

CELL NUCLEUS AND MEMBRANE RECOVERY AFTER EXPOSURE TO MICROWAVES

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Cells of human buccal epithelium of six male donors were exposed to microwave radiation (frequency $f = 36.64$ GHz, power density $W = 0.1, 1$ and 4 W/m²). Exposure time was 10 seconds. The state of chromatin in cell nucleus was estimated by a number of heterochromatin granules after staining with 2% orcein in 45 % acetic acid. Permeability of cell membranes was estimated by percentage of unstained cells after 5 min of staining the cells with vital dyes trypan blue (0.5%) and indigocarmine (5 mM). Cell exposure to microwaves induced chromatin condensation (increase of the number of heterochromatin granules) and increase of membrane permeability to trypan blue and indigocarmine. Isolated human buccal cells demonstrated the ability to recover after microwave exposure. The number of heterochromatin granules decreased to its initial level after 0.5 hour ($W = 0.1$ W/m²) and 2 hours ($W = 1$ and 4 W/m²) after cell exposure. Cell plasma membrane permeability recovered later — after 1 hour and 3 hours post exposure, respectively.

Key words: human cell, chromatin, heterochromatin, buccal epithelium, electromagnetic radiation exposure.

INTRODUCTION

The phenomenon of cell recovery after action of different external factors has been thoroughly investigated by D. Nassonov and his scientific school during 1930–1960 (Nassonov, 1930; Насонов и Александров, 1940). At present, cell recovery is being very intensively investigated in regard to mechanisms of DNA repair (Lewin, 2004). Other mechanisms of cell repair have been investigated less intensively. An example of modern study in this area may be the investigation of the effect of thymosin- β 4 treatment on human corneal epithelial cells exposed to ethanol *in vitro*, in which the cells were allowed to recover from ethanol treatment for 24 hours (Sosne *et al.*, 2004).

Detailed investigations of human cell membrane recovery after the influence of different bacterial toxins have been performed by the research team from Johannes Gutenberg University, Mainz, Germany (Walev *et al.*, 1994; Hertle *et al.*, 1999; Husmann *et al.*, 2006). The repair of pores produced in the membrane by bacterial toxins was observed to be inhibited by cycloheximide, which indicated that the repair of membrane pores is dependent on protein synthesis (Hertle *et al.*, 1999).

In the present paper, we study cell nucleus and cell membrane recovery after the influence of low-level electromagnetic cell exposure. Previously we demonstrated that microwaves induce euchromatin–heterochromatin transitions, i.e. an increase of quantity of heterochromatin granules in human cell nuclei (Shckorbatov *et al.*, 1998; Shckorbatov *et al.*, 2009b). Chromatin is a DNA-protein complex in the interphase nucleus that forms chromosomes in the phase of cell division. The main parts of chromatin are euchromatin and heterochromatin. Euchromatin mostly contains a transcriptionally active part of chromatin and heterochromatin is the transcriptionally repressed part of chromatin. Transformation of euchromatin to heterochromatin and *vice versa* is a structural representation of changes in chromatin activity. Condensation of chromatin, the so-called heterochromatinization, is associated with a decrease of functional activity of chromatin (Lewin, 2004). We have also demonstrated that low-level microwave exposure induces an increase of cell membrane permeability to vital dyes (Shckorbatov *et al.*, 2002). Trypan blue is commonly used in cell biology to access viability of cells, as normal living cells do not absorb this dye, and it penetrates only into cells with damaged plasma membrane (Hodgson, 2004). The phenomenon of cell nucleus and cell membrane recovery

after electromagnetic cell exposure has been shown earlier (Shckorbatov *et al.*, 2009a; Pasiuga *et al.*, 2009), but in this paper for the first time we test the dependence of cell recovery on the intensity of microwave exposure.

MATERIALS AND METHODS

Human cells. The studies were conducted using buccal epithelium human cells. The cells were obtained from the inner surface of donor's cheek by light scraping with a blunt sterile spatula. This operation was absolutely bloodless and painless. All the donors were informed about the purpose of investigation. Our investigations were performed in accordance with the European Convention on Human Rights and Biomedicine (1997), Declarations and Recommendations of the First, the Second and the Third National (Ukrainian) Congresses of Bioethics (Kiev, Ukraine, 2001, 2004, 2007) and Ukrainian legislation.

