

In vitro exposure to 0.57-MHz electric currents exerts cytostatic effects in HepG2 human hepatocarcinoma cells

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Received November 3, 2006; Accepted December 22, 2006

Abstract. Capacitive-resistive electric transfer (CRET) therapy is a non-invasive technique currently applied to the treatment of skin, muscle and tendon injuries that uses 0.45-0.6 MHz electric currents to transdermally and focally increase the internal temperature of targeted tissues. Because CRET electro-thermal treatment has been reported to be more effective than other thermal therapies, it has been proposed that the electric stimulus could induce responses in exposed tissues that are cooperative or synergic with the thermal effects of the treatment. Previous studies by our group, investigating the nature of the alleged electric response, have shown that short, repeated stimuli with 0.57-MHz currents at subthermal levels could provoke partial, cytotoxic effects on human neuroblastoma cells *in vitro*. The aim of the present study was to investigate the response from another human cell type, the human hepatocarcinoma HepG2 line, during and after the exposure to 0.57-MHz CRET currents at subthermal densities. The electric stimuli provoked a decrease in the proliferation rate of the cultures, possibly due to an electrically-induced blocking of the cell cycle in a fraction of the cellular population.

Introduction

Capacitive-resistive electric transfer (CRET) is a non-invasive electro-thermal therapy, based on the application of electric currents in the frequency range of 0.45-0.6 MHz. Due to the electrical resistivity of the tissues, CRET currents can induce temperature increase in the internal, targeted organs. The circulating blood dissipates the heat towards adjacent areas, allowing the temperature of the treated structures to be maintained within the desired limits and avoiding unwanted hyper-

thermia in the nearby tissues. Unlike other thermal therapies, CRET does not produce edema and avoids dermal or epidermal burns.

CRET therapy is currently used in physical rehabilitation and sports medicine to treat muscle and tendon lesions (1-4), and has been successfully applied to the treatment of asthma (5) and of vascular pathologies (6). Also, preliminary data after experimental treatment of brain tumors with CRET have provided indications that some oncological lesions could be responsive to this electro-thermal therapy (7-10). In fact, in recent years a number of thermal therapies have been applied to the treatment of oncological diseases. Such therapies that induce thermal increase at the tumoral and peritumoral areas are currently used as coadjuvants to chemotherapy and radiotherapy. Several studies have shown that hyperthermia reinforces the cytotoxic response to chemostatic agents and to ionizing radiation, improving the efficacy of those standard treatments (reviewed in refs. 11,12). Malignant tumors currently show reduced blood flow and poorly developed blood vessels, which results in hypoxia and acidosis. Treatment of tumors with moderate hyperthermia ($t=42^{\circ}\text{C}$) increases blood flow. This facilitates both the action of radiotherapy, which is particularly effective on oxygenated tumors, as well as the accessibility of the tumor to chemostatic drugs intravenously administered.

At the cellular level, hyperthermia has been reported to influence membrane fluidity, surface receptors and cytoskeletal structures. At the molecular level, hyperthermia induces aggregation and denaturalization of proteins at the nuclear matrix and inhibits the repair of radiation-induced DNA strand breaks and chromosome aberrations; which reinforces the cytotoxic and oncostatic effects of radiotherapy (13,14).

A number of clinical studies have reported that CRET therapy is more effective than other thermal therapies when applied to the treatment of muscle and tendon injuries or of vascular pathologies. It has been hypothesized that the favorable effects of electro-thermal treatments could be exerted through combined or synergic responses: a tissue or systemic response to the hyperthermia induced by the electric currents, plus a cellular or subcellular response to the currents themselves (1,3,4,6,7). The hypothesis that CRET treatments can elicit separate, though potentially cooperative thermal and electric responses, has received experimental support from the results of our previous *in vitro* studies showing that 0.57-MHz CRET-like currents at non-thermal levels can induce cytotoxic

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Key words: non-thermal, electric currents, capacitive-resistive therapy, HepG2, cytostasis

responses in the NB69, human neuroblastoma cell line (15; Hernández Bule *et al.*, Proc 24th Annu Meet Bioelectromagn Soc, Quebec, 2002; Hernández Bule *et al.*, Proc 2nd Int Workshop Biol Effects Electromagn Fields, Rhodes, 2002). The present study investigates the cellular response of HepG2 human hepatocarcinoma cells to electrical stimulation with 0.57-MHz currents. To prevent any thermal increase that could mask the investigated electrical response, the currents were administered at low-density non-thermal doses.

Hepatocarcinoma (HCC) is one of the most common malignant tumors (16), and its treatment involves the surgical removal of the affected area. Unfortunately, many patients show metastasis and other associated pathologies, such as cirrhosis, which makes resection non-viable. This fact makes the treatment of the HCC tumor difficult and highlights the urgent need to find new therapeutic strategies for this type of cancer (17). Evidence exists that electric fields can affect cell growth *in vitro* by altering different phases of the cell cycle (18-22). On the basis of that evidence, the present study investigates the potential effects of weak, 0.57-MHz electric currents on the cell cycle of hepatocarcinoma cells *in vitro*. The data show that exposure to non-thermal levels of CRET-like currents can result in decreased proliferation and partial cytostasis of HepG2 cells. Such a response is likely to be due to electrically-induced alterations observed in the progression of the cell cycle.

Materials and methods

Cell culture. Human hepatocarcinoma HepG2 cells were obtained from the European Collection of Cell Culture (ECACC, Salisbury, Wiltshire, UK). The HepG2 cells were maintained in DMEM (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-Glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). The cells were grown in culture flasks under controlled conditions of temperature (37°C) and CO₂ (5%), and were subcultured once a week, when they reached an 80-90% confluence. Tests were carried out periodically to prevent mycoplasma infection and bacterial contamination.

