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Reactive Oxygen Species Formation Is Not Enhanced by Exposure to UMTS 1950 MHz Radiation and Co-Exposure to Ferrous Ions in Jurkat Cells

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This study was designed to assess if radiofrequency (RF) radiation induces oxidative stress in cultured mammalian cells when given alone or in combination with ferrous ions ($FeSO_4$). For this purpose the production of reactive oxygen species (ROS) was measured by flow cytometry in human lymphoblastoid cells exposed to 1950 MHz signal used by the third generation wireless technology of the Universal Mobile Telecommunication System (UMTS) at Specific Absorption Rate of 0.5 and 2.0 W/kg. Short (5–60 min) or long (24 h) duration exposures were carried out in a waveguide system under strictly controlled conditions of both dosimetry and environment. Cell viability was also measured after 24 h RF exposure using the Resazurin and Neutral Red assays. Several co-exposure protocols were applied to test if RF radiation is able to alter ROS formation induced by $FeSO_4$ (RF given before or concurrently to $FeSO_4$). The results obtained indicate that non-thermal RF exposures do not increase spontaneous ROS formation in any of the experimental conditions investigated. Consistent with the lack of ROS production, no change in cell viability was observed in Jurkat cells exposed to RF radiation for 24 h. Similar results were obtained when co-exposures were considered: combined exposures to RF radiation and $FeSO_4$ did not increase ROS formation induced by the chemical treatment alone. In contrast, in cultures treated with $FeSO_4$ as positive control, a dose-dependent increase in ROS formation was recorded, validating the sensitivity of the method employed. Bioelectromagnetics 30:525–535, 2009.

Key words: radiofrequency; UMTS; lymphoblastoid cells; oxidative stress; combined exposures

INTRODUCTION

The widespread use of wireless communication devices has led to the consideration of health effects of radiofrequency (RF) radiation. A lot of attention has been devoted to the possible biological effects of the exposure to electromagnetic fields at frequencies used in second generation (2G; 900–1800 MHz, GSM signal) mobile phones. Recently, the Universal Mobile Telecommunication System (UMTS) signal, a digital technology of third generation (3G) cellular systems, has been developed as a standard, allowing multimedia services. At the moment it is the most widespread in Europe and it is expected that in the near future its use will be increased.

Although large similarities exist between 2G and 3G technologies, the low frequency spectral content

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differs significantly due to the distinct modulation techniques. The 2G signals use the Gaussian Minimum Shift Keying (GMSK) modulation, have a high coherence, extremely low frequency amplitude modulation spectra, high crest factor (pulsed signal) and a power regulation with an update in the order of seconds. In contrast, the 3G Wideband Code-Division Multiple Access (WCDMA) uses essentially Quadrature Phase Shift Keying (QPSK) modulation, has a low coherence and a broad-band extremely low frequency amplitude modulation spectrum. The latter results from the fast and dynamic power control and amplitude variations in the MHz range due to the spreading that may induce higher biological effects because of potential ''effective'' frequency windows [Penafield et al., 1997]. Taking into account the potentiality of the low frequency amplitude variation to evoke biological responses, the data already obtained on the biological effects of 2G technologies needs to be extended to 3G.

In this respect, to our knowledge, up to now few in vivo and in vitro studies have been published by employing UMTS signal and the results are still inconclusive. No effects have been detected on visual perception [Schmid et al., 2005] or on cognitive symptoms [Riddervold et al., 2008; Unterlechner et al., 2008]. Sommer et al. [2007] also failed to find effects on development of lymphoma in AKR/J mice exposed to a generic UMTS test signal at Specific Absorption Rate (SAR) of 0.4 W/kg. Furthermore, Franke et al. [2005] reported no effects on permeability of the blood brain barrier and morphology of porcine brain endothelial cells continuously exposed for up to 84 h to RF radiation with a generic UMTS signal (maximum SAR of 1.8 W/kg). The induction of DNA damage in human peripheral blood lymphocytes exposed in vitro to 1950 MHz at SAR values of 0.5 and 2 W/kg was also investigated and no effects were detected after continuous (24 h) [Sannino et al., 2006; Manti et al., 2008] or intermittent (on/off cycles) exposures [Zeni et al., 2008]. In contrast, in a recent study a significant increase in DNA Single Strand Breaks (SSB) and in micronuclei formation was detected in cultured human fibroblasts, but not in lymphocytes, after 1950 MHz RF exposure, either continuous and intermittent (5 min RF on/10 min RF off) at a SAR ranging from 0.05 and 2 W/kg [Schwarz et al., 2008]. In addition, Belyaev et al. [2009] found changes in chromatin conformation of lymphocytes exposed for 1 h to 1947.4 MHz using a UMTS signal with an output power of 0.25 W.

Among the cancer-related endpoints, oxidative stress is of crucial interest since it has been suggested as a possible mechanism of action for non-genotoxic carcinogenesis [Klauning et al., 1998; Laval et al., 1998]. Free radicals such as reactive oxygen species (ROS) are generated during a variety of biochemical reactions and cellular functions as by-products of the normal metabolism of oxygen. Exogenous factors can also generate oxygen radicals, for example, alcohol, cigarette smoke, environmental pollutants and radiation [Moller et al., 1996]. Oxidative stress occurs when the production of ROS overrides the antioxidant capability of the target cell. ROS are unstable molecules that have an unpaired electron in their outer shell. They react with several cellular components, including proteins, lipids and DNA, resulting in altered cell functions. Thus, formation of ROS is an important mechanism to take into account in risk evaluation of RF radiation exposure.

