Knee Surg Sports Traumatol Arthrosc
DOI 10.1007/s00167-014-3143-x

EXPERIMENTAL STUDY

In vitro functional response of human tendon cells to different dosages of low-frequency pulsed electromagnetic field

L. de Girolamo · M. Viganò · E. Galliera · D. Stanco · S. Setti · M. G. Marazzi · G. Thiebat · M. M. Corsi Romanelli · V. Sansone

Received: 2 October 2013 / Accepted: 12 June 2014

Abstract
Purpose Chronic tendinopathy is a degenerative process causing pain and disability. Current treatments include biophysical therapies, such as pulsed electromagnetic fields (PEMF). The aim of this study was to compare, for the first time, the functional in vitro response of human tendon cells to different dosages of PEMF, varying in field intensity and duration and number of exposures.
Methods Tendon cells, isolated from human semitendinosus and gracilis tendons (hTCs; $n = 6$), were exposed to different PEMF treatments (1.5 or 3 mT for 8 or 12 h, single or repeated treatments). Scleraxis (SCX), COL1A1, COL3A1 and vascular endothelial growth factor-A (VEGF-A) expression and cytokine production were assessed.
Results None of the different dosages provoked apoptotic events. Proliferation of hTCs was enhanced by all treatments, whereas only 3 mT-PEMF treatment increased cell viability. However, the single 1.5 mT-PEMF treatment elicited the highest up-regulation of SCX, VEGF-A and COL1A1 expression, and it significantly reduced COL3A1 expression with respect to untreated cells. The treated hTCs showed a significantly higher release of IL-1β, IL-6, IL-10 and TGF-β. Interestingly, the repeated 1.5 mT-PEMF significantly further increased IL-10 production.
Conclusions 1.5 mT-PEMF treatment was able to give the best results in in vitro healthy human tendon cell culture. Although the clinical relevance is not direct, this investigation should be considered an attempt to clarify the effect of different PEMF protocols on tendon cells, in particular focusing on the potential applicability of this cell source for regenerative medicine purpose, both in surgical and in conservative treatment for tendon disorders.

Keywords Pulsed electromagnetic field · PEMF dosage · Human tendon cells · Tendon markers · Anti-inflammatory action

Abbreviations
PEMF Pulsed electromagnetic fields
hTCs Human tendon cells

M. Viganò and E. Galliera have contributed equally to the manuscript.

L. de Girolamo (✉) · M. Viganò · D. Stanco
Orthopaedic Biotechnology Laboratory, IRCCS Istituto Ortopedico Galeazzi, Via R. Galeazzi, 4, 20161 Milan, Italy
e-mail: laura.degirolamo@grupposandonato.it

E. Galliera
Dipartimento di Scienze Biomediche, Chirurgiche ed Odontoiatriche, Università degli Studi di Milano, Milan, Italy

E. Galliera
IRCCS Istituto Ortopedico Galeazzi, Milan, Italy

S. Setti
IGEA SpA, Clinical Biophysics, Carpi, Italy

M. G. Marazzi · M. M. Corsi Romanelli · V. Sansone
Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milan, Italy

G. Thiebat
Sport Traumatology Unit, IRCCS Istituto Ortopedico Galeazzi, Milan, Italy

M. M. Corsi Romanelli
IRCCS Policlinico San Donato, San Donato Milanese, Italy

V. Sansone
Orthopaedic Department, Istituto Ortopedico Galeazzi, Milan, Italy

Published online: 24 June 2014

Introduction

Tendon disorders are a class of pathology which includes traumatic injuries as well as chronic diseases, such as tendinopathy. The recovery from these tendon pathologies can be long and is often not successful. In particular, the mechanisms underlying tendinopathy, whether chronic or acute, are still partially unclear. In fact, the debate about the possible role of inflammation in this pathology still divides clinicians and researchers. Recently, new findings have demonstrated the presence of inflammatory elements in chronic tendon disease, such as the infiltration of lymphocytes and macrophages in the peritenon, the activation of matrix metalloproteinases, and the involvement of mediators such as substance P, vascular endothelial growth factor (VEGF) and cycloxigenase type II (COX2) [26]. Pulsed electromagnetic fields (PEMF) could be an innovative approach in the management of tendon disorders [9]. In the last few years, PEMF have been demonstrated to be effective also in the treatment of several different pathologies, such as bone delayed union or nonunion and articular cartilage disease, including early osteoarthritis [1, 12, 15, 20, 34]. In vitro studies have shown that PEMF can limit the catabolic effects of pro-inflammatory cytokines on articular cartilage and promote anabolic activity of the chondrocytes [6, 25], thus suggesting that they may be used to control articular tissue inflammation and to stimulate anabolic pathways, eventually resulting in tissue repair [2, 36]. It has also been showed that PEMF are able to increase A_{2A} and A_3 adenosine receptor density and functionality in different cell lines, human neutrophils, chondrocytes and synoviocytes, resulting in the decrease of pro-inflammatory cytokines, such as IL-6 and IL-8, and on the inhibition of prostaglandin E2 (PGE_2) [23, 38, 40], and of NFκB and FGF-2 release [24, 30]. Moreover, the existence of electromagnetic-responsive DNA sequences, at least in the Hsp70 promoter, suggests that PEMF could directly modulate expression of specific proteins [29]. At cellular level, PEMF have been proved to be effective in the modulation of cell proliferation, gene expression and cytokine production also in human tendon cells (hTCs) [4, 10, 35]. However, in order to propose the optimum PEMF treatment protocol for each pathology, the conditions that obtain the most efficient dose–response effect must be defined. In vitro, physical–dynamic studies would allow researchers to find not only the best cell response, but also the minimal effective dose both in terms of daily exposure and of field intensity that could be predictive of the in vivo situation [4].

It is noteworthy that no previous in vitro studies on tendon cells have taken into account the possible different biological effects exerted by different PEMF intensity and different length of exposure. For this reason, the aim of this study was to evaluate for the first time the relationship between PEMF dosage and in vitro response of hTCs, comparing the effects of a low-intensity PEMF exposure (1.5 mT-PEMF) against double intensity (3 mT-PEMF), and with a repetition of the same treatment (R-1.5 mT-PEMF), using two different lengths of exposure (8 and 12 h). The results of this study could contribute to comprehend the effect of PEMF on tendon cells and thus on tendon disorders.

Materials and methods

hTC isolation

Tendon cells were isolated from the discarded portion of healthy semitendinosus and gracilis tendons of six donors (mean age 35 ± 12 years) after anterior cruciate ligament reconstruction by hamstring technique at Galeazzi Orthopaedic Institute under written consent. The tendon portions were minced and enzymatically digested overnight at 37 °C with 0.3 % collagenase type I (Worthington, Lakewood, NJ, USA) in DMEM (Sigma-Aldrich, St. Louis, MO, USA) (modified from [31]). The resulting nucleated cells were plated 5×10^3 cells/cm^2, in complete medium composed of DMEM, 10 % foetal bovine serum (FBS, Sigma-Aldrich), 50 U/mL penicillin, 50 U/mL streptomycin, 2 mM L-glutamine (Sigma-Aldrich) and supplemented with 5 ng/mL basic human fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA) for the cell expansion phase only and maintained in culture. When cells reached 80–90 % confluence, they were detached by trypsin–EDTA (0.05 % trypsin/0.02 % EDTA; Sigma-Aldrich) and cultured at a density of 3×10^3 cells/cm^2. The cells were used for experiments at passage 4.

