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Interactions between radiofrequencies signals and living organisms

Evaluation of the co-genotoxic effects of 1800 MHz GSM radiofrequency exposure and a chemical mutagen in cultured human cells

Évaluation des effets co-génotoxiques d'une exposition radiofréquence à 1800 MHz de type GSM et d'un agent mutagène dans des cellules humaines en culture

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ABSTRACT

We investigated the effect of a 1800 MHz radiofrequency GSM signal combined with a known chemical mutagen (4-nitroquinoline-N-oxide: 4NQO) on human THP1 cells. Comet and γ-H2AX assays were used to assess DNA damage. No heating of the cell cultures was noted during exposure (2 h). The exposure of cells to electromagnetic fields with SARs of 2 to 16 W/kg did not increase the DNA damage induced by 4NQO, whereas the number of DNA strand breaks increased with a temperature increase of at least 4 °C. In conclusion, no co-genotoxic effect of radiofrequency was found at levels of exposure that did not induce heating.

RÉSUMÉ

Nous avons étudié l'effet combiné des radiofréquences à 1800 MHz (GSM) et d'un agent mutagène connu (4-nitroquinoline-N-oxide: 4NQO) sur des cellules THP1 humaines. L'état de l'ADN a été examiné avec les tests des comètes et γ-H2AX. Les mesures de température indiquent une absence d'échauffement des cultures pendant l'exposition (2 h). Les résultats montrent que les dommages induits par le 4NQO restent inchangés sous l'effet des radiofréquences pour des SAR de 2 à 16 W/kg alors qu'une élévation de température de 4 °C induit une augmentation de ces cassures d'ADN. En conclusion, aucun effet co-génotoxique n'est observé à des niveaux non thermiques.

1. Introduction

The rapid spread of wireless communication, including mobile telephones in particular, throughout the world in the last 10 years has given rise to numerous projects in different areas of research. These have contributed to a better knowledge of the interactions between electromagnetic fields and living matter. The final objective of these studies is often to check if

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current guidelines are sufficient to ensure the safety of the population when exposed to the corresponding radiofrequency (RF) fields. The extrapolation of in vitro observations to in vivo situations is risky, but in vitro studies have proved useful for elucidating mechanisms of action and predicting certain health hazards and illnesses. Many studies have therefore investigated the effect of exposure to electromagnetic fields on isolated or cultured cells. In particular, DNA damage may lead to mutagenesis, potentially resulting in carcinogenesis if not repaired. Considerable emphasis has been placed on the question of possible mutagenic and genotoxic effects of RF exposure, because DNA damage may lead to the development of cancer, which is a major source of concern.

No mechanism for the induction of genotoxic effects by low-level RF radiation has yet been identified. Several reviews have concluded that RF radiation is not directly mutagenic and probably does not increase the genotoxicity of known physical or chemical genotoxic agents [1–3]. Furthermore, many of the studies reporting genotoxic effects present methodological and publication biases, as discussed by the authors of recent reviews [2,4–6]. Therefore, when the methodological relevance of the studies is not critically analyzed and only the numbers of positive and negative results are considered the conclusion may be more mitigated [7]. Microwave energy is not sufficient to break bonds within molecules, but an indirect influence of microwaves in an intermediate step in a complex sequence of events in mutagenesis cannot yet be ruled out.