This study was designed to investigate cell viability and ROS formation in human lymphoblastoid cells exposed to 1950 MHz, UMTS signal, for short (1 h) or long (24 h) exposure duration. The kinetics of ROS formation was also followed in the range of 5–60 min upon RF exposure. To investigate if RF radiation may modify ROS production induced by a well known oxidative stress inducer, combined exposures with ferrous ions (Fe^{2+}) were also carried out. This ion acts by producing a very reactive free radical, the hydroxyl radical ($OH^{\cdot}$), involving the Fenton's and Haber-Weiss reactions [Halliwell and Gutteridge, 1992; Smith et al., 1995].

MATERIALS AND METHODS

Reagents

RPMI-1640 and foetal bovine serum (FBS) were from Biowhittaker (Verviers, Belgium); penicillin–streptomycin and L-glutamine were from Gibco (Milan, Italy); Triton X-100, Neutral Red, DTT, Resazurin, glucose-6-phosphate and gelatin were from Sigma (St. Louis, MO). Trypan blue was from BDH (Poole, England), NaCl, acetic acid and iron (II) sulphate were from Carlo Erba (Milan, Italy) and Tris/HCl was from Applichem (Darmstadt, Germany). Ethanol and EDTA were from Baker (Deventer, The Netherlands).

Exposure System Set-Up and Dosimetry

Cell cultures were exposed or co-exposed to a microwave signal used by UMTS mobile communication systems. The experiments were carried out at 1950 MHz, which is an uplink frequency of the UMTS system, and the WCDMA standard was used, according to the 3GPP 3.5 2001-03 specifications (five power-controlled user data channels + one control channel). The SAR values investigated were 0.5 and 2 W/kg which are within the currently accepted safety limit for the cellular-telephone microwave emission (2 W/kg), as

suggested by the International Commission on Non-Ionising Radiation Protection [ICNIRP, 1998].

A standardised exposure device was designed and tested on the basis of efficiency and field uniformity criteria, as accurately described elsewhere [Calabrese et al., 2006; Sannino et al., 2006]. Briefly, the signal used was generated by an Agilent E4432B ESG-D series generator (Agilent, Santa Clara, CA), amplified (Microwave Amplifiers, AM38A-092S-40-43, North Somerset, Bristol, UK) and fed through a bidirectional power sensor (NRT-Z43, Rohde & Schwarz, Munich, Germany) into the exposure chamber: a rectangular short-circuited waveguide (WR 430: 109.2 mm $\times$ 54.6 mm; length 500 mm) whose feeding end was a coaxial waveguide adapter (Maury Microwave R213A2, VSWR:1.05, Mont Clair, CA). An identical waveguide was used for sham exposures. Both waveguides were housed in a commercial incubator at 37 °C in a 95% air/5% CO_2 atmosphere. The power sensor and signal generator were connected to a dedicated computer. Software, developed in-house, based on a commercially available program (LabVIEW 7, National Instruments, Austin, TX) provided a user-friendly system interface and continuous control of the power level that was adjusted to the required SAR. The experiments were performed in blind, monitoring and recording incident and reflected powers.

The SAR was evaluated as the ratio between the power absorbed by the sample (P_a) and its mass, with P_a being the difference between the incident (P_i) and the reflected (P_r) powers, both measured by the bidirectional power sensor. The exposure set-up is shown in Figure 1.

Exposure of more than one sample under the same electromagnetic conditions was obtained by exploiting the symmetries of the waveguide and of the unperturbed mode (TE_{10}) [Pozar, 1993] as well as those of the exposed samples. In particular, inserting four Petri dishes (each filled with 3 ml of cell suspension), a higher SAR value occurs in the inner pair of samples in comparison with the outer pair and, by properly setting the distances between the centres of the samples, a ratio of 4 can be obtained between the inner and outer SAR values (Fig. 2). In addition, because the induced electric field was essentially parallel to the sample surface, a satisfactory uniformity was achieved in the spatial distribution of the electric field along the vertical direction and across the samples [Calabrese et al., 2006; Sannino et al., 2006; De Prisco et al., 2008].

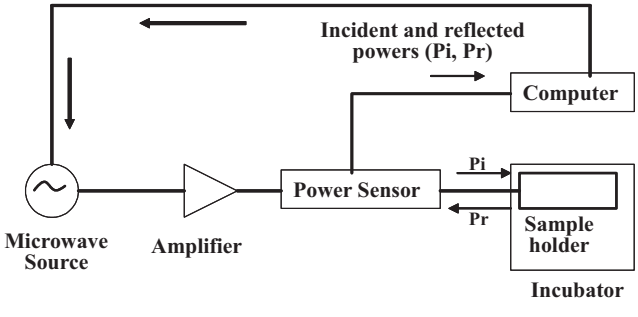

Fig. 1. Sketch of the experimental radiofrequency set-up for exposures and SAR measurements.

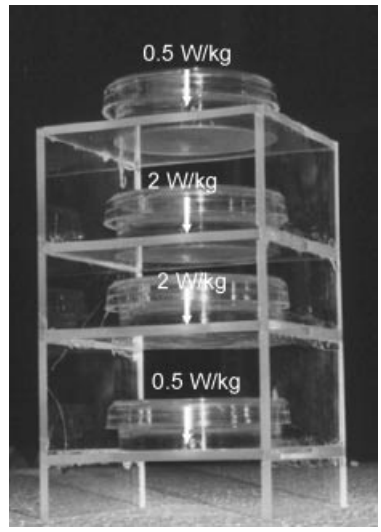

Fig. 2. Photo showing the four-dish plastic stand to host the cell cultures inside the waveguide.