The cells were placed in a 3.03 mM phosphate buffer (pH = 7.0) and 2.89 mM calcium chloride (Reachem, Moscow, Russia) solution, and stored for further experiments. 25 µl of cell suspension containing several thousand of cells were placed on a glass slide and subjected to microwave exposure. Cells were stained immediately after the exposure, 30 minutes, 1 hour, 2 and 3 hours after cell exposure with 2% orcein (Merck AG, Darmstadt, Germany) solution in 45 % acetic acid (Reachem, Moscow, Russia), 5 mM indigocarmine (Merck AG, Darmstadt, Germany) and 0.5% trypan blue (Reachem, Moscow, Russia) solutions. All cell donors were men, non-smokers. Ages of the donors A to F, respectively, were 18, 20, 24, 35, 53, and 55 years. The male donors were selected by the reasons of convenience, as in our previous experiments no gender-related differences between buccal epithelium cells in characteristics of chromatin condensation or membrane permeability was observed. For light microscopy, a microscope MIKMED-6 (var.7) with digital ocular and software produced by LOMO (St.-Peterburg, Russian Federation) was used.

Cell exposure. For electromagnetic cell exposure of frequency $f = 36.64 \pm 0.05$ GHz we applied a semiconductor generator developed at the Department of Theoretical Radiophysics of V.N. Karazin Kharkiv National University. Cell exposure was conducted at room temperature. In all experiments, cell exposure mean power density at the surface of exposed object was $W = 0.1, 1$ and 4 W/m^2 . Intensity of maximal cell sample microwave exposure 4 W/m^2 corresponds to SAR = 16.7 W/kg, according to equation (1):

$$SAR = \frac{\varepsilon_0 \varepsilon'' \omega}{\rho} |E|^2, \quad (1)$$

where ε_0 is the permittivity of free space, ε'' is the expected permittivity of water, ω is the angular frequency, ρ is the density of the irradiated solution, E is the electric field strength (rms) inside the solution obtained by numerical calculation, considering the effect of surrounding objects.

Intensities of cell exposure 0.1, 1, and 4 W/m^2 corresponded to SAR 0.418; 4.18 and 16.7 W/kg . Cell exposure time was 10 seconds. Thus, in the absence of heat exchange and evaporation, the heating of the cell sample under maximal intensity of 16.7 W/kg and exposure time 10 s was lower than $\Delta T \approx 0.04 \text{ K}$.

Chromatin state. We estimated the number of heterochromatin granules by a method described earlier (Shckorbatov, 1999). The suspended cells (25 µl) were placed on a preparation slide and exposed to microwaves. Cells were stained with 2% orcein in 45% acetic acid after cell exposure (30 s, 30 min, 1 hour and 2 hours later). Orcein is a specific dye for heterochromatin (Sanderson and Stewart, 1961). Cells were investigated at magnification 600×. Each cell sample contained several thousands of cells. In each variant of the experiment heterochromatin granule quantity (HGQ) was estimated in 30 cell nuclei and the mean HGQ was calculated. The number of cells (30) examined was determined in our previous experiments to be optimal (Shckorbatov, 1999). The variability of HGQ in a cell sample was determined as the standard error of the mean data (SEM). In our experiments, SEM was less than 5% of the mean HGQ. For each donor replication was three times, using three different cell samples obtained on different days. The heterochromatin granules in orcein stained cells are shown in Figure 1.

