Effects of PEMFs-ELFs (Pulsed Electromagnetic Fields-Extremely Low Frequencies) on Morfology and Differentiation of C2C12 Mouse Myoblast Cell Line.

F. Sereni, F. Cialdai, M. Monici

ASAcampus, ASA res Div., Dept. of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

ABSTRACT

An increasing number of reports shows the ability of ELF-PEMFs to change the behavior of cells and evoke biological responses. Therefore, the interest on the use of ELF-PEMFs in clinics is increasing and new fields of application are explored, in addition to the well-established application in the treatment of bone diseases. However, our understanding of the cellular and molecular mechanisms that underlie the clinical observations is still lacking. A better knowledge is required to improve the clinical applications and treatment parameters.

The aim of this study was to analyze ELF-PEMFs (50Hz, 2mT) effect on a myoblast model (C2C12 cell line) through morphological and molecular assays. This cell line is a well-characterized model to study muscle cell differentiation and tissue repair. To assess the effect of treatment time on the biological response we used two different time of stimulation: 15 minutes (short-treatment) and 3 hours (long-treatment). The samples were analyzed immediately after the treatment and 24 h, 72 h, 6 days later. Viability and proliferation were assessed by MTT assay. Morphology and cytoskeleton organization were analyzed by immunofluorescence microscopy. The effect of ELF-PEMFs on myoblast differentiation was investigated by analyzing the expression of markers typically expressed during myogenesis: MyoD, myogenin and MHC.

After both the treatments we found a weak decrease in proliferation but no effects on cell viability. The network of microfilaments and microtubules changed, especially after 3 h exposure to ELF-PEMFs. The expression of myogenesis markers increased and the translocation of the transcription factor MyoD to the myoblasts nucleus was observed. In conclusion the results showed that ELF-PEMFs are able to induce in the myoblast model a biological response consisting in cytoskeleton remodelling and increase in expression of myogenesis markers. The effect depended on the exposure time.

INTRODUCTION

Electromagnetic fields and magnetotherapy are commonly used in physical medicine. Generally, the EMFs applied in clinics are pulsed (PEMFs), with frequency lower than 100 Hz and field intensity ranging from μT to a few tens of mT, therefore they are classified as Extremely Low Frequency (ELF) PEMFs. Recent studies have shown that ELF-PEMFs can change cell behavior and activation by affecting biochemical and biophysical processes. The molecular and cellular mechanisms underlying the effects of ELF-PEMFs are not completely understood, but recently many progresses have been made: physical processes at the atomic and molecular level are at the basis of the biological response evoked by ELF-PEMFs, since they can affect chemical bonds, dipole orientation, charge diffusion, receptor clustering, etc……[1].

In living tissues endogenous EMFs are generated by physiological activities, for example, muscle vibrations induce mechanical strains in bone tissue and low frequency EMFs are generated both during postural muscle activity and walking, 5-30 Hz and <100 Hz respectively [2]. Muscle contraction has an important role in maintaining bone mass, since bone cells are sensitive to EMFs in the 15-30 Hz range

[3]. Thus ELF-PEMFs stimulation has been used successfully to treat a wide range of bone disorders, such as rheumatologic diseases [4] and osteoporosis, and accelerate the healing of fractures [5]. Moreover ELF-PEMFs are widely used in clinics for beneficial effects due to analgesic action, anti-oedematous activity, vasodilation and anti-inflammatory action [1]. Magnetotherapy provides a safe, non invasive and easy method to directly treat the site of injury, thus the use of this tool of physical medicine is increasing, either as single therapy or in combination with other physical devices or drugs.
It has been shown by several studies that ELF-PEMF are able to treat musculoskeletal disorders and muscle hypotrophy with faster and better results than traditional methods [6].
At cellular level, it has been demonstrated that ELF-PEMF affect plasma membrane permeability by modifying ion-channel structure and kinetics [7] and altering the concentration of intracellular ions, mainly calcium [1]. Moreover, it has been demonstrated that ELF-PEMFs affect cellular functions, such as cell proliferation and differentiation [8]. ELF-PEMFs modulate critical cellular pathways and levels of transcription of genes related to apoptosis, cell cycle, control-related proteins [9], cytochrome P450 and inducible nitric oxide synthase enzyme activity [10].
The results of preliminary studies performed in our laboratory demonstrated that ELF-PEMFs are able to trigger differentiation in SHSY5Y cells, a neuroblast model [11]. Moreover, we found that ELF-PEMFs can induce cytoskeleton rearrangement in myoblastic cells [12].The aim of this study was double: to confirm previous findings on the effects of ELF-PEMFs on myoblasts and analyze how ELF-PEMFs may affect the transcription of factors which regulate myogenesis and their intracellular distribution.