The results of the numerical and experimental dosimetry carried out for this scenario showed that, when the centre of the samples was at a distance from the short circuit of $0.56\lambda_z$, the efficiency of the system (the ratio between the absorbed power P_a and the incident power P_i, i.e., $\eta = P_a/P_i$) was about 70% and the degree of non-uniformity, numerically evaluated as the ratio between the SAR standard deviation and the average SAR, was 0.33 in all four samples.

In this work, on the basis of the biological parameters to be investigated, cell cultures were exposed in complete culture medium (RPMI), in PBS-gel (a serum-free medium made by PBS added to 0.1% gelatine [Robinson, 1993]) or in PBS-gel combined with 50 μM $FeSO_4$. The relative permittivities ε_r and effective conductivities σ_{eff} (without cells) were measured at 37 °C by wide-band measurements using a microwave vector network analyser (Wiltron-Anritsu 37269B, Kanagawa, Japan) and open-ended coaxial probe technique [Anderson et al., 1994; Bèrubè et al., 1996]. The measured values were very similar ($\varepsilon_r = 78.3$ and $\sigma_{eff} = 1.8$ S/m $\pm$ 5%), showing that the differences in the exposed samples are not appreciable in terms of permittivity.

To avoid undesired temperature increase in the exposed cultures due to electromagnetic power dissipated in the medium, preliminary experiments were carried out to monitor temperature time course during the exposure using a fibre-optic thermometer (Fiso UMI 4, FISO Technologies, Quebec, Canada) with fibre-optic temperature probe (FISO Technologies, FOT-M/2m) inserted horizontally in the culture medium. Temperature readings were taken at 5 s intervals and recorded by computer. At the maximum SAR level investigated (2 W/kg), temperature during 24 h exposure did not exceed the accuracy range of the instrument (±0.3 °C).

Cell Cultures and Experimental Design

Jurkat cells (human lymphoblastoid T-cells) [Schneider et al., 1977] were kindly provided by Dr. I. Tedesco (CNR-Institute of Food Science and Technology, Avellino, Italy). They were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and grown in a humidified 5% CO_2 atmosphere at 37 °C. Cells were seeded in complete medium at a density of $2–3 \times 10^5$ cells/ml and kept in exponential growth phase by passages at 2- to 3-day intervals. A master bank was established and, for experiments, cells from passages 4–10 were used. Each experimental run was carried out on cells split from the same parent flask.

Several experimental conditions were tested to measure ROS formation after RF exposure (SAR values of 0.5 and 2 W/kg) and co-exposure to $FeSO_4$. They are summarised in Table 1. In particular: (1) the kinetics of ROS formation after RF exposure alone was followed within 1 h. Cells were sham/RF-exposed for the last 5′, 15′, 30′, 45′ or for the entire hour; positive controls were treated with 100 μM $FeSO_4$ for the same exposure duration; (2) ROS formation was measured after 24 h sham/RF exposure; (3) the kinetics of ROS formation after RF exposure was followed within 1 h of treatment with $FeSO_4$. Cells were incubated with 50 μM $FeSO_4$ for 1 h and during this period they were sham/RF-exposed for the last 5′, 15′, 30′, 45′ or for the entire hour; (4) ROS formation induced by 1 h $FeSO_4$ in cells pre-exposed to RF for 1 or 24 h was evaluated. Cells were sham/RF-exposed for 1 or 24 h and then incubated for 1 h with 50 μM $FeSO_4$. In conditions 2–4 concurrent positive controls were provided by treating cells with 100 μM $FeSO_4$ for 1 h.

TABLE 1. Experimental Conditions Adopted for RF Exposures at Frequency of 1950 MHz (0.5 and 2 W/kg SAR Values) and Co-Exposures With 50 μM $FeSO_4$

	RF exposure	$FeSO_4$
1	From 5 min to 1 h	—
2	24 h	—
3	From 5 min to 1 h	1 h (concurrent RF)
4	1 h/24 h	1 h (after RF)

Cell viability was also investigated after 24 h sham/RF exposure at both SAR values by applying the Neutral Red and Resazurin assays. To prove the responsiveness of the cell line used in the study, for both assays positive control experiments were carried out treating cells with 5% and 10% ethanol for 1 h. For each experimental condition three independent experiments were carried out.

ROS Measurement

To quantify intracellular ROS production the fluorescent probe 2′7′-dichlorofluorescein-diacetate (DCFH-DA) was employed. It is a non-polar compound that easily penetrates in the cell membrane and is hydrolysed by intracellular esterases to its non-fluorescent polar derivate DCFH. In the presence of ROS, DCFH is oxidised to the fluorescent dichlorofluorescein (DCF) [Le Bel et al., 1990].

Cells were loaded with DCFH-DA PBS-gel. This was used because serum contains endogenous esterase activity and de-esterified DCF is cell permeable and can generate inconsistent data, as stressed by Halliwell and Whiteman [2004]. In particular, DCFH-DA (10 μM final concentration) was added to cell cultures (10^6 cells in 3 ml PBS-gel) and incubated for 1 h at 37 °C. Cells were then pelletted (1,200 rpm for 5 min), washed twice in cold PBS and DCF fluorescence was measured by a flow cytometer (FACScalibur, Becton & Dickinson, San Jose, CA) equipped with a 488 nm argon laser. For each sample, 10,000 events were acquired using CELL QUEST software and analysed as described in detail in Data Analysis Section.