State of cell membrane. We applied indigocarmine (Merck AG, Darmstadt, Germany) and trypan blue (Reachem, Moscow, Russia) as cell damage indicators. The method of trypan blue staining has been previously applied for evaluation of cell viability (Chen *et al.*, 2010; Jin *et al.*, 2010; Ko *et al.*, 2010; Yedjou *et al.*, 2010). We assessed cell viability by the method of Nasonov (Насонов и Александров, 1940). The method was performed as follows: the cells were stained with trypan blue (0.5%) and after 30 min the

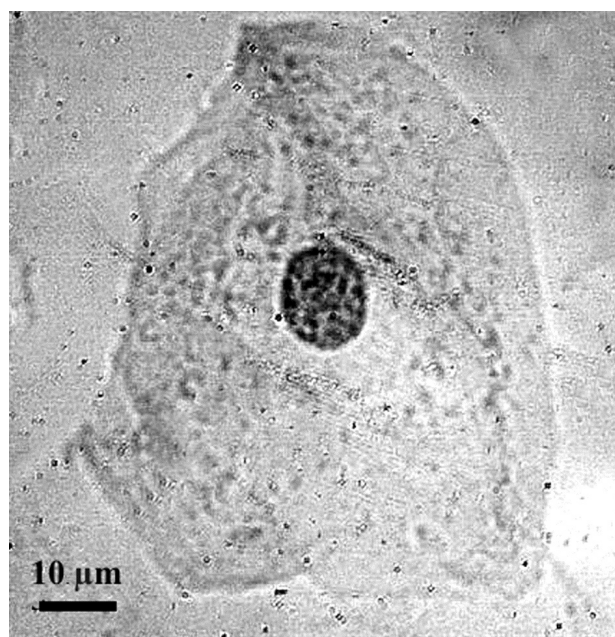


Fig. 1. Human buccal epithelium cell stained with orcein (magnification ×1000)

proportion of unstained cells was determined. Living cells retain ability to concentrate dye and their cytoplasm becomes unstained. Dead cells are stained diffusely. The method of indigocarmine staining has also been applied for this purpose (Shckorbatov *et al.*, 1995). Previously, it has been shown that cell damage induces an increase in the proportion of cells stained with indigocarmine (Shckorbatov *et al.*, 1995). Trypan blue staining can also reflect cell membrane damage. We stained cells at various times (30 s, 30 min, 1 hour, 2 hours and 3 hours) after cell exposure. We determined the percentage of unstained cells after 5 min of staining with 5 mM indigocarmine and 0.5% trypan blue solutions in the buffer solution described above. Buccal epithelium cells after staining with trypan blue are shown in Figure 2. In one experiment we analysed five cell samples exposed to irradiation independently (N1). In each cell sam-

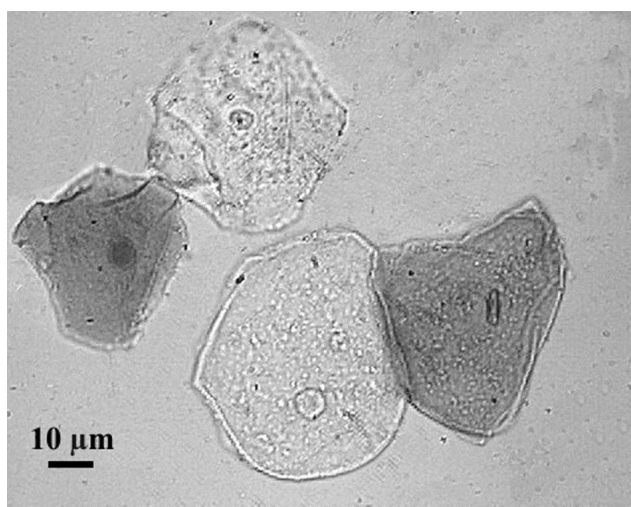


Fig. 2. Human buccal epithelium cells stained with trypan blue (magnification $\times 400$).

ple we analysed 100 cells (N2) and determined the percentage of unstained cells for each cell sample. We calculated the mean number of unstained cells for all five samples and the standard error of this value.

Statistical analysis. Significant differences were determined using the Student's t-test and ANOVA test. The probability level assumed in this paper was $P < 0.05$.

RESULTS

Figure 3 presents data on cell viability. Cells of human buccal epithelium retained their viability *in vitro* for about eight hours.