PEMF stimulation

PEMF were generated using a pair of rectangular horizontal coils as previously described [19]. The peak intensity of the magnetic field between coils was 1.5 or 3 ± 0.2 mT, measured using the Hall probe (HTD61-0608-05-T, F.W. Bell, Sypris Solutions, Louisville, KY, USA) of a gaussmeter (DG500, Laboratorio Elettrofisico, Milan, Italy) with a reading sensitivity of 0.2 %. Cells were plated the day before stimulation and exposed to a single PEMF treatment at 1.5 and 3 mT field intensity (1.5, 3 mT-PEMF) or for three 1.5 mT-PEMF (R-1.5 mT-PEMF) treatments with an interval of 48 h between the start of each exposure. For all the conditions, both 8 and 12 h of exposure were tested (Table 1).

Table 1 Experimental scheme of hTCs PEMF treatments

Treatment	Field intensity (mT)	Length of exposure (h)	No. of exposures
1.5 mT-PEMF	1.5	8	1
		12	
R-1.5 mT-PEMF	1.5	8	3
		12	
3 mT-PEMF	3	8	1
		12	

Live and dead assay

Untreated and treated cells were analysed by live/dead assay. Cells were seeded at a density of 10^5 cells/cm^2. Immediately after the end of treatment, the culture medium was removed and the cells were incubated with a solution of 2 μM calcein and 4 μM ethidium homodimer-1 (Invitrogen Ltd., Paisley, UK). The samples were observed by fluorescence microscope (OLYMPUS IX71). The percentage of live and dead cells was defined as PLive = NLive/(NLive + NDead), where NLive is the number of live cells and NDead is the number of dead cells in the same image. For each population, three different randomly selected fields of two replicate samples were analysed.

Viability and proliferation assays

Passage 4 hTCs were plated at a density of 1.5x10^4 cells/cm^2 in 96-well plates and exposed to PEMF as previously described. Measures of cell viability were performed at 0 and 2 days after the end of treatments, adding a final concentration of 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) to the culture medium and incubating for 4 h at 37 °C. After medium removal, 100 % DMSO was then added to each well. Absorbance of this solution was read at 570 nm (VictorX3, Perkin Elmer microplate, Waltham, MA, USA) [17]. DNA content analysis was performed on cells plated in 24-well plates at a density of 5x10^3 per cm^2, at 0 and 2 days after the end of treatment using CyQUANT® Cell Proliferation Assay Kit (Invitrogen Ltd.); fluorescence was read at 520 nm (excitation $\lambda = 480$ nm) (VictorX3, Perkin Elmer microplate).

Gene expression analysis

RNA from each sample was obtained using RNeasy Mini kit (Qiagen, Dusseldorf, Germany) and quantified spectrophotometrically (Nanodrop, Thermo Scientifc, Rockford, IL, USA). RNA (120 ng) was reverse-transcripted to cDNA with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Benicia, CA, USA) in a final volume of 20 μL, with reaction mix containing random hexamer primers, oligo(dT), and reverse transcriptase preblended with RNase inhibitor. The reaction mix was incubated at 25 °C for 5 min, at 42 °C for 30 min and at 85 °C for 5 min. Real-time PCR mixture was made by 10 ng cDNA template, TaqMan Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies, Grand Island, NY, USA). The reaction was performed with Applied Biosystems Step One Plus (Life Technologies) in a final volume of 20 μL. The following cycle conditions were used to perform amplification and real-time data acquisition: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), scleraxis (SCX) (Hs03054634_g1), collagen type I (COL1A1) (Hs01076777_m1), collagen type III (COL3A1) (Hs00943809_m1) and VEGF-A (VEGF-A) (Hs00900055_m1) genes were analysed. The fold change in expression of analysed genes in treated and untreated samples was normalized on expression of the GAPDH housekeeping gene. Data are expressed as fold increase on the respective controls for each gene and sample.

Cytokine and growth factor determination

The cumulative amount of soluble IL-1β, IL-6, IL-10, TNF-α, TGF-β2 released by cells in the culture medium was determined at 0, 24 and 48 h after the end of PEMF treatment by commercially available ELISA assays, according to the manufacturers' instructions (R&D System, Minneapolis, USA). For the IL-1β detection assay, the sensitivity of the test was <1 pg/mL and intra- and inter-assay coefficients of variation were 2.8 and 4.1 %, respectively. For the IL-6 detection assay, the sensitivity of the test was 2 pg/mL and intra- and inter-assay coefficients of variation were 5.8 and 3.1 %, respectively. For the IL-10 detection assay, the sensitivity of the test was <0.5 pg/mL and intra- and inter-assay coefficients of variation were 6.6 and 8.1 %, respectively. For the TNF-α detection assay, the sensitivity of the test was 1.6 pg/mL and intra- and inter-assay coefficients of variation were 5.0 and 7.3 %, respectively. For the TGF-β2 detection assay, the sensitivity of the test was 2 pg/mL and intra- and inter-assay coefficients of variation were 2.7 and 4.3 %, respectively.

The study was approved by the Institutional Review Board of the Galeazzi Orthopaedic Institute (Ref. Number IOG-2.47).

Statistical analysis

Statistical analysis was performed by GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA). All values are expressed as the mean ± SD. Normal