Recently, in vitro studies concerning the possible co-genotoxic effects of electromagnetic fields (UMTS, GSM or continuous waves) were published. We consider those in which the exposure system and dosimetry were adequately described, making it possible to determine a specific absorption rate (SAR) and consequently estimate the level of exposure of the cells. Wang et al. examined the modifications to human lymphocyte DNA induced by GSM-1800 MHz RF field radiation (SAR of 3 W/kg) combined with various chemical mutagens – mitomycin C (MMC), bleomycin (BLM), methyl methanesulfonate (MMS), and 4-nitroquinoline-1-oxide (4NQO) [8] – or with UV irradiation [9]. RF radiation was found to have no direct genotoxic effect in comet assays, but synergistic effects on DNA damage were reported with MMC and 4NQO [8,9]. The results of these studies were not coherent, with the level of DNA damage induced by UVC irradiation increasing or decreasing with the duration of exposure to the RF field. No correlation between the amount of DNA damage and UVC dose was shown, with or without RF exposure [9]. Using the comet assay, Luukkonen et al. [10] found enhanced levels of DNA damage in neuroblastoma cells exposed to menadione (free radical and DNA damage inducer) when combined with a continuous 872 MHz, but not GSM-872 MHz modulated signal (SAR of 5 W/kg), with no effect on cell viability. The temperature of the exposed cell culture medium in the experimental conditions was not mentioned although the authors stipulated that the SAR level was relatively high. Kim et al. [11] also reported a possible co-genotoxic of Korean CDMA mobile phone modulation (835 MHz, SAR of 4 W/kg) with known mutagenic agents (cyclophosphamide and 4NQO) on a fibroblast cell line in the comet assay. No effect was observed in another cellular model exposed to similar RF conditions. However, different DNA damage tests (chromosomal aberration test) and chemicals were used in the second of these studies, making comparison impossible. Other studies have failed to demonstrate co-genotoxic effects of RF fields at levels too low to generate heat (GSM-935 MHz frequency, SAR of 1 and 2 W/kg) combined with X-rays, based on several methods, including the comet assay [12]. Experiments were also conducted with classical methods, to detect mutagenesis and tumor-promoting effects in vitro in various cell lines treated with chemicals known to induce cancers. Exposure to a continuous 2450 MHz wave with a large range of SAR values (5 to 200 W/kg) led to the induction of a thermal effect at higher SAR values [13,14]. No mutagenic phenomenon was observed for very low SARs (0.08 and 0.8 W/kg), with a 2142 MHz CDMA modulated RF signal [15].

The aim of this study was to investigate whether a 1800 MHz modulated GSM electromagnetic field (GSM-1800 MHz) induced genotoxic effects in vitro when combined with a known mutagenic agent (4NQO, UV mimetic), at several SAR values: 2, 4, 8 and 16 W/kg for two hours. We used cultured human monocyte THP1 cells as the model system for this study. DNA damage was quantified with the alkaline comet assay. The number of DSBs was also assessed with a new method based on detection of the phosphorylated histone 2AX (γ-H2AX). Complementary experiments were carried out to determine the increase in temperature required to generate detectable DNA damage with these methods.

2. Materials and methods

2.1. Cell culture

The THP1 monocyte cell line was obtained from a human leukemia case [16]. It was purchased from the European Collection of Cell Cultures (ECACC no. 88081201). The cells, used between passages 20 and 40, were grown in suspension, in 75 cm^2 culture flasks containing 20 ml of RPMI 1640 culture medium plus Glutamax (Invitrogen, France), supplemented with 10% calf fetal serum (CFS; Lonza, France). Mycoplasma contamination was ruled out by testing with a commercially available detection kit (Polylabo, France). Cell viability was routinely determined by trypan blue exclusion.

2.2. Exposure set-up

Far-field exposure was performed in specially designed thermoregulated Plexiglas incubators in three identical anechoic chambers (Emerson & Cuming, Belgium) (Fig. 1A). A mechanical system was used to rotate the incubator slowly, thereby agitating the cell suspension in the Petri dishes. There were no metallic parts and motorization was external to the chamber. This system ensured the homogeneous exposure of the suspended cells to the stimuli tested. The cell cultures were kept at a constant temperature through a system based on the circulation of water and controlled by a cryostat (Huber Polystat

Fig. 1. Pictures of the laboratory (A) and, of the exposure set-up inside an anechoic chamber and numerical modeling (B), axis in cm.

Fig. 1. Photographies du laboratoire (A) et du système d'exposition dans une chambre anéchoïque ainsi que sa modélisation numérique (B), axes en cm.

Control cc2, Polylabo, France). Efficient heat conduction in the culture plates was ensured by a layer of small glass balls (diameter 1.5 mm, VWR, France).

The signal source consisted of a high-frequency power amplifier working at 1800 MHz (RFS 1800-240, RFPA, France), a function generator (Agilent 33120A, Agilent Technologies, France) and a rectangular horn antenna in a vertical position (CNP22 – 1.8–2.5 GHz, Celti, France). There were two identical emitting systems allowing exposure in two chambers simultaneously. The third was used for the sham exposed samples.