In our experiments microwave cell exposure induced a significant increase of heterochromatin granule quantity (HGQ) immediately after cell sample exposure. After a specific period of recovery HGQ gradually decreased to the control level, and the time of full recovery depended on the intensity of cell exposure (Fig. 4). In general the reaction of cells of all donors to cell exposure was similar. Immediately after cell exposure HGQ increased, and this increase was

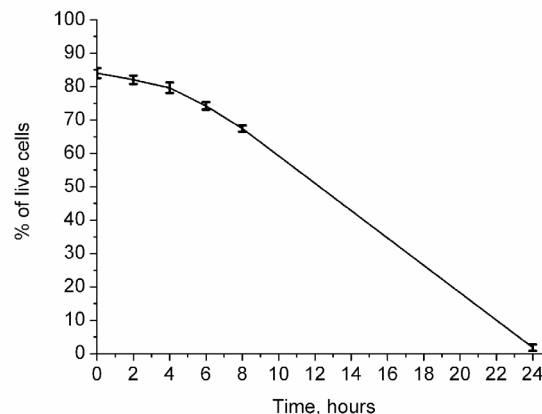


Fig. 3. Viability of human buccal epithelium cells after cell exposure (Donor F).

more pronounced after cell exposure to microwaves of intensity 1 and 4 W/m^2 , than 10^{-1} W/m^2 . After a period of recovery, HGQ decreased to the control level. Time of recovery was 0.5 hour for intensities of 0.1 W/m^2 and two hours if intensity of cell exposure was 1 and 4 W/m^2 . Cells of donor C proved to be more stable to cell exposure at intensity 0.1 W/m^2 ; the HGQ in these cells remained almost unchanged.

In the next series of experiments, we studied changes in the permeability of cell membranes to trypan blue (Fig. 5). In our experiments microwave cell exposure decreased the percentage of unstained cells, and the effect of cell exposure depended on its intensity.

The data presented in Figure 5 indicate that the control level of trypan blue staining differed in cells of different donors: in young donors (A–C) it was less than in elder donors (D–F). This observation agrees with our previous data indicating an increase of cell membrane permeability with age (Shckorbatov *et al.*, 1995). Cells recovered within one hour after cell exposure of intensity 0.1 W/m^2 , compared to three hours after exposure of intensity 1 and 4 W/m^2 .

To further assess cell membrane recovery after microwave exposure, we also studied cell membrane permeability to the vital dye indigocarmine (Fig. 6). At a low concentration this dye also penetrates damaged cells and undamaged cells remain unstained (Shckorbatov *et al.*, 1995). The reaction of cells to indigocarmine was rather similar to that of trypan blue. Cells of elder donors had a higher initial level of staining, and cell exposure induced a decrease of the proportion of unstained cells in cell samples of all donors. After a period of recovery (one hour for intensity of cell exposure 0.1 W/m^2 and three hours for intensity 1 and 4 W/m^2) the proportion of unstained cells was close to the control level.

To determine the influence of different factors, such as irradiation power, time of recovery, individual differences of donors, we applied an ANOVA test (Tables 1–2). Time of recovery (T) and power of irradiation (P) had a significant effect on the state of chromatin and cell membrane. There were some differences in cell reaction to microwave irradiation.

