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ELECTRIC STIMULATION AT 448 kHz PROMOTES PROLIFERATION OF HUMAN MESENCHYMAL STEM CELLS

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INTRODUCTION

Precursor cells play a crucial role in tissue regeneration. After proliferation the new cells regain the tissue original function. Mesenchymal stem cells (MSC) are a key cell population involved in the proliferative phase of regeneration of lesions and are present in almost all adult tissues.

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 (1-7). Among these therapies, Capacitive-Resistive Electric Transfer (CRET) is a non-invasive electro-thermal strategy, based on the application of electric currents within the radiofrequency range of 400 kHz-450 kHz. Recent in vitro results indicate that, when administered at thermal current densities, CRET causes cytotoxicity in human cancer cells, such a thermal effect being enhanced by injection of metallic microparticles within the targeted tumoral tissues (8). At the cellular level, CRET effects are not limited to the thermal ones. CRET stimulation at subthermal (non heating) 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 (9-13). These experimental results can be interpreted as supportive of the existing evidence that the effects of CRET medical therapies are not due exclusively to temperature increase, 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 (14-16). The acceleration of injury recovery due to CRET involves general reduction of the extension of the damaged area, together with anti-inflammatory processes, analgesia and recovery of muscle function (17-20).

The aim of the present study is to investigate whether cell proliferation promotion is one of those phenomena involved in CRET-induced tissue regeneration at a subthermal current density, in adipose-derived stem cells (ADSC), a type of MSC.

MATERIALS AND METHODS

Cell culture. Adipose-derived stem cells were isolated from subcutaneous fat samples from four healthy donors (two men, aged 65 and 69, and two women aged 29 and 35). ADSC from passages third to eight were used in the experiments.

CRET exposure. Cell exposure (as shown in Figure 1) to CRET currents consisted of 5-minute pulses of 448 kHz current at a subthermal density of 50 μA/mm², separated by 4-h interpulse lapses, along a total period of 48 h. The electrode pairs were connected in series to a signal generator (INDIBA® device, INDIBA SA, Barcelona, Spain). Such exposure parameters have been proven to affect cell proliferation in previous studies by our group (9-13). Cultures were grown in incubators and constantly monitored. For sham-exposure, the electrode pairs inserted in control dishes were also connected to the generator, but not energized.

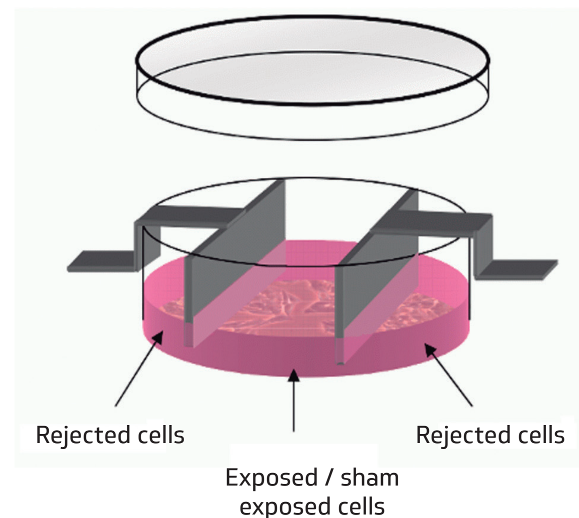

Fig. 1. In vitro exposure to a 448 kHz current flowing between two electrodes. The current density is homogeneous in the dish surface located within the electrode gap (exposed / sham exposed area; 1065 mm²). Rejected cells were scraped off the dishes and discarded immediately at the end of the treatment.

Differentiation assay for mesenchymal characterisation. To assess the differentiating multipotentiality of the obtained ADSC, cells were incubated in adipogenic, chondrogenic or osteogenic medium. At day 15 of incubation in the respective differentiating media, ADSC were fixed for assessment of adipogenic, chondrogenic or osteogenic differentiation.

The same procedure for differentiation assessment was applied to investigate whether ADSC multipotentiality could be affected by CRET exposure.

Cell proliferation assessment. The effect of CRET on cell proliferation was determined by XTT colorimetric assay and by DNA synthesis quantification through immunofluorescence detection of 5-Bromodeoxyuridine (BrdU) incorporation.

