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Exposure to 26.5 GHz, 5G modulated and unmodulated signal, does not affect key cellular endpoints of human neuroblastoma cells

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The fifth generation (5G) network is currently being worldwide spread out, raising questions about its potential health impact. The current study aimed to investigate the effects of a 26.5 GHz 5G electromagnetic field on key cellular endpoints of human neuroblastoma cells. A reverberation chamber-based exposure system was designed and realized which allowed the exposure/sham exposure of cell cultures under highly controlled exposure conditions of both electromagnetic and biological parameters. The suitability of the reverberation chambers to host cell cultures was verified by evaluating cell proliferation and cell cycle progression. The effect of 3 h exposure at specific absorption rate of 1.25 W/kg under both continuous wave and 5G modulated signal was evaluated in terms of cell cycle and DNA damage. In the latter case, the exposure was also given in combination with menadione to account for possible cooperative effects. Results showed absence of effects of exposure given alone and in combination with menadione, when both continuous wave and modulated signals were applied at the mentioned exposure level. Further investigations are needed by varying the exposure and biological parameters to strengthen the absence of effects due to 5G signals in the range of millimeter waves.

Keywords Radiofrequency fields, 5G communications, In vitro, Cooperative effects, DNA damage, Cell cycle

In the last decades a large amount of studies on possible biological and health-related effects of radio frequency electromagnetic fields (RF-EMF) in use for mobile communications (1G–4G mobile phones) has been published.

Several international and national panels, as well as review papers, critically analysed the literature on this topic at population level (epidemiological studies), at individual level (in vivo studies on humans and animals) and at level of tissues, cells and molecules (in vitro studies on cell cultures, biochemical and molecular processes). In the latter case, less complex systems are involved but interaction mechanisms can be better investigated to predict possible effects of EMFs on structures and functions at different levels of the biological organization[1]. The large majority of these panels concluded that for exposures at or below exposure limits provided by the International Commission on Non-Ionising Radiation Protection[2] or IEEE-International Commission on Electromagnetic Safety[3], the health risk for humans is weak, although long-term effects cannot be confidently excluded, and the need of additional research is always highlighted[4–6].

In addition, even in the most investigated frequency bands (800–2450 MHz), a large amount of results is controversial and inconsistent, sometimes for the low quality of the studies, that do not meet basic methodological quality criteria and/or for the lack of details for repetition/confirmation of the experiments[7]. In particular, the main limitations/criticality of the available data are related to dosimetry, lack of temperature control (especially for studies using high exposure levels) and absence of sham-exposed controls. Other variables such as humidity and pH should be controlled to provide reliable in vitro results[8].

With respect to RF-EMF signals related to previous telecommunication technologies, the recently deployed fifth-generation (5G) technology allows faster and more reliable communication, with higher data rates and

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connection density, and much lower latency. This performance is accomplished by using high carrier frequencies with massive bandwidths, extreme base station and device densities, and unprecedented numbers of antennas, in such a way to guarantee ubiquity, low-latency and adaptability required by users[9]. This is also changing the electromagnetic scenario in urban environments, already characterized by the abundant presence of RF-EMF sources providing 2G/3G/4G services[10,11].

5G technology supports two bandwidths: frequency range 1 (FR1), commonly referred to as sub-6 GHz, ranging from 450 MHz to 7.125 GHz, and frequency range 2 (FR2), commonly referred to as millimeter wave, ranging from 24.250 GHz up to 52.600 GHz[12].

While there is abundant scientific literature dealing with the evaluation of biological effects of low-level EMFs at frequencies below 6 GHz, there is a lower number of studies at higher frequencies, only a few of them dealing with 5G.

In this study, a 5G modulated signal at 26.5 GHz as representative of high 5G band currently deployed in Europe, was used to evaluate the induction of cytotoxic and genotoxic effects in a human neuroblastoma cell model (SH-SY5Y). A reverberation chamber (RC)-based exposure system operating at the frequency of interest was designed and realized to ensure reliability and reproducibility of the results. The exposure system is based on two RCs, one for sham, the other for RF-EMF exposure. To guarantee highly controlled exposure conditions, the system was characterized from both electromagnetic and biological perspectives. In particular, numerical and experimental dosimetry was carried out to assure the control of electromagnetic parameters, while the suitability of the two RCs to host cell cultures was tested in terms of cell viability and cell cycle by using both chambers in sham-configuration and having the cell culture incubator as reference control.

To assess the effect of 5G RF-EMF, cell cultures were exposed for 3 h to an EMF at 26.5 GHz, either continuous wave (CW) or 5G-modulated, at a specific absorption rate (SAR) of 1.25 W/kg. The rationale for the selected exposure conditions comes from our previous investigations carried out at 1950 MHz, UMTS signal, on the same cell model here adopted, where no effect of RF exposure alone was detected, while eliciting a consistent reduction of chemically induced damage[13].

Cell cycle progression was evaluated to account for cytotoxicity, while the comet assay was applied to test DNA damage. In addition, Comet assay was also employed to investigate whether combined treatments to 26.5 GHz and menadione, a chemical agent inducing DNA damage via reactive oxygen species formation[14], can induce cooperative effects.

Material and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and phosphate buffer (PBS) were from Domenique Dutscher (Brumath, France). GlutaMAX was from Gibco™, by Thermo Fisher Scientific Inc. (Waltham, MA, USA). Trypsin–EDTA and penicillin/streptomycin were from Biowhittaker (Verviers, Belgium). Menadione, *N*-lauryl sarcosine, propidium iodide, resazurin and triton X-100 were from SIGMA (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), EDTA, sodium citrate and sodium hydroxide were from J. T. Baker (Deventer, The Netherlands). NaCl and Tris–HCl were from Carlo Erba Reagents (Milan, Italy). Trypan blue staining solution was from Logos Biosystems (Anyang-si, South Korea). Ethidium bromide, low-melting point agarose and normal-melting agarose were from Bio-Rad Laboratories (GmbH, Munich, Germany). Ethanol was from Thermo Fisher Scientific Inc.

