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Scientific Paper

DISTURBANCE OF CELL PROLIFERATION IN RESPONSE TO MOBILE PHONE FREQUENCY RADIATION

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The aim of study was to determine the influence of mobile phone frequency radiation on the proliferation, cytoskeleton structure, and mitotic index of V79 cells after 1 h, 2 h, and 3 h of exposure. V79 cells were cultured in standard laboratory conditions and exposed to continuous-wave (CW) RF/MW radiation of 935 MHz, electric field strength of (8.2±0.3) V m^{-1}, and specific absorption rate (SAR) of 0.12 W kg^{-1}. To identify proliferation kinetics, the cells were counted for each hour of exposure 24 h, 48 h, 72 h, and 96 h after respective exposures. Microtubule proteins were determined using specific immunocytochemical methods. Cell smears were analysed under a fluorescent microscope. The study included negative and positive controls. Mitotic index was determined by estimating the number of dividing cells 24 h after exposure and dividing it with the total number of cells. In comparison to the controls, cell proliferation declined in cells exposed for three hours 72 h after irradiation ($p<0.05$). Microtubule structure was clearly altered immediately after three hours of irradiation ($p<0.05$). The mitotic index in RF/MW-exposed cells did not differ from negative controls. However, even if exposure did not affect the number of dividing cells, it may have slowed down cell division kinetics as a consequence of microtubule impairment immediately after exposure.

KEY WORDS: *continuous cell culture, microtubule structure, mitotic index, RF/MW, 935 MHz*

Radiofrequency/microwave (RF/MW) is a term which defines an electromagnetic radiation in the frequency range from 3 kHz to 300 GHz. Usually, MW radiation is considered a subset of RF radiation, although an alternative convention treats RF and MW radiation as two spectral regions. RF/MW radiation belongs to the non-ionising part of electromagnetic spectrum. This part of electromagnetic (EM) radiation has insufficient energy (<10 eV) to ionise atoms. It is known that the absorption of RF/MW energy varies with wave frequency. The primary biological effects of RF/MW energy are considered to be thermal (1). Whereas the thermal mechanism of non-ionising radiation is well known, the non-thermal remains to be elucidated (2). Among a nubmer of new devices operating in the RF/MW frequency range, cellular phones are the most common. Mobile telecommunications has boomed in recent years and has acquired an important place in society. People have become accessible at all times and places. Their benefits aside, new technologies have given rise to public concern about whether exposure to an EM field generated during wireless communication could have adverse effects on the living matter, particularly on humans.

Since 1999, a group of investigators at the Institute for Medical Research and Occupational Health, Zagreb has been investigating the non-thermal biological effects of RF/MW exposure using animal experimental models and cell cultures (3-14). This research aimed

at assessing the influence of mobile phone frequency on cell proliferation, cytoskeleton structure, and the mitotic index in a continuous V79 cell line irradiated by 935 MHz CW for 1 h, 2 h, and 3 h.

MATERIALS AND METHODS

Cell culture and exposure setup

A stable continuous line of V79 cells was used in the experiment. The cells were cultured in a RPMI 1640 medium supplemented with 10 % foetal calf serum, in a CO_2 incubator at 37 °C, following the method described by Freshney (15). The experimental design, including the exposure setup has already been described in detail elsewhere (14, 16-18). Briefly, cell samples were exposed to 935 MHz frequency radiation for 1 h, 2 h, and 3 h. The EM field was generated by a Hewlett Packard HP8657 signal generator (Pablo Alto, USA) in a Gigahertz Transversal Electromagnetic Mode Cell (GTEM-cell) manufactured by ETS Lindgren (Model 5402, ETS Lindgren, USA) (19). The temperature inside the GTEM-cell was maintained at 37 °C and verified every 10 min throughout irradiation. Negative control cells were kept in the same experimental conditions save for RF/MW exposure. Average SAR for a single cell was 0.12 W kg^{-1}. SAR was calculated by averaging the conductivity of cell macromolecules in accordance with their volume fraction (1, 20).

