

Original Paper

Electric Stimulation at 448 kHz Promotes Proliferation of Human Mesenchymal Stem Cells

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Key Words

Radiofrequency • ADSC • Proliferation • PCNA • MAPK

Abstract

Background/Aims: Capacitive-resistive electric transfer (CRET) is a non invasive electrothermal therapy that applies electric currents within the 400 kHz – 450 kHz frequency range to the treatment of musculoskeletal lesions. Evidence exists that electric currents and electric or magnetic fields can influence proliferative and/or differentiating processes involved in tissue regeneration. This work investigates proliferative responses potentially underlying CRET effects on tissue repair. **Methods:** XTT assay, flow cytometry, immunofluorescence and Western Blot analyses were conducted to assess viability, proliferation and differentiation of adipose-derived stem cells (ADSC) from healthy donors, after short, repeated (5 m On/4 h Off) *in vitro* stimulation with a 448-kHz electric signal currently used in CRET therapy, applied at a subthermal dose of 50 $\mu\text{A}/\text{mm}^2$. **Results:** The treatment induced PCNA and ERK1/2 upregulation, together with significant increases in the fractions of ADSC undergoing cycle phases S, G₂ and M, and enhanced cell proliferation rate. This proliferative effect did not compromise the multipotential ability of ADSC for subsequent adipogenic, chondrogenic or osteogenic differentiation. **Conclusions:** These data identify cellular and molecular phenomena potentially underlying the response to CRET and indicate that CRET-induced lesion repair could be mediated by stimulation of the proliferation of stem cells present in the injured tissues.

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Introduction

Tissue regeneration is a complex process involving a diversity of cell types whose functions are regulated by intricate networks of biochemical signals. One crucial phase in regeneration is that of proliferation of precursor cells. After proliferating, the progenitor cells differentiate into the tissue-specific cell types that will build up its characteristic extracellular matrix, so the original function is regained. Mesenchymal stem cells (MSC) are a key population involved in the proliferative phase of regeneration of lesions and are present in almost all adult tissues. These multipotent cells have been shown to be a useful tool in regenerative medicine due to their potential for self-renewal and differentiation into multiple cell types, and because of their role in inflammatory and immune response during injury repair [1, 2]. For these reasons the development of strategies for stimulating proliferation and differentiation of MSC present in injured tissues is currently a target of primary interest in regenerative medicine.

In another vein, cells and tissues have specific electric properties, including resting membrane potential, ionic current flow, capacitance, resistivity, permittivity and conductivity [3]. Such properties, which vary depending on the tissue type, age and physiological status, and differ between developing, normal and wounded tissues, enable biosystems to react to exogenous electric and electromagnetic fields [4-6]. Changes in endogenous electric phenomena by exposure to pharmacological or physical agents have been shown to influence the repair rate in different tissues (see Messerli and Graham, 2011 and Costin et al., 2012 for reviews) [7, 8].

Traditionally, physical therapies based on electrical or electromagnetic stimulation have been used with satisfactory results in the regeneration of traumatic or degenerative tissue lesions, as well as in aesthetic medicine [7, 9-14]. Specifically, electrical stimulation has been shown to substantially improve healing and regeneration of chronic injuries, often achieving complete closure of wounds that had failed to respond to other treatments [15-18]. At the tissue level, it has been reported that electrical stimulation can improve blood flow [19], increase elasticity of damaged tissues [20, 21] and reduce oedema [21]. At the cellular level, electrical stimulation affects adhesion, orientation and migration [22-25] and influences the regulation of morphological and phenotypic processes involved in the differentiation and proliferation of various stem cell types [3, 26-29]. Although the basic phenomena underlying tissue regeneration elicited by electric stimulation are yet to be determined, it has been proposed that in cases of torpid, blocked or reverted regeneration, an electric treatment could trigger cell responses of the same nature as those elicited by endogenous bioelectric currents involved in normal tissue repair [7, 30].

The Capacitive-Resistive Electric Transfer (CRET) is a non-invasive electro-thermal therapy, based on the application of electric currents within the radiofrequency (RF) range of 400 kHz - 450 kHz. Due to the electrical resistivity of tissues, CRET currents can induce temperature increase in the targeted organs. The circulating blood dissipates heat towards adjacent areas, allowing the temperature of the treated structures to be maintained within the desired limits and avoiding unwanted hyperthermia in nearby tissues. Recent *in vitro* results indicate that, when administered at thermal current densities, CRET causes cytotoxicity in human cancer cells, and that such thermal effect can be enhanced by injection of metallic microparticles within the targeted tumoral tissues [31]. However, evidence exists indicating that, at least at the cellular level, the CRET effects are not limited to the thermal ones. In fact, previous studies by our group have shown that CRET stimulation at subthermal doses can induce anti-proliferative and cytotoxic responses in cultured human cancer cell lines, but not in primary cultures of human peripheral blood mononuclear cells [32-36]. These results can be interpreted as an experimental support to the evidence that the effects of CRET medical therapies are not due exclusively to temperature increase in the exposed tissues, but also to direct cellular responses to the electric stimulus itself.

Regarding tissue regeneration, CRET therapy is currently used in physical rehabilitation and sports medicine to treat muscle, bone, ligament and tendon lesions [37-39] and has

been successfully applied to the treatment of asthma [40] and of vascular pathologies [41]. Unlike other thermal therapies currently applied in regenerative medicine, CRET does not induce side effects like oedema and dermal or epidermal burns. Clinical studies have shown that CRET-elicited acceleration of injury recovery involves general reduction of the extension of the damaged area, together with anti-inflammatory processes, analgesia and recovery of muscle function [42-45]. These clinical effects of CRET could be consistent with a potentiation of cellular phenomena involved in regenerative processes. The aim of the present study is to investigate whether cell proliferation promotion is one of those phenomena involved in CRET-induced tissue regeneration. To that end, we have studied the *in vitro* effects of CRET electric stimulation on the proliferation of adipose-derived stromal cells (ADSC), a type of MSC, obtained from healthy human donors. The herein described results show that repeated stimulation with short pulses of 448 kHz, sine wave CRET currents at a subthermal density of 50 $\mu\text{A}/\text{mm}^2$, promotes ADSC proliferation. Such proliferative response involves stimulation of cell cycle progression and is mediated by upregulation of the mitogen-activated protein kinase - extracellular signal-regulated kinases 1 and 2 (MAPK - ERK1/2) signaling pathway. By providing experimental evidence that supports and explains, at least in part, the effectiveness of CRET therapy in injury regeneration, these results may have a significant impact on the optimization of the standard CRET procedures, as well as on the potential extension of the applications of electro-thermal treatments based on exposure to RF currents.