The field was measured by a wide band *E* field probe (PMM OR03, EMtest, France). Emitted power was measured at the level of the antenna with an oscilloscope (Tektronics TDS 1012B, Equipement Scientifique, France) connected to a two-way coupler with a detector (Sodhy DDD 1850-N, Sodhy, France). The detector was characterized with a power meter (Anritsu ML 2488A, Anritsu, France), to link the tension measured by the detector to the incident power emitted by the horn antenna. The system was monitored under computer control, with a custom-build program (Labview, National Instrument, France). This also made it possible to record, in parallel, the temperature in the chambers and mean emitted power during each experiment. Signal modulation was typical of 1800 MHz GSM mobile phone networks (217 Hz, duty cycle 1/8).

2.3. Incubation of the cells

The cells were centrifuged and suspended in 35 mm Petri dishes (1.5×10^6 cells/ml) containing 1.5 ml of RPMI 1640 culture medium supplemented with Glutamax and 10% FCS (as for standard cell culture), plus 25 mM Hepes as a buffer to ensure the maintenance of pH during exposure. Preliminary experiments showed that cell growth was similar in classical CO_2 cell culture incubators (Jouan, France) and in the anechoic chambers, in the presence of Hepes, during the experiment (not shown).

For RF experiments: non-exposed (sham) and exposed cell culture plates were incubated simultaneously in the presence of 4NQO (1.8 µM), for 2 h, at 37 °C, with gentle stirring. The different exposure conditions were applied separated to each of the three chambers, to prevent bias related to cage effects. Two SAR levels were tested simultaneously in two anechoic chambers, with sham exposure in the third chamber. The presence or absence of the electromagnetic field was the only difference between the sham and exposed assays.

For experiments at temperatures of 37 to 43 °C, small thermostatically controlled incubators (Stuart Scientific SI 60-D, Polylabo, France) were used, giving a heating curve for the samples similar to that obtained in the samples exposed to RF radiation. The temperature was maintained by pulsed air. The plates were also placed in the anechoic chamber for gentle shaking, as in the exposed experiments. It was not possible to perform experiments at various temperatures in Plexiglas incubators in which the temperature was controlled by circulating water. It took too long for the system to equilibrate at different temperatures between two experiments and the cryothermostat was common to all the chambers, making it impossible to carry out experiments at different temperature simultaneously. In each experiment, the cells were incubated for 2 h in the presence of 4NQO (1.8 µM), in two chambers simultaneously, one at the temperature tested (39, 41 or 43 °C) and the other at 37 °C, as a control.

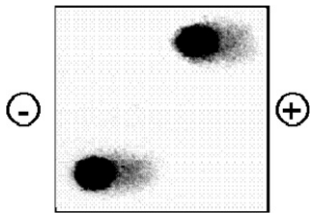

Fig. 2. Image of THP1 cells treated with 4NQO and analyzed in the comet assay. Image of the nucleus after DNA staining, permeabilization of the membranes and electrophoresis (×40). + and − indicate the anode and cathode of the electrophoresis system.

Fig. 2. Photographie de cellules THP1 traitées par le 4NQO et analysées avec le test des comètes. Image des noyaux après coloration de l'ADN, perméabilisation des membranes et électrophorèse (×40). + et − illustrent l'anode et la cathode du système d'électrophorèse.

After incubation in the experimental conditions, the cells were washed in phosphate-buffered saline solution (PBS) and centrifuged (4000 g, 10 min). The pellet was suspended in PBS for the comet assay or fixed for γ-H2AX detection (see below).

2.4. Dosimetry

Numerical dosimetry was performed with a finite difference time domain (FDTD) code, taking into account the design of the setup, the location of the Petri dishes, exposure parameters and biological parameters.

The structure of the incubator was modeled based on the precise dimensions (Fig. 1B). It was made of Plexiglas ($\varepsilon_r = 2.5$), glass balls ($\varepsilon_r = 3.6$), pipes full of liquid (30% ethylene glycol in water: $\varepsilon_r = 66.3$), and the whole system was covered and surrounded with a Plexiglas cover. A discretization of 500 µm was used to make the dosimetry more accurate. At 1800 MHz, the characteristics of the biological medium were $\varepsilon_r = 72.8$ and $\sigma = 2.29$ S/m.

