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Microscopic observation of living cells during their exposure to modulated electromagnetic fields

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Abstract

Studying cell behaviour under irradiation with radiofrequency electromagnetic fields (RF-EMF) is often impeded by the difficulty to monitor cell characteristics during irradiation. Here we report the design and the application of a complete device for continuous microscopic observation of cells exposed to modulated EMF similar to mobile phones signals. The system allows the follow up of cell progression into mitosis under controlled temperature and CO_2 environment. Protocols are proposed in which the same cells are the controls before and after the EMF exposure and we demonstrate the interest of the "before exposure" controls. The exposure system was validated by cell endocytosis measurements. While the endocytosis rate was increased, no alteration of mitosis progression and mitosis duration was observed in cells exposed to 900 MHz modulated EMF for 1 h, at 30 °C and at a Specific Absorption Rate of 2.2 W/kg.

Keywords: Mitosis; Fluid phase endocytosis; Phase contrast microscopy; TEM cell; GSM-EMF

1. Introduction

Many studies have explored the potential risks for human health linked to the use of communication technologies based on radiofrequency electromagnetic fields (RF-EMF). In the particular case of mobile phones EMF, in vitro studies have been performed on different cellular models to complement the epidemiological and in vivo studies. Meltz [1] summarized the results achieved reporting no significant genotoxicity and cell transformation induced by mammalian cell exposure to low intensity RF-EMF. He criticized the lack of rigorous dosimetry and Specific Absorption Rate (SAR) calculation, of adequate temperature control or of precise exposure protocols description in the scientific literature [1]. Since then, other studies have confirmed the absence of significant in vitro genotoxicity covering the frequency range of 800–2450 MHz [2–4] as well as the absence of apoptosis induction [5–7]. Nevertheless few reports suggested a potentiation of apoptosis suggesting that cell sensitivity to RF-EMF may be different according to the cell type [8–10]. Other studies on gene expression reported an overexpression of 38 proteins, including 2 cytoskeletal proteins [11]. Cell cycle analysis by flow cytometry and BrdU incorporation studies rather reported the absence of frequency-modulated EMF effects on the cell cycle progression of cells exposed to various SARs [12–14].

We have previously reported that the cells exposure to 900 MHz modulated EMF similar to the GSM mobile phones signals (GSM-EMF) provokes an increase of the fluid phase endocytosis. The cells were exposed for 20 min to 3.2 W/kg SAR, at 29±0.5 °C in a wire patch cell matched to the frequency of 900 MHz [15] and of Moisescu et al.(submitted). Our previous

Abbreviations: EMF, electromagnetic field; GSM, Global System for Mobile communication; RF, radiofrequency; SAR, Specific Absorption Rate; TEM cell, Transverse Electric Magnetic cell; LY, Lucifer Yellow; WPC, wire patch cell.

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microscopic observations performed during endocytosis experiments suggested that an increased number of prometaphase–metaphase round cells was present at the end of the GSM-EMF exposure comparatively to controls. In an attempt to understand the stimulation mechanism of the fluid phase endocytosis, we designed an experimental set-up to study whether or not the GSM-EMF might affect the cell mitosis too. Indeed, the changes in, for example, the cell membrane tension during endocytosis and mitosis are well documented [16,17].

Thus, a continuous follow up during the whole mitosis seemed necessary to detect not only if mitosis duration could be affected by the GSM-EMF, but also whether a particular phase of the mitosis could be either accelerated or slowed down. Other laboratories studies have been mainly focused on genotoxicity, apoptosis, cell cycle progression, but not on mitosis progression and mitosis duration during and after the exposure to the modulated RF-EMF. To perform this type of analysis, a Transverse Electric Magnetic (TEM) exposure cell with holes was developed to allow the cell microscopic visualization during 1 h GSM-EMF exposure of 2.2 W/kg SAR, at 30 °C, in culture medium not supplemented with Foetal Bovine Serum and antibiotics. The TEM cell was placed inside an incubator system adapted to an inverted microscope. Therefore, the temperature, CO_2 and humidity conditions were controlled for several hours. Consequently the same cells could be followed for hours and the cell progression through different mitosis phases could be observed in detail. The experimental set-up was validated by fluid phase endocytosis measurements under GSM-EMF exposure.