MATERIAL AND METHODS

Cell Culture

Murine myoblasts (C2C12 skeletal muscle cell line, American Type Culture Collection, Manassas, VA, USA) were routinely cultured in growing medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine and 10% fetal bovine serum (FBS). Cells were incubated at 37°C and 5% CO_2. All the reagents for cell cultures have been purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell viability

Cell viability after exposure to ELF-PEMFs was determined by a Trypan Blue assay. The dye is capable of selectively penetrate into dead cells. After treatment, cells were washed and detached with trypsin/EDTA for a few minutes, then centrifuged and resuspended in a solution of PBS and Trypan Blue (dilution factor: 2), finally counted, after 5 min of incubation, using a Neubeuer emocytometer.

MTT assay

Cell proliferation after exposure to ELF-PEMs was determined by MTT assay, following the method of Spinner [13]. Cells were cultured in 96-well plates and treated with PEMF-ELF (2 mT-50 Hz) for 15 min or 3 h. MTT assays were performed immediately, 24h, 72h and 6 days after the exposure. After the designated time period, 10 µl MTT solution was added to each well. After 4 h incubation, the supernatant was discarded and DMSO (100 µl) was added. Once the blue crystals were dissolved, the optical density (OD) was measured at the test wavelength of 595 nm and the reference wavelength of 630 nm using a plate microreader (Victor III, Perkin Elmer, VA, USA). O.D. (Optical Density) was calculated as the difference between the absorbance at the reference wavelength and the test wavelength. The percent viability was calculated as (O.D. of PEMFs-ELFs-treated sample/ control O.D.) X100.(n=3, mean ± SD).

Immunofluorescence microscopy

Control and treated samples were fixed for 5 min in cold acetone, then washed in phosphatase buffered saline (PBS). After blocking unspecific binding with PBS containing 3% bovine serum albumin, cells were incubated overnight with the specific antibodies: anti-� actin, anti-tubulin and anti-MyoD. The cells were then incubated with the fluorescein isothiocyanate (FITC) conjugated specific secondary antibody (anti-mouse IgG). Cells incubated with the anti-� actin antibody did not need incubation with the secondary antibody since a mouse anti actin Alexa Fluor® 488 conjugated was used. All antibodies were purchased from Chemicon Int, (Temecula, CA). Negative controls were obtained by omitting the primary antibodies. Samples were evaluated by an epifluorescence microscope (Nikon, Florence, Italy) at 100X magnification and imaged by a HiRes IV digital CCD camera (DTA, Pisa, Italy).

Exposure to ELF-PEMFs

Cells were exposed to an ELF-PEMF produced by a pair of coils in the configuration of Helmholtz coils. In the central region of the system, corresponding to the area where the multiwell plate is located, the intensity was constant within about 3% of its maximum value. In order to perform the experiments described in this paper, the power parameters of the coils were set in order to have a field with 2 mT intensity of and 50Hz frequency. The experiment was carried out in a heated box, specially designed to contain the coils, to ensure temperature stability (37°C) in the

volume around the samples. The control samples were placed on the bench outside the coils, in an area where the field intensity produced by the coils was negligible. In order to study the dependence of the ELF-PEMF effects on the exposure time, cells were treated for 15min or 3h and the biological response was assessed immediately, 24h, 72h and 6d after the treatments. The control samples were maintained and processed in the same conditions except for the exposure to the ELF-PEMF.

