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ORIGINAL PAPER

Low Frequency Pulsed Electromagnetic Field Affects Proliferation, Tissue-Specific Gene Expression, and Cytokines Release of Human Tendon Cells

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Abstract Low frequency pulsed electromagnetic field (PEMF) has proven to be effective in the modulation of bone and cartilage tissue functional responsiveness, but its effect on tendon tissue and tendon cells (TCs) is still underinvestigated. PEMF treatment (1.5 mT, 75 Hz) was assessed on primary TCs, harvested from semitendinosus and gracilis tendons of eight patients, under different experimental conditions (4, 8, 12 h). Quantitative PCR analyses were conducted to identify the possible effect of PEMF on tendon-specific gene transcription (scleraxis, SCX and type I collagen, COL1A1); the release of pro- and anti-inflammatory cytokines and of vascular endothelial growth factor (VEGF) was also assessed. Our findings show that PEMF exposure is not cytotoxic and is able to stimulate TCs' proliferation. The increase of SCX and COL1A1 in PEMF-treated cells was positively correlated to the treatment length. The release of anti-inflammatory cytokines in TCs treated with PEMF for 8 and 12 h was significantly higher in comparison with untreated cells, while the production of pro-inflammatory cytokines was not affected. A dramatically higher increase of VEGF-A mRNA transcription and of its related protein was observed after PEMF exposure. Our data demonstrated that PEMF positively influence, in a dose-dependent manner, the proliferation, tendon-specific marker expression, and release of anti-inflammatory cytokines and angiogenic factor in a healthy human TCs culture model.

Keywords Tendon · Tendon cells · Pulsed electromagnetic field · Tendon specific markers · Anti-inflammatory cytokines · Vascular endothelial growth factors

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Introduction

The aim of this study is to evaluate the in vitro effects of a specific low frequency pulsed electromagnetic field (PEMF) on primary human tendon resident cells (TCs) to shed light on the biologic response of tendons to biophysical stimulation, providing new evidence on the effectiveness of PEMF for the treatment of tendon disorders. Our results show that PEMF does not affect viability of TCs, but it is able to modulate their immune and angiogenic response and to stimulate TCs proliferation and tendon-specific gene transcription.

Among biophysical therapeutic modalities, electromagnetic stimulation has been studied extensively. Despite it

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has been demonstrated that they can be a successful adjuvant therapy, above all in the management of bone and cartilage disorders [1–8], all available reviews agree on the fact that the biophysical interactions between these low-energy signals and biologic tissues are still not completely understood [5, 9]. However, the most recognized results suggest that external electromagnetic stimuli interact with cells either via transmembrane receptors or ion channels, thereby initiating one or more signal transduction cascades or cell functions [10, 11]. In particular, as demonstrated by Brighton et al. [12], PEMF determines signal transduction through the intracellular release of Ca^{2+} leading to an increase in cytosolic Ca^{2+} and an increase in activated cytoskeletal calmodulin. Through this mechanism, PEMF modifies some important physiologic parameters of cells, such as proliferation, transduction, transcription, synthesis, and secretion of growth factors [10, 13]. It has been already shown that PEMF exposure is able to induce cell proliferation and a dose-dependent increase in bone differentiation and upregulation of mRNA expression of specific extracellular matrix molecules in human osteoblasts [14, 15]. Similarly, in vitro studies on cartilage have demonstrated that PEMF is able to stimulate chondrocyte proliferation [16] and to modulate production and release of cytokines and growth factors like IL-1β and insulin-like growth factor-I [17–19].

On the contrary, few and sometimes conflicting studies on the effect of PEMFs on tendons have been conducted; most of them are in vivo and demonstrate that PEMF treated groups show better collagen alignment, a greater reduction of inflammation, with a better return of tendons to histologic normality, thus suggesting a positive influence of PEMF on tendon healing [20]. Indeed, in a manner similar to bone and wound repair, tendon repair involves an inflammatory phase, angiogenesis, cell proliferation, collagen production, and remodelling stages, intrinsically via proliferation of epitenon and endotenon tenocytes, or extrinsically, by invasion of cells from the surrounding sheath and synovium [20]. So, in vitro studies exploring the effects of PEMF on human tenocytes would help in the comprehension of the possible mechanisms of action of this treatment. It is known that tendon tissue is poorly cellularized (5 % of the normal tendon tissue volume). These few tendon resident cells (TCs) represent a mixed population, made up mostly by tenocytes and tendon stem/progenitor cells, which together are responsible for the tissue homeostasis [21, 22], for the production and the remodelling of the abundant and strictly organized extracellular matrix. To our knowledge, only one study describing the behavior of tenocytes after long and continuous PEMF exposure has been published [23]. In this study, authors used a peculiar in vitro wound closure assay, investigating the effects of PEMF (0.4 mT, frequency 50 Hz) on the speed of wound closure. Our study aims to provide a more detailed analysis of the in vitro effect of PEMF with specific physical parameters, already used in clinical practice (1.5 mT, frequency 75 Hz), on the resident tendon cell clonogenic ability, viability, proliferation, and on the gene expression of specific tendon markers, such as SCX and COL1A1. Moreover, we have investigated the release of pro- and anti-inflammatory cytokines to evaluate the feasibility of activating an anti-inflammatory pathway. Since it has been recently demonstrated that vascular endothelial growth factor (VEGF) has a fundamental role in the tendon healing process, we have evaluated its release by PEMF-treated cells and compared it to untreated cells. Although Denaro et al. [23] showed positive results starting from 12 h of PEMF exposure, we chose to treat cells also for shorter periods since other studies, even if concerning other cellular models [24, 25] showed the efficacy of PEMF already after 4–8 h.

All the experiments have been performed on eight different cell populations isolated from small portions of the healthy semitendinosus and gracilis tendons of eight patients who had undergone anterior cruciate ligament (ACL) reconstruction at our Institute.