Experimental protocol and exposure system. The experimental (CRET-exposed) and control (sham-exposed) cells were seeded simultaneously on 60-mm Petri dishes (Nunc, Roskilde, Denmark) and grown for 4 days inside two identical, separate CO₂ incubators (Forma Scientific, model 3121). On day 4 the cells were CRET-exposed or sham-exposed simultaneously, for a 24-h period. In order to study the chronological progress of the cellular response, the cultures were studied at 0, 12, 24, 42, 48 or 72 h from the beginning of exposure. In those cultures to be studied during or at the end of the 24-h exposure (day 5 after seeding), the plating density was 10⁵ cells/ml. The cultures to be analyzed later on, at 42, 48 or 72 h from the beginning of exposure were plated at a lower density, 8.5x10⁴ cells/ml. In these extended term cultures, the media were renewed on day 3 after seeding.

The exposure to CRET electric currents was carried out through pairs of sterile stainless steel electrodes, designed *ad hoc* for *in vitro* stimulation. When the cultures in the Petri dishes reached a confluence of 70-80% (4th day after seeding),

the electrodes were inserted in both the experimental and the control dishes (Fig. 1A). In the experimental groups, the electrodes were connected in series to a CRET-signal generator INDIBA MD-500 (INDIBA S.A., Barcelona, Spain) modified to deliver weak sine wave currents at 0.57 MHz. The signal parameters were monitored throughout exposure. The dimensions and configuration of the electrode pair warrants homogeneity of exposure conditions and parameters to all cells located within the electrode gap. Since the population of confluent cells accumulates preferentially in the central section of the dish, it has been estimated that ≥85% of the cellular population in a Petri dish was submitted to homogeneous conditions of exposure. In each experiment, the corresponding sham-exposed samples were kept apart, inside an incubator identical to the one for the exposed group (Fig. 1B). Current densities (CD) ranging between 1 and 100 μA/mm² were assayed for cellular response. The stimulation pattern consisted of a 5-min pulse of CRET signal applied every 4 h during a 24-h period. This pattern simulates *in vitro* the exposure timing in clinical treatments with CRET (INDIBA set-up) and allows for non-thermal stimulation of the cultures, as described in the following section and in Fig. 2.

Artifact control. The experimental testing of electric or magnetic treatments *in vitro* needs accurate measurements and control of physical parameters that could induce responses in the biological samples that would otherwise be erroneously attributed to a direct effect of the CRET currents.

Influence of the electrodes. A potential influence of the presence of non-energized electrodes on the gas exchange inside the Petri dishes or on the temperature homogeneity in the culture medium might result in significant changes in the cellular population. Such a possibility was investigated and ruled out after comparative analysis of cell growth and cell viability in cultures grown for 24 h in the presence or in the absence of non-energized electrodes.

Electrochemical integrity of the electrodes. It has been described that stainless steel electrodes can degrade when used to experimentally deliver low-frequency (within the Hz-kHz range) currents at high densities. Corrosion can release iron ions to the culture medium that are cytotoxic. Such corrosive effect is reduced at higher frequencies and vanishes at the MHz range (23). Lack of corrosion has been empirically confirmed in the case of the CRET therapies, which use stainless steel electrodes to transdermally deliver high-density thermal currents in the MHz range. Nevertheless, in order to rule out electrode corrosion as a possible confounding factor in our experiments, tests were conducted periodically to detect potential release of iron ions in the medium. In these tests the electrodes were either non-energized (control condition) or stimulated for 24 h with pulses of 0.57-MHz currents at the highest thermal densities tested in the present study (400 μA/mm²). At the end of the exposure period the media were analyzed for Fe³⁺ content using an automated analyzer, the Aeroset system (Abbott, IL, USA). Additionally, three replicates of an experiment were carried out to test whether electrode microparticles potentially released during the electric stimuli could have cellular effects. Following the standard procedure, in each replicate cells were grown in two groups of 5 Petri dishes. At the beginning of the 4th day, the

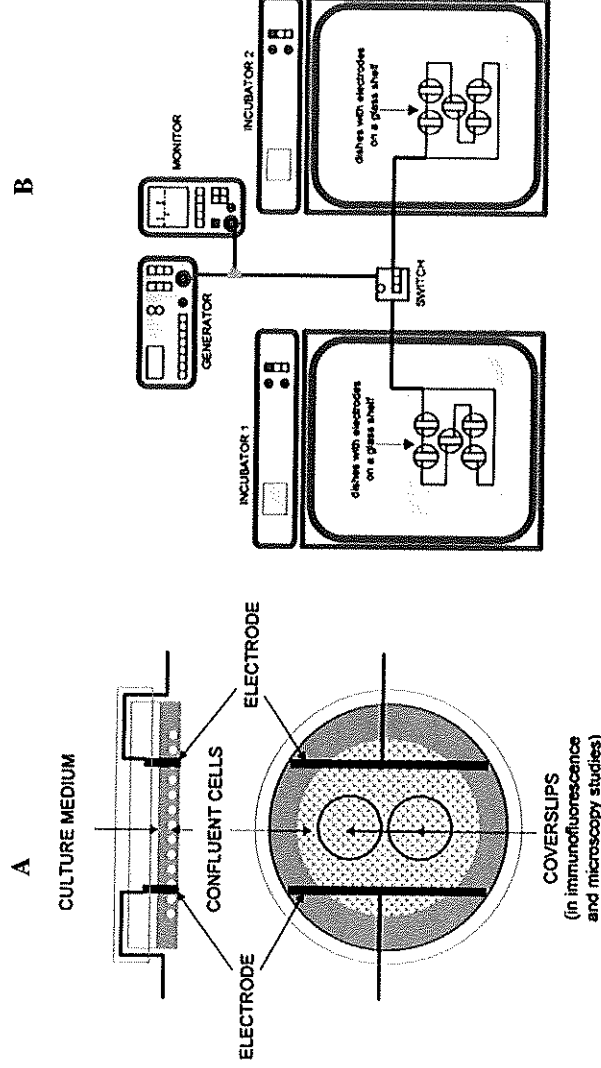


Figure 1. Exposure system. A, Petri dish with a pair of parallel stainless steel electrodes set for CRET-stimulation or sham-stimulation of confluent HepG2 cells. In experiments requiring immunofluorescence and/or microscopic analysis of the samples, two sterile coverslips were placed in the central surface of the dish before seeding. B, two identical incubators were used. In each experimental run a switch delivered in a random sequence the electric current to the Petri dishes in one of the incubators. The cultures grown inside the alternative incubator were used as sham-exposed controls of that run.