2.5. Temperature measurements

The temperature of the culture medium in the plates was measured with a microprocessor-controlled thermometer equipped with four fluoro-optic fiber probes (Luxtron, Optilas, France), making it possible to take four independent measurements simultaneously.

Temperature was measured in the conditions of the real biological experiments for the various SAR values studied (from 2 up to 16 W/kg). It was measured simultaneously in two exposed culture plates and two non-exposed plates otherwise incubated in the same conditions (sham). Temperature measurements were taken throughout the exposure period (2 h), with an interval of two seconds between consecutive readings (data not shown). We considered the mean of the last 20 points to be the final temperature in the medium after 2 h of incubation. Each experiment was repeated four times.

2.6. Alkaline comet assay

This method made it possible to detect single-strand breaks (SSBs), double-strand breaks (DSBs) and alkali-labile sites within the DNA molecule. The alkaline comet assay, also known as single cell gel electrophoresis, was performed as originally described by Singh et al. [17], with minor modifications.

In each experiment, non-exposed (sham) and GSM-1800-exposed cell culture plates (3 Petri dishes of 25 mm in diameter per set of conditions) were incubated as described above. After exposure, cells were suspended in 0.5 ml PBS and cell density was estimated with a Malassez slide for each exposure condition. An aliquot of the suspension was mixed with low-melting point agarose (0.8%, p/v in PBS) to obtain a final concentration of 25×10^4 cells/ml. We dispensed 80 µl of cells suspended in agarose onto two agarose-coated slides (high molecular weight, 1% p/v in PBS), which we then covered with a coverslip. The slides were placed on ice for five minutes and the coverslip was then gently removed. The slides were incubated (1 h, 4 °C in the dark) in lysis buffer (45 ml of buffer containing 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, with 5 ml DMSO and 0.5 ml Triton X-100, pH 10). The slides were washed twice with electrophoresis buffer (300 mM NaOH, 1 mM EDTA), for 10 min for the first wash and for 25 min for the second wash. Electrophoresis was carried out at 25 volts and 300 mA, for 37 min, at room temperature. During this step, DNA fragments migrated out of the nucleus to the positive pole (Fig. 2). The slides were washed with Tris-HCl buffer (pH 7.5) and DNA was stained with ethidium bromide (0.1 mg/ml, 50 µl per slide, $\lambda_{ex} = 525$ nm and $\lambda_{em} = 650$ nm). The slides were examined with an epifluorescence microscope equipped with a charge-coupled device (CCD) camera. The images were analyzed with the Komet 5.0 image analysis system (Kinetic Imaging). Many image analysis parameters were systematically recorded, including "comet tail length", "tail DNA content" and "tail extent moment" (TEM). No significant differences were recorded for these factors. Fragmentation is expressed here in TEM. Images of 200 randomly selected cells were analyzed for each sample (100 per slide).

Table 1
DNA damage level, expressed as mean TEM values for exposed cell cultures treated with 4NQO (4NQO + GSM) and the corresponding sham (4NQO-treated) cells without exposure (arbitrary units). For each set of exposure conditions: we analyzed 100 cells per slide, for two slides per dish from three Petri dishes. Statistical analysis of the data was performed with the Newman–Keuls test. Values are means ± SD of six independent experiments, with differences considered significant (*) for p values < 0.05 (sham *versus* exposed).

Tableau 1
Niveau d'altération de l'ADN représenté par les valeurs TEM des cellules exposées, traitées par 4NQO (4NQO + GSM) comparé aux contrôles « sham » correspondants (unités arbitraires). Pour chaque condition d'exposition : 3 boîtes de Pétri, 2 lames/boîte, 100 cellules analysées par lame. L'analyse statistique des données est faite avec le test de the Newman–Keuls. Les valeurs sont les moyennes ± SD de six expériences indépendantes ($^*p < 0{,}05$).