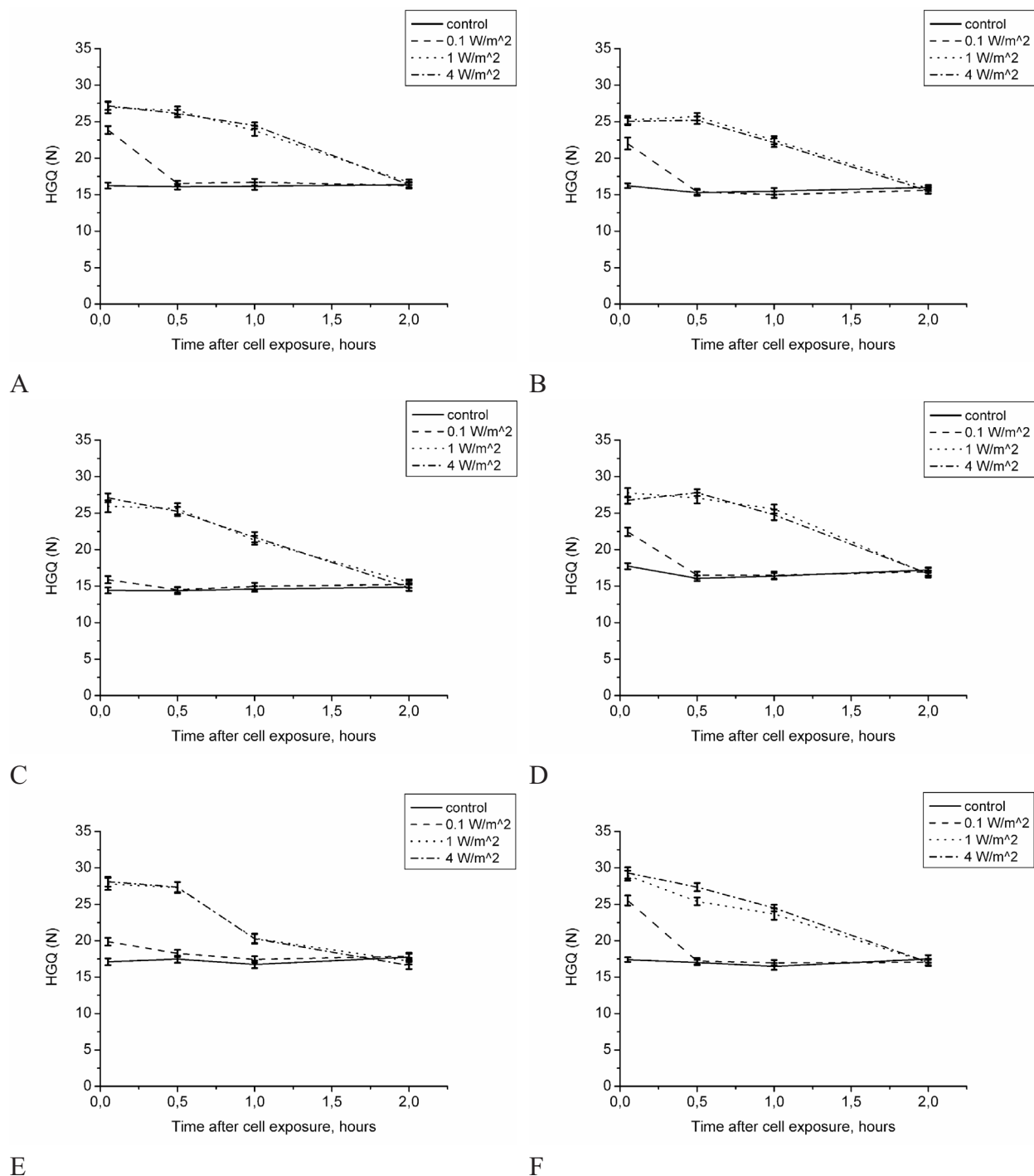


Fig. 4. Changes in heterochromatin granule quantity (HGQ) after microwave cell exposure for different donors (A–F).

tion and cell recovery after treatment. The effect of vital dyes (A) was not significant, indicating that the use of indigocarmine and trypan blue gave the same results.

DISCUSSION

In our previous work we demonstrated condensation of chromatin in granules induced by an electromagnetic field (Shckorbatov *et al.*, 1998; Shckorbatov *et al.*, 2009a; 2009b), but the process of recovery of microwave induced

changes in chromatin microscopic morphology has remained almost uninvestigated. We observed an effect of time after microwave exposure and power density of irradiation on degree of chromatin condensation (Table 1). Thus, the degree of chromatin condensation decreases with time elapsed after irradiation and depends on the energy density of irradiation. The fact that the interaction of these factors is also significant indicates that the process of chromatin recovery after microwave exposure depends on the energy density of irradiation. This process also depends on individual donors.

