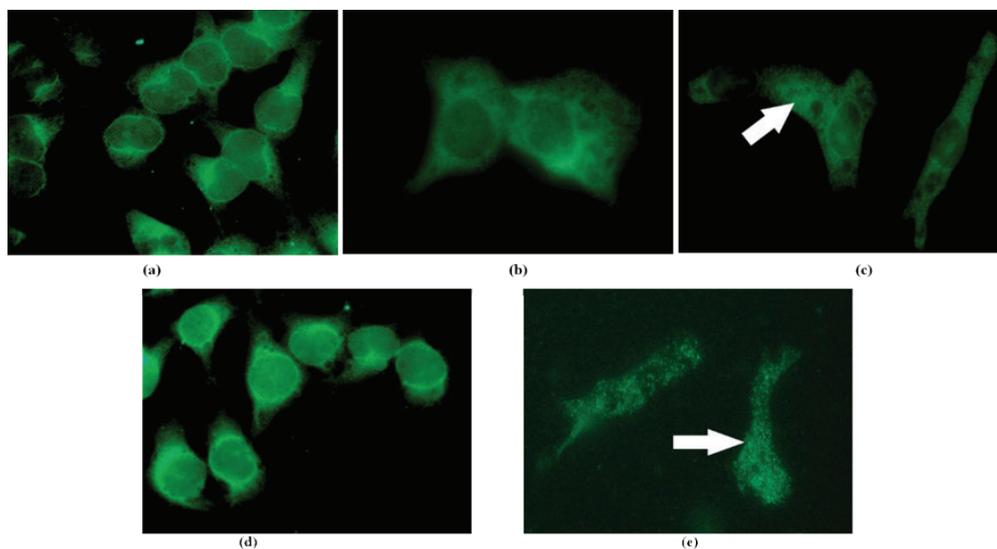


Figure 2 Photomicrography of microtubule cellular structure (400x magnification) in V79 cells after 1-hour (a), 2-hour (b), and 3-hour (c) GSM-modulated 915 MHz radiation exposure with the corresponding SAR 1.6 W/kg, including negative (d), and positive control (e). Microtubular structure impairment, visible as grainy fluorescent clusters, is indicated by arrows

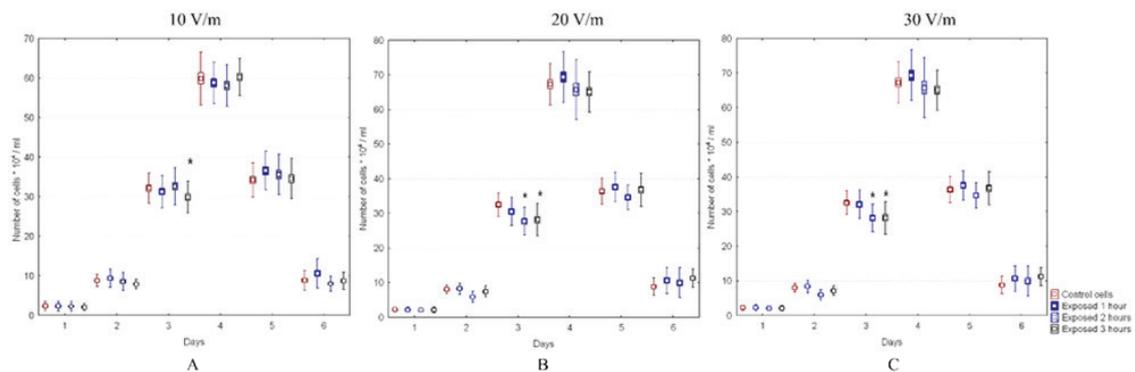


Figure 3 V79 cell proliferation rate after 1, 2, and 3 h of GSM-modulated 915 MHz radiation exposure with respective SARs of 0.23 W/kg (A), 0.8 W/kg (B), and 1.6 W/kg (C). * P<0.05 compared to control. Control cells – unexposed to radiation (sham exposure)

DISCUSSION

Our results clearly show that 915 MHz radiation impairs microtubular proteins of V79 cells in a time-dependent manner. This confirms the hypothesis that electromagnetic fields in GSM frequency range might interfere with the mechanisms driving the cytoskeleton network, as this process is based on the bipolarity of the basic protein building units (32). Microtubules, as part of the cytoskeleton, meet the basic requirements for the onset of excitation that results in vibrations theoretically predicted by Fröhlich (33–35) and for the generation of an endogenous oscillating electric field (32). In a healthy cell, this endogenous electromagnetic field is perfectly balanced, but external electromagnetic fields may disrupt it.