Cell Viability

Exponentially growing cells were seeded in coded Petri dishes (Corning, New York, NY, cat. No. 430165) at a density of 0.5×10^6/ml. For each experimental run, eight cultures were set up to test four conditions in duplicate: negative control (cultures left in a CO_2 incubator), sham exposure, exposure to RF radiation at 0.5 W/kg and exposure to RF radiation at 2.0 W/kg. After treatments, for each sample 1 ml culture was used to perform Neutral Red assay and the remaining 2 ml were used to applying the Resazurin assay.

Neutral Red assay. The Neutral Red is a cationic dye which passes through cell membrane and accumulates inside lysosomes in a process requiring cellular energy. Chemical or physical treatments that damage lysosomal

membranes or interfere with the normal energy-requiring endocytosis process will decrease the ability of cells to take up Neutral Red [Fautz et al., 1991; Putnam et al., 2002]. The Neutral Red assay was carried out as follows: samples were treated with 0.066% (v/v final concentration) Neutral Red for 3 h, washed in PBS and lysed in 200 µl of cold lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM DTT, 1% Triton X-100) containing 1% acetic acid and 50% absolute ethanol. The absorbance of lysed cells was measured spectrophotometrically at 540 nm and the absorbance of negative controls was set to represent 100% viability.

Resazurin assay. The Resazurin system measures the metabolic activity of living cells [O'Brien et al., 2000]. Resazurin (blue and non-fluorescent) is reduced to resorufin (pink and highly fluorescent) by oxidoreductases in mitochondria. Thus, measurement of resorufin fluorescence is an indicator of mitochondrial function [Zhang et al., 2004]. Therefore, a direct correlation exists between the reduction of Resazurin in the growth medium and the metabolic activity of living cells. Cells were incubated at 37 °C with 10 µg/ml Resazurin for 1 h. After centrifugation, the production of resorufin was analysed in supernatants with a fluorometer (Perkin-Elmer, LS50B, Perkin Elmer Instruments, Norwalk, CT) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The results were compared to negative controls, set to represent 100% viability.

Data Analysis

Statistical analysis of ROS data was performed comparing, for each experiment, cytometric raw data of treated and untreated samples by applying a modification of the Kolmogorov–Smirnov (KS) test, as reported in Brescia and Sarti [2008].

The ROS data are not normally distributed, thus the use of parametric tests is inadequate. Moreover, the classical non-parametric statistical tests, such as the Wilcoxon signed-rank test for repeated measurements, are not appropriate for evaluating variations among samples with different treatments. The KS test, commonly provided by most flow cytometric data-analysis programs, takes into account the entire population; however, it is too sensitive to experimental bias due to procedure or to the instrument uncertainties [Lampariello, 2000; Brescia and Sarti, 2008].

A first modification of KS test was proposed by Lampariello [2000]. In particular, given c_i $(i = 1{:}n)$ n histograms of the control, $\overline{c_i}$ the mean value of the single histogram c_i and c_m the mean value of all $\overline{c_i}$, in the Lampariello approach, for each experiment the cut-off values of the shift (S_M) occurring in the measurements is evaluated as the maximum deviation $|s_i| = |c_m - \overline{c_i}|$, as well as $|x|$ the integer nearest to the deviation $|c_m - \bar{t}|$ with $\bar{t}$ being the mean value of the histogram under test.

If $|x|$ is greater than S_M, it can be asserted that the test sample is positive; otherwise the values $\overline{r_t}$ and L should be compared, where $\overline{r_t}$ is the mean value of the ratios r_{it}, calculated for each of the n pair of histograms $c_i - t$:

$$r_{it} = \frac{1}{D_{\text{crit}}} \max_j |C_i(j - s_i) - T(j - x)| \quad i = 1 : n$$

where D_{crit} is the KS critical value, $C_i(j - s_i)$ and $T(j - x)$ are the cumulative distributions associated with the histograms c_i and t, shifted by s_i and x channels, respectively. L is defined as the cut-off value of the shape calculated as $\overline{r} + 3\sigma_r$ where $\overline{r}$ is the mean value of all ratios r_{il}:

$$r_{il} = \frac{1}{D_{\text{crit}}} \max_j |C_i(j - s_i) - C_l(j - s_l)|,$$
$$i = 1, \ldots n - 1; \quad 1 = i + 1, \ldots, n$$

and σ_r is the standard deviations of $\bar{r}$. If $\overline{r_t}$ is greater than both L and 1, the differences in the shape will be considered positive.

Brescia and Sarti [2008] showed that the Lampariello's procedure applied to ROS data are still too sensitive, due to the variability of ROS production, and the histograms' median values ($\overline{\overline{c_i}}$ and $\overline{\bar{t}}$) should be chosen as representative parameters. Thus, the shifts S_M and $|x|$ have to be calculated with respect to the median values instead of the mean values ($\overline{c_i}$ and $\bar{t}$):

$$S_M = \max\left|c_{\text{me}} + k\sigma_{\overline{\bar{c}}} - \overline{\bar{c}}_i\right| = \max\left|c_{\text{me}} - \overline{\bar{c}}_i\right| + k\sigma_{\overline{\bar{c}}}$$

where c_{me} is the mean value of all $\overline{\bar{c}}_i$, $\sigma_{\overline{\bar{c}}}$ is its standard deviation, and the term $k\sigma_{\overline{\bar{c}}}$ is included to consider the variability among the median values. It was demonstrated that this approach on one hand reduces the high variability in ROS production, detected when samples that received identical treatments are compared, and on the other hand, still permits the identification of very small differences among treatments.