Materials and Methods

TCs Isolation and Culture Expansion

All the procedures were carried out with the Institutional Review Board approval. Discarded fragments of semitendinosus and gracilis tendons were collected from 8 healthy young donors (mean age 35 ± 12 years; mean body mass index 24 ± 1) who underwent ACL reconstruction with autologous hamstrings at Galeazzi Orthopaedic Institute, Milan, under written consent. To isolate tendon cells (TCs), the tendon tissue was minced and digested enzymatically with 0.3 % type I collagenase (Worthington, Lakewood, NJ, USA) in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with continuous agitation for 15 h at 37 °C as reported by Rui et al. [22] with minor modifications. The isolated nucleated cells were then plated at 5×10^3 cells/cm^2 in complete medium composed of: DMEM, 10 % fetal bovine serum (FBS; Sigma-Aldrich), 50 U/ml Penicillin, 50 μg/ml Streptomycin, 2 mM L-glutamine (Sigma-Aldrich), and supplemented with 5 ng/ml basic fibroblast growth factor (b-FGF; Peprotech, Rocky Hill, NJ, USA) just for cell expansion. They were maintained at 37 °C in humidified atmosphere with 5 % CO_2, changing culture medium every 3 days. TCs remained quiescent for several days before starting to proliferate rapidly; when they reached 80–90 % of confluence, the cells were detached by incubation with trypsin/EDTA (0.5 % trypsin/0.2 % EDTA; Sigma-Aldrich)

and then cultured at a density of 3×10^3 cells/cm^2. Cells from passages 2–4 (P2–P4) were used for the experiments.

Fibroblast Colony-Forming Unit Assay

A colony-forming unit fibroblast (CFU-F) assay was performed as previously described [26]. TCs were plated in six-well plates at low density by limiting dilution (starting dilution: 10 cells/cm^2, final dilution: 1.5 cells/cm^2) and cultured for 14 days. After 7 days the medium was replaced, and at the end of the culture period the cells were fixed with 10 % paraformaldehyde and stained with Crystal Violet (Sigma-Aldrich). The frequency of CFU-F was established by scoring the individual colonies composed of at least 50 cells and expressed as a percentage relative to the seeded cells.

PEMF Stimulation

The TCs were exposed to PEMF generated by a pair of rectangular horizontal coils (18 × 13 cm), each made of 1,000 turns of copper wire, placed opposite each other. The culture flask was placed between this pair of coils so that the plane of the coils was parallel to the culture flasks, directly inside the incubator (Fig. 1). The coils were powered by a PEMF generator system (IGEA, Carpi, Italy) already used in previous studies [17, 19, 27, 28]. This produced a pulsed signal with the following parameters: pulse duration of 1.3 ms and frequency of 75 Hz, yielding a 0.1 duty cycle. Before starting the experiments, the peak intensity of the magnetic field and the peak intensity of the induced electric voltage were measured in air between two coils from one side to the other, at the level of the culture flasks. The peak values measured between two coils in air had a maximum variation of 1 % in the whole area in which the culture flasks were placed. The peak intensity of the magnetic field was 1.5 ± 0.2 mT and it was detected 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 %. The corresponding peak amplitude of the induced electric voltage was 2.0 ± 0.5 mV. It was detected using a standard coil probe (50 turns, 0.5-cm internal diameter of the coil probe, 0.2-mm copper diameter) and the temporal pattern of the signal was displayed using a digital oscilloscope (Le Croy, Chestnut Ridge, NY, USA). The shape of the induced electric voltage and its impulse length were kept constant.

Passage 4 cells were seeded into flasks or multiwells the day before starting the PEMF treatment. They were exposed to PEMF for 4, 8, or 12 h, under the same conditions of temperature, humidity, and CO_2 concentration as the untreated cells and then analyzed.

Fig. 1 Pulsed electromagnetic field device used for all the experiments, placed in a 5 % CO_2 and 37 °C incubator. TCs seeded in T25 culture flasks (like in the picture) or in 24-well plates were placed between the pair of coils so that the plane of the coils was parallel to the culture layer. The peak values measured between the two coils in air had a maximum variation of 1 % in the whole area in which the culture flasks were placed. Cell were exposed to PEMF for 4, 8 or 12 h

Live and Dead Assay

A live/dead assay was performed on the untreated and treated cells, seeded at a density of 10^5 cells/cm^2 in 24-well plate, after 4, 8, and 12 h of PEMF exposure. The cell culture medium was removed and a solution containing 2 μM calcein and 4 μM ethidium homodimer-1 (Invitrogen, Ltd., Paisley, UK) was added to each sample. The cells were then observed by fluorescence microscopy (Microscope OLYMPUS IX71). Live cells stained green and dead cells red. The percentage of live cells was measured and 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. Three randomly chosen fields of view were photographed for two sample of each population ($n = 3$).

Cell Apoptosis Analysis by Annexin V-FITC and Propidium Iodide (PI)

Apoptosis induced by PEMF treatment was analyzed by flow cytometry utilizing annexin V-FITC and PI staining (Sigma-Aldrich). In brief, 4.0×10^5 cells were trypsinized, washed with PBS, and resuspended with 500 μL of a specific binding buffer containing 10 μL of PI, and 5 μL of annexin V-FITC [29, 30]. After exactly 10 min of incubation in the dark at room temperature, cells were analyzed for annexin V and PI staining by flow cytometry. Each experiment was run in triplicate. Excitation wavelength was 488 nm and emitted green fluorescence of annexin V (FL-1) and red fluorescence of PI (FL-2) were collected using, respectively, a 525 and a 575 nm band pass filter. Early apoptosis and late apoptosis/necrosis were expressed

as the percentages of annexin V+/PI− and annexin V+/PI+ positive cells.