Quantitative real-time RT-PCR: expression of differentiation markers

Expression of specific markers of muscle cell maturation (MyoD, myogenin and MHC) was evaluated by quantitative real-time qRT-PCR. Total RNA was isolated from cell cultures using High Pure RNA Isolation kit (Roche) according to the manufacturer's protocol.

Extracted RNA was diluted 1:10 in MilliQ water and RNA concentration was measured at 260 nm with a spectrophotometer (Lambda 45, Perkin Elmer). RNA retrotranscription into cDNA was performed with 200 ng of RNA in a total volume of 20 μl, including 4 μl of iScriptTM Reverse Transcription Supermix (5X, Bio-Rad), containing appropriate quantities of RNaseH+, dNTPs, oligo (dT), random primers, buffer, MgCl2, and reverse transcriptase.

The synthesis program included an initial incubation at 25°C for 5 min, followed by incubation at 42°C for 45 min and 48°C for 15 min. The reaction was inactivated by heating at 85°C for 5 min, and the reaction volume was finally increased up to 200 μl with MilliQ water.

Quantitative RT-PCR was performed with a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) to determine the expression of the genes encoding for the differentiation markers considered. Results were normalized to the expression levels of a selected housekeeping gene, that is β-actin. Obtained cDNA (5μl) was mixed with 1μl of specific forward and reverse primers (8μM), 4μl of MilliQ and 10μl of SsoAdvancedTMSYBR®GreenSupermix (Bio-rad), containing appropriate quantities of hot-start Sso7f-fusion polymerase, SYBR®Green dye, dNTPs, MgCl2, and enhancers.

The thermal protocol was applied with one cycle of 30 s at 98°C for enzyme activation, followed by 40 cycles at 98°C for 3 s and 60°C for 7 s. After the last reaction cycle, the protocol provided a temperature ramp from 65°C to 95°C, at 0.5°C/s increments, to exclude unspecific products with melting curve results. All tests were carried out in triplicate. The cycle threshold (Ct) value relative of control sample was adopted as reference for the calculation of ΔΔCt (difference between ΔCt values deriving from difference between Ct of target and housekeeping genes) for the subsequent samples. The primer sequences (forward and reverse) of the investigated genes are reported below. For more details see [14].

MyoD
F: 5'-GCTCTGATGGCATGATGG-3'
MyoD
R: 5'-CACTCTTCCCTGGTCTGG-3'

Myogenin
F: 5'-TGAATGCAACTCCCACAG-3'
Myogenin
R: 5'-GCGAGCAAATGATCTCCT-3'

MHC
F: 5'- CTGGCTTCTGCTGATATTGA-3'
MHC
R:5'- CTTCTTGTTAGACATGATCTGGTA-3'

β-actin
F: 5'- CCACACCCGCCACCAGTTC- 3'

β-actin
R: 5'-GACCCATACCCACCATCACACC-3'

STATISTICS

All the experiments were carried out in triplicate. For immunofluorescence analysis, at least 30 cells per slide were scored in 10 random fields/slide, and the data were expressed as mean ± standard deviation. Statistical significance was determined using a Student's t test. A p value lower than 0.05 was considered statistically significant. For Quantitative real-time RT-PCR, analysis of the data was performed by analysis of variance (ANOVA) followed by Tukey's post-test to test for significance, which was set at 5%. Results are presented as mean value ± standard deviation.

RESULTS

Viability and proliferation

In order to verify the effect of the exposure to ELF-PEMFs on cell viability and proliferation, Trypan Blue and MTT assays were carried out immediately, 24 h, 72 h and 6 days after treatments of 15 min or 3 h.

In all the samples, both treated and controls, and for all the different times of analysis, cell viability resulted higher than 95%. Therefore we did not observe significant effects of ELF-PEMFs on cell viability and, obviously, no difference between the two treatment times.