Following this approach, in this work for each experiment the overall variability in control (sham-exposed) samples was estimated by comparing repeated measurements with increasing numbers of acquired events (5,000, 10,000, 15,000, 20,000 and 50,000) and evaluating cut-off values of the shift (S_M) and the shape (L) with a prefixed confidence level (k). Then, for each test sample (RF-exposed, positive control, co-exposed) 10,000 events were acquired and differences both in the shift and in the shape, were evaluated.

Cell viability data were analysed by applying the ANOVA univariate to compare sham-exposed and RF-exposed groups. The statistical tests were performed by R and Octave free softwares.

RESULTS

ROS Production After $FeSO_4$ Treatments

$FeSO_4$ treatment was used as positive control and for combined exposures. Preliminary experiments were carried out to test ROS formation and cell viability of $FeSO_4$ treatments in a dose-range from 50 to 200 μM. One hour treatment of Jurkat cells induced a statistically significant dose-dependent increase in ROS formation (Fig. 3, panel A) without affecting cell viability (data not shown). Moreover, when treatments of 5, 10, 15, 30, 45, and 60 min at 50 μM were performed, ROS formation increased in all cases and peaked after 15 min of treatment (Fig. 3, panel B). The results refer to the shift compared to control cultures, such as the integer nearest to the deviation between the mean value of the medians of the *n* (5) histograms of the sham-exposed and the median of the treated sample. No differences were detected between $FeSO_4$-treated and control cultures in terms of shape; therefore data related to the shape are not reported.

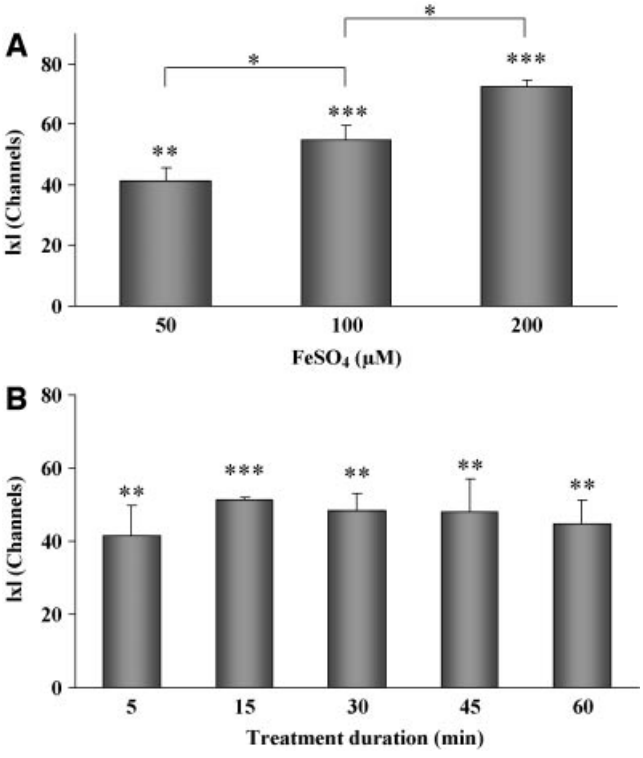

Fig. 3. ROS formation in Jurkat cells treated with $FeSO_4$: panel (**A**) 1 h treatment with 50,100 and 200 μM final concentration; panel (**B**) kinetics of ROS formation with 50 μM $FeSO_4$. Data are expressed as shift channels compared to control cultures and are reported as mean ± SD of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

ROS Production After RF Exposures and Co-Exposure With $FeSO_4$

The results of ROS formation after exposure to 1950 MHz RF radiation, alone or in combination with 50 μM $FeSO_4$, are summarised in Tables 2–5. No statistically significant differences between sham/RF-exposed cultures or between $FeSO_4$ treated and co-exposed cultures were detected for all the exposure conditions or for both SARs investigated. These findings indicate that RF radiation exposure did not alter spontaneous ROS production or the ROS production induced by $FeSO_4$ treatments. Treatments with $FeSO_4$ induced a significant and noticeable increase in ROS production in all the experiments, as expected, validating the sensitivity of the method used to detect ROS formation. In Figure 4 representative flow cytometric histograms are reported.

Cell Viability After RF Exposures

The Neutral Red uptake test and the Resazurin assay did not reveal statistically significant differences among treatments ($P > 0.05$ in all cases), indicating absence of cytotoxic effects due to 24 h exposure to RF radiation for both SAR values investigated. Positive control experiments showed that 1 h treatment with ethanol induced a dose-dependent reduction in cell viability compared to controls (Neutral Red: 38% and 90%; Resazurin: 22% and 70% for 5 and 10% ethanol, respectively) indicating the sensitivity of our assays to treatments with a well known cytotoxic agent. The results obtained are reported in Figure 5, and are expressed as % values compared to control cultures.

DISCUSSION

In this study we have investigated if RF radiation at 1950 MHz, UMTS signal, could modify ROS formation in Jurkat cells exposed or co-exposed in several experimental conditions, such as by varying the exposure duration and SAR levels. The average SARs tested were selected to evaluate conditions below or equal to the currently accepted safety limit for the mobile-phone microwave radiation emission (2 W/kg), as defined by ICNIRP [1998].