Viability and Proliferation Assay

TCs at passage 4 were plated at a concentration of 1.5×10^4 cells/cm^2 in complete medium in 96-well plates and then exposed to PEMF, according to the experimental protocol. Both untreated and treated cells were monitored immediately after exposition (0 day) and after 2, 7, and 10 days (2, 7, and 10 days), adding MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) in the culture medium at a final concentration of 0.5 mg/ml and incubated for 4 h at 37 °C. The resulting formazan precipitate was then solubilized using 100 % DMSO and the absorbance was read at 570 nm (VictorX3, Perkin Elmer microplate, Waltham, MA, USA) [31]. Similarly, 5.0×10^3 TCs/cm^2 were placed in 24-well plates and received an identical PEMF treatment. At the same time points, they were analyzed for the DNA content (Triton X-100 0.1 % in ddH_2O as lysis buffer) using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen, Ltd.); fluorescence was read at 520 nm (excitation $\lambda = 480$ nm) (VictorX3, Perkin Elmer microplate).

RNA Extraction, RT, and Real-Time PCR

Total RNA was isolated from the untreated cells and from the cells exposed for 4, 8, and 12 h to PEMF using the RNeasy Mini kit (Qiagen, Duesseldorf, Germany) and the isolated RNA was quantified spectrophotometrically (Nanodrop, Thermo Scientific, Rockford, IL, USA). 100 ng of RNA were reverse-transcripted to cDNA employing the iScriptcDNA Synthesis Kit (Bio-Rad Laboratories, Benicia, CA, USA).

The final volume of 20 μL included a 5× reaction mix containing oligo(dT), random hexamer primers, and reverse transcriptase pre-blended with RNase inhibitor. The reaction mix was incubated for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C.

10 ng of cDNA was used as a template for real-time PCR, performed using a Rotor Gene RG3000 system (Qiagen). The PCR mixture included TaqMan Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies, Grand Island, NY, USA) in a final volume of 20 μL. Amplification and real-time data acquisition were performed using the following cycle conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The genes analyzed were glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), SCX (Hs03054634_g1), COL1A1 (Hs01076777_m1), and vascular endothelial growth factor A (VEGF-A) (Hs00900055_m1). The fold change in the expression of the different genes in the control and treated cells was normalized on the expression of the housekeeping GAPDH gene.

Cytokines and VEGF Determination

Levels of soluble IL-1β, IL-6, IL-10, TNF-α, TGFβ, and VEGF-A in cell culture medium after 0, 24, and 48 h from the end of PEMF treatment were determined by commercially available ELISA assays according to the manufacturers' instructions (R&D System, Minneapolis, MN, USA). For VEGF detection assay, the sensitivity of the test was 5 pg/ml; intra- and inter-assay coefficients of variation were 6.6 and 6.7 %, respectively. For IL-1β detection assay, the sensitivity of the test was less than 1 pg/ml, intra- and inter-assay coefficients of variation were 2.8 and 4.1 %, respectively. For IL-6 detection assay, the sensitivity of the test was 2 pg/ml, intra- and inter-assay coefficients of variation were 5.8 and 3.1 %, respectively. For IL-10 detection assay, the sensitivity of the test was less than 0.5 pg/ml, intra- and inter-assay coefficients of variation were 6.6 and 8.1 %, respectively. For TNF-α detection assay, the sensitivity of the test was 1.6 pg/ml, intra- and inter-assay coefficients of variation were 5.0 and 7.3 %, respectively. For TGFβ detection assay, the sensitivity of the test was less than pg/ml, intra- and inter-assay coefficients of variation were 2.7 and 4.3 %, respectively.

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 distribution of values were assayed by Kolmogorov–Smirnov normality test, while one-way Analysis of Variance (ANOVA) for repeated measures, with the Bonferroni's correction, was used to compare data over time. Paired comparisons were performed by two-tailed t test. In the case of not normally distributed values, repeated measures were compared with the Kruskal–Wallis test with the Dunns' correction. Correlation analysis was performed by the two-tailed Pearson correlation test (Spearman's test for not normally distributed values); the same test was conducted to evaluate the correlation between the trends of these parameters across the time-points. The significance level was set at p value lower than 0.05.

Results

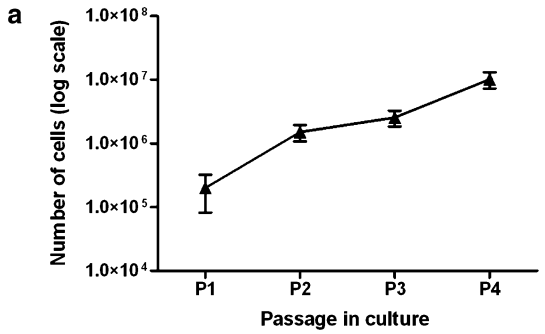
Explants of eight human hamstring tendons (1.5 ± 0.6 g) were digested and cultured in a culture flask with growth medium. The average yield of TCs was $4.5 \pm 8.5 \times 10^5$ cells/g of tissue ($n = 8$). After about 10 days of

inactivity, where the cell population was not morphologically homogeneous, typical fibroblastoid-like cells began to proliferate actively, forming a compact monolayer. In <4 weeks, from an average of $1.99 \pm 1.2 \times 10^5$ at passage 1, the TCs increased to $1.02 \pm 0.29 \times 10^7$ at passage 4 (Fig. 2a). The mean doubling time of the TCs slightly increased with the passage in culture, starting from 64 ± 27 h at passage 2 to 97 ± 28 h at passage 4 (Fig. 2b). CFU-F assay showed that an average of 12.8 ± 10.8 % of P3 cells were able to produce colonies; this ability slightly decreased with passage in culture (P4: 6.4 ± 7.4 %) (Fig. 2b).

Effect of PEMF on Viability and DNA Content

The cells at passage 4 were exposed to PEMF for 4, 8, or 12 h and then analyzed. Regardless of the length of treatment they received, the cells did not change their morphology and remained viable when compared to the untreated cells (Fig. 3). Similarly, no differences in apoptosis between the control and the treated cells were detected (Fig. 4).