Materials and Methods

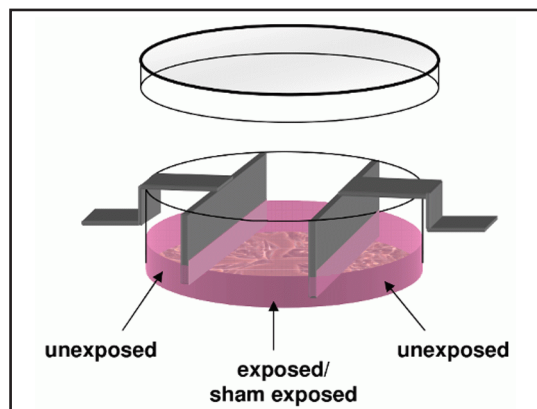
Cell culture

Adipose-derived stem cells were isolated from subcutaneous fat samples obtained, as spared tissue during general surgery procedures, from four healthy donors (two men, aged 65 and 69, and two women, aged 29 and 35). The study and procedures were evaluated and approved by the Clinical Research Ethics Committee of the University Hospital Ramon y Cajal (Madrid, Spain) and the volunteers approved tissue donation through written informed consent. Adipose tissue pieces of 0.5 - 1 cm^3 were cleaned of fibrotic tissue, visible fasciae and blood vessels and diced into small, 1 - 2 mm^3 fragments with the help of a scalpel. The fragments underwent digestion with 1 mg/ml collagenase A (Roche Applied Science, Basel, Switzerland) in Hank's Balanced salt solution (HBSS, Hyclone, South Logan, Utah, USA) for 40 m at 37 °C with gentle agitation. The collagenase activity was stopped with foetal bovine serum in high-glucose D-MEM (Biowhittaker, Verviers, Belgium). Next, cell dissociation was carried out with the help of a P1000 and MultiGuard barrier tips (Sorenson BioScience, Salt Lake City, UT, USA). Large chunks of undissociated tissue or blood vessels were allowed to settle for 2 m on the bottom of a sterile centrifuge tube. The collected cell dispersion was transferred to a different tube and centrifuged at 300 x g for 5 m to pellet the vasculostromal fraction of the cells. After suctioning the supernatant and the floating layer of adipose cells, the pellet was resuspended in MesenPro culture medium (MesenPro-RS™, Gibco, Invitrogen, Camarillo, CA, USA) supplemented with 1% glutamine (Gibco) and 1% penicillin-streptomycin (Gibco) and directly seeded on a 75 cm^2 T-flask (Falcon). After 48 h, the flask was rinsed twice with HBSS to withdraw all debris and unattached cells, and fed with MesenPro medium. Two days after, on day 4th, the medium was replaced. On day 7th, when confluent, the culture was passed by detaching the cells with 0.05% trypsin + 0.02% EDTA (Sigma, Saint Louis, MO, USA) in HBSS and seeding them in a new flask at a density of 670 cells/ cm^2 . When the culture reached confluence, cells were collected and aliquots were frozen in 10% DMSO (Sigma)/90% foetal bovine serum (Gibco, Invitrogen, Paisley, Scotland, UK). ADSC from passages third to eight were used in the experiments. The cells were plated in 60 mm-diameter Petri dishes (Nunc, Roskilde, Denmark), at a density of 725 cells/ cm^2 for cell counting study, or at 1360 cells/ cm^2 for XTT assay, immunofluorescence or Western blotting. For immunofluorescence study the cells were seeded on glass coverslips placed inside the Petri dishes.

CRET exposure

The exposure system has been described in detail in previous papers [33, 34]. Briefly, exposure to electric current was carried out by means of pairs of sterile stainless steel electrodes, designed ad hoc for

Fig. 1. *In vitro* exposure to a 448 kHz current flowing between two electrodes. The current density is homogeneous in the dish surface located within the electrode gap (exposed/sham exposed area; 1065 mm²). The cells located in spaces between each of the electrodes and the dish wall were considered unexposed. They were scraped off the dishes and discarded immediately at the end of the treatment.



in vitro stimulation, that were fitted in the Petri dishes. As shown in Figure 1, two distinct cell groups were considered within the Petri dishes in what concerns exposure. Cells growing on the dish surface within the electrode gap would be exposed (or sham exposed) to homogeneous current density, whereas those cells located in the areas delimited by each of the electrodes and the dish wall would be exposed (or sham exposed) to inhomogeneous currents. Thus, only cells grown on the rectangular surface within the electrode gap ($l = 43.5$ mm, $w = 24.5$ mm; area = 1065 mm²) were used in the present study, being the rest of the cells discarded. For CRET exposure, the electrode pairs were connected in series to a signal generator (model Indiba Activ HCR 902, INDIBA®, Barcelona, Spain). For sham-exposure, the electrode pairs inserted in control dishes were also connected to the generator, but not energized. The stimulation pattern consisted of 5-minute pulses of 448 kHz sine wave current, at a subthermal density of 50 $\mu\text{A}/\text{mm}^2$, separated by 4-h interpulse lapses, along a total period of 48 h. Such exposure parameters have been proven to affect cell proliferation in previous studies by our group [32-36]. The cultures were grown in two separate, identical CO₂ incubators (Thermo Fisher Scientific, Waltham, MA, USA). Stimulation parameters as well as atmospheric conditions inside the incubators (temperature of 37 °C, 90% relative humidity and 5% CO₂) were constantly monitored. The electromagnetic environment inside the incubators was monitored using specific magnetometers for three frequency ranges of interest: static (DC, Bartington model Mag-03, GMV associates, San Carlos, CA, USA), power frequency (50 Hz AC, EFA-3 model BN 2245/90.20, Wandel & Goltermann, Eningen, Germany) and radiofrequency (RF < 3 GHz, PMM 8053 portable field strength meter with external PMM EP-330 E-field probe, both from Narda Safety Test Solutions, Milan, Italy). The mean values obtained were $B_{\text{DC}}: 24.4 \pm 3.4$ μT rms, $B_{\text{AC}}: 5 \pm 3$ μT rms and RF: below of detection limit of the magnetometer.