In spite of the different experimental protocols used, our findings do not support the hypothesis that RF exposure, alone or in combination with a well known oxidative stress-inducer alters the ROS production in exposed/co-exposed cultures. These results have been obtained after both short (5 min–1 h) and long term

TABLE 2. Kinetics of ROS Formation in Jurkat Cells Sham/RF-Exposed to 1950 MHz at SAR Values of 0.5 and 2 W/kg

		Treatments							
Treatments duration (min)	Experiment		Sham-RF $\kappa = 0.05$	Sham-RF $\kappa = 0.01$	Sham-RF $\kappa = 0.005$		RF-exposure 0.5 W/kg	RF-exposure 2 W/kg	$FeSO_4$ (100 μM)
5	1	S_M	13	25	34	$\|x\|$	2	7	67***
		L	0.60	0.87	1.08	$\overline{r_t}$	0.37	0.39	
	2	S_M	8	16	22	$\|x\|$	2	3	44***
		L	0.59	0.84	1.27	$\overline{r_t}$	0.41	0.37	
	3	S_M	9	17	23	$\|x\|$	6	6	40***
		L	0.68	1.05	1.33	$\overline{r_t}$	0.44	0.75	
15	1	S_M	12	24	33	$\|x\|$	4	4	72***
		L	0.48	0.73	0.91	$\overline{r_t}$	0.57	0.37	
	2	S_M	13	25	34	$\|x\|$	8	11	71***
		L	1.21	2.08	2.73	$\overline{r_t}$	0.46	0.65	
	3	S_M	9	17	23	$\|x\|$	8	7	56***
		L	0.59	0.92	1.16	$\overline{r_t}$	0.44	0.52	
30	1	S_M	8	16	22	$\|x\|$	2	1	27***
		L	0.88	1.49	1.95	$\overline{r_t}$	0.49	0.54	
	2	S_M	12	24	33	$\|x\|$	8	9	64***
		L	1.04	1.72	2.23	$\overline{r_t}$	0.64	0.42	
	3	S_M	8	16	22	$\|x\|$	5	3	36***
		L	0.77	1.47	2.00	$\overline{r_t}$	0.82	0.39	
45	1	S_M	8	16	22	$\|x\|$	7	8	78***
		L	0.76	1.27	1.66	$\overline{r_t}$	0.38	0.29	
	2	S_M	17	33	45	$\|x\|$	2	2	57***
		L	0.62	0.98	1.26	$\overline{r_t}$	0.86	0.53	
	3	S_M	12	24	33	$\|x\|$	10	11	78***
		L	0.85	1.75	2.43	$\overline{r_t}$	0.59	0.63	
60	1	S_M	8	16	22	$\|x\|$	5	6	20**
		L	0.62	0.98	1.25	$\overline{r_t}$	0.53	0.45	
	2	S_M	12	24	33	$\|x\|$	6	3	35***
		L	0.48	0.73	0.91	$\overline{r_t}$	0.50	0.65	
	3	S_M	9	17	23	$\|x\|$	4	1	25***
		L	0.92	1.43	1.82	$\overline{r_t}$	0.99	0.61	

Positive controls were treated with 100 μM $FeSO_4$.
Data concerning sham-exposed samples are reported as cut-off values of the shift (S_M) and of the shape (L) for three confidence levels (k); data on treatments are expressed as shift channels ($|x|$) and shape ($\overline{r_t}$) compared to sham-exposed samples.
**$P < 0.01$.
***$P < 0.005$.

TABLE 3. ROS Formation in Jurkat Cells After 24 h Sham/RF Exposure at SAR of 0.5 and 2 W/kg

		Treatments						
Experiment		Sham-RF $\kappa = 0.05$	Sham-RF $\kappa = 0.01$	Sham-RF $\kappa = 0.005$		RF-exposure 0.5 W/kg	RF-exposure 2 W/kg	$FeSO_4$ (100 μM)
1	S_M	13	25	34	$\|x\|$	9	12	65***
	L	0.67	1.03	1.30	$\overline{r_t}$	0.50	0.58	
2	S_M	17	33	45	$\|x\|$	13	15	47***
	L	0.83	1.29	1.63	$\overline{r_t}$	0.47	0.41	
3	S_M	13	25	34	$\|x\|$	12	9	61***
	L	0.69	1.11	1.43	$\overline{r_t}$	0.73	0.39	

Positive controls were treated with 100 μM $FeSO_4$ for 1 h.
Data concerning sham-exposed samples are reported as cut-off values of the shift (S_M) and of the shape (L) for three confidence levels (k); data on treatments are expressed as shift channels ($|x|$) and shape ($\overline{r_t}$) compared to sham-exposed samples.
***$P < 0.005$.