2. Materials and methods

2.1. Cell culture

Metastatic murine B16F10 melanoma cells were cultured in 5% CO_2 humid atmosphere at 37 °C, in Minimum Essential Medium with Earle's Salts w/1 mM L-Glutamine (MEM) supplemented with 10% Foetal Bovine Serum, and 1% antibiotic mixture (10 000 UI/ml Penicillin G sodium, 10 000 μg/ml Streptomycin sulphate in 0.85% saline) (MEMs). For endocytosis experiments, 24 h before, $5–7\times10^5$ cells were seeded per 35 mm Petri dish (TPP, Switzerland) in order to obtain a homogeneously distributed cell monolayer in exponential growth. For mitosis experiments, $1–2\times10^5$ cells were seeded per dish to get half-filled cellular monolayer where the morphological aspects typical to various mitosis phases could be easily visualised. Cell culture products were from Gibco-Invitrogen, UK.

2.2. Fluid phase endocytosis determination

Cells were incubated for 30 min, at 30±0.5 °C with 3.5 ml of 2 mM Lucifer Yellow (LY) in MEM and simultaneously exposed or not to GSM-EMF. Then, cells were washed three times with 3 ml of 0.9% NaCl, trypsinized for 1 min, harvested in 3 ml phosphate buffer, centrifuged (200 g, 10 min, room temperature) and the cellular pellets discarded in 1 ml lysis buffer (Promega Lysis Reagent kit). Lysate fluorescence was measured at 423 nm excitation/525 nm emission wavelengths, using a spectrofluorometer (SFM 25, Kontron Instruments). The baseline was obtained using LY untreated cells. Total protein dosage was performed on 20 μl of cell lysis (MicroBCA Protein Assay kit, Pierce) to normalise the measured fluorescence intensity. Lucifer Yellow dipotassium salt and phosphate buffer were from Sigma-Aldrich, Germany.

2.3. EMF exposure system

EMF exposures were performed into a Transverse Electric Magnetic cell (TEM) mounted in a specially designed holding frame of an inverted microscope (AxioVert S100, Zeiss, Germany). The TEM cell was placed inside an incubator chamber covering the microscope stage to control the culture conditions during exposure (Fig. 1).

To allow living cells follow up under the microscope, holes were perforated into the three metal plates of the TEM cell (Fig. 2).The 35 mm Petri dish with attached cells in culture was centered above the lower plate hole.

A RFS 900-60 generator (RFPA, Bordeaux, France) was used to deliver 900 MHz modulated signals similar to the GSM mobile phones signals (GSM-EMF). Its amplitude was modulated by rectangular pulses (pulse width 576 μs) with a repetition of 217 Hz and a duty cycle of 1:8. The generator could deliver powers ranging from 0 to 8 W (mean incident powers, corresponding to 0–64 W peak incident powers). A powermeter (E4417A, Agilent, USA) connected to a bidirectional coupler (BC J000 30 N, Universal Microwave Components Corp., USA) through two power sensors

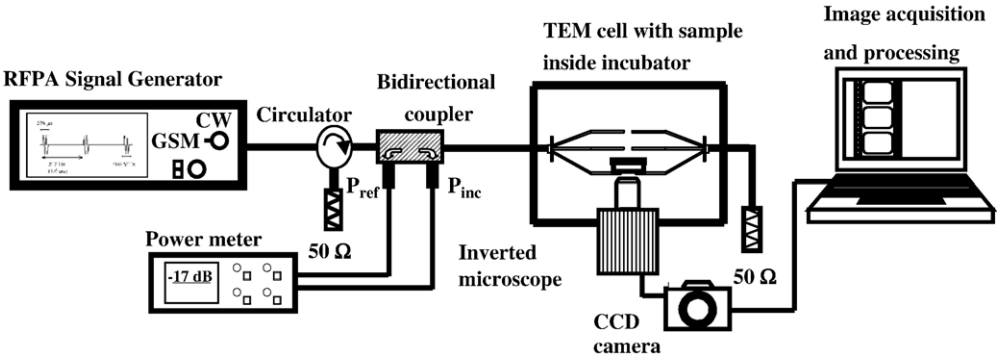

Fig. 1. Experimental set-up drawing. P_{inc}/P_{ref} = incident/reflected power.