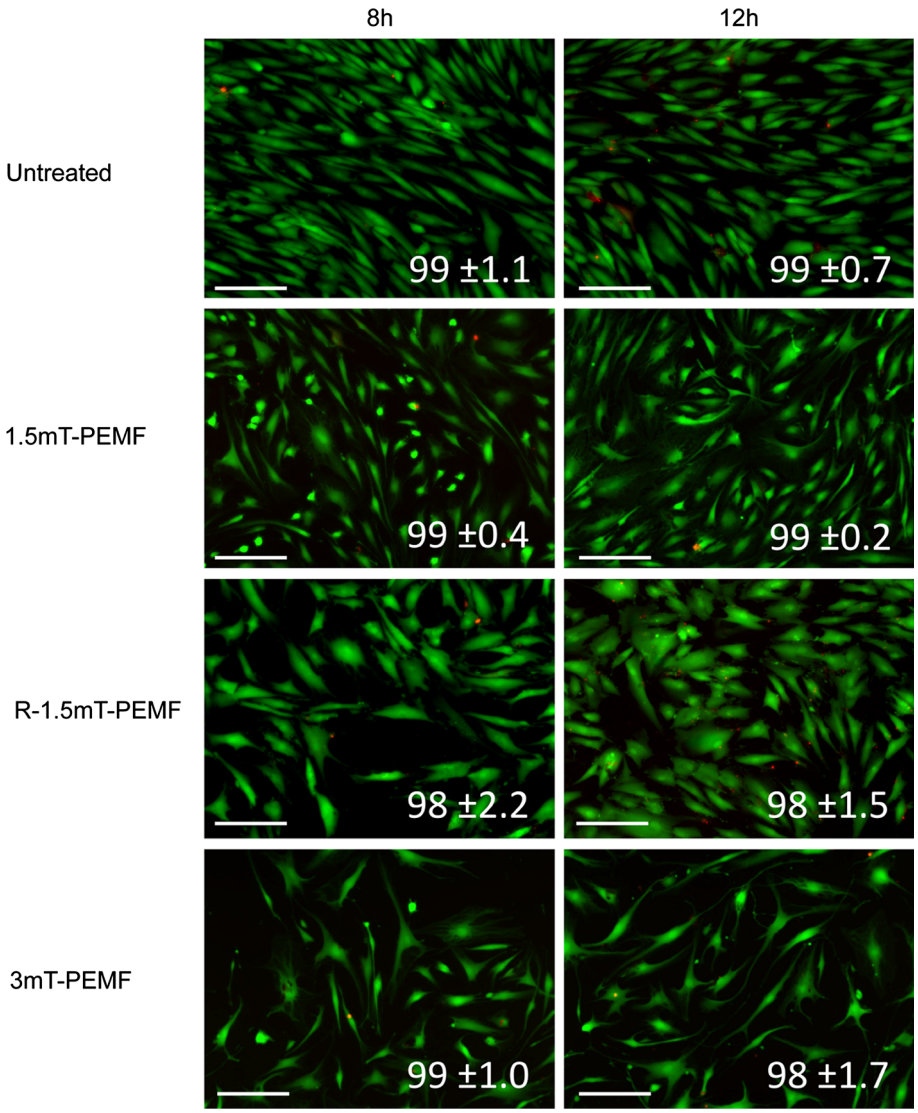

Fig. 1 Live and dead staining of untreated and PEMF treated cells for 8 and 12 h (*green* viable cells, *red* dead cells). Percentages of viable cells (expressed as mean ± SD) are reported for each group (fluorescence microscopy, *scale bar* 200 μm, merged images)

distribution of values was assayed by Kolmogorov–Smirnov normality test, while one-way analysis of variance (ANOVA) for repeated measures, with Bonferroni's correction, was used to compare data over time. Paired or unpaired comparisons were performed by two-tailed t test. In the case of not normally distributed values, repeated measures were compared with the Mann–Whitney test. p values <0.05 were considered statistically significant.

Results

hTCs treated with 1.5- and 3 mT-PEMF showed a similar cell viability and DNA content

As shown by live and dead assay, no combination of treatment provoked any cell death event, as in all cases, more than 98 % of cells were viable, without any differences between untreated and treated hTCs (Fig. 1). The viability of hTCs exposed to PEMF treatments was also confirmed by MTT assay; the only decrease was observed in 8 h R-1.5 mT-PEMF treated cells at 2 days with respect to untreated cells (−19 %, $p < 0.05$) (Fig. 2a). On the other hand, all the samples treated for a longer time period (12 h) showed an increase of hTC viability with respect to untreated cells at day 2, with statistically significant increases for the 1.5- and 3 mT-PEMF treatments (+17 %, $p < 0.05$ and +13 %, $p < 0.01$, respectively) (Fig. 2a). Indeed, immediately after 8 h treatment (0 day), 1.5 mT-PEMF treated cells, as well as R-1.5 mT-PEMF and 3 mT-PEMF hTCs, showed an increase in DNA content of 25, 24 and 14 % with respect to untreated cells, although only the first one was significant ($p < 0.05$; Fig. 2b). At day 2, all treated cells showed a DNA content comparable to that of the untreated ones. A divergent behaviour was observed when cells were exposed to the

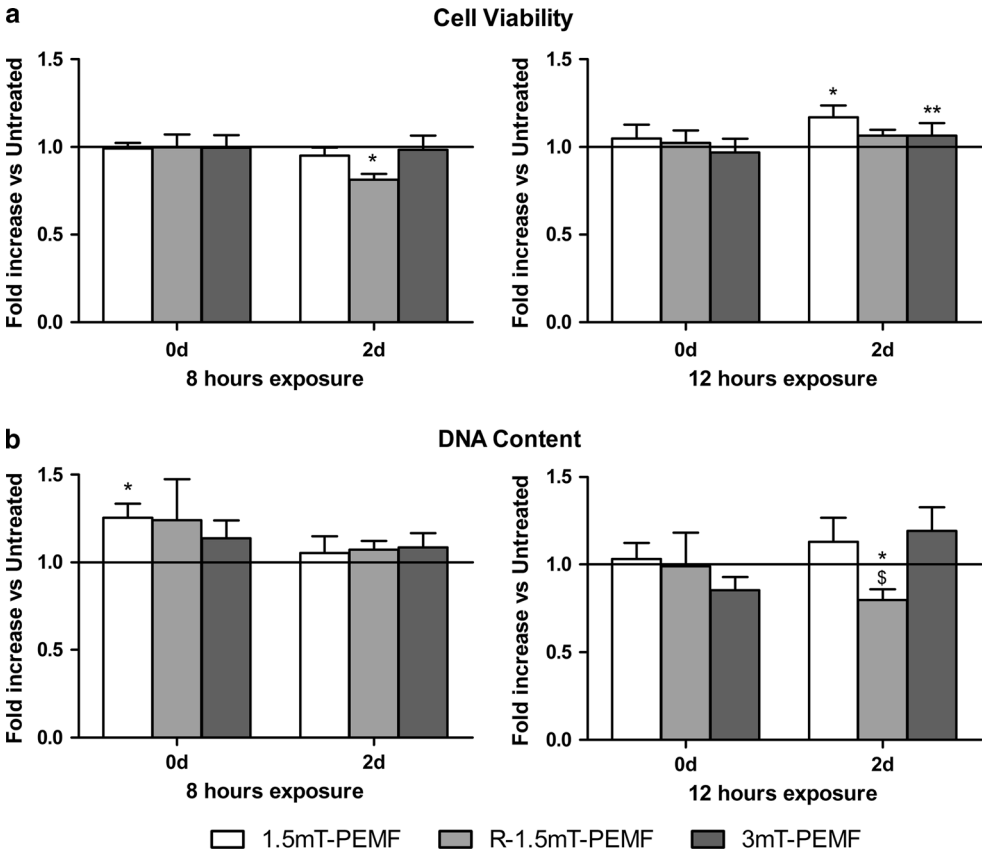

Fig. 2 Cell viability (**a**) and DNA content (**b**) measured at 0 and 2 days after 8 and 12 h of PEMF treatments ($n = 6$), assessed by MTT and CyQUANT® Cell Proliferation assays, respectively. Data are expressed as average fold increase ± SD respect to untreated cell (fixed line set at 1). Levels of significance: $^{*}p < 0.05$; $^{**}p < 0.01$ versus untreated cells; $^{\$}p < 0.05$ versus 3 mT-PEMF

different PEMF treatments for 12 h: at day 0, the DNA content of hTCs was not affected by the stimulation, whereas after 2 days both 1.5- and 3 mT-PEMF induced an increase with respect to the untreated cells, although it was not significant. Conversely, the repeated stimulation (R-1.5 mT-PEMF) induced, at day 2, a significant decrease of DNA content with respect to untreated cells (−25 %, $p < 0.05$).

hTCs treated with a single 1.5 mT-PEMF treatment showed higher VEGF-A, SCX, COL1A1 and COL1A1/COL3A1 ratio transcript levels

SCX expression seemed to be positively influenced in a dose-dependent manner by the length of exposure for all types of treatments, in particular for the 1.5 mT-PEMF treatment (8 h, +54 %, $p < 0.05$; 12 h +79 %, n.s.) (Fig. 3a). Nonsignificant slight increases were observed for R-1.5 mT- and 3 mT-PEMF treatments after 12 h of exposure at day 0 (+12 and +10 %, respectively). Comparing the three groups of treatment, hTCs treated for 8 h with R-1.5 mT-PEMF and assessed at day 0 showed a significantly lower SCX expression in comparison with that observed in 1.5 mT-PEMF treated cells (−46 %, $p < 0.05$).