Cell culture conditions

Human SH-SY5Y neuroblastoma cell line (ATCC, Cat. No. CRL2266, Rockville, MD, USA) were cultured in 4.5 g/L glucose DMEM, supplemented with 10% heat-inactivated FBS, 1% Glutamax, 100 U/mL penicillin, and 100 mg/mL streptomycin and were routinely checked for mycoplasma infection. A master bank was established. Cell cultures were maintained under standard conditions in an incubator (Forma Scientific, model 311, Freehold, NJ, USA) at 37 °C temperature and 5% CO_2 humidified atmosphere. Fresh culture medium was supplied every 72 h, and cells were harvested twice a week by 200 mg/mL trypsin treatment. The experiments were conducted using cells between passages 3 and 10.

For the experiments, 10^6 cells were seeded in 35 mm Petri dishes (Corning Inc., NY, USA) and grown for 72 h in 3 mL of complete medium.

Exposure system set-up and dosimetry

The set up for exposure of cell cultures to 26.5 GHz is based on a RC, i.e. an environment with metallic walls whose dimensions are very large compared to the wavelength, so that a large number of modes can be excited, and a spatially uniform power density is obtained by mechanical stirring[15].

The RC was designed by numerical simulations in CST Studio Suite (Dassault Systèmes, Vélizy-Villacoublay, France) platform, to (1) obtain high uniformity of the electric field distribution inside the samples; (2) maximize the number of samples that can be exposed at the same time, and (3) be hosted inside standard incubators. According to Hill's theory, the field distribution in a RC can be represented as a superposition of plane waves, whose Cartesian components magnitude has a Rayleigh distribution. Consequently, simulations were performed by considering a suitable number of plane waves impinging on the samples (35 mm Petri dish filled with 3 mL of culture medium) with both polarizations, and different incoming directions, and by superposing the computed fields[16]. Average SAR values were calculated and the coefficient of variation (CV, the ratio of standard deviation of SAR to average SAR) was derived as an indication of degree of uniformity of SAR distribution (a CV of less of 30% was considered acceptable[17]). The mesh used in the simulations was as fine as 0.010 mm in the lower 100 μm of DMEM, corresponding to the cell monolayer, and 0.1 mm in the higher part of DMEM. As an example, in

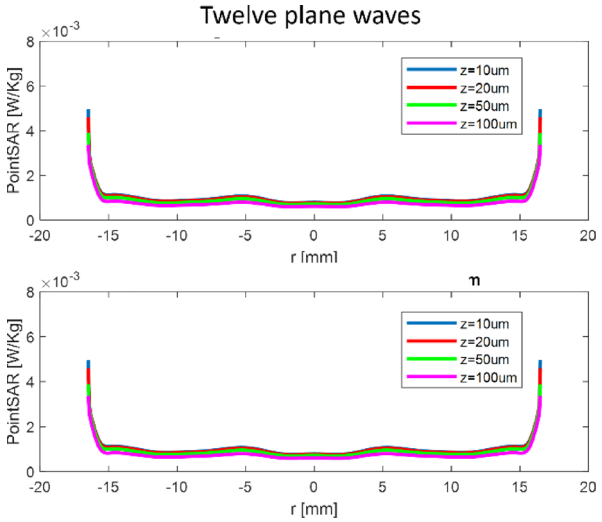

Fig. 1. An example of the SAR distribution, along two different radial directions, at different locations within the monolayer when 12 plane waves are considered, with z = 0 being the bottom of the Petri dish.

Fig. 1 the SAR distribution at different locations within the monolayer of the cell sample is plotted, in the case of 12 incident plane waves: the CV in different slices of the cell monolayer ranges between 27.7 and 31.5%.

Based on the simulation results, a RC with inner dimensions of 404 mm × 419 mm × 375 mm (compatible with the dimension of standard cell culture incubators), and wall thickness of 4 mm was realized by anticorodal (aluminium-magnesium-silicon) alloy. An opening (8 cm diameter) with a metal net (200 µm mesh) was inserted into the top wall to guarantee constant environment (temperature, relative humidity, and CO_2) when the RC is inserted into the incubator. The stirrers were realized by using two rectangular metallic crosses rotating parallel to the right and the bottom walls at independently controlled speed. Two WR-28 open-ended waveguides were used as transmitting and receiving antennas (for monitoring purposes) on the walls opposite to the stirrers.

Two identical RCs, one for RF- and one for sham-exposure, were realized and hosted inside two standard cell culture incubators.

Each RC hosted four samples, as reported in Fig. 2, Panel B (chamber 1, positions 1–4; chamber 2, positions 5–8).

The RC used for RF exposure was connected to a Rohde&Schwarz, SMW200A signal generator with SMW-K144 and SMW-K148 options enabled to generate a 5G compliant signal. The delivered power was measured during the exposure by means of a power meter (Rohde&Schwarz, NRP-Z85) connected to port #2 of the RC, and power values were monitored and acquired through a Rohde&Schwarz Power Viewer software. A picture of the exposure system is presented in Fig. 2 (Panel A), where the stand with housings for Petri dishes opportunely engraved to allow a reproducible positioning of samples is also shown for both RCs (Panel B).

To rule out thermal increase due to EMF exposure, the temperature profile was measured in Petri dishes filled with 3 mL culture medium and RF-exposed/sham-exposed for 24 h. To this purpose, each RC has been loaded with four samples, according to Fig. 2, Panel B, and the temperature was measured in one of them by using a FISO Technologies (Quebec, Canada) UMI4 thermometer equipped with a fiber optic sensor (FOT-L). The result of this measure is plotted in Fig. 3, where the RF is turned on after a lag period of 3 h, time required for the samples to reach the thermal regimen (about 36.7 °C). The temperature change in the exposed and sham exposed samples was in the range of the thermometer accuracy (± 0.3 °C).