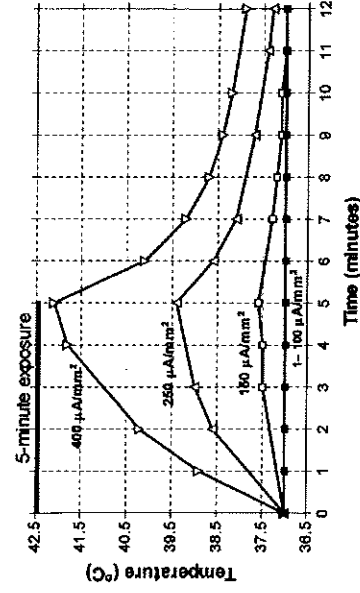


Figure 2. Temperature monitoring of the culture medium during and after a 5-min pulse of exposure to current densities of 1, 5, 10, 50, 100, 150, 250 and 400 $\mu\text{A}/\text{mm}^2$. Significant linear increases of temperature were observed at densities $>100 \mu\text{A}/\text{mm}^2$.

culture medium in one of the two groups was replaced by new medium previously stimulated for 24 h with a 0.57-MHz current at a density of 100 $\mu\text{A}/\text{mm}^2$. The other group of 5 dishes received new medium that had not been stimulated. Simultaneously, electrode pairs were inserted in all the dishes and the cultures were grown for an additional 24 h. At the end of that period the cultures were analyzed for cell growth and cell viability following the standard procedures described below.

Influence of electromagnetic fields. 0.57-MHz currents passing through the energized electrodes and the wires connecting the different electrode pairs emit radiofrequency electromagnetic fields that might affect the behavior of control cells if grown in the same incubator as the exposed samples. To obviate such a potential influence, the sham-exposed samples were incubated in a separate incubator, identical to

the one used for CRET-exposed cultures and with the same temperature and gas conditions.

Electrophoretic effects. The 0.57-MHz CRET electric currents having a frequency in the MHz range are not expected to induce significant electrophoretic displacement of charged particles or molecules in the culture medium. Consequently no cellular changes attributable to CRET-induced electrophoretic effects in the media would be expected in the present experiments. Nevertheless, potential changes in the concentration of hydrogen and other biologically relevant ions, including Na^+ , K^+ , Cl^- , Ca^{2+} in cultures treated with CRET currents for 24 h were studied through analysis of pH and of simple ion levels in the media (Aeroset system).

Thermal effects. With regard to identifying potential electrically-induced thermal effects, a study was carried out using digital thermometers HIBOX 16 (Hibox, Taiwan) for recording temperature changes in the media in response to exposure current densities in the 1-400 $\mu\text{A}/\text{mm}^2$ range.

Blind protocols. All the experimental procedures in the present study were carried out in blind conditions for treatment.

Cell viability and time-dependent growth processes. A chronological study was carried out through analysis of the samples before treatment (0 h), during the treatment (12-h exposure) and after treatment (24, 42, 48 or 72 h from the exposure onset). For both the CRET-exposure and sham-exposure conditions, the cells were harvested through trypsinization with 0.25% trypsin (Gibco). The fractions of alive and dead cells in the samples were quantified through Trypan blue dye-exclusion method (24), using 0.4% Trypan blue (Sigma, St. Louis, MO, USA) in PBS (1:8 dilution).

Spectrophotometric analysis of protein and DNA. Analysis of protein and DNA levels was performed to evaluate the

potential effects of the electric exposure on cell proliferation. The analyses were carried out at the end of a 24-h period of CRET-exposure or sham-exposure incubation. Protein concentration was quantified through the method of Lowry *et al* (25), using albumin from bovine serum (Sigma, Steinheim, Germany) as a standard. DNA quantification was carried out through Burton's methodology (26), using 2-deoxy-D-ribose (Sigma, Steinheim, Germany) as a standard.

5-bromodeoxyuridine (BrdU) assay. The BrdU labeling technique was used for evaluation of cell proliferation. This method is based on the incorporation of 5-bromo-2deoxyuridine, which partially replaces thymidine in DNA-synthesizing cells. The dividing cells are subsequently labeled with anti-BrdU antibodies conjugated to fluorescent markers. Cells were cultured as described above, except that they were plated in Petri dishes containing two sterile coverslips of 12-mm diameter, for further analysis of the cellular response through fluorescence microscopy (Fig. 1A). BrdU (Sigma, Steinheim, Germany) at a 3-mM concentration was added to the culture medium at the beginning of the CRET exposure ($t=0$ h). At the end of the 24-h exposure, the cells were fixed with 4% paraformaldehyde and permeabilized with 95/5 ethanol/acetic acid. Cells were incubated overnight with monoclonal primary antibody anti-BrdU (Dako, Denmark) at 4°C. Anti-mouse Ig fluorescein-linked whole antibody (Amersham, Buckinghamshire, UK), the secondary antibody, was added to the cells and incubated at room temperature for 1 h. Preparations were counterstained and mounted in Hoechst-Non-Fade (Sigma, Steinheim, Germany) and studied through a Nikon Eclipse TE300 fluorescence microscope. Images from a total of 28000 cells exposed and an equivalent number of control cells corresponding to three experimental replicates were recorded and computer-assisted analyzed with AnalySIS 3.1 (Soft Imaging Systems GmbH, Münster, Germany).