1.5 mT-PEMF treatment was able to significantly up-regulate VEGF-A expression at day 0 (8 h, +100 %, $p < 0.05$; 12 h: +75 %, $p < 0.05$), while other PEMF treatments did not significantly affect it. Cell treated for 8 and 12 h showed a similar trend, with just slightly higher increases of VEGF-A expression at day 0 after 12 h compared to 8 h (Fig. 3b).

Among the different PEMF treatments, only 1.5 mT-PEMF was able to up-regulate COL1A1 expression with respect to untreated cells at least at day 0, both after 8 h (+140 %) and 12 h (+133 %), although, due to the high inter-donor variability, this increase was not statistically significant (Fig. 4a). hTCs exposed to R-1.5 mT-PEMF for 8 h showed a significantly lower COL1A1 expression in comparison with 1.5 mT- and 3 mT-PEMF treated ones (−34 and −42 %, respectively, both $p < 0.05$). The hTCs exposed to 3 mT-PEMF treatment behaved differently, since a slight up-regulation of COL1A1 expression with respect to untreated cells (+46 and +22 % for 8 and 12 h, respectively, n.s.) was observed only 2 days after treatment.

Interestingly, PEMF treatments did not significantly affect the hTC COL3A1 expression, with the exception of those exposed for 8 h to a single 1.5 mT-PEMF treatment; indeed, at day 0, a significant reduction of collagen type III

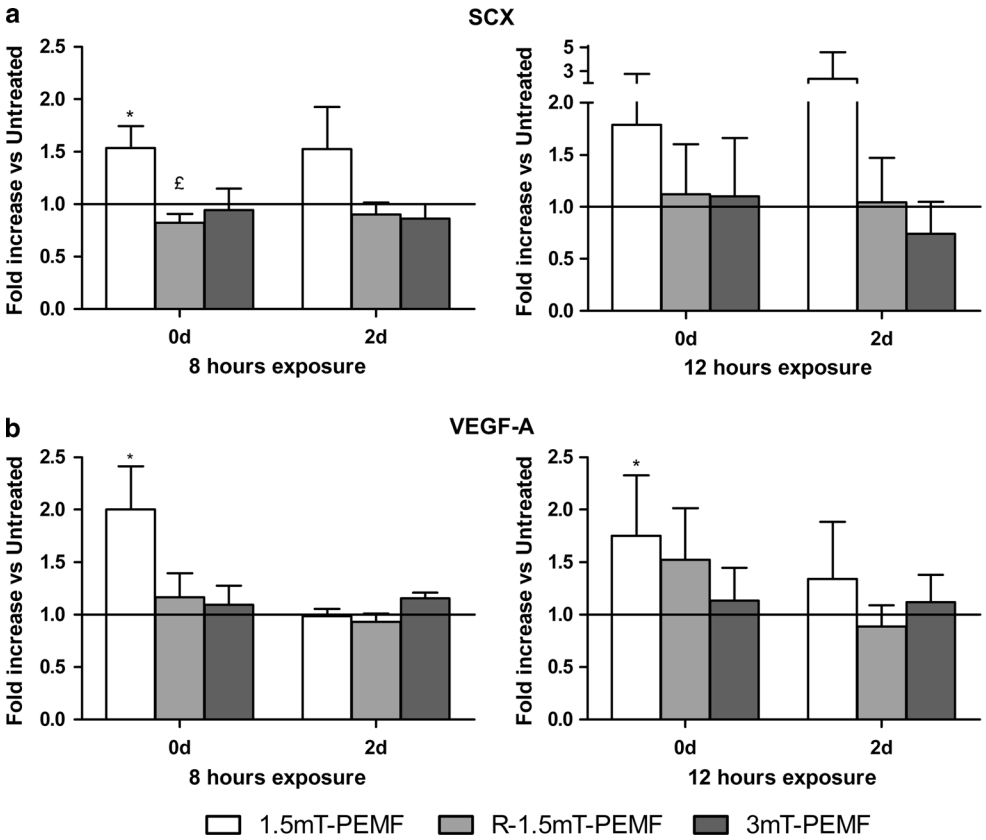

Fig. 3 Gene expression of SCX (**a**) and VEGF-A (**b**) at 0 and 2 days after 8 and 12 h of PEMF treatments ($n = 6$), assessed by real-time PCR. Data are expressed as average fold increase ± SD respect to untreated samples (fixed line set at 1), normalized on GAPDH expression. Levels of significance: $^{*}p < 0.05$ versus untreated cells; $^{£}p < 0.05$ versus 1.5 mT-PEMF

expression with respect to untreated cells was observed (−50 %, $p < 0.05$; Fig. 4b). In general, the resulting COL1A1/COL3A1 expression ratio was higher in all the PEMF treated cells compared to untreated ones, with the exception of those exposed to R-1.5 mT-PEMF. In particular, it was much higher in the single 1.5 mT-PEMF treated cells for both 8 h (+218 %, n.s. and +47 %, $p < 0.05$ at 0 and 2 days, respectively) and 12 h (+181 and +143 % at 0 and 2 days, respectively, both n.s.) with respect to untreated cells (data not shown).

R-1.5 mT-PEMF treatment induced a higher IL-10 release

All the PEMF treatments were able to increase the production of IL-1β in hTCs at each time point in a field intensity-dependent manner (Fig. 5a). However, at day 0, the increase of IL-1β, both after 8 and 12 h of exposure to 1.5 mT-PEMF single treatment, was not significant in comparison with untreated cells. In general, when the cells were treated for 8 h, the maximum levels of IL-1β were observed 1 day after the exposure and then reduced over time. Conversely, when cells were treated for a longer time, 12 h, the IL-1β release was significantly higher in comparison with the amount released by untreated cells, starting immediately at the end of the treatment up to 2 days.

TNF-α release was only slightly modulated by all the PEMF treatments, with the exception of 3 mT-PEMF and of R-1.5 mT-PEMF treatments, which were able to significantly up-regulate its release after 8 h of exposure at 2 days (+17 %, $p < 0.05$) and after 12 h at day 1 (+30 %, $p < 0.05$), respectively (Fig. 5b).

The release of IL-6 by hTCs was significantly up-regulated by all the PEMF treatments and at each time point (Fig. 5c). Very similar levels, as well as temporal trends, were observed after 8 and 12 h of exposure; indeed, in both cases, IL-6 was immediately released by the cells (0 day) and then reached the maximum and stable levels 1 day after treatment. R-1.5 mT-PEMF treated cells seemed to be less affected by the treatment in terms of IL-6 production, in particular at 2 days after 12 h of exposure, although their amount of IL-6 production was always significantly higher than the controls.