TABLE 4. Kinetics of ROS Formation in Jurkat Cells Treated for 1 h With 50 μM $FeSO_4$ and Sham/RF-Exposed at SAR of 0.5 and 2 W/kg

		Treatments						
Treatment duration (min)	Experiment		Sham-RF $FeSO_4$ (50 μM)			RF-exposure + $FeSO_4$ (50 μM)		$FeSO_4$ (100 μM)
			$\kappa = 0.05$	$\kappa = 0.01$		0.5 W/kg	2 W/kg	
5	1	S_M	12	24	$\lvert x \rvert$	5	3	12*
		L	0.48	0.69	$\overline{r_t}$	0.44	0.51	
	2	S_M	12	24	$\lvert x \rvert$	5	2	13
		L	0.43	0.56	$\overline{r_t}$	0.85	0.98	
	3	S_M	8	16	$\lvert x \rvert$	2	1	14*
		L	0.98	1.64	$\overline{r_t}$	0.47	0.92	
15	1	S_M	8	16	$\lvert x \rvert$	1	3	12*
		L	0.68	1.10	$\overline{r_t}$	0.67	0.70	
	2	S_M	9	17	$\lvert x \rvert$	3	2	10*
		L	0.56	0.89	$\overline{r_t}$	0.46	0.65	
	3	S_M	12	24	$\lvert x \rvert$	2	1	15*
		L	0.53	0.76	$\overline{r_t}$	0.48	0.28	
30	1	S_M	9	17	$\lvert x \rvert$	4	6	15*
		L	0.54	0.80	$\overline{r_t}$	0.51	0.35	
	2	S_M	9	17	$\lvert x \rvert$	0	0	12*
		L	0.80	1.29	$\overline{r_t}$	0.38	0.45	
	3	S_M	12	24	$\lvert x \rvert$	3	2	14*
		L	0.63	1.05	$\overline{r_t}$	0.42	0.42	
45	1	S_M	8	16	$\lvert x \rvert$	8	1	11*
		L	0.72	1.12	$\overline{r_t}$	0.55	0.48	
	2	S_M	13	25	$\lvert x \rvert$	3	2	20*
		L	0.90	1.49	$\overline{r_t}$	0.67	0.57	
	3	S_M	9	17	$\lvert x \rvert$	8	2	15*
		L	1.05	1.87	$\overline{r_t}$	0.97	0.61	
60	1	S_M	8	16	$\lvert x \rvert$	1	4	12*
		L	0.73	1.14	$\overline{r_t}$	0.39	0.43	
	2	S_M	8	16	$\lvert x \rvert$	3	5	14*
		L	0.58	0.91	$\overline{r_t}$	0.54	0.63	
	3	S_M	9	17	$\lvert x \rvert$	7	7	16*
		L	0.99	1.73	$\overline{r_t}$	0.51	0.53	

Positive controls were treated with WITH 100 μM $FeSO_4$ for 1 h.
Data concerning sham-exposed samples are reported as cut-off values of the shift (S_M) and of the shape (L) for two confidence levels (k); data on treatments are expressed as shift channels ($|x|$) and shape ($\overline{r_t}$) compared to sham-exposed samples.
*$P < 0.05$.

(24 h) exposure duration and the lack of combined effects has been assessed when RF exposure was given before or in combination with $FeSO_4$ treatments. The absence of ROS production induced by RF exposure alone is also supported by our findings on cell viability.

Measurement of ROS has been conducted by flow cytometry that, compared to the methods currently in use, is more sensitive and allows one to quantitatively examine a large number of individual cells in a short time [Sarkar et al., 2006]. The data analysis has been carried out by applying a new statistical approach recently proposed by our research group [Brescia and Sarti, 2008] that reduces the high variability in ROS production detected when samples that received identical treatments are compared. Moreover, it still permits identification of very small differences among treatments.

In the literature a limited number of in vitro studies is reported on oxidative stress induced by RF exposure alone or given in combination with known oxidative stress-inducers, in the frequency range of 800–2000 MHz. Most of them indicate no effects in several cell types, evaluated with different oxidative stress parameters. In particular, no variations in terms of antioxidant enzyme activities and nitric oxide production was reported in J774.16 mouse macrophages cells exposed to 935.62 MHz frequency modulated continuous wave (CW) or to 847.74 MHz code division multiple access and co-exposed to γ-interferon and bacteria lypopolysaccharide (LPS) [Hook et al., 2004]. Lantow and co-workers failed to find superoxide radical anion production and increase in ROS formation in human Mono Mac 6 and K562 cells exposed to 1800 MHz (CW and several typical modulations of

TABLE 5. ROS Formation in Jurkat Cells Sham/RF-Exposure for 1 h or 24 h at SAR of 0.5 and 2 W/kg and then Treated for 1 h With 50 μM $FeSO_4$

		Treatments						
			Sham-RF + $FeSO_4$ (50 μM)			RF-exposure + $FeSO_4$ (50 μM)		
Treatment duration (h)	Experiment		$\kappa = 0.05$	$\kappa = 0.01$		0.5 W/kg	2 W/kg	$FeSO_4$ (100 μM)
1	1	S_M	9	17	$\|x\|$	3	2	14*
		L	0.472	0.656	$\overline{r_t}$	0.344	0.489	
	2	S_M	8	16	$\|x\|$	7	1	15*
		L	0.590	0.919	$\overline{r_t}$	0.543	0.602	
	3	S_M	12	24	$\|x\|$	3	1	16*
		L	0.821	1.220	$\overline{r_t}$	0.735	0.705	
24	1	S_M	8	16	$\|x\|$	1	3	20*
		L	0.592	0.914	$\overline{r_t}$	0.414	0.485	
	2	S_M	13	25	$\|x\|$	3	1	18*
		L	0.845	1.328	$\overline{r_t}$	0.360	0.443	
	3	S_M	12	24	$\|x\|$	7	6	23*
		L	0.752	1.188	$\overline{r_t}$	0.327	0.659	