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. To evaluate cells undergoing S and G2 cell cycle phase it was used the proliferating cell nuclear antigen (PCNA) which is a DNA polymerase-associated protein marker (21).

RESULTS

Adipogenic, chondrogenic and osteogenic differentiation of ADSC. ADSC showed clear patterns of differentiation into the three studied cell lineages: adipocytes, chondrocytes or osteocytes (Fig. 2)

CRET proliferation. CRET effect was dependent on the culture passage. ADSC treated 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). 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 (Fig. 3B).

The proportion of BrdU+ cells in the treated group was significantly increased by 38 % over that in controls ($p < 0.001$).

Cell cycle analysis. About CRET effects on the cell cycle, in passages P3 to P5 there was 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 4). These data were reinforced by PCNA counting of positive cells, where CRET elicited a statistically significant increase (35%) over controls.

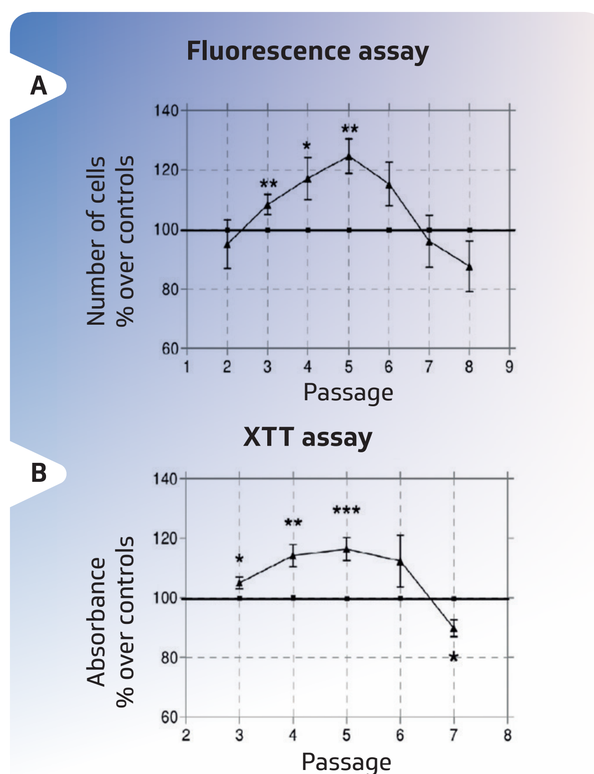

Fig. 3. Proliferation assays. **(A)** Fluorescence microscopy count of bisBenzimide stained cell nuclei in culture passages P2 to P8. Data are normalized over the respective control samples. **(B)** XTT assays for cell proliferation in culture passages P3 to P7. *: $0.01 \le p \le 0.05$; **: $0.001 \le p \le 0.01$: ***: $p < 0.001$ (Student's t test).