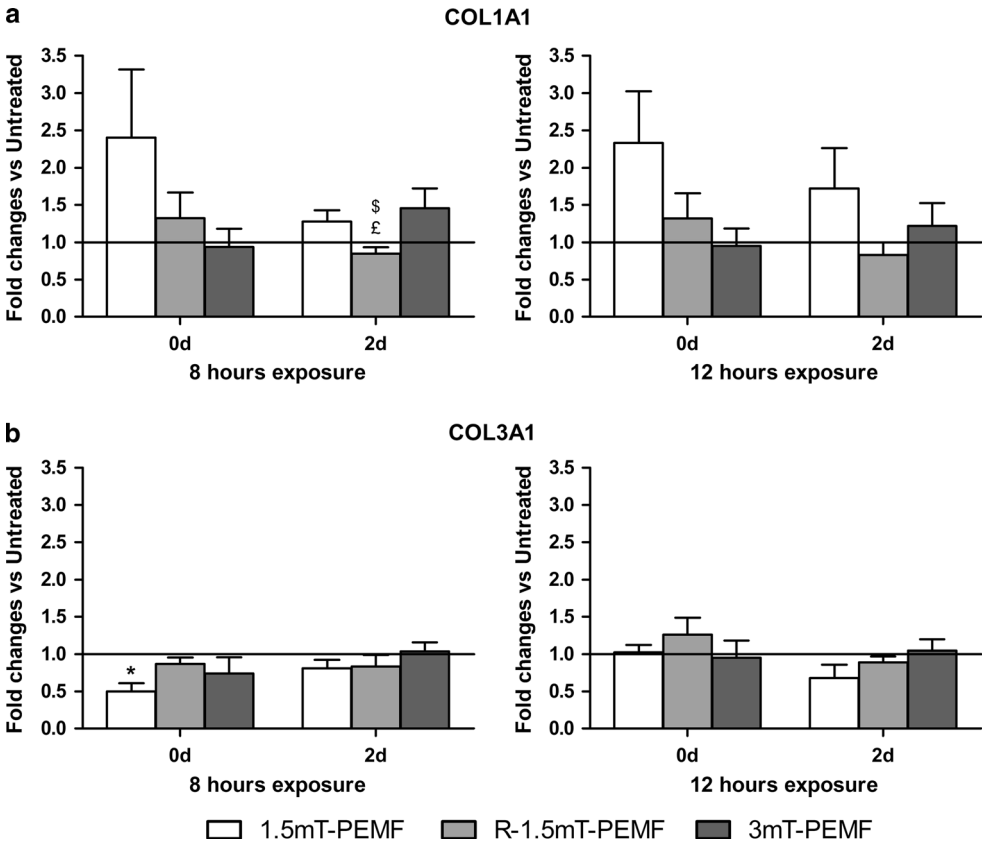

Fig. 4 Gene expression of COL1A1 (**a**) and COL3A1 (**b**) at 0 and 2 days after 8 and 12 h of PEMF treatments ($n = 6$), assessed by real-time PCR. Data are expressed as average fold increase ± SD respect to untreated samples (fixed line set at 1), normalized on GAPDH expression. Levels of significance: $^{*}p < 0.05$ versus untreated cells; $^{£}p < 0.05$ versus 1.5 mT-PEMF; $^{\$}p < 0.05$ versus 3 mT-PEMF

A similar scenario was observed for IL-10: also, in this case, all the PEMF treatments were able to increase the production of this cytokine by hTCs, with increases ranging from +35 to +300 % in comparison with untreated cells, and no significant differences were found between cells treated for 8 and 12 h (Fig. 6a). However, diverging from our observations for IL-6, hTCs treated with R-1.5 mT-PEMF showed the highest increase in terms of IL-10 release in comparison with the other treated cells, particularly at day 0, both after 8 and 12 h of exposure (+70 and 80 % vs. 1.5 mT-PEMF for 8 and 12 h, respectively, $p < 0.01$ and +68 and +83 % vs. 3 mT-PEMF for 8 and 12 h, respectively, $p < 0.05$).

Also, TGF-β levels were found to be significantly higher in all groups at all time points, with fold increases ranging from 6 to 11 with respect to untreated ones (Fig. 6b) and with the highest release of this growth factor was observed in cells exposed to a single 1.5 mT-PEMF treatment; this effect was particularly evident after 12 h of exposure, when, at both 1 and 2 days after treatment, 1.5 mT-PEMF treated cells showed a significantly higher amount of TGF-β with respect to both 12 h R-1.5 mT- (1 day, +27 %, $p < 0.001$; 2 days, +25 %, $p < 0.05$) and 3 mT-PEMF (1 day, +43 %, $p < 0.01$ and 2 days, +39 %, $p < 0.05$, respectively).

Discussion

The most important finding of the present study is that the length of exposure, the field intensity and the number of PEMF treatments differently affect tendon cell proliferation, gene expression and release of pro- and anti-inflammatory cytokines. Our results could improve knowledge about the role of PEMF in the treatment of tendon pathologies. Indeed, to date, only a few studies on the clinical effect of PEMF on tendon or ligament repair have been reported [9] and these are sometimes conflicting, probably due to the different PEMF dosages used. Moreover, from the basic science point of view, the mechanism and effects of PEMF are far from being clearly defined, even if the cell membrane is often considered to be their main target [21], as they could affect membrane-mediated signal transduction processes, modulating cell proliferation, gene expression, and activity of cytokines and growth factors involved in the inflammatory response and in tissue repair, such as FGF-2, VEGF, NFkB and IL-1β [3, 30, 39–41, 43]. Recently, it has been observed that a single exposure to PEMF positively influences gene expression of tendon-specific markers and the release of anti-inflammatory cytokines and growth factors of hTCs [4, 8]. The main aim of this study was to evaluate the different response of