TCs viability was slightly affected by PEMF stimulation; in particular, 12 h of exposure was able to produce mild, not significant increases (+17 %) in viability when compared to the untreated cells, 2 days after treatment. Shorter PEMF treatment (4 and 8 h) did not affect TCs viability (Fig. 5). Eight hours of PEMF treatment provoked a prompt significant increase of the TCs total DNA content (+22 %, $p = 0.020$) immediately after treatment (day 0). Analyses performed at the following time points (2 and 7 days) showed more pronounced effects, although not significant, in cells treated with PEMF for 12 h, as already observed for the viability parameter (Fig. 5), while shorter treatments didn't affect DNA content at these time points. However, the effect of PEMF on cell viability and DNA content progressively decreased as the time from the exposure increased, and at 10 days no effects were observed, probably because cells reached 100 % confluence.



b

	P2	P3	P4
Doubling Time (h)	64±27	86±52	97±28
Percentage of clonogenicity (%)	-	12.8±10.8	6.4±7.4

Fig. 2 Features of human TCs used in the experiments. Proliferation ability, expressed as number of cells (**a**) and average doubling time (**b**) during passages in culture. Percentage of clonogenic TCs at passage 3 and 4 (**b**). Data are expressed as mean ± SD. For all data, $n = 8$

Effect of PEMF on Gene Expression

The expression of transcripts of SCX, collagen I (COL1A1) and (VEGF-A) was determined by real-time PCR at two time points (0 and 2 days from the end of the treatment). The fold change in the different gene expression in the treated and untreated TCs was normalized on the expression of the GAPDH gene. Immediately after the treatment (day 0), the SCX up-regulation was clearly dependent on the length of PEMF treatment: the TCs treated for 4 h showed a mild increase with respect to the untreated cells (+25 %); this effect was then more pronounced in cells treated for 8 h (+58 %) and 12 h (+95 %, $p = 0.0156$) (Fig. 6). The same trend was observed for COL1A1 expression, where 4, 8, and 12 h of treatment were able to induce increases of 22, 49, and 97 %, respectively, although, due to the interdonor variability, this effect was not statistically significant (Fig. 6).

Both SCX and collagen I expression in PEMF-treated cells remained slightly upregulated 2 days after the end of the exposure (day 2), even if these increases were not statistically significant (Fig. 6). VEGF-A expression of TCs was also upregulated by PEMF at day 0, but the increases were statistically significant only after 8 and 12 h of exposure (+41 %, $p = 0.0187$ for 8 h; +34 %, $p = 0.0303$ for 12 h) (Fig. 6). On the other hand, at day 2 VEGF-A expression of all the PEMF-treated cells returned to basal level (Fig. 6).

Effect of PEMF on Cytokine Release

Cytokines and growth factors were assayed in the conditioned medium of cells exposed for 8 and 12 h to PEMF. IL-1β and TNFα levels were greatly variable among the different cell populations. Both IL-1β and TNFα were not significantly affected by the treatments (both 8 and 12 h) at all the time points (day 0, 1, and 2) with the exception of a small, although significant, increase observed for IL-1β after one day after 12 h PEMF treatment (Fig. 7). On the contrary, the release in the culture medium of IL-6 by both the cells exposed to 8 and to 12 h was significantly higher compared to the control cells, starting from day 1 and especially at day 2 (360 %, $p < 0.001$ for 8 h; 427 % and $p < 0.001$ for 12 h, respectively) (Fig. 7). IL-10 production was also strongly upregulated by PEMF: already at day 1, the levels were significantly increased both for the TCs

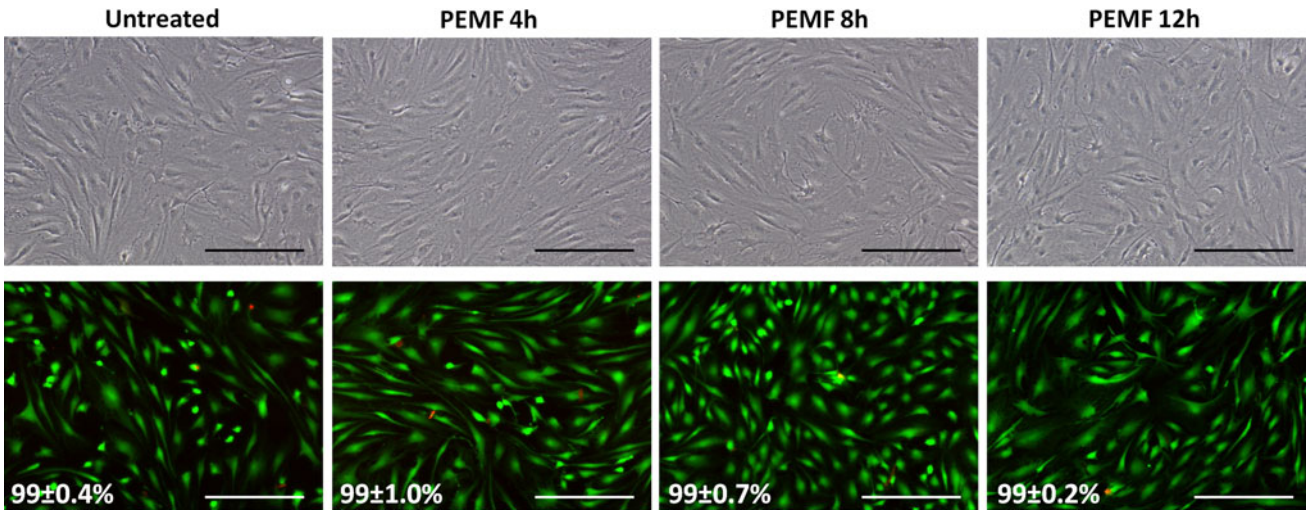


Fig. 3 Morphology of untreated cells and of cells exposed to PEMF for different duration (4, 8, or 12 h) (optical microscopy ×10, *scale bar* 200 μm) (*upper panel*); Live and Dead staining for viable (*green*) and dead cells (*red*) in treated and untreated cells (fluorescence microscopy, ×10, *scale bar* 200 μm, merged images). *Percentages* in pictures report rates of viable cells indicated as mean ± SD ($n = 3$) (Color figure online)