Western blot quantification of proliferating cell nuclear antigen expression. The proliferating cell nuclear antigen (PCNA) is a polymerase-associated protein that is currently used as a marker for cells in S phase of the cell cycle (27,28). At 0, 12 or 24 h from the exposure onset the cells were scraped from the dishes, re-suspended in PBS and centrifuged for 5 min at 4000 rpm. The pellet was lysed at 4°C in buffer with 10 mM Tris-HCl, pH 7.6, 10 mM KCl, 1 mM Dithiothreitol, 1 mM EDTA, 1 mM PMFS, 10 µg/ml Leupeptin, 5 µg/ml Pepstatin, 100 mM NaF, 20 mM B-Glycerophosphate, 20 mM Sodium Molybdate, 0.5% Triton X-100 and 0.1% SDS. The lysates were centrifuged at 10000 rpm for 5 sec at room temperature and the protein concentration in the supernatant was determined through Bradford's method (29). Proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond ECL nitrocellulose membranes (Amersham) using semidry transfer methodology (Bio-Rad Labs., Richmond CA, USA). Membranes were blocked in TBS-Tween (1% TBS pH 7.6; 0.1% Tween-20) containing 5% non-fat, dried milk and incubated overnight at 4°C in TBS-Tween containing polyclonal anti-PCNA antibody (Santa Cruz-Technologies, CA, USA) and 5% non-fat dried milk. Following incubation, the membranes were washed three times with TBS-Tween and then incubated with the

secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The blot was developed by chemiluminescence following the ECL Western blot analysis system protocol (Amersham). The blots were analyzed with a PDI Quantity One-4.5.2 imaging system (Bio-Rad, Hercules, CA, USA).

Light microscopy. The cells were grown and exposed on coverslips in dishes. At the end of the 24-h treatment, the cells on coverslips were fixed in 4% paraformaldehyde, permeabilized with acetone, dyed with hematoxylin and eosin (Merck, Darmstadt, Germany) and mounted in Entellan (Merck, NJ, USA). The preparations were studied with a Nikon TE300 microscope and quantitative analyses of metaphases, telophases/anaphases and aneuploid cells were performed using photomicrographic images of ~12000 exposed cells, plus an equivalent number of control cells, in 3 experimental replicates.

Cell cycle analysis. Different phases of the cell cycle of CRET-exposed cultures were analyzed by flow cytometry (FACScan Mod. FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA) and compared to the corresponding controls. At 0, 12, 24, 42, 48 or 72 h of the exposure onset, the cultures were trypsinized and both floating and adherent cells were collected. Samples of 3×10^6 cells were centrifuged at 1200 rpm for 5 min, fixed in 70% ethanol and stained with 3.4 mM sodium citrate, 20 µg/ml propidium iodide (Boehringer, Mannheim, Germany) and 100 µg/ml RNase A (Boehringer) solution. CellQuest 3.2 software was used for data acquisition (20000 events per sample) and analysis.

Statistical analysis. Data were analyzed using unpaired, two-tailed Student's t-test. Differences $p < 0.05$ were considered statistically significant.

Results

Artifact control. The obtained results confirmed that neither the electrical current nor the presence of electrodes altered the physical/chemical properties of the culture medium. No significant changes were detected in cell growth or in cell viability that could be attributed to the presence of non-energized electrodes in the dishes (data not shown). The pH and the ionic concentration (Na^+ , K^+ , Cl^- , Ca^{2+} and Fe^{3+}) in the medium were studied at the end of the 24-h exposure, both in treated and in control samples. No changes were observed in those parameters that could be attributable to a direct effect of the electric currents on the culture medium or to the release of cytotoxic ions to the medium because of electrically-induced deterioration of the electrodes (data not shown). No changes were observed either, in the growth rate or in the cell viability of cultures grown for 24 h in the presence of electrically stimulated ($100 \mu\text{A}/\text{mm}^2$) medium when compared to their corresponding controls, grown simultaneously in non-stimulated medium (data not shown).

Electrically-induced hyperthermia. The objective of the present study is to test the hypothesis that the effects of CRET therapies are due in part to the response of the biological system to the

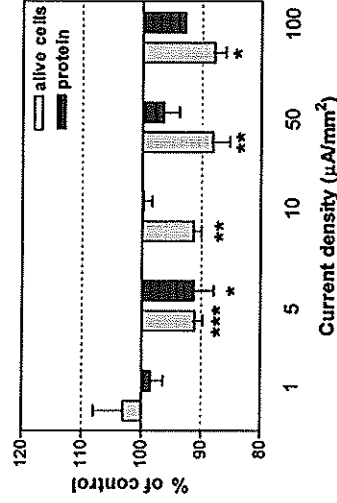


Figure 3. Alive cells and protein concentration (mg/dish) after 24-h exposure to weak non-thermal current densities (1-100 $\mu\text{A}/\text{mm}^2$). Percents over the respective controls. The histograms represent means \pm SEM of five experimental replicates. Five CRET-exposed dishes vs. five sham-exposed dishes per experimental replicate and per current density tested. *** $p < 0.001$; ** $0.001 \leq p \leq 0.01$; * $0.01 < p < 0.05$ (Student's t-test).

electrical stimulus itself. In order to select current densities that could guarantee non-thermal exposure conditions, a test was conducted recording temperature changes in the medium of Petri dishes exposed to current densities in the 1-400 $\mu\text{A}/\text{mm}^2$ range. At the end of the 5-min exposure pulses, current densities $\text{CD} \geq 150 \mu\text{A}/\text{mm}^2$ induced in the medium thermal increases $> 0.5^\circ\text{C}$. No thermal changes were observed at $\text{CD} \leq 100 \mu\text{A}/\text{mm}^2$ (Fig. 2).

Cell viability, protein levels and time-dependent cell-growth processes. Athermal current densities between 1 and 100 $\mu\text{A}/\text{mm}^2$ caused significant reduction in the proportion of alive cells at the end of a 24-h exposure period, when compared to the respective controls (Fig. 3). This reduction tended to be maximal, $\sim 12\%$, for the highest current densities tested, 50 and 100 $\mu\text{A}/\text{mm}^2$. For protein levels, a general decrease in the protein content was observed after treatment with the different current densities tested. However, only the effect induced by the 5- $\mu\text{A}/\text{mm}^2$ dose reached statistical significance, indicating that this cellular response is not linearly-dependent on the current density of the stimulus. In control conditions, the proportion of necrotic cells was low ($2.0 \pm 1.0\%$), and corresponded to the spontaneous necrosis reported by others in HepG2 cells. None of the assayed current densities induced significant changes in the incidence of necrosis (data not shown). Therefore, the described reduction in the alive cellular population and protein concentration are not due to increased rates of necrosis.