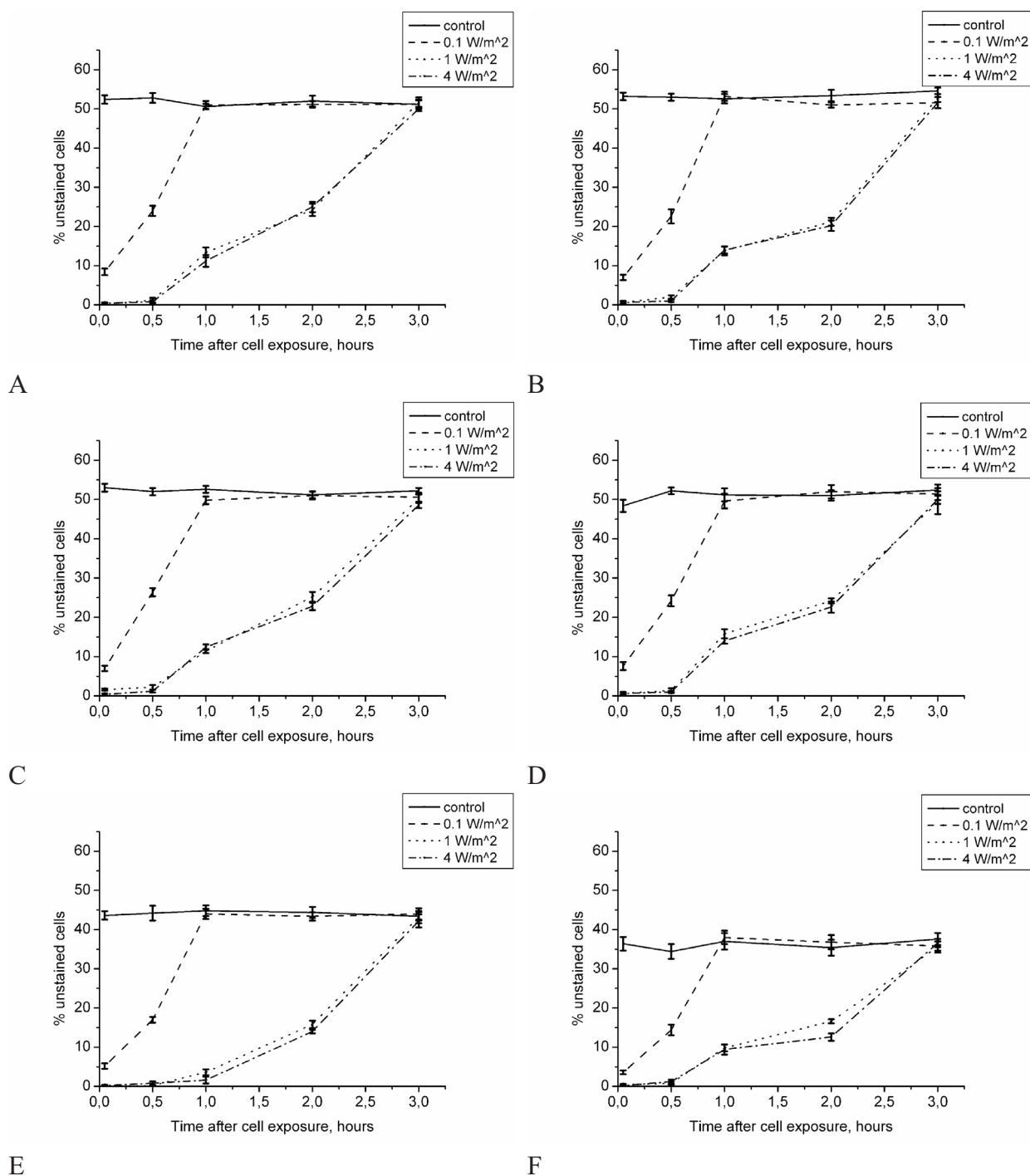


Fig. 5. Changes in cell membrane permeability for trypan blue after microwave cell exposure for different donors (A–F).

Similar results were obtained when using vital dyes trypan blue and indigocarmine, (Table 2), indicating that the effect has common character. Naturally, changes in membrane permeability depended on density of irradiation energy and time elapsed after irradiation. Individual differences in cell reaction of different donors were also significant. The used tests show potential in further studies on the determination of individual resistance of cells of different donors to electromagnetic radiation.