For experiments, cell cultures were exposed for 3 h to 26.5 GHz at 1.25 W/kg SAR at either CW or 5G modulated. A 5G signal was employed, which is compliant with 3GPP TS.38.211 and had OFDM/QPSK modulation with a 100 MHz bandwidth centred at 26.5 GHz. In the following, such signal will be referred briefly to as 5G signal.

Experimental procedures

Assessment of suitability of RCs to host cell cultures

Experiments were carried out to assess whether differences occur in cell cultures hosted for 24 h in RCs set with no field (sham-sham configuration) and the standard cell culture incubator (negative control). To this purpose, cell viability and cell cycle progression were measured in three independent experiments. For each endpoint, 10 cultures were set up as follows: a negative control, eight samples hosted in the two RCs (four samples per chamber, according to Fig. 2B) and a positive control (treatment with 2% ethanol and 0.5% FBS for viability and cell cycle, respectively).

Radiofrequency-exposure protocol

RF/sham exposure at 26.5 GHz was given for 3 h, from 48 to 51 h after cell seeding, at 1.25 W/kg SAR to test the effect of CW and 5G-modulated signal.

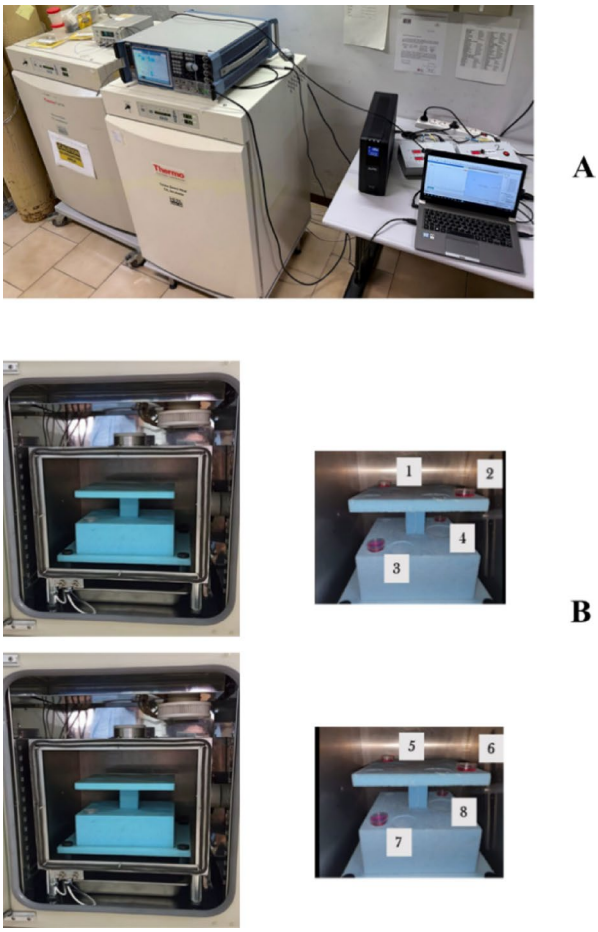

Fig. 2. Panel (**A**) Reverberation chamber-based 5G exposure system set up; Panel (**B**) the two RCs located inside cell culture incubators (left side) and the housings for 35 mm Petri dishes on the top and bottom level of each stand (right side).

Four sets of experiments were carried out to evaluate the effect of RF exposure on cell cycle progression and on DNA damage. In the latter case, the effect of co-exposure with MD was also evaluated.

For cell cycle analysis, each experimental run included four randomly assigned cultures as follows: (1) untreated control (incubator); (2) sham control (Sham); (3) RF-exposed (RF); (4) 0.5% FBS (positive control).

To evaluate DNA damage following RF-EMF exposure and co-exposure, six randomly assigned cultures were set up, such as (1) untreated control (incubator); (2) sham control (Sham); (3) RF-exposed (RF); (4) menadione-treated (MD); (5) sham control and MD-treated (Sham + MD); (6) RF-exposed and MD-treated (RF + MD).

MD (10 µM final concentration, dissolved in DMSO) was given from 71 to 72 h and also served as positive control for DNA damage.

All the experiments were carried out blinded, that is, samples were coded by the operator performing the exposure. In such a way, the operator involved in the analysis was not aware of the treatment, and codes were broken after the analysis[18].

Assay procedures

Cell viability

The Resazurin assay provides information on cell viability and cell number[19]. Resazurin (non-fluorescent) is reduced to resorufin (highly fluorescent) by oxidoreductases in mitochondria, and a direct correlation exists between resorufin release in the growth medium and metabolic activity of living cells. Cells were incubated at 37 °C with 10 µg/mL resazurin for 1 h, until the appearance of color change. After centrifugation, the production of resorufin was measured in supernatants with a fluorometer (Perkin-Elmer, LS50B, Perkin Elmer Instruments, Norwalk, CT) at excitation and emission wavelengths of 530 and 590 nm, respectively. Resorufin production was expressed as Relative Fluorescence Unit (RFU). Cells treated for 40 min before harvest with 2% ethanol were used as positive control.

Cell cycle analysis

Propidium Iodide (PI) staining was used to evaluate DNA content of permeabilized cells, according to the protocol of[20]. Briefly, after 72 h of growth, cell cultures were trypsinized, collected by centrifugation (1200 rpm, 5 min)