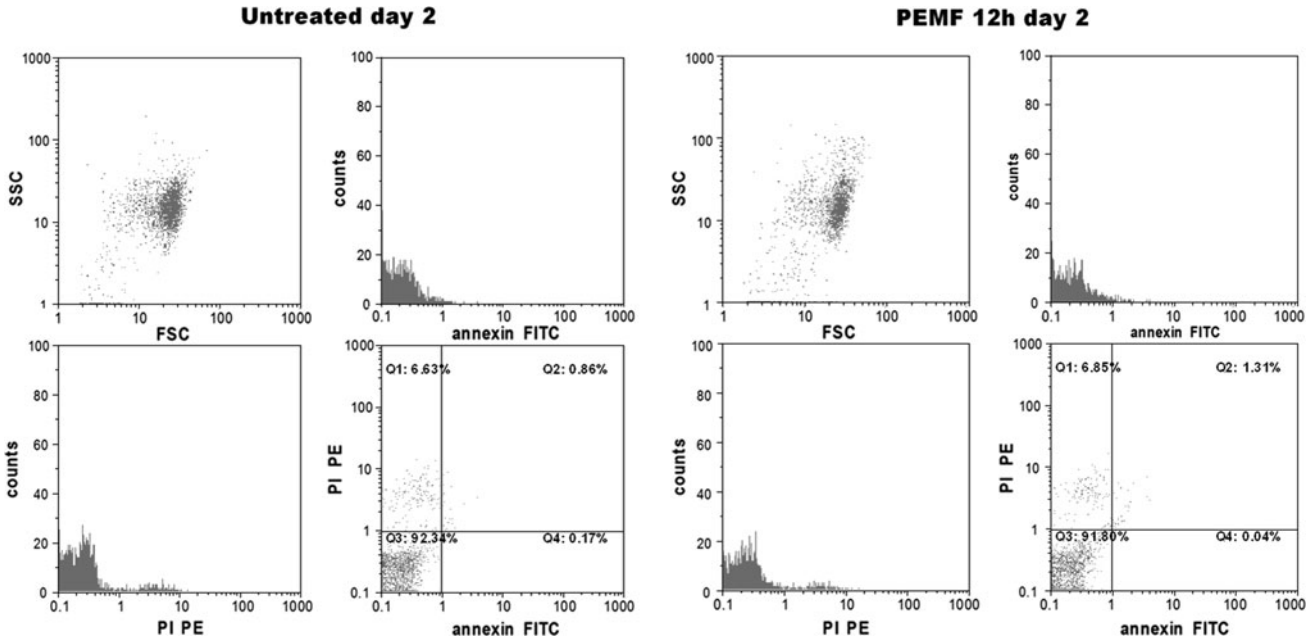


Fig. 4 Apoptosis analysis by flow cytometry at day 2 of untreated and treated cells after 12 h of PEMF exposure. Cells were detected using annexin V-FITC and PI probes. Cell populations FITC−/PI− (Q3), FITC+/PI− (Q4), and FITC+/PI+ (Q2), FITC−/PI+ (Q1) were living, early apoptotic, and late apoptotic/necrotic cells and necrotic cells, respectively ($n = 3$)

treated for 8 and 12 h (133 and 191 %, $p < 0.001$, respectively) and this effect was maintained until day 2 for both treatments (162 % for 8 h and 197 % for 12 h, $p < 0.001$) (Fig. 7). The increase of TGF-β concentration in medium was faster and more evident: the differences between treated and untreated TCs progressively increased, starting immediately after treatment and showing a more than 11-fold increase both for the 8 and 12 h treated cells after 2 days ($p < 0.001$) (Fig. 7).

No significant difference in IL-6, IL-10, or TGF-β was observed between the TCs treated with PEMF for 8 or 12 h.

The same scenario was observed for VEGF-A, which immediately after the PEMF treatment (day 0) was released in a significantly higher amount by cells treated both for 8 and 12 h, compared to untreated cells. This difference accelerated over time with more than 60- and 90-fold increases observed both for 8 and 12 h treated cells after 1

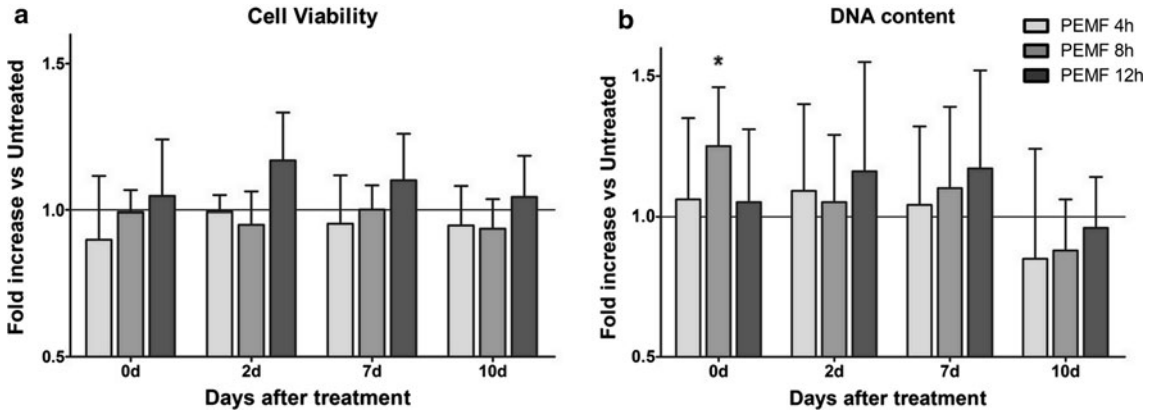


Fig. 5 Effect of PEMF exposure on cell viability (**a**) and DNA content of TCs after 0, 2, 7, and 10 days (**b**). Values are indicated as fold increase respect to untreated cells represented by a fixed line set at 1; data are expressed as mean ± SD. *$p < 0.05$, PEMF-treated cells versus untreated cells, $n = 8$