Differentiation assay for mesenchymal characterisation

In order to assess the differentiating multipotentiality of the obtained ADSC, the cells were seeded at a high density of 2270 cells/cm² in 60 mm-diameter Petri dishes, and incubated in adipogenic, chondrogenic or osteogenic medium. The three media had a common basis composed of high-glucose D-MEM (Biowhittaker) supplemented with 10% foetal bovine serum (Gibco), 1% glutamine and 1% penicillin-streptomycin (Gibco). The adipogenic medium was supplemented with 0.25 mM 3-isobutyl-1-methylxanthine (IBMX, Gibco), 200 μM indomethacin (Sigma), 10 $\mu\text{g}/\text{ml}$ insulin (Sigma) and 1 μM dexamethasone (Sigma). The specific supplement in the chondrogenic medium consisted of 150 nM ascorbic acid-2 phosphate (Sigma), 10 ng/ml TGF- β 1 (Peprotech, Rocky Hill, NJ, USA), 10 $\mu\text{g}/\text{ml}$ insulin and 100 nM dexamethasone. For osteogenic differentiation, the basic medium was supplemented with 10 ng/ml BMP-2 (Invitrogen, Camarillo, CA, USA), 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate and 10 mM β -glycerophosphate (Calbiochem, Darmstadt, Germany). At day 15 of incubation in the presence of the respective differentiating media, the ADSC were fixed in 4% paraformaldehyde and stained with Oil Red O (Sigma) for assessment of adipogenic differentiation, with Alcian Blue (BDH, Poole, UK) for chondrogenesis or with Alizarin Red S (Sigma) for osteogenesis.

The same procedure for differentiation assessment was applied to investigate whether ADSC multipotentiality could be affected by CRET exposure. After the electric or sham treatment, the cultures were incubated for two weeks in adipogenic, chondrogenic or osteogenic media, and then fixed and stained with Oil Red, Alcian Blue or Alizarin Red, respectively.

Cell counting

Cultures at passages between two and eight were used. In each experimental run, 5 CRET-stimulated and 5 sham-exposed cultures were plated in 60 mm Petri dishes. After 48 hours of treatment, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton in PBS and the cell nuclei were fluorescence stained with 10^{-5} M bisBenzimide H 33258 (Sigma). The number of cells in the dish surface located within the 1065 mm² rectangle delimited by the two electrodes was assessed through an Olympus IX-70 fluorescence microscope. Using a 10x objective, 24 microscope fields (840 μ m x 630 μ m; area = 0.5292 mm²) spaced an average distance of 1.25 mm, were randomly selected, photographed and analyzed. Nuclei that had at least half of their cross-area included inside one field were counted and the total cell number within the exposed area was estimated from such counting.

XTT proliferation assay

The effect of CRET on cell proliferation was determined by XTT assay (Roche). Cells from passage third to seventh were used. In each experimental run, 10 dishes were plated (5 for CRET exposure and 5 controls). After 48 h of CRET or sham treatment, the cells grown within the area of homogeneous exposure were incubated with the tetrazolium salt XTT for 3 hours in a 37 °C and 6.5% CO₂ atmosphere. XTT was reduced by the metabolically active cells into colored formazan compounds that were quantified with a microplate reader (TECAN, Männedorf, Switzerland) at a 492 nm wavelength. The obtained values were directly correlated to the number of active cells. A total of 28 experimental replicates were conducted with different culture passages.

Bromodeoxyuridine incorporation assay

The effects of the electric stimulation on cell proliferation and DNA synthesis were assessed through immunofluorescence detection of 5-bromodeoxyuridine (BrdU) incorporation. Cells from passages third to fifth were seeded in 60 mm Petri dishes, on pairs of 12-mm diameter coverslips placed within the electrode gap. During the last 6 h of sham or CRET treatment the cultures were incubated in the presence of 3 mM BrdU (Sigma). Afterwards, the cells were fixed with 4% paraformaldehyde and permeabilized with ethanol:acetic acid (95:5) for 10 min at 4 °C. The coverslips were immunofluorescence stained through overnight incubation with a mouse monoclonal anti-BrdU antibody (1:20, Dako, Glostrup, Denmark) at 4 °C, followed by 1 h incubation at room temperature with anti-mouse IgG conjugated to Alexa Fluor 568 (1:500, Molecular Probes, Invitrogen, Camarillo, CA, USA). Cell nuclei were fluorescence stained with bisBenzimide H 33258 (Sigma) added to mounting medium containing anti-fade p-phenylenediamine (Sigma). The coverslips were analyzed in a Nikon Eclipse TE300 fluorescence microscope. Four experimental repeats were conducted with 4 coverslips per experimental group. The numbers of total nuclei and of BrdU-positive (BrdU+) cells were counted in fields selected by systematic random sampling. A total of 15 fields were studied per coverslip. Images were recorded and analyzed with AnalySIS 3.1 software (Soft Imaging Systems GmbH, Münster, Germany.)

Cell cycle analysis

The potential effects of the treatment on the cell cycle were evaluated by flow cytometry using cultures at passages P3 and P4. At the end of the electric or sham treatments, the cells growing within the electrode gap were detached with trypsin (Sigma), harvested in eppendorf tubes and fixed overnight at 4 °C in 1 ml of 70% ethanol. Samples of approximately 1×10^5 cells per dish were washed twice in PBS and incubated for one hour in the dark and at room temperature with 20 μ g/ml propidium iodide staining solution (Boehringer, Ingelheim, Germany) supplemented with RNase A (200 ng/ml; Boehringer) in 3.4 mM citrate buffer. The cells were analyzed in a flow cytometer (FACScalibur, BD Biosciences, San Jose, CA, USA). Ten thousand events per sample were acquired using CellQuest 3.2 software (BD Biosciences). A total of 4 experimental replicates were conducted. Ten samples (5 CRET-treated and 5 sham-exposed) were analyzed per replicate.