Cell proliferation measurement

To determine the kinetics of cell proliferation, cells were seeded on 24-well plates in the concentration of $1x10^4$ cells mL^{-1}. For each hour of exposure the cells were counted with a hemocytometer under a light microscope (400x) 24 h, 48 h, 72 h, and 96 h after exposure (21).

Microtubule structure examination

To determine microtubule proteins in irradiated, negative, and positive control cells we used an indirect immunocytochemical method (22). Positive control cells were treated with colchicine in the concentration of 0.1 mmol L^{-1} (Colchicine, Sigma Chemical Co., St. Louis, USA). Colchicine inhibits microtubule polymerisation by binding to tubulin, one of the main constituents of microtubules. The availability of tubulin is essential for mitosis, and therefore colchicine is an effective "mitotic poison" or spindle poison. Cell culture samples in the total volume of 5 mL were initially seeded at a concentration of $2.5x10^4$ cells mL^{-1}. After irradiation, the samples were washed and permeabilised with 0.5 % Triton X-100 (Sigma Chemical Co., St. Louis, USA). Subsequently, cell preparations were fixed and microtubule proteins marked by a primary antibody (IgG anti-beta-tubulin, Sigma Chemical Co., St. Louis, USA) and a secondary antibody (a conjugate of anti-mouse IgG and fluorescein isothiocyanate, Imunološki zavod, Zagreb, Croatia). The preparations were embedded in a fluorescent mounting medium. To examine the microtubule network configuration, at least 500 cells per slide were analysed using a fluorescent microscope (1000x). The microtubule structure was impaired if there were granular fluorescent clusters. In addition, damaged cells were also characterised by increased vacuolization and changes in cell shape.

Cells with impaired microtubule structure are expressed as a percentage of total irradiated, colchicin–treated, or positive and negative control cells.

Mitotic index

The cells were seeded at the concentration of $2.5x10^5$ cells mL^{-1}, exposed to RF/MW radiation, and incubated for 24 h. Then they were detached using 0.25 % Trypsin/EDTA solution (Sigma Chemical Co., St. Louis, USA), washed and fixed with methanol-acetic acid (5:1), air dried, and then stained with 5 % Giemsa in PBS.

The mitotic index is a measure of the proliferation of a cell population. It is defined as the ratio between the number of cells in mitosis and the total number of cells. A cell population grows as cells pass through interphase and mitosis to complete the cell cycle. Many cells lose the capacity to divide, if their maturation or proliferation is affected by internal or external reasons. In this study, MI was obtained by scoring 1000 cells per slide using a light microscope (400x).

Statistical analysis

The collected data were analysed using a Statistica 7.0 (StatSoft Inc., USA). Each bar in Figure 1 shows the proliferation of cells exposed to RF/MW radiation, expressed as the percentage of proliferation of negative control cells with standard deviation (SD). Figures 2 and 3 show the mean values ± SD. Pairwise comparisons between groups of data

were performed using the analysis of variance with multiple comparison tests. Statistical difference was determined at $p<0.05$.

RESULTS

Figure 1 shows the proliferation of V79 cell cultures after one, two, or three hours of exposure to 935 MHz irradiation. Seventy-two hours after exposure, a significant difference was found in the proliferation of cells exposed for three hours in comparison with negative controls ($p<0.05$).

Figure 2 shows changes in microtubule fibres after 1 h, 2 h, and 3 h of exposure to RF/MW radiation in comparison with negative and positive control cell samples. Irregularities were observed in both cell morphology and microtubule protein structure (see Figures 4, 5, and 6). Frequent cytoplasm membrane knobs and bubbles are considered phenotypic changes caused by irradiation. Nearly all microtubules exposed for one and two hours were normal, and did not differ from negative controls. We did not observe any damage of the mitotic spindle or irregularities in the distribution of microtubule proteins.