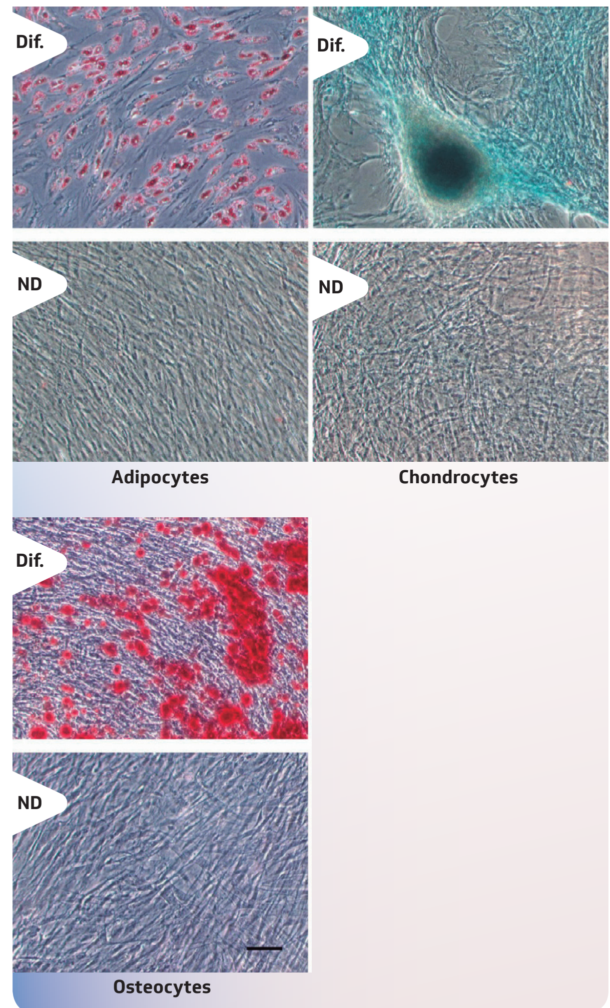

Fig. 2. When supplemented with adipogenic, chondrogenic or osteogenic media (Dif.) the cells isolated from fat tissue differentiated into the corresponding cell lineages, whereas in the absence of supplement (ND) cells remained undifferentiated. Scale bar = 100 µm.

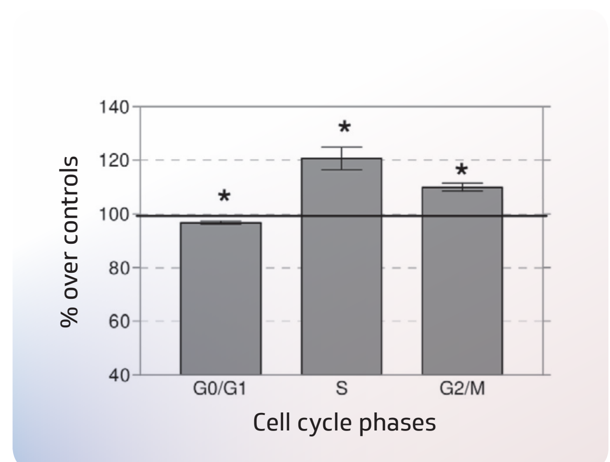

Fig. 4. CRET effects on cell number in different cycle phases; percentage over controls (100%). *: $0.01 \le p \le 0.05$.

ADSC multipotentiality after CRET treatment. After two weeks of post-exposure incubation in the presence of the corresponding differentiating media the differentiation patterns in the CRET exposed samples did not differ significantly from those in controls (Fig. 5).