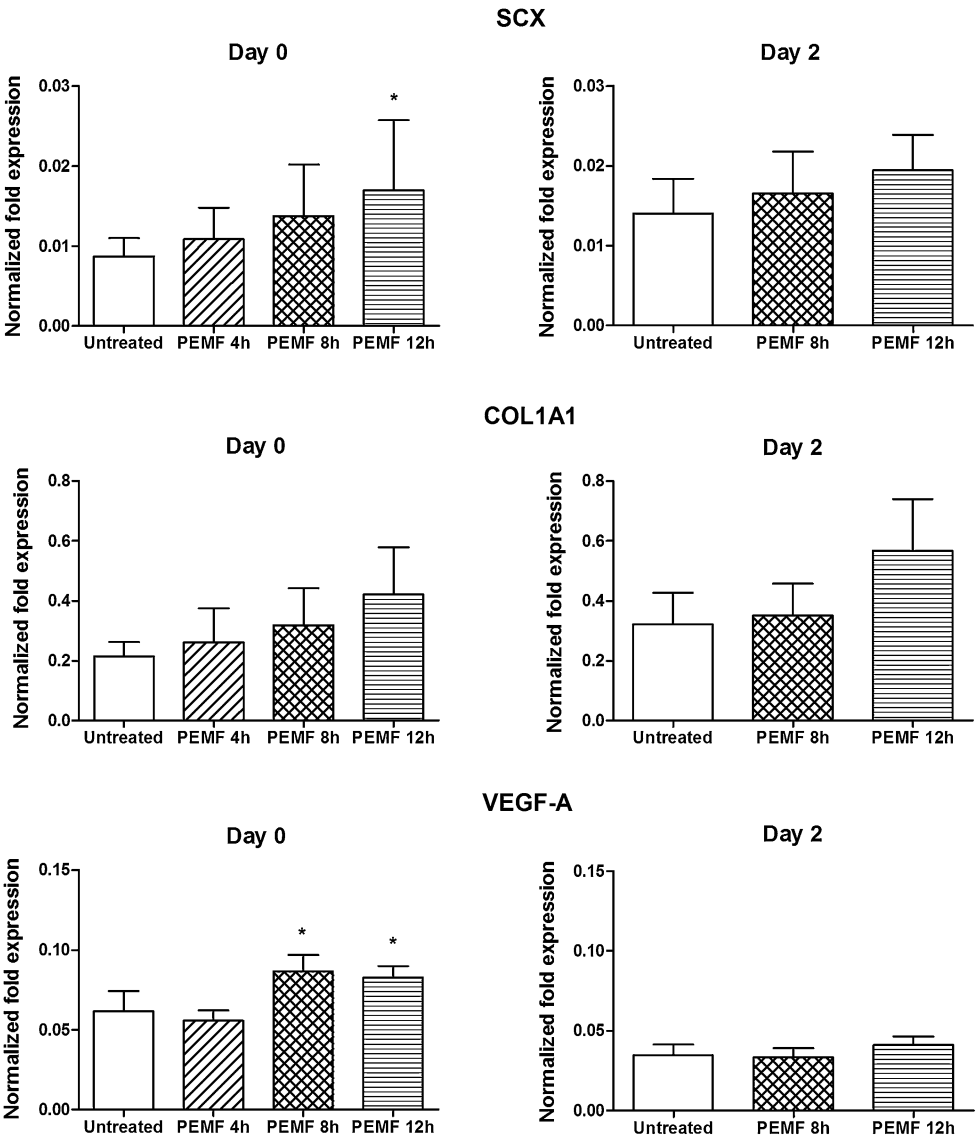


Fig. 6 Effect of PEMF exposure on scleraxis (SCX), collagen type I (COL1A1), and vascular endothelial growth factor A (VEGF-A) gene expression, determined by quantitative real-time PCR at day 0 and day 2 from the end of treatment, both in untreated and treated TCs. Data were normalized on the expression of the housekeeping GAPDH gene and are expressed as mean ± SD. *$p < 0.05$, PEMF 12-h treated cells versus untreated cells, $n = 8$