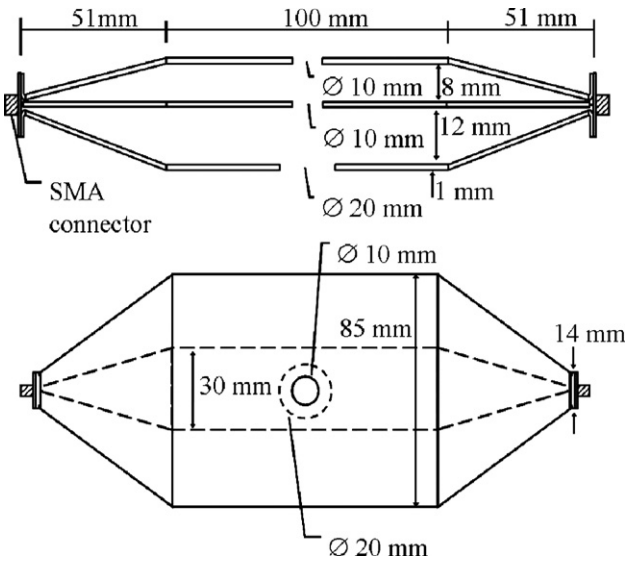

Fig. 2. Schematic drawings of TEM cell, upper and lateral views.

(E4412A, Agilent) allowed the simultaneous measurement of incident and reflected powers during exposure (Fig. 2). The good 900 MHz matching ($S_{11} < -17$ dB) of the TEM cell was monitored on the powermeter.

For endocytosis experiments, the cells attached to Petri dishes were subjected to either GSM-EMF or sham exposure, under identical experimental conditions. For mitosis experiments, the same cell population served as control "before exposure" and control "after exposure".

2.4. SAR evaluation

The Specific Absorption Rate is defined as

$$\mathrm{SAR} = \sigma E^2/\rho, \quad (1)$$

where σ is the conductivity of the radiated system, ρ the mass density, here taken as 1 g/L, and E is the local field at the molecular sites where the radiation energy absorption process takes place, say water molecules bound at molecular interfaces, including those in catalytic centers of proton transfers [18]. The Specific Absorption Rate (SAR) assessments have been performed solving the Maxwell's equations (FDTD method) with a 0.1 mm spatial resolution. The characteristic values of the medium were a relative permittivity ε_r of 75, a conductivity σ of 1.88 S/m (experimentally measured at 30 °C) and a medium density ρ of 1000 kg/m^3. The geometry of the Petri dish, including the thickness of the plastic, as well as the presence of the microscope objective under the dish, was taken into account.

2.5. Temperature measurements for EMF exposure

The TEM cell was placed inside the incubator chamber of the microscope at least 2 h before the beginning of the experiments. This incubator device included plexiglass chamber, heated microscope stage and temperature/CO_2 digital controllers (Temp-control 37-2 and CTI Controller 3700, Zeiss). The controllers were adjusted 0.5 °C above the 30 °C experiment temperature to compensate the heat loss. The bulk increase of the temperature in the sample dishes was measured (i) immediately before and after either real or sham exposures, using a fast digital thermometer P600 with thin thermocouple probe (Dostmann Electronic GmbH, Germany), or (ii) during the exposure, using an optical fiber thermometer (Luxtron One, Luxtron, USA), that is immune to the EMF. The temperature variations were never larger than 1 °C in either real or sham exposure. Even though endocytosis is known as a temperature sensitive process, the 1 °C variation resulted in no or insignificantly small variations of endocytosis rate (Moisescu et al — submitted).

Before the GSM-EMF or sham exposures, the cell culture medium (MEMs) was changed with incubation medium (MEM w/wo LY) under a hood, at room temperature. To prevent the sample temperature decrease due to this manipulation, the incubation media were heated around 5 °C above the 30 °C and hence a fast temperature equilibration was achieved.