Exposure conditions	2 W/kg	4 W/kg	8 W/kg	16 W/kg
4NQO + GSM	5.24 ± 0.46	5.10 ± 0.55	5.77 ± 0.38	6.30 ± 0.40
4NQO (corresponding sham cells)	5.24 ± 0.47	5.07 ± 0.39	5.90 ± 0.34	6.51 ± 0.43

2.7. γ-H2AX indirect immunofluorescence and detection by cytometry

In each experiment, non-exposed (sham) and exposed cell culture plates (4 Petri dishes for each set of conditions) were incubated as described above. The following protocol was developed in the laboratory. The cells (approximately 3×10^5 per sample, in duplicate) were then fixed in 1% paraformaldehyde in PBS, washed twice in PBS and permeabilized by incubation with permeabilization buffer (20 mM Hepes, 50 mM NaCl, 3 mM $MgCl_2$ ($6H_2O$), 300 mM sucrose, 0.5% Triton X-100) for five minutes on ice. Cells were washed three times in 0.2% Tween in PBS (PBS-Tween) and blocked by incubation with 2% BSA in PBS (PBS-BSA) for 30 min at room temperature. Cells were washed with PBS-Tween and incubated for 1 h at room temperature with a mouse monoclonal antibody directed against phosphorylated H2AX (Ser139; Clone JBW301, Upstate, France) diluted to a concentration of 1.4 µg/ml in PBS-BSA. Cells were washed four times with PBS-Tween and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (F(ab′)2) secondary antibody (Molecular Probes, France) diluted to a concentration of 4 µg/ml in 1% BSA in PBS, for 1 h at room temperature. Cells were then washed four times with PBS-Tween and once with PBS. The cells were collected by centrifugation (4000 g, 10 min) and the pellet was suspended in PBS and analyzed in a BD LSRII flow cytometer (Becton Dickinson, San Jose, CA). The mean fluorescence, expressed in arbitrary units, was determined for 20,000 cells.

2.8. Statistical analysis

For each experiment, the values presented are means ± standard deviation, for three or four separate Petri dishes (comet assay, H2AX). Each experiment was reproducibly repeated four or six times. Statistical analysis of the data was based on analysis of the variance for repeated measurements (ANOVA). Newman–Keuls unpaired t test was used to determine the statistical significance of differences. p values lower than 0.05 were regarded as statistically significant.

3. Result

3.1. Dosimetry and temperature measurements

The mean SAR in the four dishes used for biological experiments at 1800 MHz was 0.82 ± 0.4 W/kg for 1 W incident.

The final temperature measured (mean ± SEM) in exposed and sham Petri dishes was 36.77 ± 0.37 and 36.97 ± 0.28 °C respectively. No temperature increase was observed, whatever the SAR, by comparison with sham plates.

3.2. Alkaline comet assay

3.2.1. Effect of exposure to 1800 MHz GSM on the genotoxic effect of 4NQO

The cells were incubated as described above, to investigate the effect of exposure to GSM-1800 MHz, at four different SAR levels (2–4–8–16 W/kg), on the genotoxic effect of 4NQO. The comet assay was performed immediately after the exposure period. The results for exposed samples were always compared with those for sham samples containing identical cells and incubated in parallel, because the level of DNA damage may differ slightly from one day to the next. No statistically significant difference was detected between the different exposure conditions and DNA damage levels were not modified by the exposure of cells to a GSM-1800 MHz electromagnetic field (Table 1).

3.2.2. Effect of temperature on the genotoxic effect of 4NQO

We also investigated the effect of temperature on DNA damage in the comet assay, using temperatures from 37 to 43 °C. The extent of DNA damage at 43 °C differed significantly from that at 37 °C. Thus, an increase of more than 4 °C was required to modify the number of DSBs induced by the mutagenic agent (Table 2).

Table 2
Mean TEM values (arbitrary units) for THP1 cells treated with 4NQO at several incubation temperature and for the corresponding controls incubated at 37 °C. For each set of conditions, we analyzed 100 cells per slide, for two slides from three Petri dishes. Statistical analysis of the data was performed with the Newman–Keuls test. Values are means ± SD of four independent experiments, with differences considered significant (*) for p values < 0.05 (tested temperature versus 37 °C).

Tableau 2
TEM moyens (unités arbitraires) obtenus avec des cellules THP1 traitées par le 4NQO à différentes température d'incubation, comparés aux contrôles correspondants incubés à 37 °C. Pour chaque condition d'exposition : 3 boîtes de Pétri, 2 lames/boîte, 100 cellules analysées par lame. L'analyse statistique des données est faite avec le test de Newman–Keuls. Les valeurs sont les moyennes ± SD de 4 expériences indépendantes.