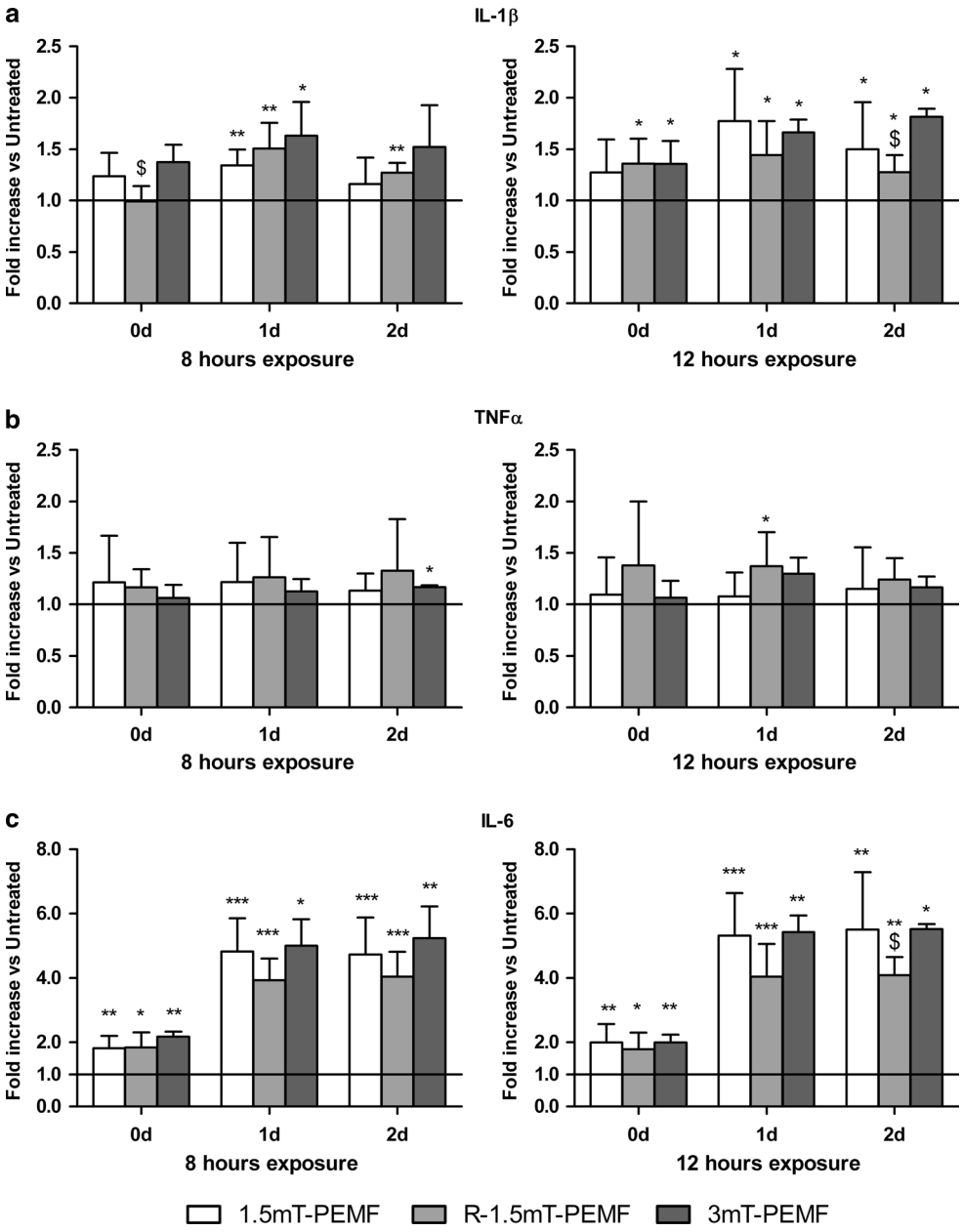

Fig. 5 IL-1β (**a**), TNF-α (**b**) and IL-6 (**c**) production in hTCs at 0, 1, and 2 days from PEMF treatments ($n = 6$), assessed by ELISA assay. Data are expressed as average fold increase ± SD respect to untreated cells (fixed line set at 1). Levels of significance: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus untreated cells; $^{\$}p < 0.05$ versus 3 mT-PEMF

tendon cells to different PEMF treatments and to eventually identify the more performing one, in order to translate these findings into the clinical practice. Indeed, in our opinion, these in vitro "physical–dynamic" studies should allow to establish the best cell response together with the minimal effective PEMF dose both in terms of daily exposure and field intensity. We previously reported that a short length of exposure (4 h) was not sufficient to trigger a functional hTC response and that the greater PEMF effects were associated with longer exposure time such as 8 and 12 h [4]. For this reason, using these durations of exposure, in this study, we compared the effects of different field intensities and length and number of exposures.

No apoptotic or necrotic events were observed after any treatment, demonstrating that PEMF are not toxic at a cellular level. hTC viability and proliferation were slightly enhanced by both 1.5 mT-PEMF (single or repeated) and 3 mT-PEMF treatments but with different kinetics, although a decrease was observed in R-1.5 mT-PEMF. However, this may be explained by a bias due to the longer culture time of these samples. As revealed by the gene expression analysis, only the 1.5 mT-PEMF single treatment was able to positively influence both the tendon-specific markers and VEGF which are known to participate in the regenerative processes of tendons; in particular, an increase of collagen type I/type III ratio was observed,

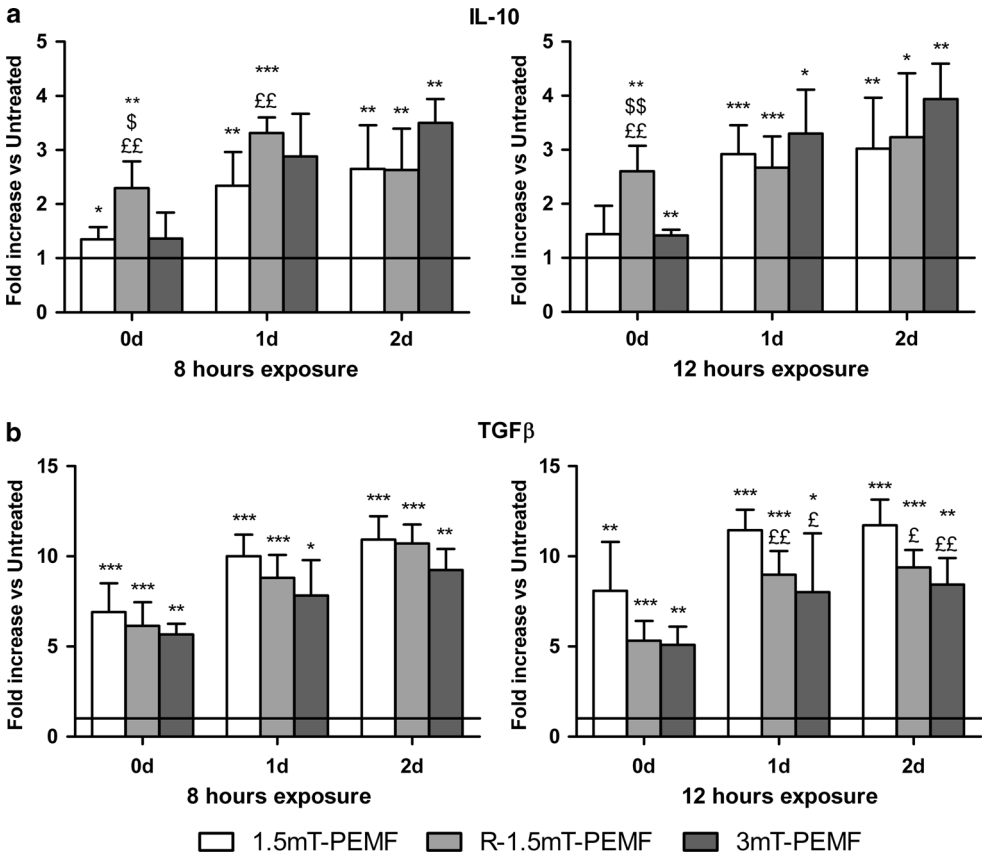

Fig. 6 IL-10 (**a**) and TGF-β (**b**) production in hTCs at 0, 1, and 2 days after different PEMF treatments ($n = 6$), assessed by ELISA assay. Data are expressed as average fold increase ± SD respect to untreated cells (fixed line set at 1). Levels of significance: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus untreated cells; $^{£}p < 0.05$, $^{££}p < 0.01$ versus 1.5 mT-PEMF; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$ versus 3 mT-PEMF

suggesting a pro-healing effect of PEMF treatment as this ratio has been reported to be in favour of collagen type III in tendinopathy, together with other changes in total collagen composition [5, 7, 16, 18, 19, 28]. In agreement with our results, other authors have already observed that, within a range of magnetic field intensity of 0.1–3.5 mT (75 Hz frequency), 1.5 mT gave the best results in terms of proteoglycans synthesis in cartilage explants compared to other field intensities [32].