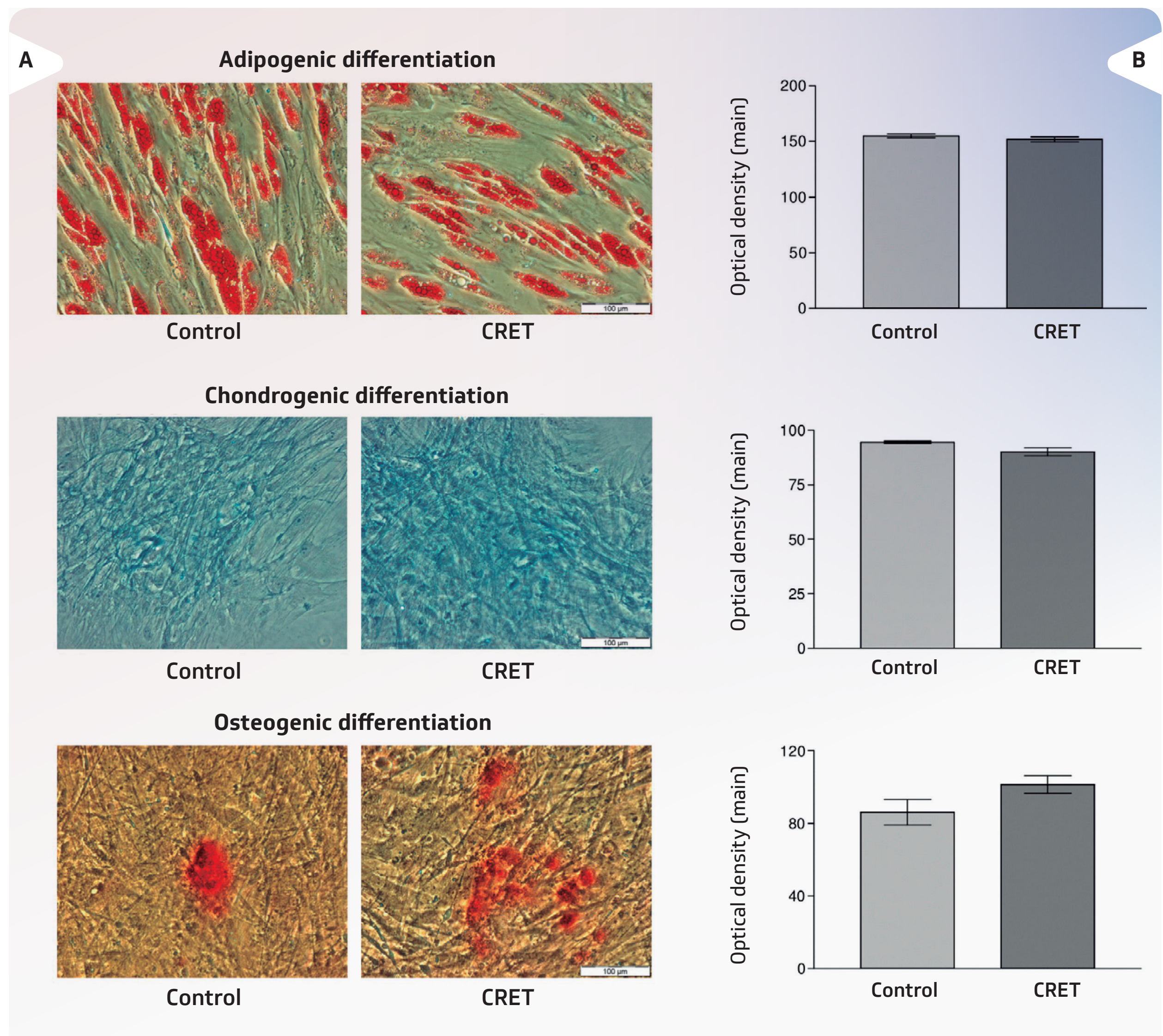

Fig. 5. ADSC multipotentiality after CRET treatment. After CRET- or sham- exposure, cell cultures in passages P3 and P4 were grown for 14 days in the presence of differentiating media. **(A)** Representative micrographs of samples maintained in adipogenic (top), chondrogenic (center) or osteogenic media (bottom) and stained with Oil Red, Alcian Blue or Alizarin Red, respectively. Scale bar: 100 µm. **(B)** ADSC differentiation was assessed by staining quantification through computer assisted image analysis. Histograms show that the means ± SEM of the optical densities in the CRET exposed samples did not differ significantly from those in the corresponding controls ($p > 0.05$).

CONCLUSIONS

The results revealed significant improvements in OA-related pain and function in participants treated with active CRMRF, when compared to those who were treated with exercise and advice only. The fact that the sham group also improved at a greater rate than the control group notwithstanding the effect size being smaller than that of the active treatment indicated the presence of a placebo effect. This is unsurprising given that significant placebo effect may normally exist with perceived intervention, mainly due to expectation [26].

The present results show that intermittent exposure to a 448 kHz electric stimulus applied in Capacitive-Resistive Electric Transfer (CRET) therapies increases the percents of cells in phases S, G2 and mitosis, and promotes proliferation in human mesenchymal stem cells. The obtained results indicate that CRET electric treatment could promote tissue regeneration by activating proliferation of quiescent Adipose Derived Stem Cells (ADSC) present in the damaged area, without compromising the stem cell multipotentiality for subsequent adipogenic, chondrogenic or osteogenic differentiation. These data, together with previously published experimental evidence, strongly support the hypothesis that molecular and cellular mechanisms other than the thermal ones can be crucial to the therapeutic efficacy of CRET treatments, including those applied to tissue repair.

In sum, taken together, the herein released results suggest that CRET electrical treatment could promote or accelerate lesion repair by stimulating proliferation of the already expanding stem cells. From this it can be proposed that CRET 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. CRET might also be useful in anti-inflammatory treatments.

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