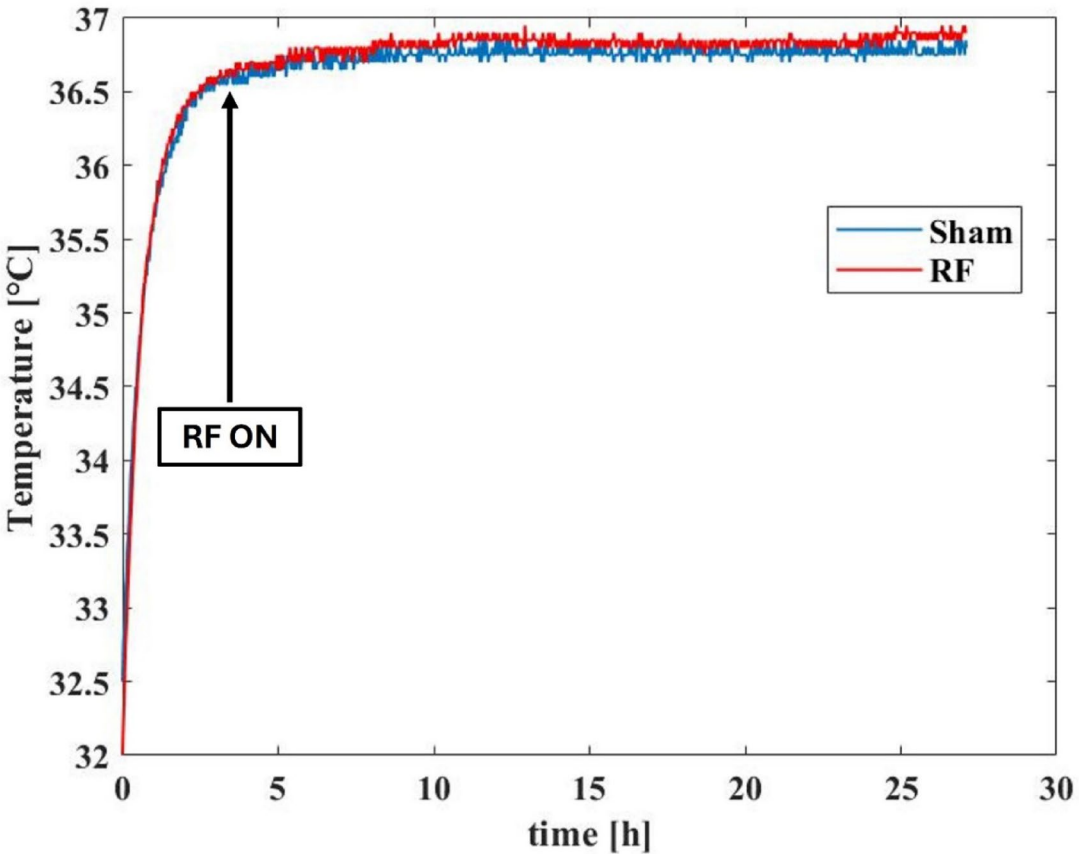

Fig. 3. Temperature profile in samples RF/sham exposed for 24 h, after a lag period of 3 h.

and 5×10^5 cells were washed twice with PBS and loaded for 30 min at 4 °C in the dark with a permeabilizing staining solution (50 µg/mL PI; 33 mM sodium citrate, pH 8, and 0.1% Triton X-100) diluted 1:2 in DMEM. Data from 20,000 events per sample were acquired by using a flow cytometer (FACSCalibur®; BD Biosciences) and analysed via CellQuest software (BD Biosciences). The percentage of cells in G0/G1, S, and G2/M stages of the cell cycle was determined by using FlowJo analysis program (TreeStar, OR, USA). Cell cultures grown in medium with reduced FBS content (0.5%) were used as positive controls. Serum reduction is a standard method to reduce the amount of cells entering the cell cycle[21].

Comet assay

The induction of DNA strand breaks was evaluated in the alkaline comet assay, performed according to the protocol described in[22], with minor modifications to obtain a consistent DNA migration in control cells and a subsequent higher assay sensitivity[23]. Briefly, after treatments, cells were collected by trypsinization, and cell viability was assessed using the Trypan blue dye exclusion method (TBDE). For each treatment, 1×10^5 cells were resuspended in low melting point agarose (LMA; 0.5% (w/v); 37 °C) and sandwiched on microscope slides between a lower layer of 1% normal melting agarose (NMA; 1%, w/v) and an upper layer of LMA (0.5%, w/v). Following 1 h immersion in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 1% *N*-laurylsarcosine, 1% Triton X-100 and 10% DMSO), slides were placed in a horizontal gel electrophoresis tank with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2 EDTA, pH 13) for 40 min at 4 °C for equilibration and DNA unwinding. Using the same buffer, electrophoresis was carried out for 40 min at 30 V by using an Amersham Pharmacia Biotech power supply (Uppsala, Sweden) and by adjusting the current to 340 mA. The slides were rinsed three times with 0.4 M Tris–HCl, pH 7.5, and rinsed again in distilled water and air-dried in the dark. Immediately before analysis, slides were stained with 12 µg/mL ethidium bromide. For each treatment, images of 500 randomly selected nuclei (250 from each duplicate slide) were analyzed by using a computerized image analysis system (Delta Sistemi, Rome, Italy) fitted with a Leica DMBL fluorescence microscope (Leica Microsystems, Mannheim, Germany) at 200× magnification. DNA integrity was measured as percentage of DNA migrated in the comet tail.

Statistical analysis

For each condition tested three to four independent experiments were carried out.

The two-tailed unpaired Student's *t* test was used to compare negative (incubator) and positive controls. To compare sham-exposed samples, RF-exposed samples and negative controls the one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference) was applied using MATLAB software

(The MatWorks, Natick, MA). Differences were considered statistically significant for p values lower than 0.05 with: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

Reverberation chambers are suitable to host cell cultures for RF exposure

To assess if differences occurred in cell cultures hosted in a standard cell culture incubator (control) and in the two RCs set with no field (sham–sham configuration), cell viability and cell cycle progression were tested.

Despite a slight decrease in cell viability in cultures sham exposed for 24 h compared to incubator controls was recorded, both among samples hosted in the four positions of the same RC, and between the ones in the two RCs, it resulted not statistically significant.

The results of resorufin production are reported in Fig. 4, Panel A, as mean ± SD of three independent experiments. Treatments with 2% ethanol served as positive control and resulted in a 75% reduction in cell viability, as expected (two tailed unpaired Student's t test: $p < 0.01$).