2.6. Evaluation of mitosis phases

Mitosis follow up images were acquired using a cooled CCD camera (AxioCam HRc, Zeiss) and processed with AxioVision 4.3 software (Zeiss). Images of 50–100 ms exposure time were acquired in time lapse mode, 1 image/minute, for 3 h or 5 h, depending on the experiment. The images were taken with a small ×5 objective to cover large optical fields with sufficiently numerous cells. In a very slow speed movie mode, the cells progressing into mitosis (generally 100–200 mitosis events) were identified and marked all over the frames Then, under digital magnification (190–220%) every cell mitosis was analyzed frame by frame regarding the morphological modifications of mother and daughter cells. When morphological aspects were indicating the passage from one mitosis phase to the next one, the frames' numbers were recorded and thus, the duration of every phase could be calculated.

2.7. Statistics

For endocytosis experiments the fluorescence of each sample was first referred to the protein content of the sample. Baseline of fluorescent unexposed cells (cell autofluorescence) was then

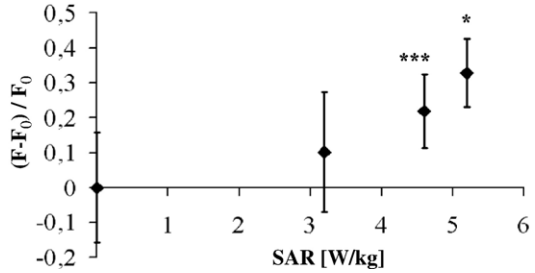

Fig. 3. Relative changes in fluorescence ($\Delta F/F$)± SD for different SAR (W/kg) exposures. B16F10 intracellular fluorescence was measured after 30 min of incubation with 2 mM LY, at 30 °C, under sham or GSM-EMF exposures into the TEM with holes. Statistical significance was determined using unpaired t-test: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

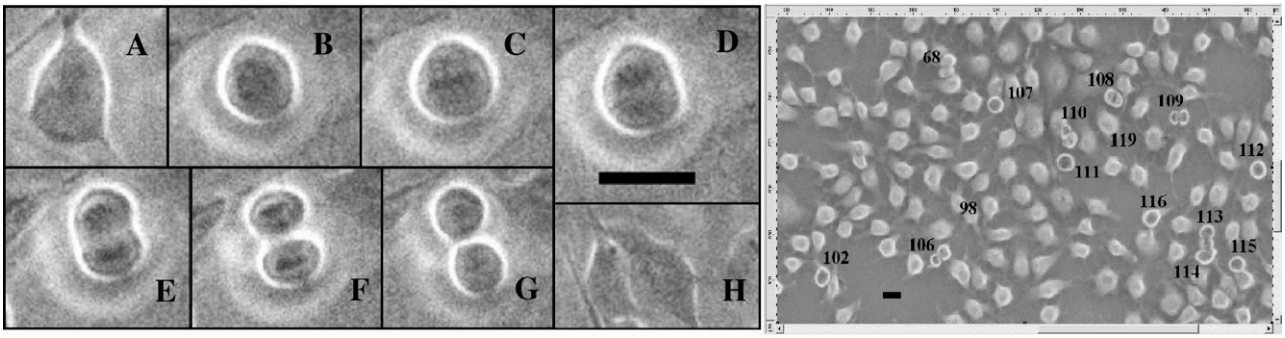

Fig. 4. Left image: progression of cell morphological appearances in different mitotic phases (light microscopy, phase contrast objective (×10), scale bar=20 μm): A) beginning of Prophase: cellular projections start to retract and the cell rounds off; B) beginning of Prometaphase: the cell is round and the genetic material condenses in visible chromosomes; C) Metaphase: the chromosomes align in the middle line referred as the metaphase plate; D) beginning of Anaphase: the cell starts to elongate while the paired chromosomes separate and move to opposite sides of the cell; E) beginning of Telophase: the partitioning of the cell starts thanks to the cytokinesis ring that visibly starts to pinch off the cell into two daughter cells; F) end of Telophase: the cytokinesis is complete and the doublet cells are two smaller separate round cells with visible genetic material; G) beginning of Interphase: the two daughter cells are separated, start to flatten and the chromosomes disperse; H) Interphase: the daughter cells are adherent to the plastic and present cytoplasmic projections. Right image: part of a working screen with cells in different mitotic phases; the numbers mark the cells that have been, are or will be in mitosis at some time within the 5 h of image acquisition (scale bar=20 μm).

subtracted. Samples values were calculated as percentage of the average value of controls performed during the same day with the same batch of cells. They are reported as average values± standard deviation (SD). Statistical significance of differences between data groups was assessed using unpaired Student's *t*-test (NS = not significant, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$).