PCNA immunofluorescence

The proliferating cell nuclear antigen (PCNA) is a DNA polymerase-associated protein that is currently used as a marker for cells undergoing S and G2 phases of the cell cycle [46]. Cultures at passages third to fifth were seeded on coverslips and CRET- or sham-exposed as described above. The cells were fixed with 4% paraformaldehyde and permeabilized in ethanol:acetic acid, incubated overnight at 4 °C with anti-PCNA

antibody (Santa Cruz Biotechnologies, TX, USA) and fluorescence stained with anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes) for 1 h at room temperature. Cell nuclei were counterstained with bisBenzimide H 33258. Four experimental repeats were conducted, each with 4 coverslips per experimental group. The percentages of PCNA-positive cells were estimated as described above for BrdU assay.

Western Blotting

Cells at passages third to fifth were used. At the end of the CRET- or sham-exposure interval, the cells located within the electrode gap were scrapped off the dishes, re-suspended in PBS and centrifuged for 5 min at 1200 rpm. The resulting pellet was lysed at 4 °C in buffer containing 10 mM Tris-HCl, 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 β-glycerophosphate, 20 mM sodium molybdate, 0.5 % Triton X-100 and 0.1% SDS. The lysates were centrifuged at 10000 rpm for 5 seconds at room temperature and the protein concentration in the supernatant was determined through Bradford's method. Proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) using semi-dry transfer methodology (Bio-Rad, Hercules, CA, USA). Membranes were blocked in PBS containing 5% nonfat dry milk, and incubated overnight at 4 °C in rabbit anti-PCNA antibody or rabbit anti-phospho-ERK1/2 antibody (Invitrogen), both diluted 1:1000, and mouse anti β-actin (1:5000; Sigma), as loading control, in blocking buffer (0.1% Tween and 5% nonfat dry milk in PBS). The membranes were then washed four times with PBS-Tween and subsequently, incubated for one hour at room temperature with goat anti-rabbit IgG conjugated to IRdye 800 CW (1:10000 dilution, LI-COR Biosciences, NE, USA) and anti-mouse IgG conjugated to IRdye 680 LT (1:15000 dilution, LI-COR Biosciences). The fluorescent intensity of the blots was measured with a LI-COR Odyssey scan (LI-COR Biosciences) and evaluated using Quantity One software, version 4.6.7, from Bio-Rad. Five experimental replicates for PCNA protein and three replicates for phosphorylated ERK1/2 (p-ERK1/2) were conducted. Eight samples (4 sham- and 4 CRET-exposed) were analyzed per replicate. PCNA and p-ERK1/2 values were normalized with respect to the loading control.

Statistical Analysis

Except where otherwise stated, the data were analyzed by two-tailed unpaired Student's t-test using GraphPad Prism software, San Diego, CA, USA. Differences between samples were considered statistically significant at $p < 0.05$.

Results

Adipogenic, chondrogenic and osteogenic differentiation of ADSC

The ADSC were assessed for capability to differentiate into adipocytes, chondrocytes or osteocytes. After two weeks in differentiating medium, clear patterns of differentiation into the three cell lineages were observed (Fig. 2).

CRET effects on cell number

The count of bisBenzimide labelled nuclei revealed that the CRET effect was dependent on the culture passage. Indeed, cultures treated with CRET in passages P3 to P5 showed statistically significant increases in cell number, reaching up to a 25% raise over controls sham-exposed in passage P5 (Fig. 3A). By contrast, no increase in cell count was noticeable at very early passages (P2), and even a decrease in cell number might occur at late passages (P7, P8) at which the cells started to show signs of senescence. The XTT colorimetric assay confirmed the cell number increase of up to 20% with respect to controls in cultures treated at passages P3 to P5. Also, a statistically significant decrease in cell number was detected in samples exposed to CRET in passage P7 (Fig. 3B).

Assessment of CRET effect on cell proliferation through BrdU incorporation into DNA

The amount of proliferative cells after 48 h of CRET treatment or sham exposure was determined by BrdU incorporation. The proportion of BrdU+ cells in the treated group

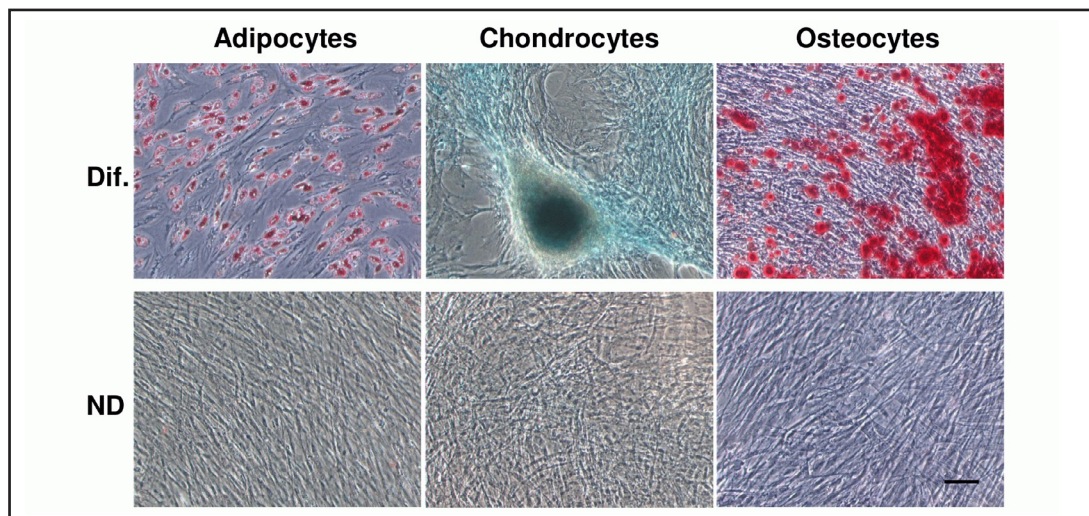


Fig. 2. Multipotentiality assay for ADSC characterization. When supplemented with adipogenic, chondrogenic or osteogenic media (Dif.) the cells isolated from fat tissue differentiated into the corresponding cell lineages, whereas in the absence of supplement (ND) the cells remained undifferentiated. Scale bar = 100 μ m.