In comparison with negative controls, no significant changes were observed in microtubule structure in cells irradiated for one or two hours. However, the difference between negative controls and cells irradiated for three hours was statistically significant (Figure 2) ($p<0.05$).

Figure 3 shows that the mitotic index of V79 cells exposed to 935 MHz RF/MW irradiation for one, two, or three hours did not significantly differ from negative controls.

DISCUSSION

Non-ionising electromagnetic radiation involves the physics of the electromagnetic field. This field exerts a force on particles with electric charge, and is in turn affected by the presence and motion of those particles. A changing magnetic field produces an electric field. Similarly, a changing electric field generates a magnetic field. Because of this interdependence of the electric and magnetic fields, it makes sense to consider them as a single coherent entity - the electromagnetic field (23). Alternating electric fields have a wide range of effects on living systems (24). At extremely low frequencies, electric fields stimulate excitable tissues through membrane depolarisation (25), stimulate bone growth, and accelerate fracture healing (26, 27). As the electric field frequency increases, the stimulatory effect disappears, ending in the so-called thermal effect, when tissue heating becomes a dominant event (28). In-between the desired stimulatory and undesired

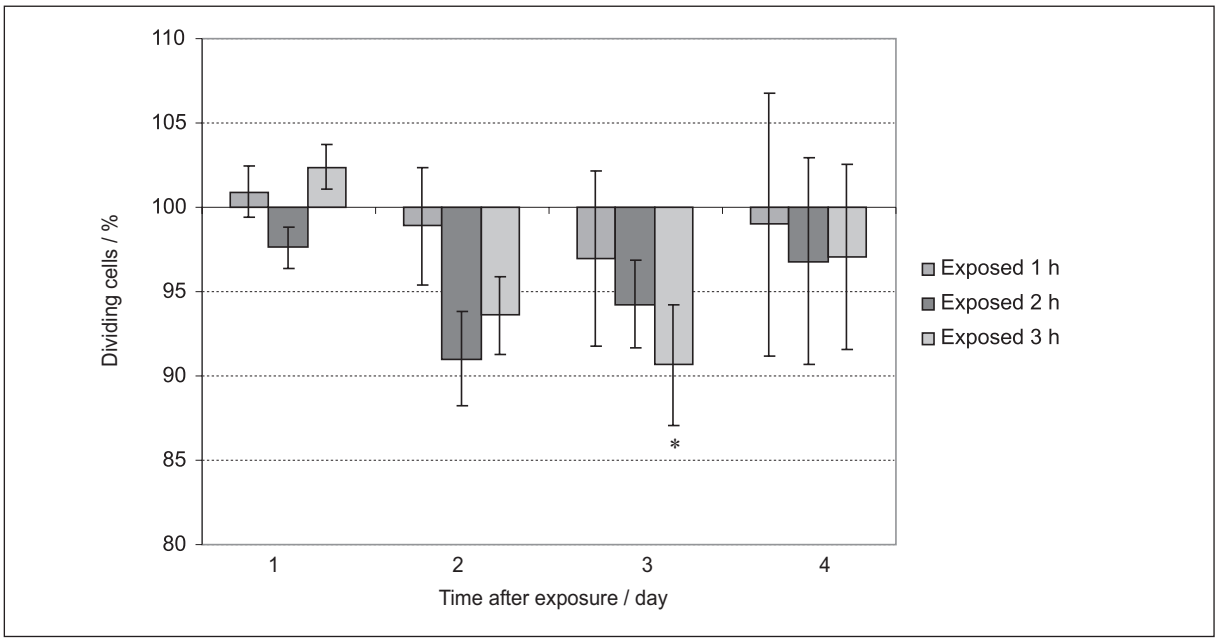

Figure 1 *Cell proliferation after 1 h, 2 h, and 3 h of RF/MW radiation established on each of the four post-irradiation days (The 100 % line corresponds to negative control results)(*p<0.05)*