The chronological analysis of the cellular response in samples exposed to 50 $\mu\text{A}/\text{mm}^2$ (Fig. 4) confirms the reduction in the number of alive cells observed at the end of the 24-h exposure (Fig. 3) and indicates that the maximal reduction ($\sim 20\%$ below the respective controls) occurred during the post-exposure period, at 42 and 48 h from the onset of the treatment. These decreases in the number of cells at 42 and 48 h after the onset of a 24-h treatment are chronologically coincident with the starting of the stationary phase of the culture, which occurs earlier than in controls (Fig. 4). The lack of effect at 72 h was likely due to depletion of the medium, which could no longer support the high growth rates of the control cultures. Therefore, the treatment with

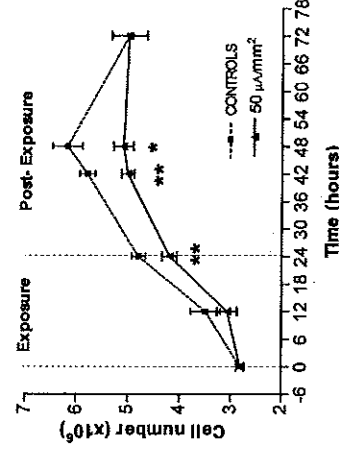


Figure 4. Chronological progress in the cellular population during (12 h) and after (24, 42, 48 and 72 h) the exposure to CRET currents at 50 $\mu\text{A}/\text{mm}^2$. The currents affect the growth kinetics of HepG2 cells. The points represent means \pm SEM of five experimental replicates. Five CRET-exposed dishes vs. five sham-exposed dishes per time lapse and per experimental replicate. ** $0.001 \leq p \leq 0.01$; * $0.01 < p < 0.05$ (Student's t-test).

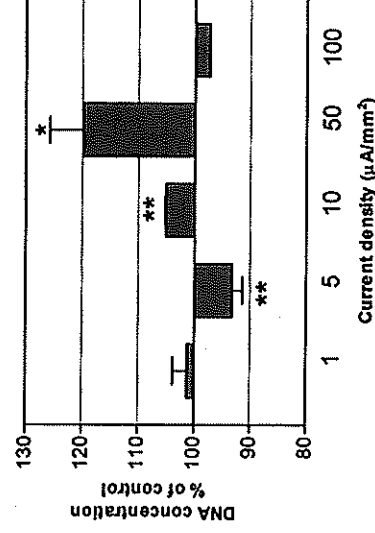


Figure 5. Spectrophotometric quantification of DNA concentration (mg/dish) in cultures at the end of a 24-h exposure to weak non-thermal current densities (1-100 $\mu\text{A}/\text{mm}^2$). Percents over the respective controls. Histograms represent means \pm SEM of five experimental replicates. Five CRET-exposed dishes vs. five sham-exposed dishes per experimental replicate and per current density tested. * $0.001 \leq p \leq 0.01$; * $0.01 < p < 0.05$ (Student's t-test).

50 $\mu\text{A}/\text{mm}^2$, besides reducing the number of viable cells during and after exposure, affected the growth kinetics of HepG2, stopping the cell growth 20 h after exposure.

Effects on cell proliferation. Analyses were conducted to determine whether the significant reduction in the cellular population after the treatment, described above, could be due to alterations in the proliferation rate. In the present context, the term 'proliferation' describes an orderly progression through the cell cycle that results in normal cellular division. The spectrophotometric measurement of DNA levels in the samples at the end of the 24-h exposure revealed changes that are not linearly correlated to the applied current density (Fig. 5). Samples exposed to 5 $\mu\text{A}/\text{mm}^2$ showed a significant decrease in DNA levels, whereas a 50- $\mu\text{A}/\text{mm}^2$ CD induced DNA increase (20% over controls, statistically significant) stronger than that obtained after treatments with lower (10 $\mu\text{A}/\text{mm}^2$) or higher (100 $\mu\text{A}/\text{mm}^2$) levels. As illustrated in Fig. 6, in cells exposed to 50 $\mu\text{A}/\text{mm}^2$ the increase in DNA levels was accompanied with a significant increase of BrdU incorporation in the nuclei (17% average over controls; $p=0.04$). These

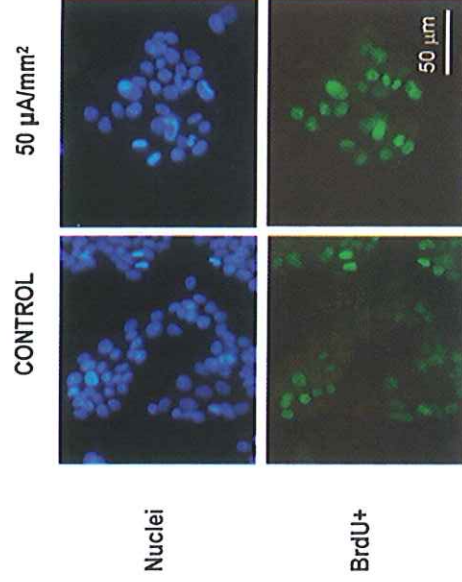


Figure 6. Representative images of BrdU-immunofluorescent labeling at the end of a 24-h treatment with CRET currents at $50 \mu\text{A}/\text{mm}^2$. BrdU⁺, fraction of cells incorporating BrdU in their DNA (fluorescein-green, lower photographs) out of the total cellular population, revealed by their nuclei (Hoechst blue-stained, upper pictures). Higher proportions of BrdU-positive cells were observed in the CRET-exposed samples when compared to their controls.

results and those described above, namely, that the $50\text{-}\mu\text{A}/\text{mm}^2$ CD was found to efficiently and consistently elicit cellular, non-thermally induced responses, caused us to select the $50\text{-}\mu\text{A}/\text{mm}^2$ current density as a standard exposure dose in the forthcoming experiments.