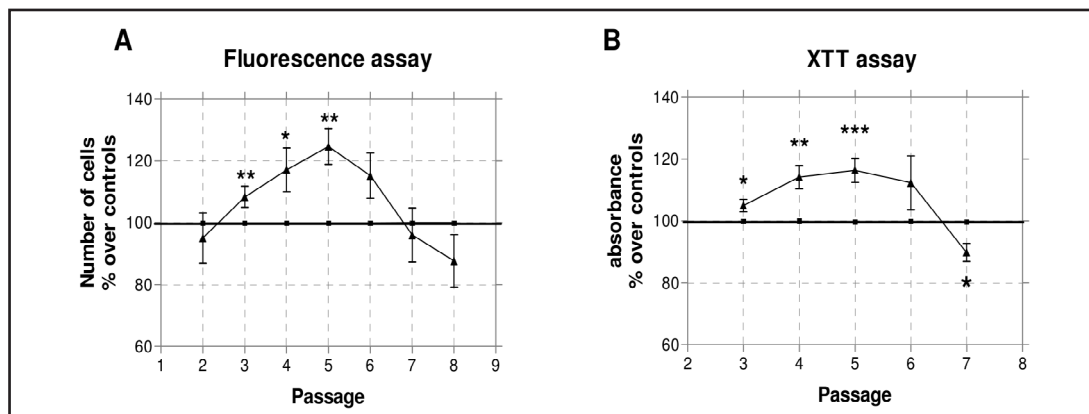


Fig. 3. Proliferation assays. (A) Fluorescence microscopy count of bisBenzimide stained cell nuclei in culture passages P2 to P8. Between 2 and 4 experimental replicates were conducted per passage. Points represent Means \pm SEM of cells per microscope field; data are normalized over the respective control samples. (B) XTT assays for cell proliferation in culture passages P3 to P7. Between 3 and 8 experimental replicates per passage. Means \pm SEM in percents over the corresponding controls. *: $0.01 \leq p \leq 0.05$; **: $0.001 \leq p \leq 0.01$; ***: $p < 0.001$ (Student's t test).

was significantly increased by 38% over that in controls ($p < 0.001$, Student's t test, Fig. 4), indicating that the increase in cell number induced by CRET and revealed by the bisBenzimide and XTT assays, would be mediated, at least in part, by stimulated cell proliferation.

CRET effects on the cell cycle

The potential effects of CRET treatment on the cell cycle were assessed by flow cytometry with propidium iodide. Analysis of the data obtained from 4 experimental replicates using cells in passages P3 to P5, revealed a modest (3%) but statistically significant decline in the proportion of cells in G0/G1 phase, accompanied by statistically significant increases (21% and 10% over controls) of cells at phases S and G2/M, respectively (Fig. 5). These data reinforced the preceding results on cell proliferation and indicate that CRET treatment stimulates cell cycle progression.

Fig. 4. BrdU immunofluorescence assay. The fraction of cells which incorporated BrdU into their DNA (Alexa Red), out of the total cell population, whose nuclei were stained with bisBenzimide, was quantified in cultures in passages P3 to P5. (A) Representative micrographs; scale bar: 100 μ m. (B) Results in percents over controls. Means \pm SEM of 4 experimental replicates. ***: $p < 0.001$ (Student's t test).

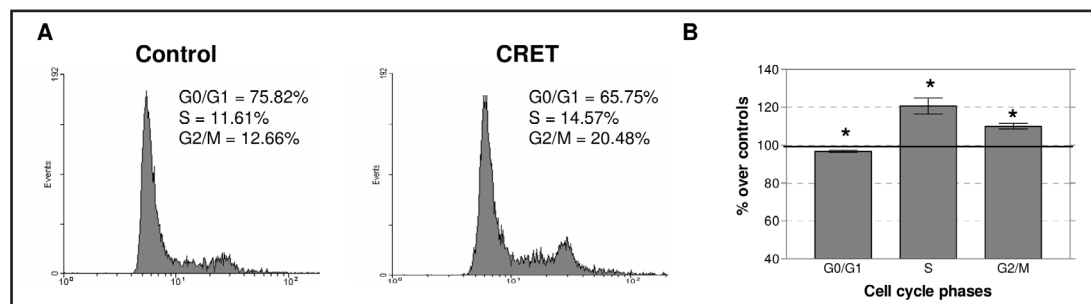
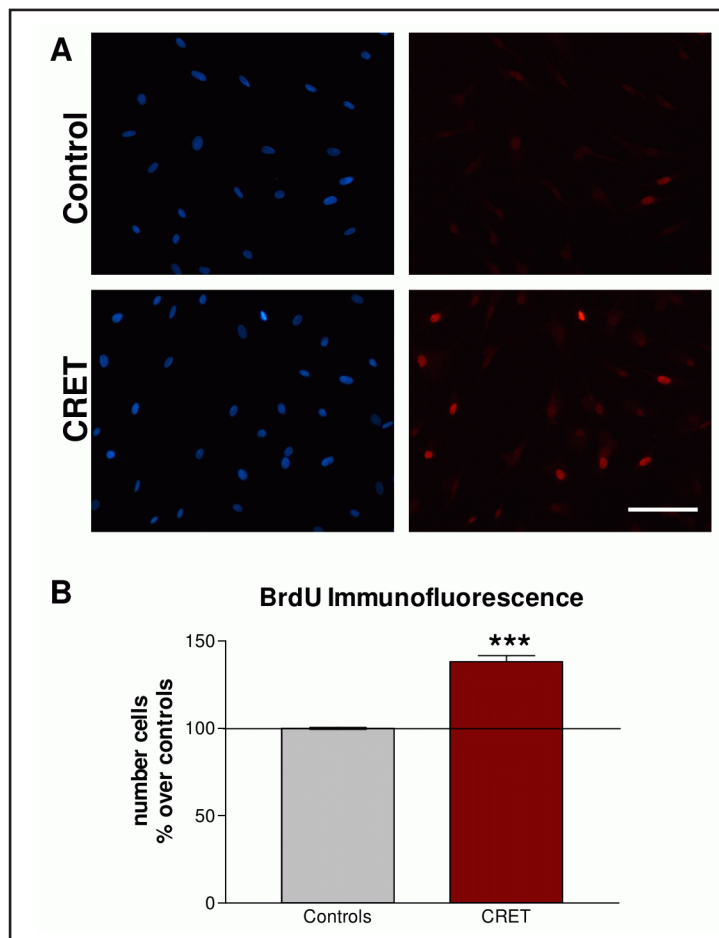


Fig. 5. Flow cytometry analysis of the cell cycle. Cells in passages P3 and P4 were harvested at the end of the CRET- or sham- exposure interval and stained with propidium iodide. (A) Representative results from one single repeat. Each graph represents the analysis of 10000 events obtained from the corresponding samples. (B) Propidium iodide determination of the fractions of cells in different phases of the cell cycle. Percents over controls. Means \pm SEM of 4 experimental replicates. *: $0.01 \leq p \leq 0.05$ (Student's t test).