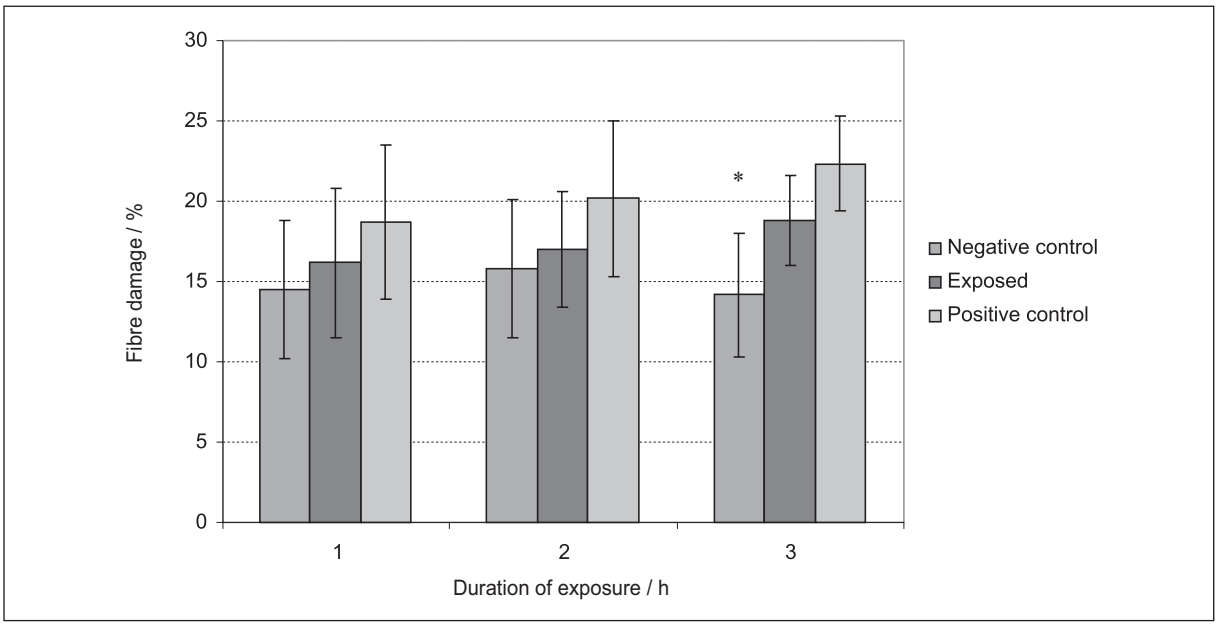

Figure 2 *Microtubule fibre damage in cells exposed to RF/MW radiation for 1 h, 2 h, and 3 h, compared to negative and positive control (colchicine-treated) cells (*p<0.05)*