As shown in Fig.1, immediately after 15 min exposure, a moderate but significant decrease in cell proliferation was found. At the subsequent evaluation times (24h, 72h and 6 days), no significant differences were observed between treated samples and controls. As regard the longer treatment (3h), data obtained at all the times considered did not show any significant difference between treated and control samples and controls (Fig.2)

Cytoskeleton

Cytosketelon is an important cell structure since it allows both movement and shape

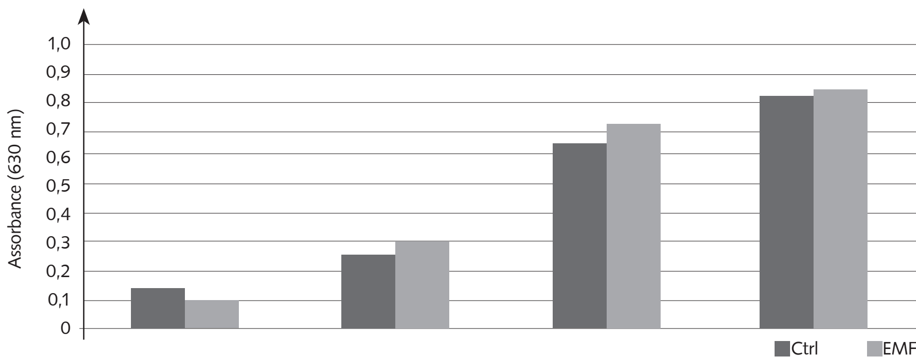

Figure 1: Cell proliferation assessed at 0h, 24h, 72h and 6days after 15 min of exposure to EMFs. Data were obtained by MTT assay.

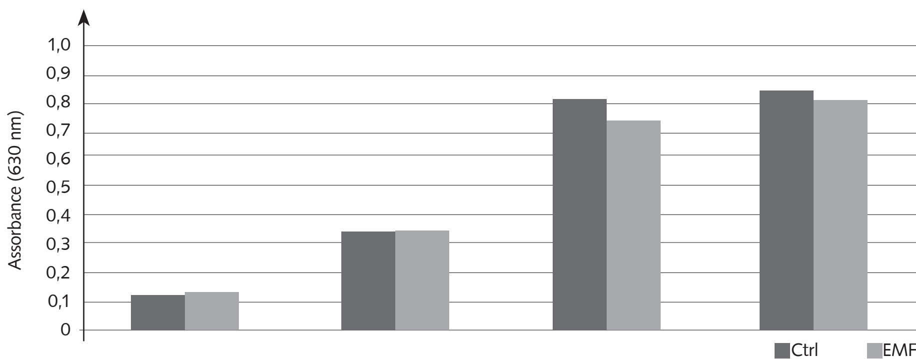

Figure 2: Cell proliferation assessed at 0h, 24h, 72h and 6days after 3h of exposure to EMFs. Data were obtained by MTT assay.

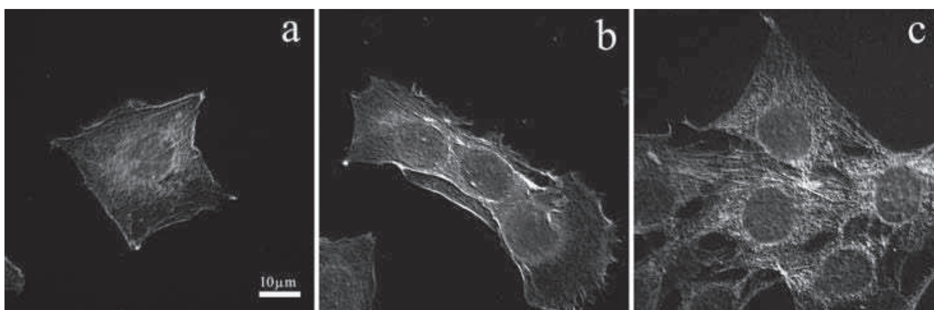

Figure 3: Actin expression assessed by immunofluorescence microscopy. Control (a) at 0h (b) and 72h (c) after 15 min of exposure to EMFs.

