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Pulsed magnetic therapy increases osteogenic differentiation of mesenchymal stem cells only if they are pre-committed

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ABSTRACT

Aim: Pulsed electromagnetic field (PEMF) therapy has been documented to be an effective, non-invasive, safe treatment method for a variety of clinical conditions, especially in settings of recalcitrant healing. The underlying mechanisms on the different biological components of tissue regeneration are still to be elucidated. The aim of the present study was to characterize the effects of extremely low frequency (ELF)-PEMFs on commitment of mesenchymal stem cell (MSCs) culture system, through the determination of gene expression pattern and cellular morphology.
Main methods: Human MSCs derived from adipose tissue (ADSCs) were cultured in presence of adipogenic, osteogenic, neural, or glial differentiative medium and basal medium, then exposed to ELF-PEMFs daily stimulation for 21 days. Control cultures were performed without ELF-PEMFs stimulation for all cell populations. Effects on commitment were evaluated after 21 days of cultures.
Key findings: The results suggested ELF-PEMFs does not influence ADSCs commitment and does not promote adipogenic, osteogenic, neural or glial differentiation. However, ELF-PEMFs treatment on ADSCs cultured in osteogenic differentiative medium markedly increased osteogenesis.
Significance: We concluded that PEMFs affect the osteogenic differentiation of ADSCs only if they are pre-commitment and that this therapy can be an appropriate candidate for treatment of conditions requiring an acceleration of repairing process.

1. Introduction

Pulsed electromagnetic field (PEMF) therapy is successfully in use in modern medicine [1]. It is based on the physical effect sorted from the combination of an electric with a magnetic field, generating waves that propagate at the speed of light [2]. Human clinical trials and animal experiments have shown that electromagnetic stimulation accelerates wound healing and bone formation, carrying the promise of a safe, non-invasive treatment for challenging conditions such as non-healing ulcers, bone mal-union, and chronic inflammatory diseases [3–7]. Extremely low frequency (ELF)-PEMF instruments, like Therapeutic Magnetic Resonance (TMR®) device, generate shorts bursts of electrical current which have been demonstrated to increase cell proliferation and extracellular matrix (ECM) component synthesis [8–12]. This enhances the tissue regenerative potential and wound healing response, as growth factor signaling is influenced by low frequency electromagnetic signals.

Although the positive effects exerted by PEMFs are well documented, the reasons for the reactions of the biological systems still remain unidentified. The biological response could be driven by a stem cell-mediated action, with mesenchymal stem cells (MSCs) possibly playing the greatest role. MSCs are hosted in specific microenvironments referred to as "niches", which constitute basic physical units where cell-cell and tissue-cell signals are integrated, ultimately determining stem cells response [13,14]. Some theories hypothesize the induction of a mechanical response of MSCs to electromagnetic fields, which would induce stem cells to emerge from the niches promoting tissue regeneration [15]. Other studies have described a possible effect on stem cells commitment, with electromagnetic therapy modulating MSCs ability to "switch" among different phenotypes forming cartilage, fat, myogenic, and neural-like tissue [13–18]. However, the effect of PEMFs on stem cells as key players in tissue regeneration remains to be investigated.

This study focuses on the effects of ELF-PEMFs on commitment of a ubiquitous MSCs subpopulation, adipose-derived stem cells (ADSCs),

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in an *in vitro* model. The results may contribute to enhancing the knowledge in the field of pulsed magnetic therapy and may support further stem cell-therapeutics and clinical application.

2. Materials and methods

2.1. Isolation of ADSCs

Adipose tissue samples were obtained from the abdominal region of six healthy patients (aged 35–58) undergoing abdominoplasty, after acquiring written consent, according to the Guidelines provided by the Clinic of Plastic Surgery of Padua University Hospital. The samples were washed in phosphate-buffered saline (PBS, EuroClone, Milan, Italy) and cut into 1 cm^2 pieces. The tissue was digested with a solution of 0.075% collagenase from Clostridium hystolyticum Type II (Sigma-Aldrich, St. Louis, MO, USA) in Hank's Balanced Salts Solution (HBSS, Lonza, Milano, Italy) and placed under stirring for 3 h at room temperature. After digestion was completed, collagenase activity was quenched with an equal volume of basal medium composed of Dulbecco's modified Eagle's medium (DMEM, Lonza), 10% fetal bovine serum (FBS, EuroClone) and 1% Penicillin/Streptomycin (P/S, Lonza). Indigested material was removed by filtration, and eluate was centrifuged at 1200 rpm for 4 min. Cells were seeded in basal medium into 75 cm^2 tissue culture flask, and incubated at 5% CO_2 and 37 °C. Culture medium was changed every 2 days.

2.2. Adipogenic and osteogenic differentiation of ADSCs

ADSCs, amplified up to passage 2, were harvested by trypsin treatment (trypsin/EDTA, EuroClone). Cells were counted under Bürker Chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), then seeded at a density of 15×10^3 cells/cm^2 in 24-well plates. The cultures were incubated in adipogenic or osteogenic differentiation medium up to 21 days. Adipogenic differentiation medium was made of DMEM high glucose (Lonza) supplemented with 10% FBS, 1% P/S, 10 μg/mL insulin (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (IBMX, Sigma-Aldrich), 0.1 mM indomethacin (Sigma-Aldrich), and 1 μM dexamethasone (Sigma-Aldrich). Osteogenic differentiation medium was composed of DMEM high glucose supplemented with 10% FBS, 1% P/S, 10 ng/mL Fibroblast Growth Factor 2 (FGF-2, ProSpec, East Brunswick, NJ, USA), 10 mM β-glycerophosphate (Sigma-Aldrich), and 10 nM dexamethasone. Experiments were performed with three different cell preparations and repeated three times.