3. Results

3.1. Validation of experimental set-up for GSM-EMF exposure

The stimulation of LY uptake by GSM-EMF was used to validate the experimental TEM cell with holes installed on the

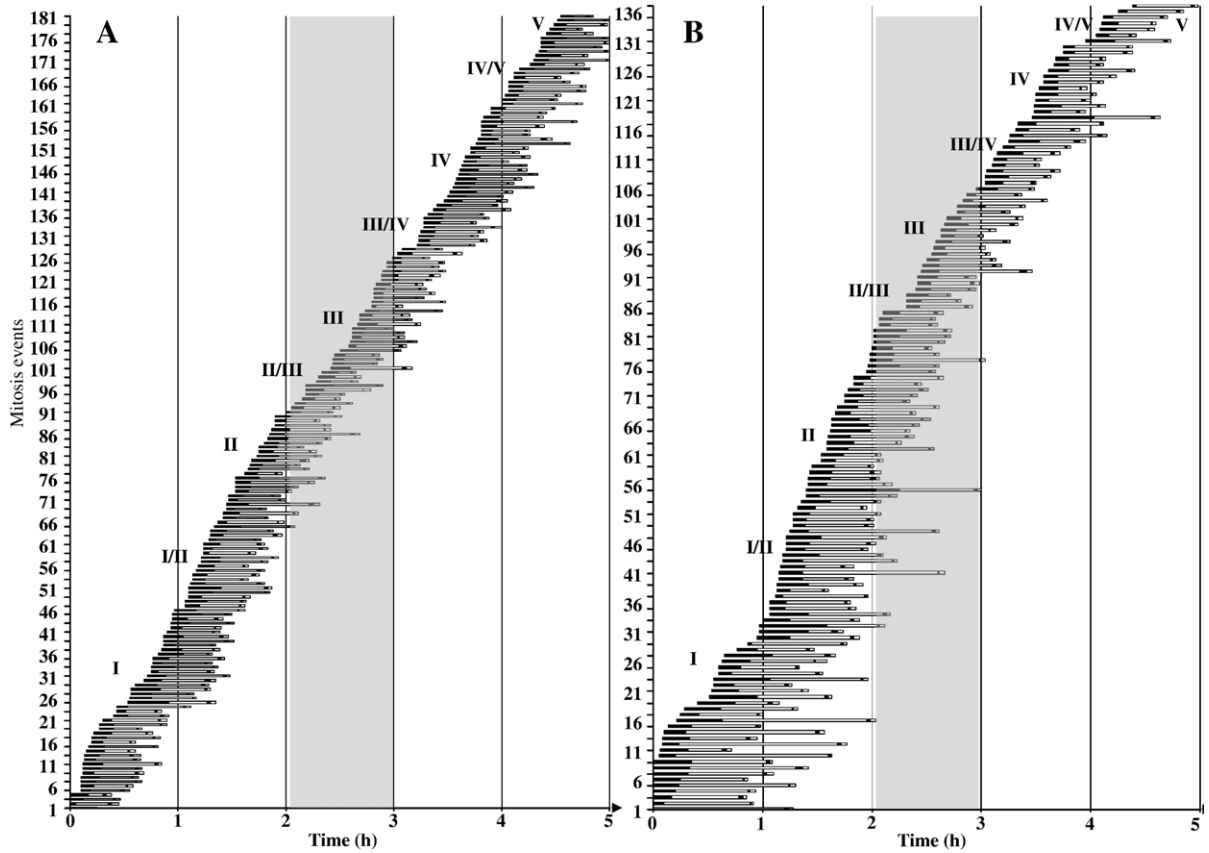

Fig. 5. Sequence of mitosis events during two records of 5 h long acquisitions. The cells were exposed for 1 h to 2.2 W/kg SAR in 3.5 ml MEM, at 30 °C during the 3rd hour of incubation (grey area). Every cell mitosis is presented as a bar with 4 segments proportional to the 4 mitosis phases duration: ▬▭▬▭, where the closed/open segments stand consecutively for Prophase (P), Prometaphase+Metaphase (M), the Anaphase (A) and the Telophase (T). Symbols I, II, III, etc. stand for mitotic events that take place strictly during the 1st, 2nd, 3rd, etc. hour of image acquisition, while the I/II, II/III, etc. stand for mitotic events that straddle the limit between the 1st and 2nd hour, the 2nd and 3rd hour, etc.

microscope stage. The B16F10 cells were incubated for 30 min with 3.5 ml of 2 mM LY in MEM. For 4.2±4.15 and 4.7±4.65 W/kg average SAR (±SD) values calculated at the bottom of the Petri dishes (corresponding respectively to 5 and 5.6 W incident), the cells displayed LY uptake increases of 22% and 33% respectively, statistically significant with respect to the control cells (Fig. 3).

3.2. Follow up of mitosis phases

During the mitosis follow up experiments, the cells were incubated with 3.5 ml MEM, at 30 °C in humid 5% CO_2. Fig. 4 illustrates the cellular morphological aspects that were used to define the following mitosis phases: P = Prophase (Fig. 4A), M = Prometaphase (Fig. 4B) +Metaphase (Fig. 4C), A = Anaphase (Fig. 4D) and T = Telophase (Fig. 4E and F). The duration of every phase and the total mitosis duration were measured.