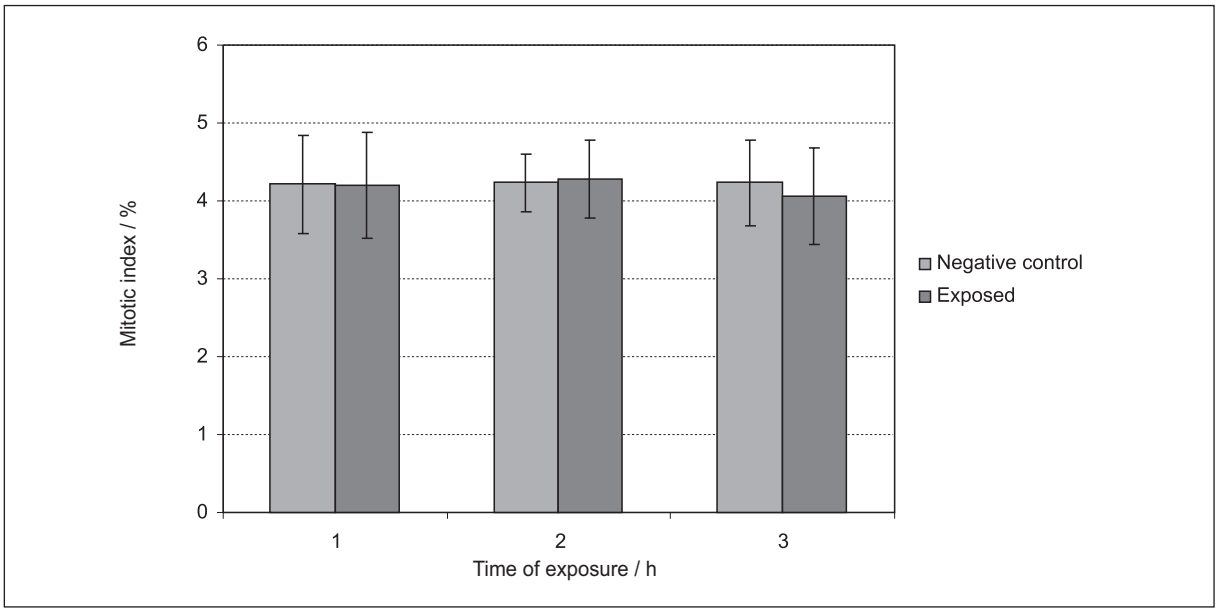

Figure 3 *Mitotic index in V79 cells exposed to RF/MW radiation for 1 h, 2 h, and 3 h, compared to negative control cells*

heating effects, there is this mostly uncharted biological potential of RF/MW non-thermal radiation. In our research, the EM field was generated in a high-quality, certified radiation apparatus, which ensures a stable and uniform field and steady temperature of 37 °C throughout irradiation. The conditions inside the GTEM-cell and low SAR of 0.12 W kg^{-1} made sure that any changes observed in the exposed cells were the consequence of non-thermal RF/MW irradiation.

Seventy-two hours after irradiation, we observed a significant decrease in cell proliferation in cells irradiated for three hours ($p<0.05$) (see Figure 1). These cells also showed impaired microtubule structure immediately after exposure (Figures 2, 4, 5, and 6). Microtubules are structural cell components, and are involved in many cellular processes including mitosis, cytokinesis, and vesicular transport. One of the most important structures involving microtubules is the mitotic spindle in dividing cell (29). The