2.3. Neuronal and glial differentiation of ADSCs

Cells obtained from the digestion of adipose tissue were seeded at a density of 10^5 cells/cm^2, and cultured in proliferation medium at 5% CO_2 and 37 °C for 14 days. Proliferation medium consisted of DMEM-HAM's F12 (3:1, EuroClone), 10% FBS, 1% P/S, 20 ng/mL Epidermal Growth Factor (EGF, ProSpec), and 40 ng/mL FGF-2. In order to induce neuronal and glial differentiation, cells were then seeded at a density of 15×10^3 cells/cm^2 onto poly-D-lysine 24-well plates and cultured for 21 days alternatively with neuronal or glial differentiation medium. The neuronal differentiation medium consisted of NeuroBasal Medium (Gibco by Thermo Fisher Scientific, Waltham, MA, USA) containing 1% FBS, 1% P/S, 2% b27 serum-free supplement (Gibco), 10 μg/mL human recombinant beta nerve growth factor (bNGF, ProSpec), 100 ng/mL human recombinant brain-derived neurotrophic factor (BDNF, ProSpec), and 20 ng/mL human recombinant neurotrophin-3 (NT3, ProSpec). Glial differentiation medium was made of NeuroBasal Medium containing 1% FBS, 1% P/S, 1% N2 supplement (Gibco), 4 μM forskolin (Merck Millipore, Darmstadt, Germany), and 10 ng/mL human recombinant heregulin-β (ProSpec). Experiments were performed with three different cell preparations and repeated three times.

2.4. TMR® treatment

Cell cultures were exposed to ELF-PEMFs from DIAPASON® device (Thereson S.p.A., Vimercate, MB, Italy). As previously described [8], the DIAPASON® device is composed of a console that generates electrical signals and an emitter connected to the console that converts the electrical signals into PEMFs. The emitter comprises two solenoids with 36 turns of copper wire of 0.8 mm diameter. The signal comprises a plurality of base pulses grouped in pulse packets and in pulse trains, in which each pulse packet consists of a series of base pulses followed by a first pause, in which each pulse train consists of a series of pulse packets followed by a second pause. The control circuit is configured to reverse the polarity of the base pulses after a given time interval. The frequency of the base pulses is 100 Hz, and the repetition frequency of the pulse packet is 2.89–25.9 Hz. The repetition frequency of pulse trains is 0.3–2.8 Hz, while the time interval is 120–180 s. The repetition frequency of the train sets is 0.1–0.3 Hz. The average amplitude of the generated magnetic field is <40 μT (comparable to the Earth's magnetic field). The treatment consisted of a daily stimulation of the cell cultures up to 21 days. In detail, cells cultured in 24-well plates were positioned in correspondence of the two solenoids of the DIAPASON® device, then exposed to ELF-PEMFs for 24 min at room temperature. Control cultures were positioned on the device in the same manner as the exposed cultures but without receiving ELF-PEMFs stimulation.

2.5. Real time PCR

Total RNA was isolated from each cell culture by using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), including DNase digestion with the RNase-Free DNase Set (Qiagen). For the first-strand cDNA synthesis, 500 ng of total RNA of each sample was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Human primers were selected for each target gene with Primer 3 software. Real-time PCRs were carried out using the designed primers at a concentration of 300 nM and FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Thermal cycling conditions were as follows: 15 minute denaturation at 95 °C; followed by 40 cycles of denaturation for 15 s at 95 °C; annealing for 30 s at 60 °C; and elongation for 20 s at 72 °C. Cell cultures that were not subjected to ELF-PEMFs stimulation were used as control condition. Values were normalized to the expression of the transferrin receptor (TFRC) internal reference, whose abundance did not change under our experimental conditions.

2.6. Lipid content

Oil Red O quantification was used to evaluate the intracellular lipid content. Oil Red O stock solution was prepared by dissolving 3.5 mg/mL Oil Red O (Sigma-Aldrich) in isopropanol. Working solution consisted of 3:2 Oil Red O stock in ddH_2O. Cell cultures were incubated in 0.5 mL of Oil Red O working solution for 15 min at room temperature. After washing in ddH_2O, Oil Red O was extracted with 0.25 mL 100% isopropanol. For each sample, optical density (O.D.) values at 490 nm were recorded using a multilabel plate reader (Victor 3 Perkin Elmer, Milano, Italy).

2.7. Alkaline phosphatase assay

The intracellular Alkaline phosphatase (ALP) activity was detected with a colorimetric Alkaline Phosphatase Assay Kit (Abcam, Cambridge, UK). Cells were washed with PBS, homogenized with ALP Assay Buffer, then centrifuged at 13,000 rpm for 3 min to remove insoluble material. A standard curve was drawn using the corrected absorbance values of standards, and the pNP concentration was identified for each sample. ALP activity was calculated as follows: ALP activity (U/mL) = (A / V) / T,