Incubation conditions	39 °C	41 °C	43 °C
4NQO	5.96 ± 1.01	6.92 ± 0.67	9.14 ± 1.75*
4NQO at 37 °C (control)	5.76 ± 0.82	6.55 ± 0.40	6.51 ± 0.54

* $p < 0.05$.

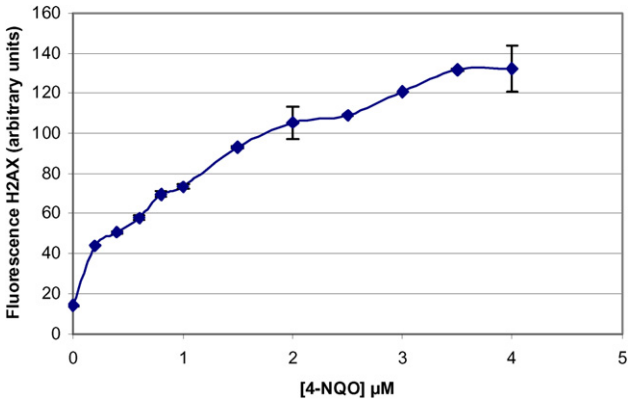

Fig. 3. Flow cytometry calibration. Mean fluorescence intensity was calculated from THP1 cells treated with various doses of 4NQO. The cells were incubated for 2 h at 37 °C in the presence of chemical mutagen (Petri dishes, in duplicate). The graph illustrates one representative experiment. The experiment was reproducibly repeated three times (values are mean ± SEM).

Fig. 3. Calibration en cytométrie de flux. Intensité de fluorescence moyenne calculée pour des cellules THP1 traitées par des doses croissantes de 4NQO. Les cellules sont incubées pendant 2 h à 37 °C en présence de l'agent mutagène (boites de Pétri, en double). Le graphe illustre une expérience représentative. L'expérience a été répétée de façon reproductible trois fois (valeurs moyennes ± écart moyen).

3.3. Phosphorylated γ-H2AX detection

3.3.1. Validation of the technique

We first validated the technique, with 4NQO, in our experimental conditions. THP1 cells were incubated for 2 h with various concentrations of chemical agent. We previously tried to quantify DNA damage by counting the number of foci in the cells under an Olympus fluorescence microscope. The number of foci increased with the dose of chemical agent, but this method was not sensitive enough for the quantification of small changes in DNA damage.

We therefore quantified γ-H2AX by flow cytometry. The intensity of THP1 cell fluorescence as a function of 4NQO concentration (0 to 4 µM) is illustrated in the representative experiment shown in Fig. 3. Fluorescence intensity was found to be proportional to 4NQO concentration. A concentration of 1.8 µM 4NQO generated a moderate response useful for our purpose, as reported for the comet assay. We also assessed the variability and reproducibility of the results obtained by this method in preliminary experiments (data not shown). Both were found to be satisfactory.

Another control before the RF exposure experiment involved determining how long after cell incubation γ-H2AX detection should be carried out. We found that focus detection was optimal immediately after treatment (data not shown).

3.3.2. Effect of exposure to 1800 MHz GSM on the genotoxic effect of 4NQO

The effect of exposure to 1800 MHz GSM combined with 4NQO was compared with the effect of 4NQO alone. The cells were incubated, as described in the materials and methods, at SAR levels of 2, 4, 8 and 16 W/kg. We carried out γ-H2AX detection immediately after incubation. No difference was found between cells treated with the chemical agent with and without exposure to RF, for the various SAR values tested (Table 3).

3.3.3. Effect of temperature on the genotoxic effect of 4NQO

The co-genotoxic effect increasing the temperature from 37 to 43 °C, in cells treated with 4NQO treatment of the cells was assessed with the comet assay. The genotoxic effects observed at 41 and 43 °C were significantly different from that at 37 °C. No effect was observed when the incubation was carried out at 39 °C. Thus, an increase in temperature of more than 2 °C was required to modify the genotoxic effects observed with the mutagenic agent alone (Table 4).