In our opinion, the ability of cell membrane to recover after microwave cell exposure is associated with the general abil-

ity of cell to repair damage produced by external factors. The molecular basis of cell membrane recovery in our experiments remains unknown. We speculate that this process may be associated with rearrangement of lipid molecules in the plasma cell membrane. It is possible that the process of membrane recovery depends on protein synthesis, as shown by Hertle (Hertle *et al.*, 1999). However, further investigations are necessary to reveal the exact mechanism of this process.

In our opinion, the process of cell chromatin condensation induced by microwave radiation is associated with changes

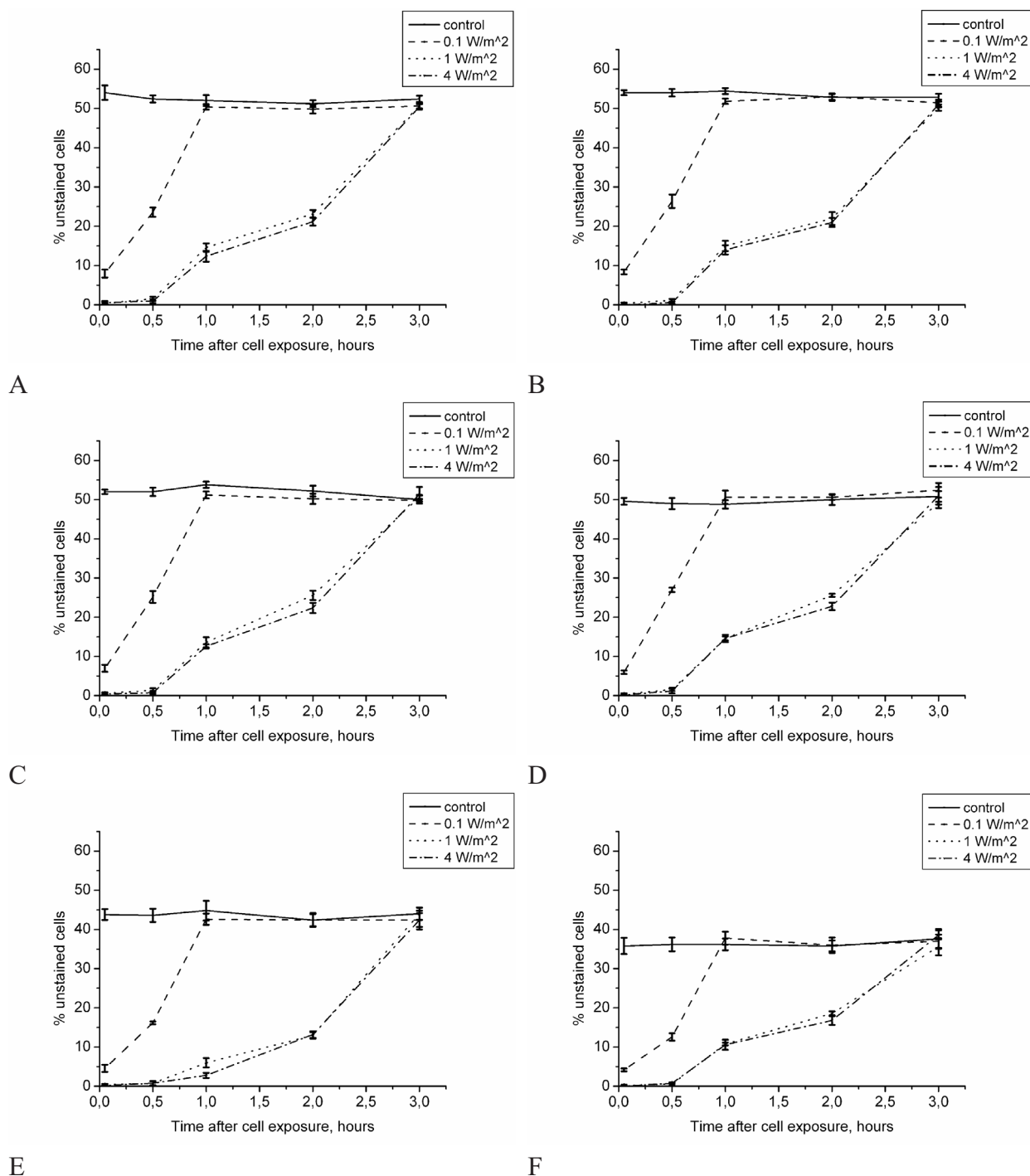


Fig. 6. Changes in cell membrane permeability for indigocarmine after microwave cell exposure for different donors (A–F).