In order to further investigate the kinetics of the reported increase in DNA synthesis in response to the treatment, the PCNA expression in the exposed ($50 \mu\text{A}/\text{mm}^2$) and control samples was analyzed at 12 and 24 h of treatment. The quantification of PCNA expression was through densitometric analysis of Western blots. The results showed a significant increase in the PCNA protein expression at 12 h, followed by a decreased (statistically non-significant) expression at 24 h (Fig. 7), which indicates that the above described increase in the DNA synthesis occurred within the initial 12 h of exposure. These results were confirmed by a complementary study with computer-assisted image analysis of PCNA expression in photomicrographs of samples grown in coverslips (data not shown). In sum, the data in Figs. 5 and 7, show increases in the total levels of DNA at the end of the treatment and in PCNA levels at 12 h of treatment, respectively, that did not result in increased cell proliferation (Fig. 4).

Quantification of aneuploidy in the culture. The observed increase in the DNA levels in the treated samples may be due to an increase in the rate of cells entering S phase, of DNA synthesis, or alternatively, to an anomalous replication of DNA that could result in an excess of aneuploid cells. The subsequent progression of those aneuploid cells through the cell cycle could be arrested at the mitosis phase. A total of 12000 cells, hematoxylin and eosin-stained after 24-h treatment with $50 \mu\text{A}/\text{mm}^2$, were analyzed for aneuploidy and compared to an equivalent number of control cells. A slight excess in the rate of aneuploid mitoses (5.88% over controls, statistically non-significant) was observed in the exposed samples at the end of the treatment. In additional experiments, the flow

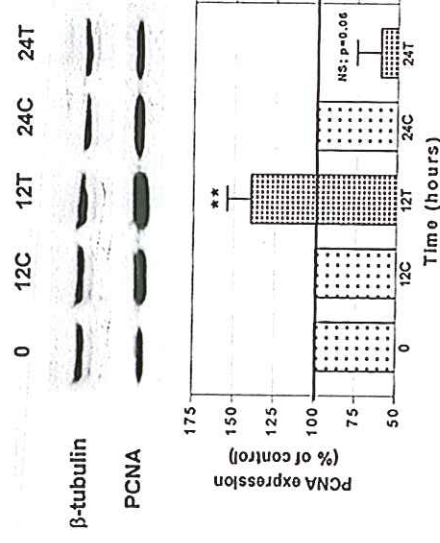


Figure 7. Western blot analysis of PCNA expression at the onset (0 h), during (12 h) and at the end (24 h) of exposure to CRET currents. T, samples treated with a $50 \mu\text{A}/\text{mm}^2$ current density; C, sham-exposed controls. A representative blot with densitometric analysis, using β -tubulin as load control, is shown at the upper part of the figure. SDS-polyacrylamide gel electrophoresis and Western blot assays were conducted on $30\text{-}\mu\text{g}$ extracts, using a PCNA polyclonal antibody as described in Materials and methods. The values in the histogram (normalized over controls) represent means \pm SEM of optical density in three experimental replicates. Five CRET-exposed dishes vs. five sham-exposed dishes per experimental replicate, per exposure period and per current density tested. ** $0.001 \leq p \leq 0.01$ (Student's t-test).

cytometry analysis of exposed samples revealed a similar increase in the rate of aneuploidy (5.92% over controls, non-significant) at the end of the treatment with a $50\text{-}\mu\text{A}/\text{mm}^2$ CD. These results do not provide sufficient support for the hypothesis that the observed increase in DNA levels could lead to a significant excess of aneuploid cells.

Analysis of the cell cycle. As shown before, treatment with a $50\text{-}\mu\text{A}/\text{mm}^2$ CD induced an increase in DNA synthesis followed by a decrease in the cellular population. The results described in the above paragraphs indicate that such a decrease in the cellular population is not likely to be due to increased rates of necrosis or aneuploidy, but rather to a treatment-induced impairment of the progression of the cell cycle. The analyses of the data from flow cytometry show that, when compared to controls, the percent of cells in G0/G1 phase was slightly increased during and after the treatment (Table I and Fig. 8); the increase being statistically significant at the end of the 24-h exposure. The cellular population in S phase increased significantly during the treatment (12 h) and decreased significantly to below that of controls at 24 and 48 h. These results are in agreement with the data on PCNA expression, which show that DNA synthesis peaks during the treatment and falls to control values at the end of the 24-h exposure (Fig. 7). As a whole, those results indicate that the exposure to $50\text{-}\mu\text{A}/\text{mm}^2$ currents could arrest the progression of the cell cycle in a fraction of the cellular population.

Quantitative analysis of cells in different phases of mitosis. To complete the study of the potential effects on the cell cycle, additional experiments were conducted studying the percent of cells in metaphase and in anaphase/telophase at the end of the 24-h exposure to $50\text{-}\mu\text{A}/\text{mm}^2$ currents. The