Table 3
γ-H2AX detection for different SAR levels. Fluorescence intensity is expressed as mean fluorescence intensity values for cell cultures treated with 4NQO and exposed to RF (4NQO + GSM) and for the corresponding sham cells without exposure (4NQO only). For each set of exposure conditions, we analyzed 20,000 events per sample, for two samples per dish from four Petri dishes. Values are means ± SD of six independent experiments. Statistical analysis of the data was performed with the Newman–Keuls test, with differences considered significant (*) for p values < 0.05 (sham versus exposed).

Tableau 3
Détection de γ-H2AX à différents niveaux de DAS. Intensité de fluorescence moyenne des cellules traitées par le 4NQO (4NQO + GSM) et exposées aux RF comparée aux contrôles « sham » correspondants (4NQO). Pour chaque condition d'exposition : 4 boites de Pétri, 2 échantillons par boîte, 20 000 évènements analysés par échantillon. Les valeurs sont les moyennes ± SD de 4 expériences indépendantes. L'analyse statistique des données est faite avec le test de Newman–Keuls.

Exposure conditions	2 W/kg	4 W/kg	8 W/kg	16 W/kg
4NQO + GSM	36.49 ± 0.40	36.66 ± 0.31	37.46 ± 0.22*	37.25 ± 0.20*
4NQO (corresponding sham)	36.50 ± 0.23	36.59 ± 0.45	36.59 ± 0.45	36.74 ± 0.59

* $p < 0.05$.

Table 4
γ-H2AX detection at different temperatures. Fluorescence intensity of THP1 cells treated with 4NQO (4NQO) at several incubation temperatures and for the corresponding control incubated at 37 °C (4NQO treatment only). For each set of conditions, we analyzed 20,000 events per sample, for two samples per dish from four Petri dishes. The values presented are means ± SD of 6 independent experiments. Statistical analysis of the data was performed with the Newman–Keuls test, with differences considered significant (*) for p values < 0.05 (tested temperature *versus* control at 37 °C).

Tableau 4
Détection de γ-H2AX à différentes températures. Intensité de fluorescence moyenne des cellules traitées par le 4NQO et incubées à différentes températures comparée aux contrôles correspondants à 37 °C. Pour chaque condition d'exposition : 4 boîtes de Pétri, 2 échantillons par boîte, 20 000 évènements analysés par échantillon. L'analyse statistique des données est faite avec le test de Newman–Keuls, les valeurs sont les moyennes ± SD de 6 expériences indépendantes.

Incubation conditions	39 °C	41 °C	43 °C
4NQO	71.37 ± 13.07	93.11 ± 14.61*	120.06 ± 5.35*
4NQO at 37 °C (control)	67.61 ± 17.83	64.78 ± 13.61	77.82 ± 18.07

* $p < 0.05$.

4. Discussion

Two methodological approaches were used to study the co-genotoxic effect of GSM-1800 MHz electromagnetic field exposure in human cells also exposed to a known chemical mutagen. The SARs studied ranged from 2 to 16 W/kg. The lowest value corresponds to the upper limit of the acceptable range for local exposure to the human head according to the International Commission on Non-Ionizing Radiation Protection (ICNIRP) guidelines for public exposure [18].

The THP1 leukemia cell line was used because these cells have several advantages for studies of this type. They are human immune cells with residual characteristics of normal cells, able to differentiate into macrophages in response to stimulation. Furthermore, these cells do not adhere to the Petri dish, making it possible to ensure that the exposure is uniform throughout the sample. Indeed, during exposure, adhesion to the Petri dish might lead to SAR levels varying several-fold throughout the dish. It is also difficult to determine the precise distribution of temperatures in cell culture. We avoided the introduction of such experimental biases, in which different cells might be exposed to different SARs, by using cells that naturally grow in suspension. Gentle stirring of the cell culture during exposure thus ensured the uniform exposure of all the cells present.

The direct assessment of DNA strand breaks in the alkaline comet assay (DSBs and SSBs) was compared with the indirect assessment of DSBs based on H2AX phosphorylation at serine 139. DSBs are the most toxic form of DNA damage, because they are the most difficult to repair and may lead to the loss of DNA fragments, resulting in chromosome damage and, possibly, even cell death. It is widely accepted that chromosomal aberration is strongly associated with mutagenic and carcinogenic events.