It has been recently reported that tendon disorders, in particular in chronic tendinopathies, are characterized by an inflammatory state which involves COX2, substance P, MMPs and the infiltration of immune cells in the peritenon [26]. However, in the physiologic tendon healing process, the inflammatory response also plays an important role, and it is orchestrated in different consequential phases. In the first phase, the increase of the pro-inflammatory cytokine IL-1β induces the immunoregulatory cytokine IL-6 activation in tendons, triggering the inflammatory reaction. Once IL-1β and IL-6 have reached the peak of production, they are themselves promoters of the production of an anti-inflammatory cytokine, IL-10, which counteracts the inflammatory reaction, thereby concluding the inflammation process [11, 32]. This effect is particularly evident in our study comparing the single 1.5 mT-PEMF treatment with the repeated treatment. The early increase of IL-1β, in particular after 8 h exposure, indicates that 1.5 mT-PEMF treatment was able to boost the early inflammatory phase responsible for the healing process. A similar effect was observed for IL-6, which lies under the direct regulatory control of IL-1β [37]; in fact, lower levels of IL-6 in R-1.5 mT-PEMF treated cells were found compared to 1.5 mT-PEMF ones, indicating that the timing of the response to the repeated treatment exceeded the initial inflammatory phase and entered the second, anti-inflammatory phase. A similar response has been reported in a study by Gomez-Ochoa, where a decrease of IL-1β and IL-6 and an increase of IL-10 at late time points after PEMF treatment were observed [13]. This is clearly confirmed in our study by the increase of IL-10, the recognized anti-inflammatory mediator, in parallel with the decrease of IL-1β and IL-6 at 1 and 2 days after exposure. IL-10 does not only control the "self-regulating" anti-inflammatory loop of the final phases of the inflammatory response, but is also reported to be closely connected with the tendon healing process [27] and to affect the proliferation of connective tissue cells [33]; moreover, an up-regulation of IL-10 correlates with a superior healing process compared to controls in a murine model of patellar tendon lesion [32]. Therefore, the further increase of IL-10 in the repeated

treatment indicates that the repetition of 1.5 mT-PEMF treatment represents an additional potential advance in the healing and remodelling process. The positive effect of the 1.5 mT-PEMF treatment, both as single exposure and repeated, is also confirmed by the significant increase of TGF-β, which is known to be strongly related to the tendon healing process by stimulating the production of fibronectin by tendon cells [42]. Conversely, by increasing the field intensity, no further advantages were observed on these parameters, but rather it provoked a slightly significant increase of TNF-α, an inflammatory cytokine involved in tendon degeneration [14].

The main limitation of this study is the lack of daily observations of the parameters during the R-1.5 mT-PEMF treatment. This could probably explain why we did not observe a specific gene expression up-regulation in cells exposed to repeated PEMF treatments as compared to those just treated once. It has to be considered, however, that the cumulative effect exerted by repetitive exposure to electromagnetic fields is hardly detectable in gene expression since it is a short-term event and is often characterized by a negative feedback mechanism. The same could be said for the cytokine release analyses, where we decided to observe the cumulative effect in cytokine production rather than the daily release. In this case, this choice allowed us to clearly identify the stronger anti-inflammatory action of the repetition of PEMF treatment, as a net effect of a higher IL-10 production, confirming the beneficial effects of similar protocols already used in clinical practice [2, 20, 22, 44]. Furthermore, our study was performed starting from healthy tissues, while cells isolated from pathological tissues could better represent the in vivo condition for which PEMF treatment is used, helping clarify the kinetics of inflammatory and anti-inflammatory cytokine production involved in tendon repair.

Conclusion

1.5 mT-PEMF treatment was able to give the best results in in vitro healthy human tendon cell culture in term of cell proliferation, up-regulation of tendon-specific gene expression and release of anti-inflammatory cytokines and growth factors. In addition, the repetition of this low-intensity treatment leads hTCs to significantly increase their IL-10 production, which is closely involved in the anti-inflammatory pathways. These "physical–dynamic" findings could be used to define the minimal effective dose both in terms of daily exposure and field intensity to be applied in preclinical and clinical studies. Although the clinical relevance is not direct, this investigation should be considered as the first attempt to clarify the effect of different PEMF protocols on tendon cells, in particular focusing on the potential applicability of this cell source for regenerative medicine purpose, both in surgical and in conservative treatment for tendon disorders.

Acknowledgments The Authors thank Dr. Alessandra Colombini for her precious help in RT-PCR analyses. The study has been partially supported by IGEA SpA, Clinical Biophysics, Carpi (Italy), and by the Italian Ministry of Health (Ricerca Corrente L1006).

References

1. Assiotis A, Sachinis NP, Chalidis BE (2012) Pulsed electromagnetic fields for the treatment of tibial delayed unions and nonunions: a prospective clinical study and review of the literature. J Orthop Surg Res 7:24
2. Benazzo F, Cadossi M, Cavani F, Fini M, Giavaresi G, Setti S, Cadossi R, Giardino R (2008) Cartilage repair with osteochondral autografts in sheep: effect of biophysical stimulation with pulsed electromagnetic fields. J Orthop Res 26:631–642
3. Brighton CT, Wang W, Seldes R, Zhang G, Pollack SR (2001) Signal transduction in electrically stimulated bone cells. J Bone Joint Surg Am 83-A:1514–1523
4. de Girolamo L, Stanco D, Galliera E, Vigano M, Colombini A, Setti S, Vianello E, Corsi Romanelli MM, Sansone V (2013) Low frequency pulsed electromagnetic field affects proliferation, tissue-specific gene expression, and cytokines release of human tendon cells. Cell Biochem Biophys 66:697–708
5. De Mattei M, Fini M, Setti S, Ongaro A, Gemmati D, Stabellini G, Pellati A, Caruso A (2007) Proteoglycan synthesis in bovine articular cartilage explants exposed to different low-frequency low-energy pulsed electromagnetic fields. Osteoarthr Cartil 15:163–168
6. De Mattei M, Pasello M, Pellati A, Stabellini G, Massari L, Gemmati D, Caruso A (2003) Effects of electromagnetic fields on proteoglycan metabolism of bovine articular cartilage explants. Connect Tissue Res 44:154–159
7. de Mos M, van El B, DeGroot J, Jahr H, van Schie HT, van Arkel ER, Tol H, Heijboer R, van Osch GJ, Verhaar JA (2007) Achilles tendinosis: changes in biochemical composition and collagen turnover rate. Am J Sports Med 35:1549–1556
8. Denaro V, Ruzzini L, Barnaba SA, Longo UG, Campi S, Maffulli N, Sgambato A (2011) Effect of pulsed electromagnetic fields on human tenocyte cultures from supraspinatus and quadriceps tendons. Am J Phys Med Rehabil 90:119–127
9. Dingemanse R, Randsdorp M, Koes BW, Huisstede BM (2013) Evidence for the effectiveness of electrophysical modalities for treatment of medial and lateral epicondylitis: a systematic review. Br J Sports Med 48:957–965
10. Fassina L, Visai L, Benazzo F, Benedetti L, Calligaro A, De Angelis MG, Farina A, Maliardi V, Magenes G (2006) Effects of electromagnetic stimulation on calcified matrix production by SAOS-2 cells over a polyurethane porous scaffold. Tissue Eng 12:1985–1999
11. Galliera E, Corsi MM, Banfi G (2012) Platelet rich plasma therapy: inflammatory molecules involved in tissue healing. J Biol Regul Homeost Agents 26:35S–42S
12. Ganesan K, Gengadharan AC, Balachandran C, Manohar BM, Puvanakrishnan R (2009) Low frequency pulsed electromagnetic field—a viable alternative therapy for arthritis. Indian J Exp Biol 47:939–948
13. Gomez-Ochoa I, Gomez-Ochoa P, Gomez-Casal F, Cativiela E, Larrad-Mur L (2011) Pulsed electromagnetic fields decrease