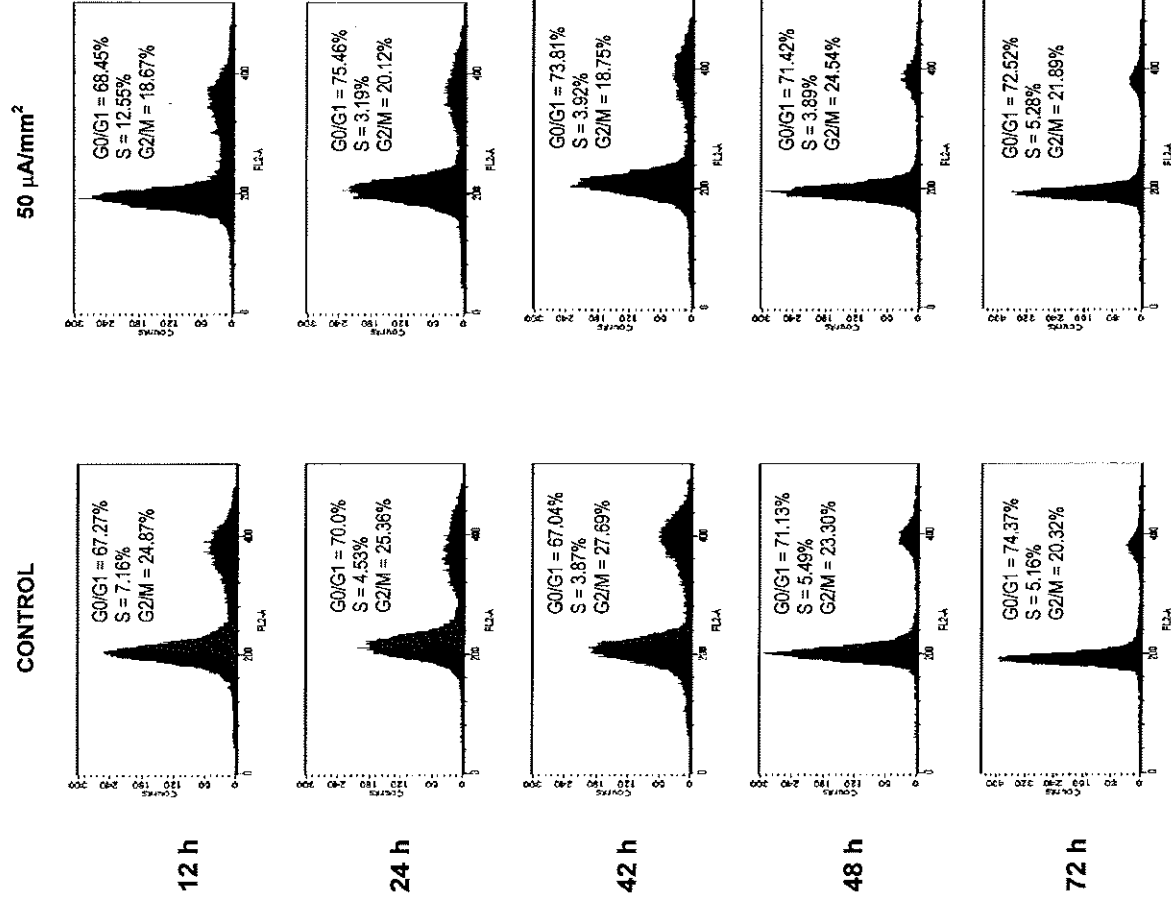


Figure 8. Flow cytometry analysis of the cell cycle in samples exposed for 24 h to 50- $\mu\text{A}/\text{mm}^2$ CRET currents. Cells were harvested during (12 h) and after exposure (24, 42, 48 and 72 h) and stained with propidium iodide for DNA quantification. Representative results obtained from one single experiment. Each histogram represents the analysis of 20000 events obtained from the corresponding samples (five exposed dishes vs. five controls).

Table I. Treatment-induced changes in the cell cycle.

Time (h)	No. of experimental replicates	G0/G1 phases	S phase	G2/M phases
12	3	101.3 \pm 1.2	123.6 \pm 5.7 ^a	87.7 \pm 4.6
24	3	103.0 \pm 0.5 ^b	82.0 \pm 6.0 ^a	93.9 \pm 2.4
42	3	103.3 \pm 1.5	96.9 \pm 12.5	90.7 \pm 8.2
48	2	104.5 \pm 8.3	75.2 \pm 7.5 ^a	104.3 \pm 19.4
72	2	96.5 \pm 4.8	105.2 \pm 9.8	84.6 \pm 10.0

Changes in the percent of cells in the different phases of the cycle during CRET exposure (12 h) and after exposure (24, 42, 48 and 72 h; flow cytometry). The values are percents over the respective controls and represent means \pm SEM of 2 or 3 replicates of the experiment illustrated in Fig. 8. ^a0.01<p<0.05 (Student's t-test). ^b0.001 \le p \le 0.01.

analysis through light microscopy of cells grown on coverslips revealed a significant increase in the proportion of metaphases in the exposed samples (173% over the respective controls) accompanied with a reduction (non-significant statistically) in the percent of cells in anaphase/telophase (56.21% of that in controls; Fig. 9). This could indicate that the cell cycle of a fraction of the cellular population was arrested in metaphase. However, such an arrest, if confirmed, seems to affect only a modest fraction of the population, because the effect was too weak to be detected by flow cytometry (Fig. 8 and Table I).

Discussion

In recent years a number of electric therapies have been applied to the treatment of tumors (30,31). Preliminary clinical assays have provided indications that the electro-thermal CRET therapy could exert oncostatic effects in humans (7-10). *In vitro*, the stimulation with electric fields and electric currents can

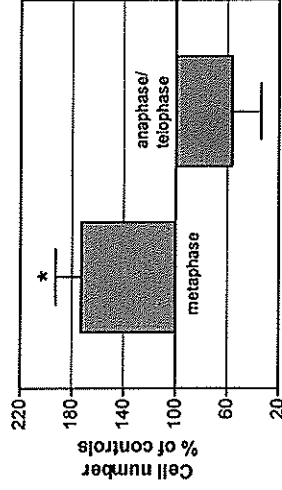


Figure 9. Changes in the proportion of cells in metaphase and in anaphase/telescope after 24-h exposure to $50 \mu\text{A}/\text{mm}^2$; percent over controls. Quantitative data obtained by computer-assisted image analysis of hematoxylin and eosin-stained cells. Histograms represent means \pm SEM of three experimental replicates; five exposed dishes vs. five controls per replicate. Number of cells analyzed: 750 cells per replicate. * $0.01 < p < 0.05$; Student's t-test.

influence cell viability and cell proliferation in a variety of cell types (18-22). Although traditionally, living systems have been considered to be non-responsive to exposure to weak intermediate-frequency electric stimuli, recent results indicate that low intensity, 0.1-0.6 MHz, alternating electric currents or electric fields can significantly inhibit cell growth (20-100% below controls) in different cancer cell lines from human or rodent origin (15,21). The data in the present study indicate that exposure to weak non-thermal electric currents of CRET frequency, 0.57 MHz, can induce decreased cellular population (Figs. 3 and 4) and protein content (Fig. 3) in the human hepatocarcinoma cell line HepG2. The responses could be time-dependent, with respect to cell population, for which the maximal reduction compared to controls was attained about 24 h after the end of the exposure period ($t=42-48$ h; Fig. 4). However, as shown in Fig. 3, the general pattern of the response does not seem to be linearly-dependent on the applied current density.