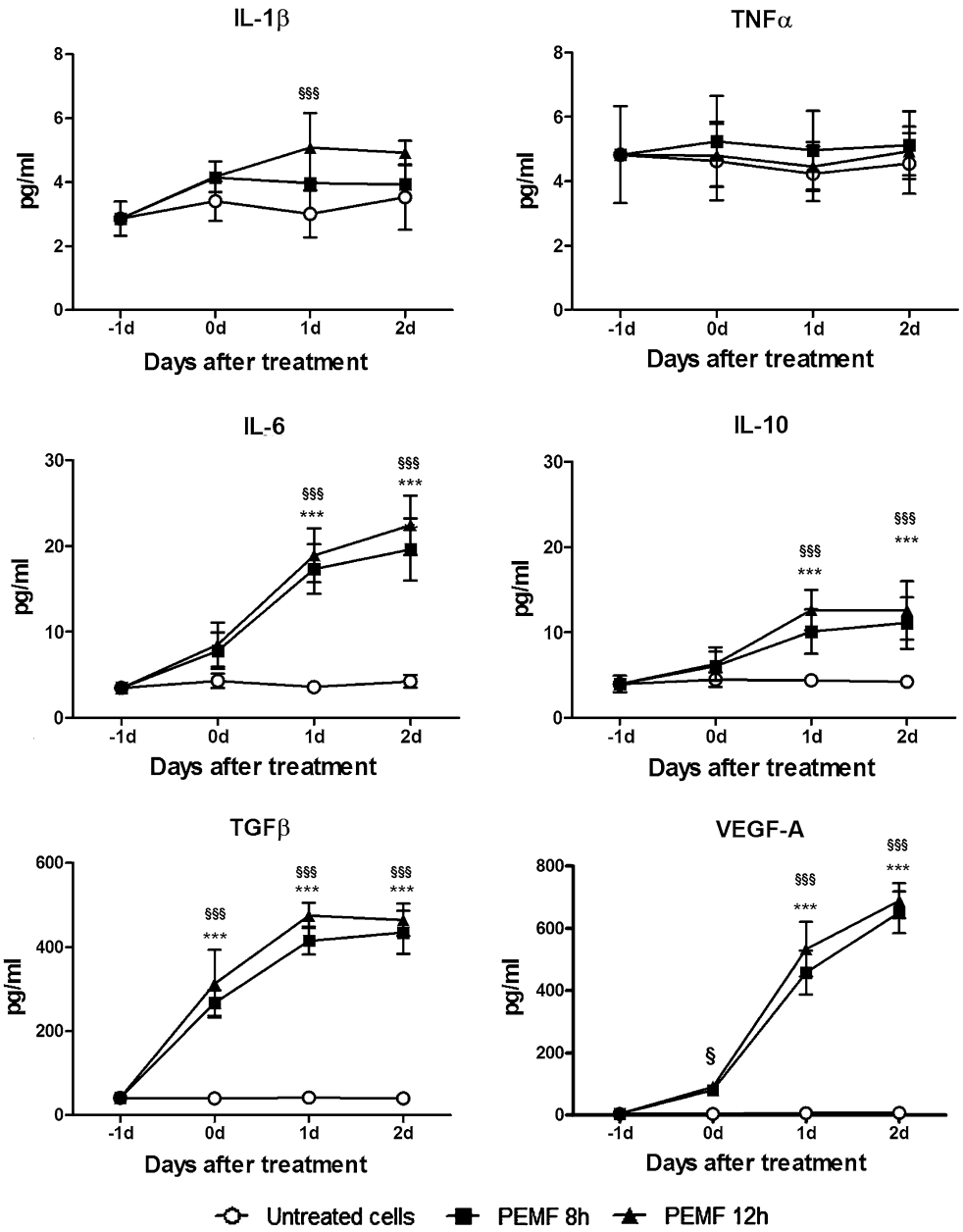


Fig. 7 Release of cytokines and VEGF-A in cell culture medium of untreated and treated TCs (PEMF 8 and 12 h), after 0, 1, and 2 days from the end of the treatment. Data are expressed as mean ± SD. ***, $p < 0.001$ PEMF 8-h treated cells versus untreated cells. §, $p < 0.05$; §§§, $p < 0.001$ PEMF 12-h treated cells versus untreated cells, $n = 8$

and 2 days, respectively ($p < 0.001$), but without any significant differences between the two groups of treated cells (Fig. 7).

Discussion

The major finding of our paper is that 8-h PEMF exposure (1.5 mT, 75 Hz) is able to induce the modulation of TCs' proliferation, tendon-specific marker expression, and the release of anti-inflammatory cytokines and angiogenic factor.

Over the last decades, extensive in vitro, pre-clinical, and clinical researches have revealed some insights into the biologic effects of electromagnetic stimulation and the effectiveness of PEMFs treatment in bone and joint disorders. However, most of these studies lack homogeneity since they present a high variability in terms of magnetic flux density (the component of the magnetic field passing through a surface), signal type, frequency, duration, and the number of treatment sessions [20, 23, 32, 33]. In our study, we used the same parameters of pulsed electromagnetic field that was used in other ones, which showed that this extremely low frequency PEMF (1.5 mT, 75 Hz) was able to stimulate anabolic processes and limit IL-1β catabolic effects in chondrocytes and cartilage explants [17, 19] and, to enhance the proliferation and the tissue-specific marker expressions in osteoblasts [14, 34, 35]. Moreover, as shown

in preclinical studies, these parameters of PEMF preserve the morphology of articular cartilage and retard the development of osteoarthritic lesions in guinea pigs [1]. Finally, in humans, this stimulation positively affected the recovery after surgical procedures of the knee by reducing the joint inflammation [36, 37]. Despite all these extensive researches, this is one of the first studies which aimed to investigate the possible effects of PEMF on TCs. Since mechanostimuli play an essential role in tendon homeostasis, tendon healing, and tenocyte survival [38, 39], it is interesting to evaluate the mechanoresponsiveness of TCs to biophysical stimuli. Resident cells make up around only 5 % of the normal tendon tissue volume; this scarcity is confirmed by the low yield of cells from tendon digestion that we obtained from our experiments. Recently, stem cells have been discovered in human and mouse tendon tissue (TSPCs, tendon stem progenitor cells); they exhibit universal stem cell characteristics, including clonogenicity, self-renewal, and multi-lineage differentiation capacities, even after extended expansion in vitro [21, 22]. In particular, human hamstring tendons contain 3–4 % of TSPCs with respect to the total nucleated cells [21]. Since we did not use any specific methods to select only tenocytes, our experiment was conducted on a non-homogeneous cell population, which probably better reflects the physiologic tendon cell environment. The presence of a subpopulation of tendon stem cells could explain the high proliferation rate and the corresponding short doubling time that we observed in our experiment, together with a consistent percentage of clonogenic cells in our cell cultures (12 %).