Earlier studies, such as the one by Ortner et al. (36) showed no effect of 2450 MHz microwave radiation during *in vitro* polymerisation of purified microtubular proteins. Still, small differences were seen in the dynamics of light scattering curves out of the standard error of the mean of measurement. A more recent study (37), on the other hand, showed that 24-hour intermittent exposure to 1800 MHz RF radiation affected gene expression in rat neurons associated with multiple cellular functions, including cytoskeleton. In another study (38), mobile-phone radiation was found to affect the expression of a cytoskeleton protein vimentin and of stress fibres forming F-actin. Similarly, in another study (39) one-hour 902.4 MHz irradiation with SAR of 0.6 W/kg affected fibroblast morphology through increased expression of genes coding for structural proteins. Furthermore, changes in actin-binding proteins were observed in human astrocytoma cells exposed to 835 MHz at power density of 40 mW/cm², while changes in cell proliferation were detected at power density of 8.1 mW/cm² (40). All these findings support the hypothesis that cytoskeleton responds to RF radiation.

Our proliferation rate findings complement previous research. We believe that the marked drop three days after 2- and 3-hour exposure was the consequence of damage done to microtubular structure. These changes seem to be reversible, however, as the count grew back to normal on day 4 post-exposure, indicating that the cells did not lose their ability to divide. This implies that the observed microtubule damage was not significant for the cell cycle. Comparable findings were reported by Ballardina et al. (41) for V79 cells exposed to 15-minute 2.45 GHz exposure. It induced changes in the mitotic apparatus, apoptosis, and a moderate drop in proliferation rate. The authors suggested that spindle alterations were not permanent and that the length of the exposure was insufficient to make them irreversible. It is interesting to note that a similar mechanism of microtubule structure impairment is seen at low-intensity, intermediate-frequency (100–300 kHz) radiation. These fields arrest cell proliferation by interfering with the formation of mitotic spindle and cause rapid disintegration

of dividing cells. Their use in tumour treatment has been approved by the US Food and Drug Administration (42).

CONCLUSION

Our study shows that V79 cells are sufficiently sensitive to be affected by GSM RF radiation, as it caused time-dependent microtubule protein impairment and, consequently, lower proliferation rate. It has also established that radiation effects on microtubules did not depend significantly on electric field strength and corresponding SAR, which calls for further investigation. A more detailed analysis of microtubule structure is also warranted to cover the entire cell cycle and look into the effects on mitotic spindle. This will be in the focus of our future research.

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Promjena strukture mikrotubula nakon izloženosti GSM-moduliranom RF zračenju

Cilj istraživanja bio je ispitati narušava li GSM modulirano radiofrekvencijsko zračenje (RF) frekvencije 915 MHz strukturu mikrotubula te utječe li na rast stanica. Stanice V79 izložene su GSM-moduliranom polju unutar gigahercne transverzalne elektromagnetske komore (GTEM-komora) tijekom jednog, dva i tri sata. Generator signala u kombinaciji s pojačalom i modulatorom signala generirao je elektromagnetsko polje (EMF). Jačina električnoga polja namještena je na vrijednosti od 10, 20 i 30 V/m, a izračunane vrijednosti specifične brzine apsorpcije (SAR) u stanici bile su 0,23, 0,8 i 1,6 W/kg. Struktura mikrotubularnih proteina utvrđena je posrednom imunocitokemijom, a stanični rast određen je na temelju broja stanica izmjerenih za svako vrijeme izloženosti zračenju tijekom šest dana nakon ozračivanja. Značajne promjene u strukturi mikrotubula zabilježene su nakon tri sata zračenja, neovisno o jakosti električnoga polja. Također, značajno smanjen rast stanica zabilježen je tri dana nakon trosatne izloženosti zračenju. Navedene promjene uočene su bez obzira na primijenjenu jakost električnoga polja. Značajno smanjen rast stanica zabilježen je također tri dana nakon dvosatne izloženosti zračenju pri jakosti električnoga polja od 20 i 30 V/m. Možemo zaključiti da je učinak GSM-moduliranoga RF zračenja frekvencije 915 MHz na proteine mikrotubula i stanični rast ovisan o trajanju izloženosti zračenju. Zabilježene promjene bile su izraženije pri višoj jakosti električnoga polja.

KLJUČNE RIJEČI: 915 MHz; citoskelet; *in vitro*; stanični rast; zračenje mobilne telefonije