proinflammatory cytokine secretion (IL-1beta and TNF-alpha) on human fibroblast-like cell culture. Rheumatol Int 31:1283–1289
14. Hosaka Y, Sakamoto Y, Kirisawa R, Watanabe T, Ueda H, Takehana K, Yamaguchi M (2004) Distribution of TNF receptors and TNF receptor-associated intracellular signaling factors on equine tendinocytes in vitro. Jpn J Vet Res 52:135–144
15. Ibrahim MS, Khan MA, Nizam I, Haddad FS (2013) Peri-operative interventions producing better functional outcomes and enhanced recovery following total hip and knee arthroplasty: an evidence-based review. BMC Med 11:37
16. Ireland D, Harrall R, Curry V, Holloway G, Hackney R, Hazleman B, Riley G (2001) Multiple changes in gene expression in chronic human Achilles tendinopathy. Matrix Biol 20:159–169
17. Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G (2007) Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Exp Neurol 207:267–274
18. Lui PP, Chan LS, Lee YW, Fu SC, Chan KM (2010) Sustained expression of proteoglycans and collagen type III/type I ratio in a calcified tendinopathy model. Rheumatology (Oxf) 49:231–239
19. Maffulli N, Ewen SW, Waterston SW, Reaper J, Barrass V (2000) Tenocytes from ruptured and tendinopathic achilles tendons produce greater quantities of type III collagen than tenocytes from normal achilles tendons. An in vitro model of human tendon healing. Am J Sports Med 28:499–505
20. Marcheggiani Muccioli GM, Grassi A, Setti S, Filardo G, Zambelli L, Bonanzinga T, Rimondi E, Busacca M, Zaffagnini S (2013) Conservative treatment of spontaneous osteonecrosis of the knee in the early stage: pulsed electromagnetic fields therapy. Eur J Radiol 82:530–537
21. Markov MS, Colbert AP (2000) Magnetic and electromagnetic field therapy. J Back Musculoskelet Rehabil 15:17–29
22. Moretti B, Notarnicola A, Moretti L, Setti S, De Terlizzi F, Pesce V, Patella V (2012) I-ONE therapy in patients undergoing total knee arthroplasty: a prospective, randomized and controlled study. BMC Musculoskelet Disord 13:88
23. Ongaro A, Varani K, Masieri FF, Pellati A, Massari L, Cadossi R, Vincenzi F, Borea PA, Fini M, Caruso A, De Mattei M (2012) Electromagnetic fields (EMFs) and adenosine receptors modulate prostaglandin E(2) and cytokine release in human osteoarthritic synovial fibroblasts. J Cell Physiol 227:2461–2469
24. Pan Y, Dong Y, Hou W, Ji Z, Zhi K, Yin Z, Wen H, Chen Y (2013) Effects of PEMF on microcirculation and angiogenesis in a model of acute hindlimb ischemia in diabetic rats. Bioelectromagnetics 34:180–188
25. Pezzetti F, De Mattei M, Caruso A, Cadossi R, Zucchini P, Carinci F, Traina GC, Sollazzo V (1999) Effects of pulsed electromagnetic fields on human chondrocytes: an in vitro study. Calcif Tissue Int 65:396–401
26. Rees JD, Stride M, Scott A (2013) Tendons—time to revisit inflammation. Br J Sports Med. doi:10.1136/bjsports-2012-091957
27. Ricchetti ET, Reddy SC, Ansorge HL, Zgonis MH, Van Kleunen JP, Liechty KW, Soslowsky LJ, Beredjiklian PK (2008) Effect of interleukin-10 overexpression on the properties of healing tendon in a murine patellar tendon model. J Hand Surg Am 33:1843–1852
28. Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE, Hazleman BL (1994) Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. Ann Rheum Dis 53:359–366
29. Rodriguez-De la Fuente AO, Alcocer-Gonzalez JM, Heredia-Rojas JA, Rodriguez-Padilla C, Rodriguez-Flores LE, Santoyo-Stephano MA, Castaneda-Garza E, Tamez-Guerra RS (2010) Effect of 60 Hz electromagnetic fields on the activity of hsp70 promoter: an in vivo study. Cell Biol Int Rep 19:e00014
30. Ross CL, Harrison BS (2013) Effect of pulsed electromagnetic field on inflammatory pathway markers in RAW 264.7 murine macrophages. J Inflamm Res 6:45–51
31. Rui YF, Lui PP, Li G, Fu SC, Lee YW, Chan KM (2010) Isolation and characterization of multipotent rat tendon-derived stem cells. Tissue Eng Part A 16:1549–1558
32. Schulze-Tanzil G, Al-Sadi O, Wiegand E, Ertel W, Busch C, Kohl B, Pufe T (2011) The role of pro-inflammatory and immunoregulatory cytokines in tendon healing and rupture: new insights. Scand J Med Sci Sports 21:337–351
33. Schulze-Tanzil G, Zreiqat H, Sabat R, Kohl B, Halder A, Muller RD, John T (2009) Interleukin-10 and articular cartilage: experimental therapeutical approaches in cartilage disorders. Curr Gene Ther 9:306–315
34. Shi HF, Xiong J, Chen YX, Wang JF, Qiu XS, Wang YH, Qiu Y (2013) Early application of pulsed electromagnetic field in the treatment of postoperative delayed union of long-bone fractures: a prospective randomized controlled study. BMC Musculoskelet Disord 14:35
35. Sollazzo V, Palmieri A, Pezzetti F, Massari L, Carinci F (2010) Effects of pulsed electromagnetic fields on human osteoblastlike cells (MG-63): a pilot study. Clin Orthop Relat Res 468: 2260–2277
36. Strauch B, Patel MK, Rosen DJ, Mahadevia S, Brindzei N, Pilla AA (2006) Pulsed magnetic field therapy increases tensile strength in a rat Achilles' tendon repair model. J Hand Surg Am 31:1131–1135
37. Suzuki K, Nakaji S, Yamada M, Totsuka M, Sato K, Sugawara K (2002) Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. Exerc Immunol Rev 8:6–48
38. Varani K, Gessi S, Merighi S, Iannotta V, Cattabriga E, Spisani S, Cadossi R, Borea PA (2002) Effect of low frequency electromagnetic fields on A2A adenosine receptors in human neutrophils. Br J Pharmacol 136:57–66
39. Vianale G, Reale M, Amerio P, Stefanachi M, Di Luzio S, Muraro R (2008) Extremely low frequency electromagnetic field enhances human keratinocyte cell growth and decreases proinflammatory chemokine production. Br J Dermatol 158:1189–1196
40. Vincenzi F, Targa M, Corciulo C, Gessi S, Merighi S, Setti S, Cadossi R, Goldring MB, Borea PA, Varani K (2013) Pulsed electromagnetic fields increased the anti-inflammatory effect of A(2)A and A(3) adenosine receptors in human T/C-28a2 chondrocytes and hFOB 1.19 osteoblasts. PLoS One 8:e65561
41. Werner S, Grose R (2003) Regulation of wound healing by growth factors and cytokines. Physiol Rev 83:835–870
42. Wojciak B, Crossan JF (1993) The accumulation of inflammatory cells in synovial sheath and epitenon during adhesion formation in healing rat flexor tendons. Clin Exp Immunol 93:108–114
43. Yost MG, Liburdy RP (1992) Time-varying and static magnetic fields act in combination to alter calcium signal transduction in the lymphocyte. FEBS Lett 296:117–122
44. Zorzi C, Dall'Oca C, Cadossi R, Setti S (2007) Effects of pulsed electromagnetic fields on patients' recovery after arthroscopic surgery: prospective, randomized and double-blind study. Knee Surg Sports Traumatol Arthrosc 15:830–834