CRET effect on the expression of the proliferating cell nuclear antigen (PCNA)

In order to additionally investigate the basis of the induced proliferative response, we studied the potential effects of CRET on the expression of the proliferating cell nuclear antigen, PCNA by counting of positive cells (PCNA+) and by Western Blotting. The electric treatment elicited a statistically significant increase (35%) of PCNA+ cells over controls (Figs. 6A and 6C). As for the densitometry of PCNA immunoblots, normalized with respect to β -actin as loading reference, it revealed that PCNA expression in the CRET-treated samples was increased by 35% over that in sham-exposed controls ($p < 0.05$; Paired t test, Figs. 6B and 6C).

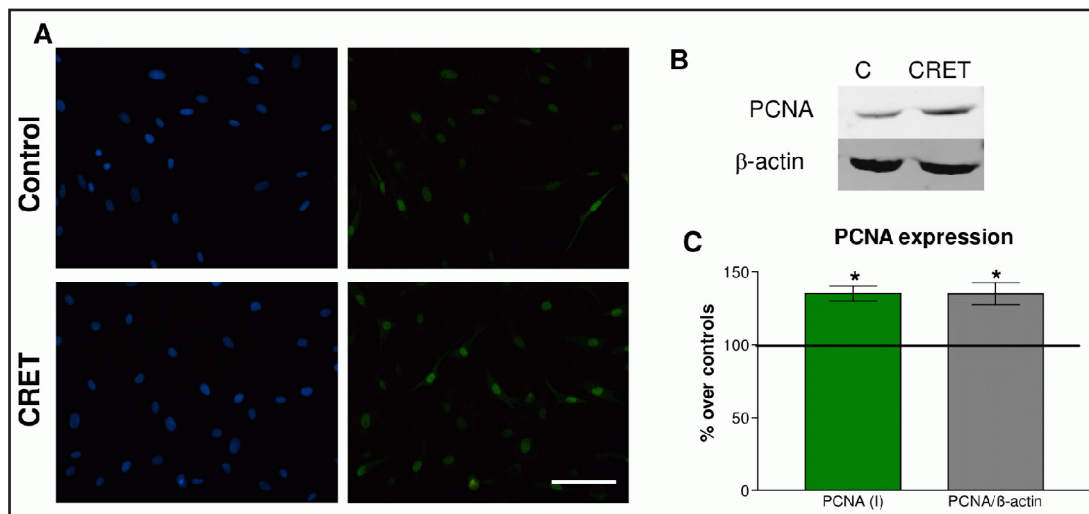
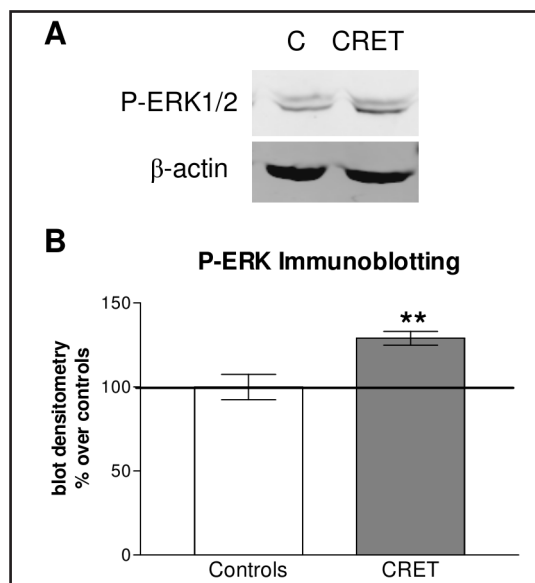


Fig. 6. PCNA expression. (A) Micrographs of immunofluorescence for PCNA antigen. PCNA+ cells were revealed with Alexa Green, whereas the nuclear DNA of the entire cell population was blue stained with bis-Benzimide. Scale bar: 100 μ m. (B) Representative blot of PCNA expression using β -actin as loading control, 30 μ g protein/lane. Cells from passages P3 to P5. C: sham exposed controls; CRET: electrically stimulated samples. (C) Results of immunofluorescence (PCNA (I)) and densitometric analysis of immunoblots for PCNA (PCNA/ β -actin ratio). Percents over controls. Means \pm SEM (immunofluorescence) of 4 experimental replicates, and means \pm SD (immunoblots) of 5 experimental repeats. *: $0.01 \leq p \leq 0.05$ (Paired t test for immunoblot analysis).

Fig. 7. p-ERK1/2 expression. (A) Representative blot of p-ERK1/2 expression, using β -actin as loading control, 30 μ g protein/lane. Cells in passages P3 to P5. C: sham exposed controls; CRET: electrically stimulated samples. (B) Densitometric analysis of immunoblots; p-ERK1/2/ β -actin ratio. Percents over controls; means \pm SD of 3 experimental repeats. **: $0.001 \leq p \leq 0.01$ (Paired t test).



CRET effect on the ERK1/2 signaling pathway

The activity of diverse regulatory systems of the cell proliferation is orchestrated by activation of ERK1/2 [47]. Thus, since CRET stimulation promotes ADSC proliferation, it is possible that such effect was mediated by an action of CRET on the ERK signaling pathway. The expression of p-ERK1/2 was assessed by immunoblotting of ADSC at passages P3 and P4. Band densitometry normalized to β -actin revealed a 43% increase of p-ERK1/2 expression in the CRET-treated samples with respect to sham-exposed controls ($p < 0.01$; Paired t test, Fig. 7).