Cell cycle progression also resulted unaffected by comparing cell cultures grown for 24 h in the RCs and in a standard incubator, while positive control treatments with reduced FBS content (0.5%) induced a reduction of cells in G2/M stage ($p < 0.01$), accompanied by a slight increase of cells in G0/G1 stage ($p < 0.05$). The results of three independent experiments are reported in Fig. 4, Panel B where the relative percentage of cells in different stages of cell cycle is provided as mean ± SD.

RF exposure does not induce alterations in cell cycle progression

After 3 h exposure to 26.5 GHz, 1.25 W/kg SAR, no change in cell cycle progression was detected. The results, expressed as mean ± SD of four independent experiments, are reported in Fig. 5, where the relative percentage of cells in different stages of cell cycle is provided for both CW exposure (Panel A) and 5G modulated signal (Panel B). Treatment with 0.5% FBS served as positive control and worked properly, inducing a significant reduction of cells in G2/M stage ($p < 0.01$) and a slight increase of cells in G0/G1 stage ($p < 0.05$).

RF exposure does not induce DNA damage and does not modify the MD-induced damage

The alkaline comet assay was applied to investigate DNA damage in SH-SY5Y cultures exposed for 3 h to 26.5 GHz, 1.25 W/kg SAR, either alone or in combination with MD. The results, expressed as % of DNA in the tail, are shown in Fig. 6 as mean ± SD of four independent experiments. Panel A refers to CW exposure, while in Panel B the results of 5G-modulated signal are reported. At both signals investigated, RF exposure alone did not alter the spontaneous DNA damage (RF vs. Sham) and, in combined treatments, pre-exposure to both signals investigated did not change the MD-induced damage (RF + MD vs. Sham + MD), indicating lack of effects due to combined exposure. Statistically significant increase of DNA damage was detected in all the MD-treated samples compared to the corresponding controls, as expected (MD vs. control; Sham + MD vs. Sham, RF + MD vs. RF: $p < 0.001$).

Discussion

Despite the worldwide deployment of the fifth-generation of cell phone technology in the different frequency range since 2019, very few research has been published on its biological or health-related effects.

In this study, a reverberation chamber-based exposure system was suitably designed and realized to expose cell cultures to 26.5 GHz under strictly controlled conditions in terms of both dosimetry and biological parameters. For the sake of methodological quality, and thus to assure the reliability and robustness of the experimental results, we consider electromagnetic and biological aspects with equal importance to reduce errors and ensure the reproducibility of the RF experiments by controlling the major conditions. The exposure system was numerically and experimentally characterized from a dosimetric point of view, demonstrating a high degree of SAR uniformity in the exposed samples in terms of CV. The suitability of the reverberation chambers made up by metallic boxes, used as RF applicators to host cell cultures without affecting their physiological condition, was proven by analyzing cell viability and cell cycle. To this purpose, the two RCs were set in sham configuration having the incubator as reference control.

The appropriateness of the biological procedures to detect effects, if induced, was also verified by establishing positive control cultures with treatments inducing the effect under investigation. Finally, blind analysis was assured, i.e. the researchers involved in sample processing were not aware of the exposure/treatment, and data were decoded after completion of the analyses.

The above mentioned aspects are of primary importance given that the effect of RF exposure on cell cultures is predicted to be weak, and small variation of physiological status of the cell cultures can contribute skewing the results.

Despite the importance of conducting good quality investigations has been highlighted for many years, it appears evident that it has not entered into common practices[7,18,24]. As a matter of fact, a large number of investigations suffer from methodological issues which greatly affect the reliability of the results.

Under the experimental conditions here adopted, we demonstrated that RF exposure of SH-SY5Y human neuroblastoma cells for 3 h to 26.5 GHz, 1.25 W/kg SAR, was not able per se to affect cell cycle progression and to induce DNA damage, consistently with the majority of published results on several cell models and different frequencies/signals of previous mobile phone technologies[7,25,26]. Moreover, moving from our previous in vitro investigations in the lower frequency bands which demonstrated that RF-EMF is able to modify the effects of other physical or chemical agents, we also tested here this possibility by using menadione as chemical treatment administered after RF exposure, with negative results. The lack of effects following co-exposure was also detected when V79 Chinese hamster fibroblasts were pre-exposed to 1950 MHz, LTE signal for 3 h but not for 20 h, and