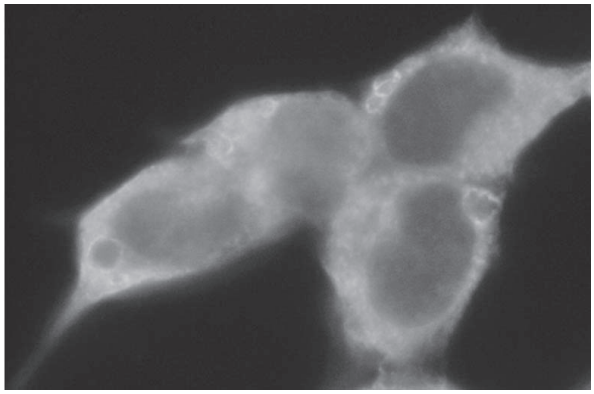

Figure 4 *Microtubule structure in cells irradiated for 3 h*

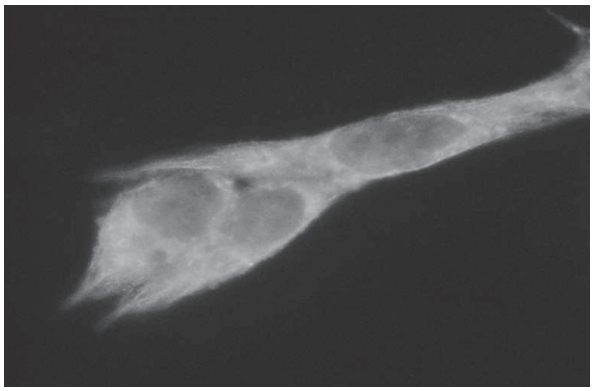

Figure 5 *Microtubule structure in negative control cells*

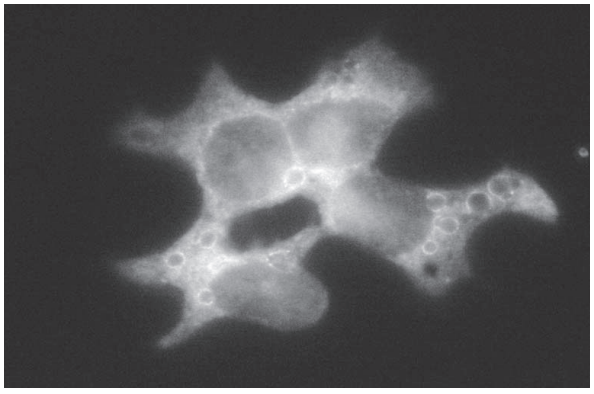

Figure 6 *Microtubule structure in positive control (colhicine-treated) cells*

microtubules are an extremely dynamic structure whose functioning depends on dynamic instability induced by internal electromagnetic forces (30).

It seems that significantly lower proliferation of cells exposed for three hours, found on the third post-irradiation day, is a consequence of the microtubule structure damage observed immediately after RF/MW irradiation.

By calculating the mitotic index, we were able to see how the cells differed in their capability to divide. It turned out that irradiation did not significantly affect the mitotic index (Figure 3).

CONCLUSION

The results obtained in this investigation indicate that cell proliferation kinetics may depend on microtubule impairment observed immediately after exposure to radiation at a mobile phone frequency.

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Sažetak

PROMJENE STANIČNOG RASTA KAO ODGOVOR NA ZRAČENJE FREKVENCIJE MOBILNE TELEFONIJE

Istraživanje je provedeno s namjerom utvrđivanja brzine rasta, strukture citoskeleta i mitotskog indeksa (MI) u stanicama izloženim radiofrekvencijskom/mikrovalnom (RF/MW) zračenju u trajanju od 1 h, 2 h i 3 h. Kultura V79-stanica održavana je u standardnim laboratorijskim uvjetima. Stanice su bile izložene zračenju kontinuiranih valova (CW) frekvencije 935 MHz, jakosti električnog polja od (8,2±0,3) V m^{-1} i prosječne brzine apsorpcije (SAR) od 0,12 W kg^{-1}. Kako bi se odredila kinetika stanične diobe, stanice su za svaki sat izlaganja brojene tijekom četiri dana nakon zračenja. Struktura mikrotubula bila je određena imunocitokemijskom metodologijom. Stanični razmazi bili su analizirani s pomoću fluorescentnog mikroskopa. Negativno i pozitivno kontrolni uzorci stanica bili su uključeni u studiju. MI je određen brojem stanica u diobi dvadeset četiri sata nakon izlaganja. Pad staničnog rasta primijećen je u uzorcima zračenim tri sata, ali sedamdeset i dva sata nakon izlaganja ($p<0,05$). U odnosu na kontrolne uzorke, odmah nakon tri sata izlaganja primijećene su jasne promjene u strukturi mikrotubula ($p<0,05$). MI u stanicama izloženim RF/MW-zračenju nije se razlikovao od MI kontrolnih uzoraka. Čini se da je usporena kinetika diobe stanica posljedica oštećenja mikrotubula izazvanih zračenjem, što nije utjecalo na broj stanica u diobi.

KLJUČNE RIJEČI: *brzina stanične proliferacije, mitotski indeks, RF/MW 935 MHz, struktura mikrotubula, trajna kultura stanica*

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