The duration of the phases before the beginning of the chromosomes separation and of the phases after this point, were compared and reported respectively as pre-division phase and post-division phase. The pre-division phase duration was determined by the number of frames between the beginning of the prometaphase and the beginning of the anaphase (Fig. 4, B and D). Similarly the post-division phase duration was considered between the beginning of the anaphase and the end of the telophase (Fig. 4, D and F). Then, the ratio of the pre-division to the post-division duration was calculated separately for every cell. The averages of these ratios were made for the following time domains: I, I/II, II, II/III, III, III/IV, IV, IV/V and V, where I, II, III, etc. domains cover the mitotic events that took place strictly during the 1st, 2nd, 3rd, etc. hour of image acquisition, while the I/II, II/III, etc. domains cover the mitotic events that straddled the limit between the 1st and 2nd hour, the 2nd and 3rd hour, etc. For each recorded mitosis the duration of every P, M, A, T mitosis phase is reported in proportional piled up bars (P and A — closed bars, M and T — open bars). The ratios of average duration of the pre-/post-division phases are reported as histograms.

3.3. Evaluation of mitosis duration and progression

The cells were exposed to 2.2 W/kg SAR, for 1 h at the beginning or in the middle of image acquisition (local SAR corresponding to 5 W incident, calculated at the bottom of the Petri dish center). Following exposure protocols were used: (i) 1 h GSM exposure+2 h control after exposure (1 h GSM+2 h C) and (ii) 2 h control before exposure+1 h GSM exposure+2 h control after exposure (2 h C+1 h GSM+2 h C).

Fig. 5A is an example of a detailed presentation of all individual mitosis events recorded during a typical experiment under the protocol 2 h C+1 h GSM+2 h C. The cells were exposed to 2.2 W/kg SAR for 1 h in the middle of 5 h image acquisition (grey area). No significant perturbation of total mitosis duration (P+M+A+T) or every mitosis phase duration was found during the 1 h GSM-EMF exposure. These values were similar to those measured during the 2 h of control "before exposure" and 2 h of control "after exposure". The average mitosis duration of all the cells that progressed into mitosis during the 5 h of image acquisition was 33±8 min.