Positive controls were treated with 100 μM $FeSO_4$ for 1 h.
Data concerning sham-exposed samples are reported as cut-off values of the shift (S_M) and of the shape (L) for two confidence levels (k); data on treatments are expressed as shift channels ($|x|$) and shape ($\overline{r_t}$) compared to sham-exposed samples.
*$P < 0.05$.

the GSM) at SARs ranging from 0.5 to 2 W/kg and co-exposed to phorbol-12-myristate-13-acetate (PMA), LPS [Lantow et al., 2006a] or ultrafine particles [Simko et al., 2006]. Similar results were obtained when ROS production was measured in human umbilical cord blood-derived monocytes and lymphocytes [Lantow et al., 2006b] after RF exposure and co-exposure to PMA. Lack of ROS formation was also reported by Zeni et al. [2007] in L929 murine fibroblastoma cells exposed to 900 MHz RF radiation (CW or GSM modulation) at 0.2 and 1 W/kg SAR values and co-exposed to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone. Zmyslony et al. [2004] failed to find ROS increase in rat lymphocytes exposed to 930 MHz CW RF fields (power density 5 W/m^2) alone, but a significant increase was detected when cells were co-exposed with ferrous chloride. Similar results were reported by Höytö et al. [2008] who exposed human SH-SY5Y neuroblastoma and mouse L929 fibroblasts to 871 MHz RF radiation (either CW and GSM modulation) alone or in combination with tert-butylhydroperoxide. They did not find variation in reduced

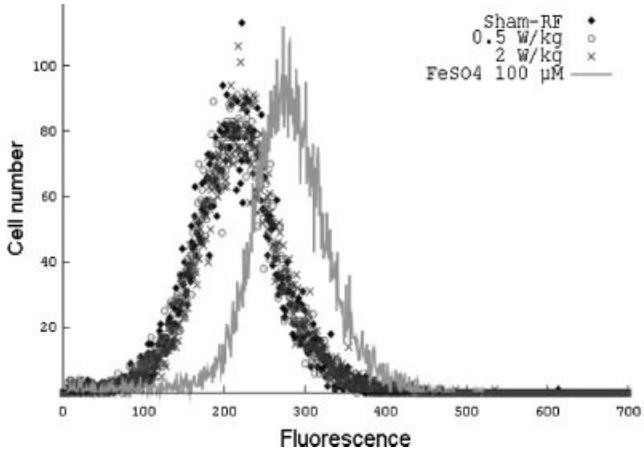

Fig. 4. Representative flow cytometric histograms of Jurkat cells sham-exposed, exposed to RF radiation (0.5 and 2 W/kg) and treated with $FeSO_4$ 100 μM. Figure refers to data reported in Table 1 for 5 min treatment.

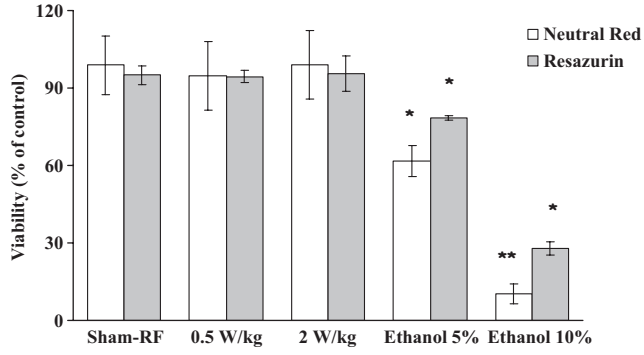

Fig. 5. Cell viability in Jurkat after 24 h RF radiation exposure (1950 MHz, UMTS signal), evaluated with the Neutral Red uptake test and the Resazurin assay. Positive controls were provided by treating cells with ethanol 5% and 10% for 1 h. Data are expressed as percentage of viable cells compared to negative controls (incubator) and are reported as mean ± SD of three independent experiments, performed in duplicate. *$P < 0.05$; **$P < 0.01$.

GSH levels and lipid peroxidation after RF exposure alone, but a significant increase in lipid peroxidation was recorded in GSM modulation co-exposed cells. No cooperative effects were detected when CW was tested. The only paper reporting effects on oxidative stress after RF exposure alone refers to Rat1 and HeLa cells exposed to 875 MHz (0.07 mW/cm^2). The authors did not directly measure ROS production, but concluded that enhanced NADH oxidation activity was found to activate ROS production [Friedman et al., 2007].

The results reported in the present study are consistent with most of the above-mentioned data; nevertheless, we would like to point out that they are not directly comparable due to the substantial difference in the employed modulation (3G vs. 2G technologies). In fact, it has been suggested that the biological effects of RF radiation, when present, may be related not only to the frequency and SAR level investigated, but also to the type of modulation applied [Penafield et al., 1997].

In conclusion, we have shown that exposures to 1950 MHz RF radiation with UMTS modulation are able to alter neither cell viability nor ROS formation in Jurkat cells. Moreover, such electromagnetic fields are not able to modulate ferrous ion-induced ROS production. The absence of effects detected after RF exposure alone extend our previous results indicating no increase in DNA migration [Sannino et al., 2006] and those published by a collaborating group who reported absence of chromosomal aberration [Manti et al., 2008] in human peripheral blood lymphocytes exposed for 24 h in the same experimental conditions employed in this study, including the RF exposure set up.

At present, to our knowledge this is the only study available on the induction of ROS after RF exposures/co-exposures employing the UMTS signal. Because we cannot rule out the possibility that other oxidative stress parameters could be altered by RF exposure/co-exposure, further studies are needed to investigate other endpoints involved in the oxidative cell metabolism and to use different stressors.

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