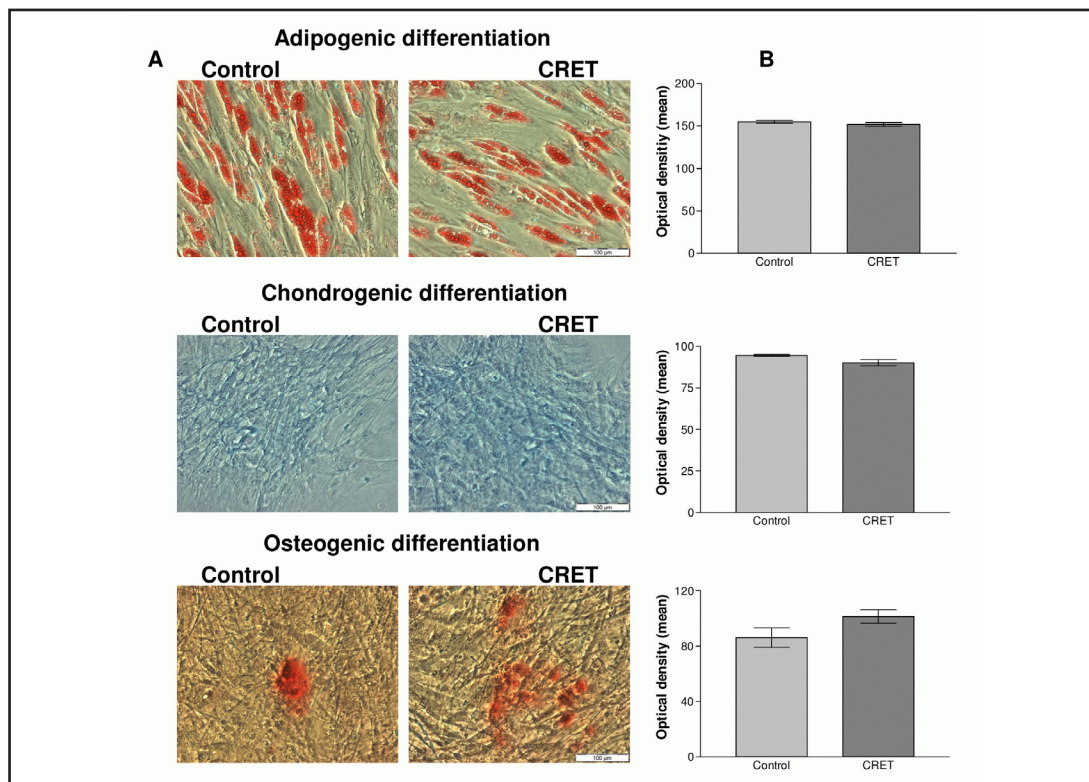


Fig. 8. Assessment of ADSC multipotentiality after CRET treatment. After CRET- or sham- exposure, cell cultures in passages P3 and P4 were grown for 14 days in the presence of differentiating media. (A) Representative micrographs of samples maintained in adipogenic (top), chondrogenic (center) or osteogenic medium (bottom) and stained with Oil Red, Alcian Blue or Alizarin Red, respectively. Scale bar: 100 μ m. (B) ADSC differentiation was assessed by staining quantification through computer assisted image analysis of the relative optical density of photomicrographed samples. Histograms show that the means \pm SEM of the optical densities in the CRET exposed samples did not differ significantly from those in the corresponding controls ($p > 0.05$). Three experimental replicates, 4 treated dishes and 4 controls per replicate.

ADSC maintain multipotentiality after CRET treatment

Cytochemical analysis of the adipogenic, chondrogenic and osteogenic differentiation post-treatment was carried out in order to elucidate whether the multipotentiality of ADSC might be affected by CRET exposure. After two weeks of post-exposure incubation in the presence of the corresponding differentiating media, intracellular lipid vesicles, GAG and calcium spots were present in the adipogenic, chondrogenic and osteogenic cultures, respectively. The differentiation patterns in the CRET exposed samples did not differ significantly from those in controls, kept in the corresponding media for two weeks after sham exposure (Fig. 8). This indicates that CRET treatment does not affect the multipotentiality of ADSC in what concerns their ability for adipogenic, chondrogenic and osteogenic differentiation.

Discussion

Most studies aimed at promoting proliferation of stem cells and progenitors of various cell lineages through exposure to physical stimuli, have used very low frequency electromagnetic fields. For instance, *in vitro* exposure to pulsed electromagnetic fields (PEMF) at extremely low frequencies (ELF) of 15 Hz or 50 Hz, has been shown to significantly

increase cell proliferation by 20% to 60% over controls, in human [48] and rat BMSC [49]. Also, 50 Hz sine wave fields of 0.5 to 1 mT have been reported to promote proliferation of rodent BMSC [29, 50]. Although regenerative therapies applying signal frequencies within the intermediate-to-radiofrequency spectrum have expanded in recent years, no experimental evidence has been obtained to date that electric or electromagnetic stimulation at such frequencies could promote stem cell proliferation. The results of the present study show that repeated stimulation with subthermal doses of CRET radiofrequency currents stimulates ADSC proliferation, this effect involving cell cycle changes that are mediated by stimulation of the expression of phosphorylated ERK1/2.

The counting of bisBenzimide labelled nuclei revealed that CRET stimulation increases the cell number in ADSC cultures treated at intermediate passages. The maximal proliferative effects were reached at passages P3 to P5, at which the cell number in CRET-treated cultures was up to 25% higher than that in sham-exposed controls (Fig. 3A). By contrast, such electrically induced increases in cell number were not observed neither in passages earlier than P3 nor in those later than P6. These results obtained by nuclei counting were confirmed through XTT assay (Fig. 3B).

Passage-dependence of the proliferative response to weak, electric or electromagnetic stimuli has also been observed by other authors. For instance, Diniz et al. [51] reported that a 15 Hz, 7 mT PEMF stimulates cell proliferation in the osteoblast line MC3T3-E1, only when exposure takes place at intermediate passages. When grown in MesenPro medium under the herein described conditions of low-density seeding and one passage per week, our ADSC appear to be insensitive to CRET stimulation for the initial two weeks. This lack of response would be attributable to the characteristic insensitivity of the still immature cells in early passages. From week/passage 3rd to 6th, when sham-exposed cultures expand at a rate of 30-50 fold per week (data not shown) the cells become sensitive and their proliferation rate increases in response to the electric stimulus. As for the null or even potentially antiproliferative response of late passages (Fig. 3), it might be due to the fact that from passage 7 on, ADSC cultured in MesenPro-RSTM enter replicative senescence. Indeed, our late passage cells showed a number of genetic abnormalities, including aneuploidy (data not shown) that are characteristic of senescent cultures. The possibility that CRET elicits antiproliferative effects in senescent ADSC could take partial support from previous studies by our group, which report that CRET treatment causes antiproliferative effects in hepatocarcinoma HepG2 and neuroblastoma NB69 [32-36], two human cancer cell lines known to carry a variety of genetic alterations [52, 53].