Fig. 5B presents another complete 5 h long mitosis follow up. This experiment interest consists in "before exposure" mitosis (time domains I, I/II and II) longer than exposed mitosis (time domains III) or "after exposure" ones (time domains IV, IV/V and V) mainly because of the extension of the prometaphase+metaphase segment duration. A careful a posteriori analysis of the experimental conditions revealed that the incubation medium had been transferred from a bottle of medium opened 3 weeks earlier, with a basic pH (pH=7.8–8). Fig. 5A experiment with no variable mitosis duration during the all image acquisition, was performed using an incubation medium transferred from a freshly open bottle, with equilibrated chemical characteristics. In ulterior experiments made on purpose, we verified that the pH returns to 7.4 after 2 1/2 h under the 5% CO_2 atmosphere of the microscope incubator. But even in Fig. 5B cases, once the incubation conditions are stabilized, the mitosis duration as well as the duration of mitosis different phases were similar during both the exposure and the "after exposure" control. The average mitosis duration of the cells that

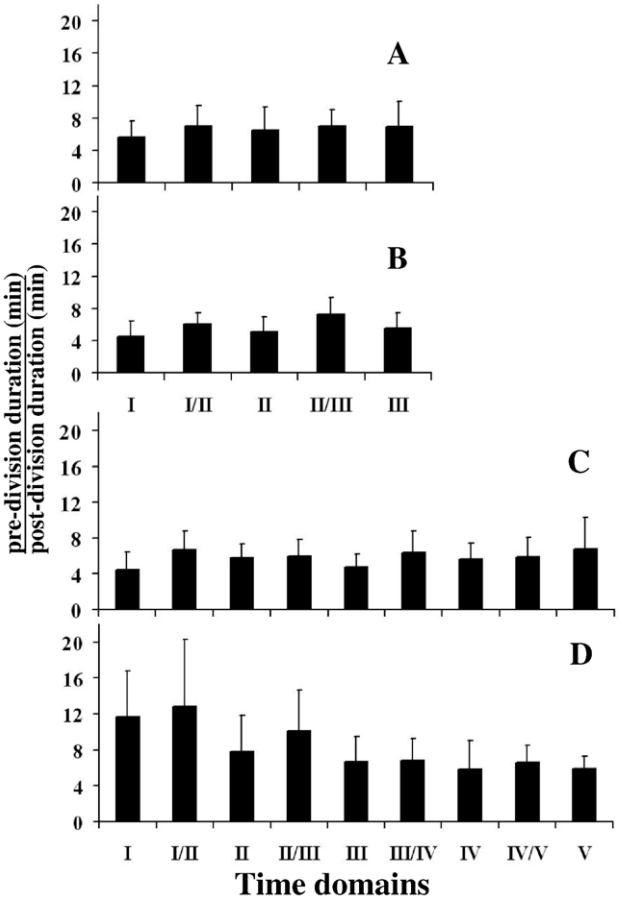

Fig. 6. Duration ratio of the pre-/post-division phases during consecutive time domains. Panel A: 3 h control, without any exposure, Panel B: 1 h GSM+2 h after exposure control performed with the same batch of cells as in Panel A, Panels C and D: 2 h control before+1 h GSM exposure+2 h control after exposure. The cells were exposed to GSM for 1 h to 2.2 W/kg SAR, at 30 °C, in 3.5 ml MEM during the 1st hour of image acquisition (domain I — Panel B) and 3rd hour (domain III — Panels C and D).

progress into mitosis during the first 2 h was 56±18 min. Then, during the last 3 h, it decreased to 38±9 min.

Actually, the first protocol employed 1 h GSM+2 h C did not include a control image acquisition before the GSM-EMF exposure period (Fig. 6B). The same day, under the same conditions, less the GSM exposure, a 3 h control was performed (Fig. 6A). The ratio of the pre-division phase duration to the post-division phase one was similar all over the time domains, with or without GSM-EMF exposure. The average mitosis duration of the cells that progressed into mitosis during the 3 h of image acquisition was 32±8 min for the 1 h GSM+2 h C experiment and 34±7 min for the 3 h control experiment.