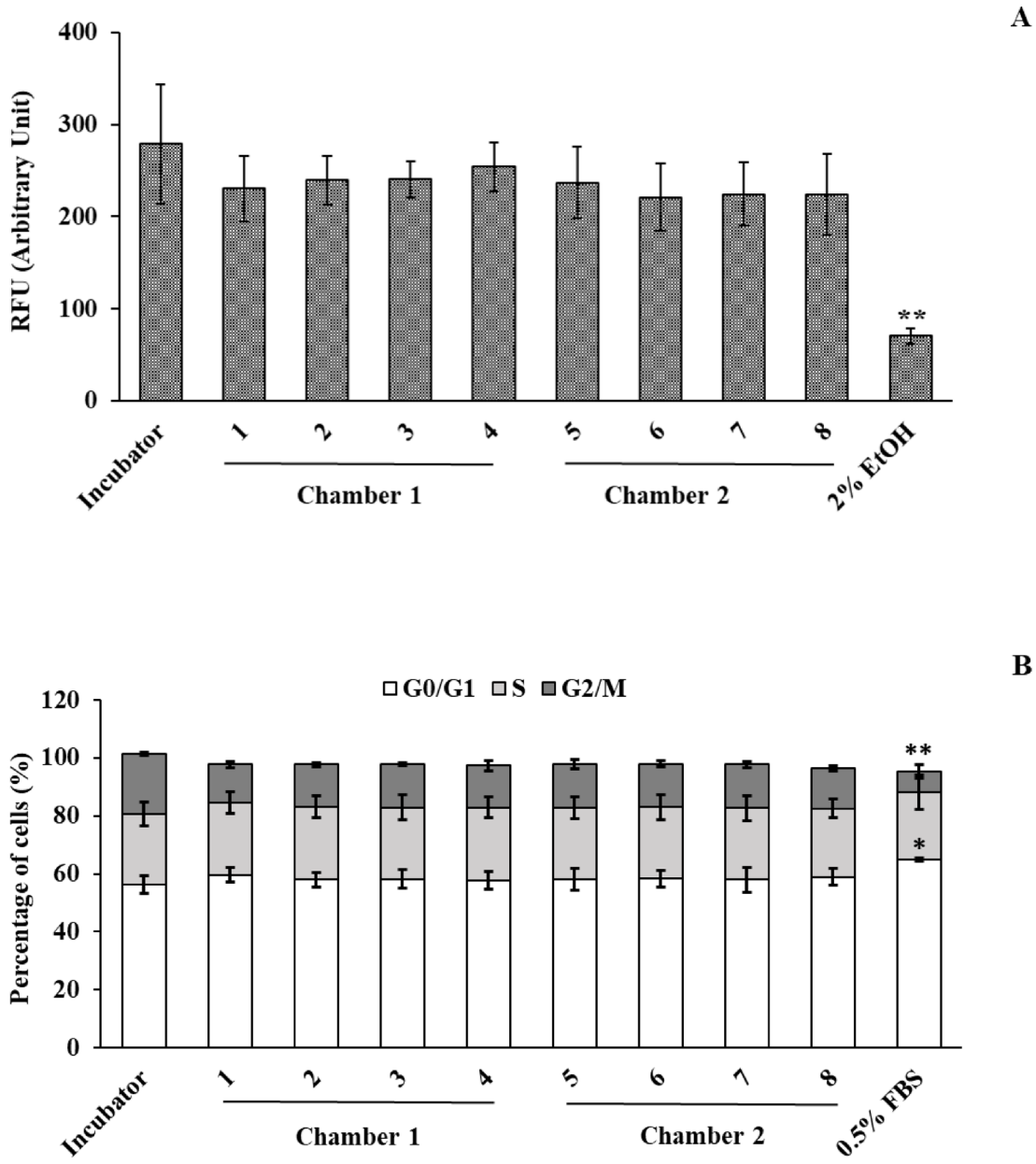

Fig. 4. Cell viability (Panel **A**) and cell cycle progression (Panel **B**) of SH-SY5Y cells sham exposed for 24 h in the two reverberation chambers and hosted in four different positions (chamber 1, position 1–4; chamber 2, position 5–8). Results of treatments with 2% ethanol (EtOH) and 0.5% FBS are also reported as positive control for cell viability and cell cycle progression, respectively. Data are expressed as mean ± SD of resorufin production in arbitrary units (RFU) and percentage of cells in the different stages of cell cycle (three independent experiments). Two-tailed unpaired Student's *t* test: $^{*}p < 0.05$; $^{**}p < 0.01$ versus incubator.

subsequently treated with mitomycin-C[25]. On the contrary, when SH-SY5Y cells were pre-exposed for several exposure durations from 1 to 20 h and treated with MD, a consistent reduction of the MD-induced effect was detected for all the exposure durations investigated[13]. These observations definitely highlight the role of cell model and electromagnetic conditions in eliciting cooperative effects.

The lack of effects of RF exposure and co-exposures detected in the present investigation were achieved when either CW or 5G modulated signal were used.

It is worth mentioning that this comparison is missing in the majority of the investigations available in the literature, although several authors pinpoint that the presence of signal modulation exerts a critical role in eliciting a biological response with respect to CW[27,28].

Concerning in vitro investigations addressing the effects of 5G technology, to the best of our knowledge data available in the literature deal with both FR1 and FR2.

The FR1 band was investigated by a French research group. Canovi and co-workers found absence of effects on neuronal activity of cultures from embryonic cortices subjected to exposure duration and SAR levels