The data of BrdU incorporation into nuclear DNA showed that CRET stimulation actually increases cell proliferation, by an average of 38% over sham-exposed controls, in cultures at intermediate passages (Fig. 4); this result being potentially corroborated by the immunocytochemical and immunoblot analyses of PCNA expression (Fig. 6). Since PCNA is a DNA polymerase-associated nuclear protein, its expression increases during cycle phases S and G2, as well as during DNA repair. Consequently, parallelism between PCNA expression and BrdU incorporation, as shown by the present results, is currently interpreted as a suitable indicator of DNA replication [46]. Alternatively, increased PCNA levels might be suggestive of DNA damage due to CRET exposure. However, the fact that the increases in PCNA expression are accompanied of increases in cell number, together with the BrdU incorporation data, indicates that the CRET-induced proliferative effect would be mediated by stimulation of the cell cycle progression in phases S/G2 (Fig. 5).

Comparison between the responses of the ADSC cultures obtained from the four donors of different ages and gender, did not reveal significant differences in the proliferative effects of CRET when administered at intermediate passages. Also, the fact that CRET treated cultures conserve their ability to differentiate into a variety of cell types including adipocytes, chondrocytes and osteocytes (Fig. 8), suggests that the multipotentiality of proliferating ADSC is not affected by the electric stimulus. Thus, in what concerns the clinical applications of CRET currents, these results suggest that the electrical treatment could promote or accelerate lesion repair by stimulating proliferation of the already expanding stem cells.

Such proliferative effect does not appear to be dependent on the patient's age or gender, at least *in vitro*, and would not affect the ADSC multipotentiality to subsequently differentiate into a number of cell lineages.

Evidence exists indicating that the proliferative response to electric or electromagnetic stimuli could be related with increased proportions of cells at specific, proliferative cycle phases. In this regard, it has been reported that a 50 Hz, 0.5 mT field induces proliferation in mouse BMSC by increasing the proportion of cells entering phases S and G2 of the cycle [29]. Also, human BMSC exhibit a proliferative response to *in vitro* treatment with 15 Hz PEMF through increased proportions of cells undergoing phase G2/M during exposure, and of cells in phase G0/G1 at the end of the stimulation interval [48]. Recently, Li et al. [49] have reported that exposure to 50 Hz, 10 mT PEMF increases proliferation in rat BMSC and blocks cell cycle in phase G1, what is interpreted by the authors as a potential cytodifferentiating effect of the stimulus. In the present study, flow cytometry analysis revealed that after CRET exposure, the cultures showed increased percentages of cells in phases S, G2 and mitosis, accompanied by a limited but statistically significant drop in the rate of cells in the non proliferative phase G0/G1 (Fig 5). Thus, it can be hypothesized that under treatment with CRET electric currents of 448 kHz, quiescent stem cells present in damaged tissues can be stimulated to enter proliferative phases of the cell cycle, thereby triggering the process of stem cell renewal that will lead to tissue repair.

As for molecular mechanisms underlying the proliferative response elicited by CRET, the main candidates are those signaling pathways that, being involved in cell proliferation and cell cycle regulation, are also potentially susceptible to electric stimulation. Among these pathways, the mitogen-activated protein kinases (MAPK-ERK1/2) are molecules typically involved in transduction of proliferative signals from extracellular origin. The family of serine/threonine kinases to which ERK belong, can be activated by a large variety of chemical [47] and physical stimuli [54-56]. ERK1 and ERK2 are fully activated through phosphorylation by MEKs (MAPK/ERK kinases). It has been reported that static, DC electric fields can regulate the regeneration of the lens epithelium through activation of the MAPK signaling cascade ERK1/2 [57]. Other physical therapies could also promote injury recovery through cell proliferation induced by activation of ERK1/2, as observed in ultrasound-treated human skin fibroblasts [58]. The present results show that the expression levels of phosphorylated ERK1/2 were significantly increased in the CRET-treated ADSC when compared to sham-exposed controls (Fig. 7). This is consistent with the existing evidence that weak electric or electromagnetic stimuli can activate the Ras/Raf/MEK/ERK pathway, and provides substantial support to the hypothesis that the proliferative action of CRET on human mesenchymal cells can be exerted, at least in part, by activation of such signal transduction pathway.

Conclusion

In sum, the herein reported results show that intermittent exposure to a 448 kHz electric stimulus currently used in electrothermal CRET therapies, induces upregulation of the ERK1/2 signaling pathway and promotes proliferation in mesenchymal stem cells obtained from healthy human donors. Since the electric stimulus was applied at a subthermal current density of 50 $\mu\text{A}/\text{mm}^2$, these results reveal that molecular and cellular mechanisms other than the thermal ones can be crucial to the therapeutic efficacy of CRET treatments for tissue repair. The present results are also suggestive that CRET treatment could promote tissue regeneration by activating proliferation of the ADSC present in the damaged area. Furthermore, the proliferative response elicited by the treatment would not compromise the stem cell multipotentiality for subsequent adipogenic, chondrogenic or osteogenic differentiation. If extrapolable to the medical practice, these results are suggestive that CRET electrotherapy could be applied as an efficacious adjuvant to the recovery of a variety of tissular and vascular lesions, or as an optional treatment for patients that are sensitive to the

side effects of some chemical therapies. Also, since mesenchymal stem cells directly intervene in the control of inflammatory processes by secreting anti-inflammatory interleukins in the damaged region [2], CRET might be useful in anti-inflammatory treatments through its ability to increase the local population of mesenchymal stem cells. Studies are in progress investigating the possibility that CRET could stimulate stem cell differentiation towards connective tissue.

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Disclosure Statement

The authors declare that no competing interest exists.

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