No significant changes in the rate of necrosis were observed to be caused by the electric treatment that could explain the decrease of the cellular population observed in CRET-exposed samples. In order to determine whether the potential origin of this effect could be due to alterations in the rate of cell proliferation, spectrophotometric quantification of DNA and analysis of bromodeoxyuridine (BrdU) incorporation were carried out. The results revealed increased DNA concentration in the exposed samples (Fig. 5) accompanied with increased BrdU incorporation in the DNA (Fig. 6), which could be interpreted as an indication that the treatment promotes proliferation in HepG2 cell cultures. In fact, exposure to electric currents has been reported to promote DNA synthesis (32-36), which generally leads to increased cell proliferation (37,38). However, in the present study the analysis of the expression of the antigen of cell proliferation (PCNA), which is synthesized during the G1/S phase of the cell cycle (27,28), revealed increased DNA synthesis during the first half of the exposure period ($t=12$ h), followed by decreased DNA synthesis at the end of the treatment ($t=24$ h; Fig. 7). At that time a reduction in the fraction of alive cells occurs (Figs. 3 and 4). Different phenomena may be responsible for the observed electrically-induced increase in DNA levels followed by a subsequent decrease of cell proliferation. One possibility

is that the CRET non-thermal currents could induce cells in S phase to erroneously duplicate DNA, giving rise to aneuploid cells. Such an aberrant replication would result in increased DNA content together with reduced proliferation due to blockage of the cell cycle progression. In mitotic cells, the exposure to electric currents of intermediate frequencies has been reported to interfere with the normal process of polymerization/depolymerization occurring in forming microtubules (21). As a result, changes in the microtubule orientation, polyploid prophase, multi-spindle metaphases and increased number of cells in metaphase occur. The results of the present study, showing increased proportions of cells in metaphase (Fig. 9) together with a modest excess of aneuploidy (6% over controls, non-significant statistically) at the end of the treatment, could provide partial support for the above hypothesis. Nevertheless, the incidences of those anomalies in the treated samples are modest and cannot explain by themselves the significant decrease in cell population observed after CRET exposure.

An alternative explanation for the results is that the increase in the DNA synthesis detected during the treatment ($t=12$ h) could be related to alterations in the progression of the cell cycle. The cytometric results are consistent with that hypothesis. According to the data in Fig. 8 and Table I, during the first half of the exposure period the electric treatment significantly increased the fraction of cells in the S phase (DNA synthesis). This increase was accompanied with a decrease in the proportion of cells in the G2/M phase. At the end of the 24-h treatment, a decrease in the rate of cells in the S phase was observed, together with a moderate, though significant, increase in the proportion of cells in the G0/G1 phase. The effect could remain for several hours after the end of the exposure ($t=48$ h). Taken together, these responses could indicate an arrest of the cell cycle at the G0/G1 phase in a potentially sensitive fraction of the cellular population which would result in a subsequent decrease in cellular proliferation, as observed here several hours after the end of the treatment ($t=42$ and 48 h). Such arrest of the cell cycle could be mediated by electrically-induced alterations in the expression of one or more proteins involved in the regulation of the cell cycle. These proteins are the cyclins, the cyclin dependent kinases (CDKs) and the cyclin kinase inhibitors (CKIs), including proteins p21 and p27 among others (39). In fact, Wang *et al* (18) have reported that exposure to electric fields of physiological intensities can inhibit proliferation in endothelial cells through a blocking of the cell cycle caused by a reduced expression of cyclin E and an overexpression of p27. Studies are in progress in our laboratory investigating potential changes induced by CRET treatment in the expression of these proteins and the influence of such changes on the cellular response.

In conclusion, the exposure to non-thermal levels of short repeated pulses of electric currents of CRET frequency induces a cytostatic effect in HepG2 cells from human hepatocarcinoma. Such an effect could occur by blocking the cell cycle in an electrically sensitive fraction of the cellular population. The data obtained in this study strongly indicate that the cellular responses to the weak intermediate frequency currents assayed are not linearly dependent on the applied current density. Non-linearity in the dose-response pattern is a common observation in studies investigating biomedical

effects of weak non-thermal electric and magnetic stimuli (40.41). The lack of linearity in the present *in vitro* response would provide additional support to the current evidence that, at least at the cellular level, the clinical treatments with CRET therapy can elicit dual electrical and thermal responses. Depending on the biological target and on the parameters of the stimulus, such responses, though potentially independent in nature, could exert co-operative or intermodulatory actions that are of potential therapeutic interest. Extended research is necessary to better characterize the cellular response to non-thermal 0.57-MHz electric currents. The understanding of the cellular mechanisms of response could contribute to improve the efficiency of CRET-like treatments and to extend the applications of the electro-thermal therapies, including those for hepatocarcinoma, one of the most lethal malignant tumors, for which the current therapies are often of limited efficacy.

Acknowledgments

The authors wish to express their appreciation to Dr C.F. Blackman for helpful suggestions and critical reading of the manuscript; to Dr E. Roldán for expert technical advice in the studies with flow cytometry; to Dr A. Lobo for technical support in the analysis of ionic content in culture media; to engineers J. Calbet, J. Buisán and V.J. García-Rey for technical support in the design and setting of the exposure system and in the study of the thermal response of the media. M.L. Hernández-Bule was supported by an INDIBA, S.A. grant for Ph.D. students. This study was performed under assignment and supervision of the Spanish Ministry of Health.

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