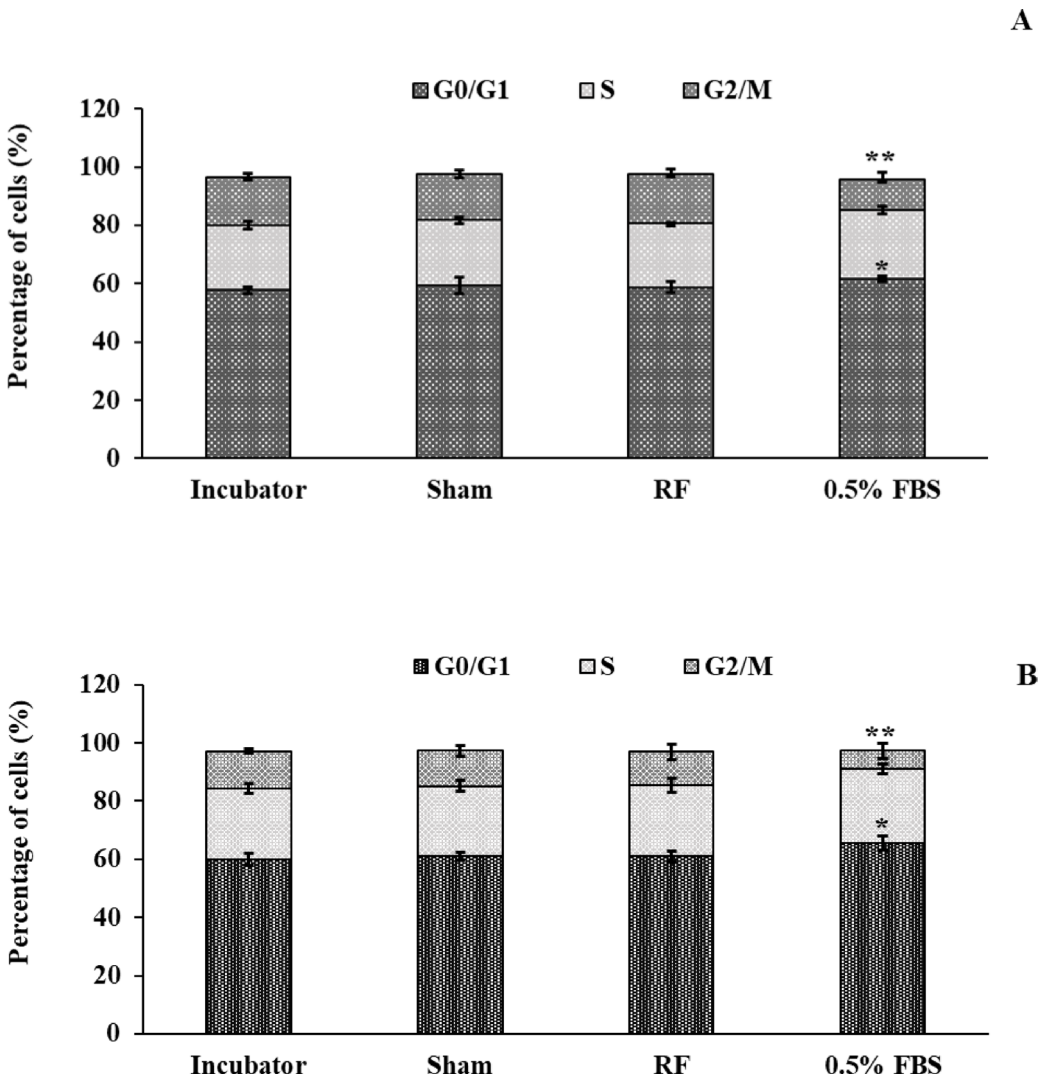

Fig. 5. Cell cycle progression of SH-SY5Y cells exposed for 3 h to 26.5 GHz, 1.25 W/kg SAR, to CW (Panel **A**) and 5G signal (Panel **B**). Results of cultures grown with reduced FBS content (0.5% FBS) are also reported as positive control. Data are expressed as mean ± SD of four independent experiments. Two-tailed unpaired Student's *t* test: $*p < 0.05$; $**p < 0.01$ versus incubator.

comparable to the ones here adopted at both CW and 5G modulated signal at 3.5 GHz. Instead, when 28 W/kg SAR was used, a decrease in bursting and total firing rates during exposure was elicited[29]. Moreover, the same research group also reported absence of effects on molecular pathways involved in environmental cell-stress responses in human keratinocytes and fibroblasts exposed and co-exposed with MG132, a proteasome inhibitor, for 24 h to 3.5 GHz, CW and modulated signal at 0.25, 1, and 4 W/kg SAR levels[30].

In a more recent study, human fibroblasts (Xp6eb cells) and keratinocytes (KHAT cells) were exposed to 3.5 GHz, 5G-modulated signal for 24 h at 0.25, 1, and 4 W/kg SAR, and co-exposed with UVB light. The authors did not detect effects on mitochondrial membrane potential and apoptosis for all the experimental conditions investigated. On the other hand, MitROS resulted decreased in Xp6eb cells exposed to RF alone at 1 W/kg and increased in KHAT cells co-exposed to RF at 0.25 and 1 W/kg and UVB[31].

A Korean research group focused on FR2 band. Kim et al. exposed a murine melanoma cell line (B16F10) and human melanoma cells (MNT-1) to evaluate the effect on cell viability and skin pigmentation, respectively after 4 h/day exposure for 2 days to 28 GHz, 10 W/m^2, 5G radiation. The results indicated the lack of effects. The absence of effects on skin pigmentation was also confirmed in MNT-1 and HaCaT co-cultures[32,33].

In a follow-up study, the authors examined the effect of the same exposure conditions for a longer exposure duration (4 h/day for 16 days), in presence and in absence of α-MSH, a melanin synthesis inducer. The results gave absence of effects of RF alone, while co-exposures resulted in a decrease of α-MSH-induced skin pigmentation, in absence of alteration in cell viability. The authors hypothesized an attenuation of ROS as possible mechanism underneath the observed effect[33].

We highlight that, in the two studies by Kim and co-workers, the authors did not specify if CW or modulated 5G radiation was used and did not include sham exposures as reference control.