in intracellular calcium concentration. Microwave cell exposure induces an increase in intracellular calcium concentration (Pazur and Rassadina, 2009). An intranuclear increase of calcium concentration has been shown to induce chromatin condensation (Martin and Cardoso, 2010). A direct influence of magnetic field on chromatin structure via concentrating of nucleosome nanoparticles and aggregates (germs) in gradient magnetic fields has also been demonstrated (Norina, 2007).

The observed process of recovery of chromatin structure and membrane integrity showed that chromatin structure re-

newal was faster than membrane re-structuring. After cell exposure at intensity 0.1 W/m² chromatin structure recovered after 0.5 hour and the membranes attained initial state after 1 hour. After cell exposure at intensity 1 and 4 W/m² the nucleus recovered after two hours and membranes after three hours. Thus, nuclear recovery precedes membrane recovery, and possibly nuclear recovery is necessary for membrane recovery.

In conclusion: human cell exposure to a low-level microwave irradiation induced chromatin condensation (increase of the number of heterochromatin granules) and increase of

Table 1

ANOVA TEST FOR THE INFLUENCE OF MICROWAVE IRRADIATION ON THE STATE OF CHROMATIN AND ITS RECOVERY

	F	p
T	1973.2*	0.00
P	3131.9*	0.00
T*P	471.9*	0.00
B	168.2*	0.00
B*T	13.7*	0.00
B*P	9.2*	0.00
B*T*P	10.2*	0.00

T, independent factor, time of recovery: 1 – 30 s, 2 – 30 min, 3 – 1 h, 4 – 2 h, 5 – 3 h; P, independent factor, power of irradiation: 1 – control, 2 – 10^{-6} W/m², 3 – 10^{-5} W/m², 4 – 10^{-4} W/m²; B, dependent factor, individual differences of donors)

Table 2

ANOVA TEST FOR THE INFLUENCE OF MICROWAVE IRRADIATION ON THE CELL MEMBRANE PERMEABILITY AND ITS RECOVERY

	F	p
A	0.9	0.34
T	7440.3*	0.00
P	12434.0*	0.00
A*T	0.9	0.45
A*P	0.7	0.57
T*P	1328.2*	0.00
A*T*P	0.7	0.75
B	441.3*	0.00
B*A	1.0	0.45
B*T	17.7*	0.00
B*P	32.2*	0.00
B*A*T	1.2	0.22
B*A*P	0.8	0.71
B*T*P	4.7*	0.00
B*A*T*P	0.5	0.99

A, independent factor, vital dye: 1 – indigocarmine, 2 – trypan blue; T, independent factor, time of recovery: 1 – 30 sec, 2 – 30 min, 3 – 1 h, 4 – 2 h, 5 – 3 h; P, independent factor, power of irradiation: 1 – control, 2 – 10^{-6} W/m², 3 – 10^{-5} W/m², 4 – 10^{-4} W/m²; B, dependent factor, individual differences of donors

membrane permeability to vital dyes. These changes proved to be transitory, and the number of heterochromatin granules decreased to the initial level after 0.5 hour and two hours, depending on cell exposure intensity. The membrane permeability at the same exposure levels recovered later, after one hour and three hours.

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ŠŪNU KODOLU UN MEMBRĀNU ATJAUNOŠANĀS PĒC APSTRĀDES AR MIKROVIĻŅIEM

Eksperimentāli noteikta vīriešu epitēlija šūnu atjaunošanās dinamika atkarībā no mikroviļņu apstarošanas devas.