For the comet assay, the tail extent moment (TEM) was used as a criterion because this parameter takes into account both tail length and the percentage of DNA in the comet tail [19]. It is known to provide an accurate representation of the level of DNA damage. Nevertheless, this assay has several limitations and may give false positive results if the effect is subtle. It was therefore important to assess DNA damage by another technique, in addition to TEM assessment, in this study. We therefore also carried out γ-H2AX detection. This histone is rapidly phosphorylated and is colocalized with other repair proteins when DNA is altered [20,21]. Many γ-H2AX molecules present at the site of damage may form foci, amplifying the DNA damage signal and facilitating its detection. This method has a detection threshold one hundredth that of the comet assay. We used an improved version of the method, not based on the detection of γ-H2AX by the counting of foci under a microscope as frequently described in previous studies. Instead, flow cytometry was used to quantify fluorescence. This technology is increasingly being used for γ-H2AX detection [22,23], making it possible to analyze a larger number of cells for each sample than in the classical approach and providing a more powerful statistical analysis of the results.

In preliminary experiments, we first assessed the dose-dependent effect of 4NQO alone on THP1 cells after 2 h of incubation, to check that the choice of the chemical agent was pertinent and to determine the most appropriate concentration for

use in combination with RF exposure. This concentration of mutagen should induce moderate DNA damage, making it possible to detect small positive or negative changes induced by the electromagnetic field in both comet and γ-H2AX detection assays. Concordant results were obtained, demonstrating that the same concentration could be used for both techniques. We found that, in the experimental conditions, a final concentration of 1.8 μM 4NQO in the culture medium was required to induce about 50% fragmentation (data not shown). Furthermore, it was in the concentration range usually employed for in vitro studies [8].

When cells were exposed to GSM-1800 MHz radiofrequency combined with 4NQO, RF exposure had no significant effect on DNA damage, which remained unchanged for SARs of 2 to 16 W/kg. Similar results were obtained with both the methods used: the comet assay and γ-H2AX detection. Further experiments were carried out to determine the increase in temperature required to induce an increase in DNA damage. The comet and the γ-H2AX assays were performed with 4NQO-treated cells incubated at temperatures of 37 to 43 °C.

A temperature increase of 6 °C was required to induce a positive effect observable in the comet assay, whereas such an effect was obtained with an increase of 4 °C when DNA damage was assessed by the γ-H2AX detection method. We found that the temperature of the culture medium did not increase significantly after exposure to RF at the various SAR levels tested (from 2 to 16 W/kg). This lack of increase in temperature may be accounted for by the continual stirring of the culture medium, dissipating any increase in temperature by effective exchange at the surface and preventing heat accumulation in the sample. This confirmed the absence of a thermal effect, and the absence of a non-thermal co-genotoxic effect of GSM-1800 MHz in our experiments. Our results also provided further evidence confirming that the detection threshold of the γ-H2AX detection assay is lower than that of the alkaline comet assay.

We did not investigate the effect on DNA damage of temperature alone, in the absence of a mutagenic agent. Ideally, such investigations should have been carried out, but, in the absence of an effect of the four SAR levels tested, exceeding standard limits, we felt that this would have been beyond the scope of the study. However, further investigations should be performed to compare the effect of higher levels RF power, generating higher SAR values and possibly giving rise to a temperature increase of up to 4 or 6 °C. Such studies would make it possible to determine whether thermal effect on 4NQO-induced DNA damage, as assessed by γ-H2AX and comet assays, is similar for all sources of heat, whether electromagnetic or conductive in nature.

Finally, we can conclude that the exposure of human cells to mobile phone GSM-1800 MHz radiofrequencies at SARs of up to 16 W/kg does not increase the level of DNA damage induced by the known mutagenic agent 4NQO. There was therefore no co-genotoxic effect in our experimental conditions. These results do not bring news arguments for the hypothesis that radiofrequency exposure may play a role favoring the development of cancer at non-thermal level.

Abbreviations

GSM: Global system for mobile communication
CDMA: Code-division multiple access
UMTS: Universal Mobile Telecommunication System

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