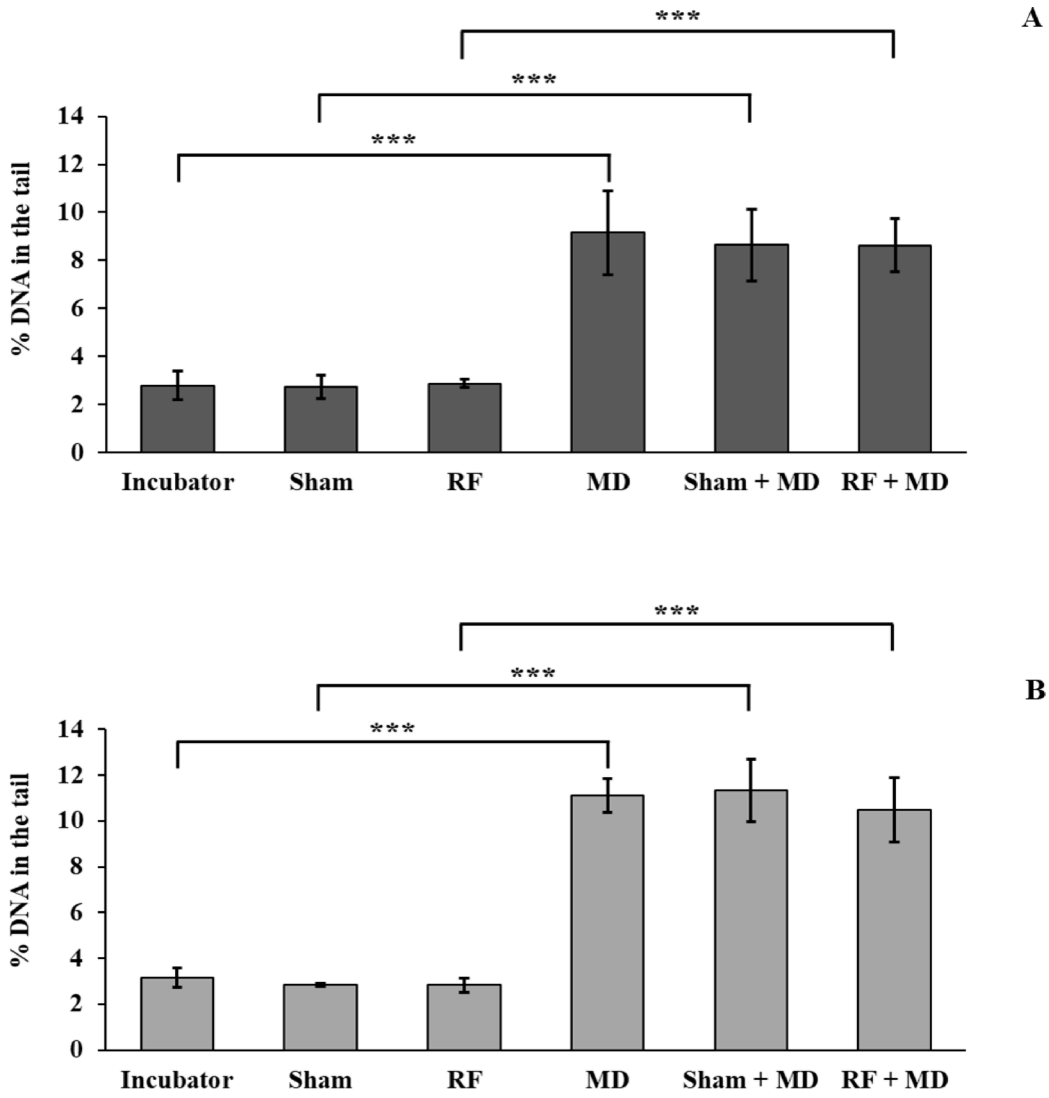

Fig. 6. Percentage of DNA in the tail in SH-SY5Y cells exposed for 3 h to 26.5 GHz, 1.25 W/kg SAR, or co-exposed to 10 μM menadione (MD). Panel (**A**) and (**B**) refer to CW and 5G signal, respectively. For each condition 500 nuclei were examined. Results are expressed as mean ± SD of four independent experiments; one way ANOVA for repeated measurements followed by post-hoc Tukey test (***$p < 0.001$).

A few other in vitro studies are available in the literature which we did not include here owing to flaws related to electromagnetic characterization of exposure.

A higher number of in vivo investigations is available in the literature. Nine studies used murine models exposed to CW in the range 2.4–4.9 GHz while only one study used 28 GHz, and one a 5G modulated signal. Mainly, long intermittent (on/off cycles) exposure durations were tested at SAR levels ranging from 0.059 to 10 W/kg. All the studies employing CW exposure gave alteration in several parameters including oxidative stress[34], temperature[35], metabolic profile[36], perturbation in the descendants after gestational exposure[37] and several behavioral parameters[38–41]. On the contrary, the study employing the 5G modulated signal gave no effect on physiological parameters and cognitive abilities after a 5 week long whole body exposure of rats at SAR of 0.0076 and 0.0059 W/kg[42].

Four studies have been carried out on Zebrafish exposed to CW RF in the range 700 MHz-27 GHz and 1.1 and 8.27 W/kg SAR for several exposure durations to assess effects on fertilized eggs and on several developmental stages, with negative results[43–46]. Two studies have been carried out on Drosophila Melanogaster with effects on thermal stress, oxidative stress and humoral immunity after 3 days exposure at 3.5 GHz and 0.1, 1 and 10 W/m^2 power density[47,48].

We want to highlight that in most of the above-mentioned animal studies 5G modulated was not used. Moreover, some of them suffer of adequate dosimetry and do not use sham exposure as reference control, which greatly affect the reliability of the results.

One study deals with the effects on healthy volunteers where no alteration in brain activity was detected after 26 min at 3.5 GHz, 2 V/m 5G modulated RF exposure followed by 17 min post exposure[49].

In conclusion, to the best of our knowledge this is the first study which addresses the effects of 26.5 GHz RF exposure and co-exposure in mammalian cells and compares the effect of CW and 5G modulated signal under the same experimental conditions.

While highlighting the strength of this study which relies on the attention paid to both biological and technical aspects with equal importance to reduce errors and ensure the reproducibility of the experiments by controlling the major conditions in the experiments, and the direct comparison between CW and 5G modulated signal, it is worth mentioning that here preliminary assessment of the effect of exposure/co-exposure to 5G signal in the FR2 frequency band is reported, which lays the foundations for more extended investigations. As a matter of fact, neuroblastoma cells were used as biological model and menadione as agent for co-exposure to have a direct comparison with our previous results on this cell model in different frequency bands. Cell models from superficial tissues are even more interesting due to the low penetration depth of RF-EMF in the sub-millimeter wave band. Also, a 3 h exposure duration was tested in this study since it is more realistic than longer durations in a 5G scenario. On the other hand, longer exposure periods are also interesting from a mechanistic point of view, as well as other 5G modulation schemes. Cell cycle progression and primary DNA damage were tested due to their critical role in the cellular processes but other endpoints are of interest as well by moving from the results available in the literature and related to the lower frequency band.

Data availability

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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Author contributions

M.R.S., O.Z., G.P. and S.R. conceived the experiments. A.S., M.A., V.P, and G.C. conducted the experiments. A.S., S.R., O.Z., M.A., F.S. and M.R.S. analyzed the data. O.Z. S.R. and M.R.S wrote and edited the manuscript. All authors reviewed the manuscript. Funding acquisition: M.R.S., F.S. and O.Z.

Declarations

Competing interests

The authors declare no competing interests.

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