Because nevertheless, some variations were observed at the beginning of the incubations, the adopted protocols were those when the cells are being followed before the GSM-EMF exposures as well (the 2 h C+1 h GSM+2 h C protocol). The corresponding analysis of Fig. 5A case revealed no variation, under optimal conditions, of the pre-division phase duration relative to the post-division phase before, during, after exposure, as well as for the mitosis overlapping the periods with and without GSM-EMF (Fig. 6C). The fluctuations of the mitosis duration, if detectable, are more the consequence of medium parameters disequilibrium, rather than a GSM-EMF effect. The corresponding analysis of Fig. 5B case is presented in Fig. 6D.

4. Discussion

In summary, we can conclude that 1) the 900 MHz GSM-EMF signals of 2.2 W/kg SAR applied for 1 h do not affect either the mitosis progression or the mitosis duration in our experimental conditions, 2) the incubation parameters (for example the incubation medium pH) may actually affect the mitosis progression and duration, and 3) our exposure chamber allows to circumvent perturbations of the initial experimental conditions, to correct them (if necessary) and consequently to get rigorously controlled observations. The protocols including 2 h observation before and after the GSM-EMF exposure are not only more complete but also more suitable to obtain reliable data.

We highlight that a new exposure system consisting in a TEM cell with holes for microscopic observation was developed for living cells studies during the GSM-EMF exposure. The system includes the incubation parameters control during microscope observation. Consequently the same cells could be followed for hours, allowing the detailed observation of cell progression through different mitosis phases.

Concerning the cells exposure to GSM-EMF, the experimental set-up was validated by LY uptake measurements under GSM exposure. Indeed the fluid phase endocytosis rate was increased by 30 min exposures to 4.2±4.15 and 4.7±4.65 W/kg average SAR (±SD) values at the bottom of the Petri dishes, as we previously reported using another exposure system [15], (Moisescu et al. — submitted). In these previous reports a wire patch cell (WPC) was mounted into a usual cell incubator and the LY endocytosis was increased under 1.8–4.5 W/kg SAR exposures. The WPC was designed to get a homogeneous SAR distribution [19], while the present TEM cell with holes designed for microscopic observation, not for a homogenous SAR distribution inside the Petri dish. Despite this in present experiments the endocytosis was actually stimulated, validating the use of the TEM cell for mitosis microscopic observations and validating the numerical calculations of the SAR. Calculated SAR at the center of the Petri dish bottom (the actual location of the cells observed under the microscope was 2.2 W/kg, value in the SAR range values that actually stimulate the fluid phase endocytosis in the WPC [15].

In the experiments here reported the cells were incubated at 30 °C with culture medium not supplemented with FBS and antibiotics in order to respect the conditions of endocytosis stimulation. Indeed, the goal of the experiments here reported was to determine whether the GSM-EMF that perturb the endocytosis could also perturb the mitosis progression or duration. In another respect, the phases used here to describe the mitosis progression are the conventional ones. The duration of these phases was based on morphological criteria and we considered that enough precision was achieved with a time lapse recording procedure of 1 image/minute.

We found that the sequence of mitotic events, the mitosis total duration and the duration of each of the mitosis phases were not modified by the cells exposure for 1 h to 2.2 W/kg SAR. Moreover, we compared the duration of the phases before the beginning of the chromosomes separation and after this point, which we reported respectively as pre- and post-division phases. No change in the duration ratio of the pre-/post-division phases was observed under any of the experimental protocols. This result suggests that the GSM-EMF signals do not accelerate nor retard the beginning of the cytokinesis. Indeed, effects of the modulated RF-EMF on linear polymers (like the spindle pole body microtubules) or on the DNA itself could affect the start of the movement of the chromosomes at the limit metaphase/anaphase. A potential effect on mitosis progression could not cause prolonged total mitosis duration because compensatory mechanisms could accelerate the post-division phases. Thus, it was important to check every phase duration independently of the mitosis total duration.

Finally, here we report an improved experimental set-up which allows the continuous microscope cell observation during their exposure to GSM-EMF under well controlled conditions. The system described in this manuscript also offers the possibility to monitor fluorescent changes in living cells exposed to GSM-EMF. The possibility to have the best of the controls, namely the same cells before and after the cells exposure, is one of the most attractive features of this experimental system. In particular, we recommend a 2 h "before the exposure" control during which the cells will overcome the normal manipulation stress and reliable data on GSM-EMF effects only are obtained.

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