PEMF exposure did not exert any cytotoxic effects on TCs, as demonstrated by the absence of apoptotic cells and the similarity in terms of viability of treated cells from untreated ones. Immediately after treatment, a significant increase in DNA content of 8-h PEMF-treated cells was observed; this result is particularly important since PEMF treatment could be able to stimulate cell proliferation with a consequent positive effect on tendon recovery. However, this effect on cell proliferation was very short-lasting, probably also due to the experimental model where cells progressively reached 100 % confluence and were not able to further grow. In any case, regardless of the length of exposure, the effect on viability and DNA content lasted for a week at most, thus suggesting that repeated daily PEMF exposures could be probably necessary to achieve a durable result. For this reason, it would be interesting to re-treat the same cells to assess if this is able to prolong or enhance the observed effects. It is important to note that 4 h of treatment did not induce any response in the TCs. Moreover, we hypothesize that the lack of considerable differences between PEMF-treated and untreated cells could be due to the origin of the cells: indeed, since they were derived from healthy hamstring tendons, the effect of PEMF on viability and DNA content could have been masked since these parameters were already optimal in untreated TCs. Our results are partially in contrast with previously published data on the in vitro effect of PEMF on tenocytes [23], which did not show any positive influence of PEMF on cell proliferation. This difference could be due to the different features of PEMF treatment, in term of dosage and physical parameters since they used a lower magnetic intensity (0.4 mT) and the cells were exposed to an uninterrupted stimulation for longer time, until for seven continuous days. As already demonstrated, PEMF exposure is also able to act on the expression of specific tissue markers [40–42] and thus we assessed the expression of SCX and collagen I, both specific markers of tendon. In particular, collagen I gives tendon its resilience and biomechanical stability, whereas SCX is a transcription factor family continuously expressed through differentiation into the mature tenocytes and ligament cells [43, 44], involved in the regulation of growth and differentiation of numerous cell types [45] and expressed by the tendon progenitor population that forms the fourth somitic compartment (the "syndetome"). In our experiment, immediately after PEMF treatment, we observed a progressive, dose-dependent upregulation in both the SCX and COL1A1 expression, suggesting a very rapid and strong effect of PEMF. The similar behavior of PEMF-induced expression of these two tendon markers could be explained as SCX regulates transcription of COL1A1 through binding to tendon-specific element 2 [46]. Two days later, the upregulation of mRNA synthesis appeared to be maintained although it was no longer statistically significant. At this time point, the TCs treated with PEMF for 4 h were not assayed since they did not show any relevant differences from the untreated cells not only in terms of SCX and COL1A1 expression but also in terms of viability and proliferation. This implies that a short length of exposure is not sufficient to trigger a functional response in this cell model. For the same reason, the release of cytokines in cells treated with PEMF for 4 h was not analyzed.

As for other tissue and organs, tendon healing is characterized by an initial inflammatory response [47]. In our study, the release of the main inflammatory, anti-inflammatory, and regulatory cytokines, involved in tissue healing, was positively affected by PEMF exposure. Normally, in vivo most of the cytokines are produced by infiltrating leukocytes and resident macrophages and fibroblasts although an endogenous production of several cytokines by TCs has been recently demonstrated [48]. We did not observe any increase in TNFα level, which is usually involved in several aspects of tendon degeneration [49]. The absence of a PEMF-related increase of this cytokine indicates that PEMF does not damage tenocytes, in accordance with the absence of apoptosis and the increase

of cell proliferation after PEMF treatment discussed above. We observed similar findings for IL-1β: in this case, there was a small, but significant, increase one day after 12 h of PEMF treatment. IL-1β induces the production of IL-6, a multifunctional Th2 cytokine which exhibits immunoregulatory functions in tissues and plays an essential role in tissue healing, as it is involved in cell proliferation and survival [48, 50]. Thus, the increase of IL-6 correlates with the amount of cell viability and proliferation observed after PEMF treatment. IL-6 also has a stimulatory effect on IL-10 production. IL-10 is not only the most effective anti-inflammatory Th2 cytokine but it also has been demonstrated to affect connective tissue cells such as fibroblasts and chondrocytes [51]. In tenocytes, upregulation of IL-10 correlates with an increase in healing in murine models [52]. Over the last decades, research has clarified the role of IL-10 in tissue healing, indicating a role in extracellular matrix remodeling, in particular in elastin upregulation [48]. We also observed a strong increase of the transforming growth factor (TGF-β); it has already been demonstrated that it is strongly correlated with fibronectin production by TCs [53], thus confirming the positive effect of PEMF treatment on TCs. Moreover, it is also produced in osteoblasts and in synovial fibroblasts after PEMF treatment [54, 55].

PEMF has been used for several years for bone healing and have been advocated to promote extracellular matrix production through the regulation and promotion of neo-angiogenesis [56]. In our study, we observed a marked increase in the, VEGF. This is in accordance with the observed increase of IL-6, which is the main VEGF promoter. VEGF production has been demonstrated to have a fundamental role in the tendon healing process [57, 58] even though it has been observed that an excess of vascularization induced by VEGF upregulation can be detrimental in some tendon degenerative diseases [59]. It has already been demonstrated that PEMF promotes angiogenesis in bone marrow, by the overexpression of angiopoietin-2 mRNA [56], but to our knowledge this is the first evidence of the secretion of VEGF by tenocytes after PEMF treatment.

Taken together, these results indicate that PEMF treatment has a beneficial effect at the cellular level. Not only can it increase cell viability and proliferation but also can contribute to the process of "tenocyte activation" [60], promoting the production of the immunoregulatory cytokines, growth, and angiogenic factors involved in the tendon healing process. Nevertheless, further investigations are required to confirm these in vitro observations, in particular in pre-clinical models.

In a future study, it would be interesting to investigate the effect of PEMF on TCs derived from pathological tendons, and compare their response with that of healthy cells.

In conclusion, despite these further findings on PEMF-induced physiologic effects on tendon resident cells, the mechanisms of action by which TCs perceive physical stimuli and how this information is transduced into the synthesis and organization of extracellular matrix remain to be better established. However, our data demonstrated that PEMF positively influences proliferation, tendon-specific marker expression, and the release of anti-inflammatory cytokines and angiogenic factor in a healthy human TCs culture model in a dose-dependent manner.

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