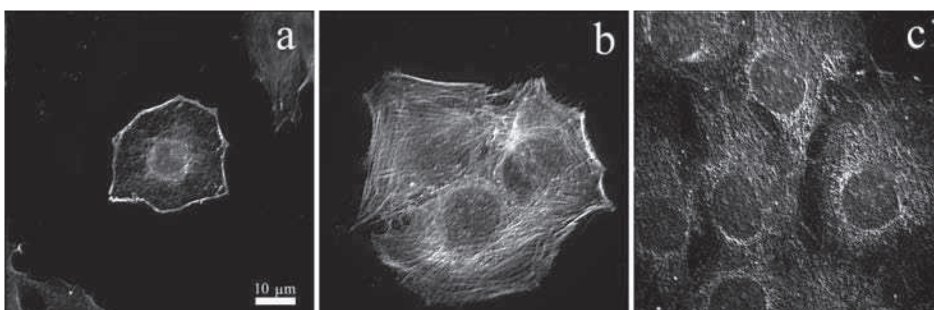

Figure 4: Actin expression assessed by immunofluorescence microscopy. Control (a), at 0h (b) and 6days (c) after 3h of exposure to EMFs..

modifications and has an important role in intracellular transport and signalling.
The morphological analysis of major cytoskeleton components, actin microfilaments and microtubules, which was performed by immunofluorescence microscopy, showed evident architectural alterations of both the cytoskeleton components considered in the samples exposed to ELF-PEMFs.
As expected, in control samples the actin microfilaments were distributed mostly in the perinuclear area and under the plasmamembrane, where they formed a thick layer (actin ring) (Fig.3a)
In cells exposed to ELF-PEMFs for 15 min, analyzed immediately after the treatment, the actin expression increased, in particular in the perinuclear area, and cells showed a higher number of filopodia (Fig.3b). In samples analyzed 72h after the treatment (15 min), cells showed some stress fibers arranged in bundles that allowed connections with neighboring elements (Fig.3c).
For longer exposure (3 h), an increase in actin stress fibers was observed immediately, 24 and 72 h after the treatment; cells merged to form syncytia-like structures (Fig.4b). In cells analyzed 6days after a 3 h exposure to ELF-PEMFs, actin resulted homogeneously distributed, the stress fibers and actin ring disappeared (Fig.4c).
As regards the microtubule network, in control samples the cells showed the expected organization pattern: microtubules radially distributed from the microtubule organizing centre, near the nucleus, towards the periphery of the cell (Fig.5a). In contrast, in treated cells analyzed immediately and 24h after the exposure, the microtubules were not radially distributed but formed a dense network (Fig.5b). 72h after 15min exposure to ELF-PEMFs, tubulin expression further increased (Fig.5c). In samples that had undergone the longer treatment (3 h) the tubulin expression strongly increased

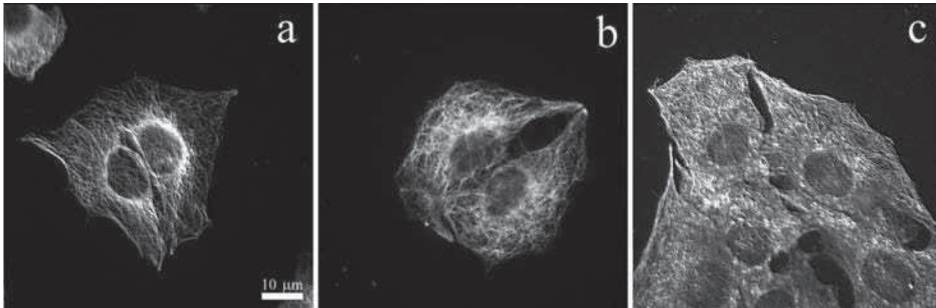

Figure 5: Tubulin expression assessed by immunofluorescence microscopy. Control (a) at 24h (b) and 72h (c) after 15 min of exposure to EMFs.

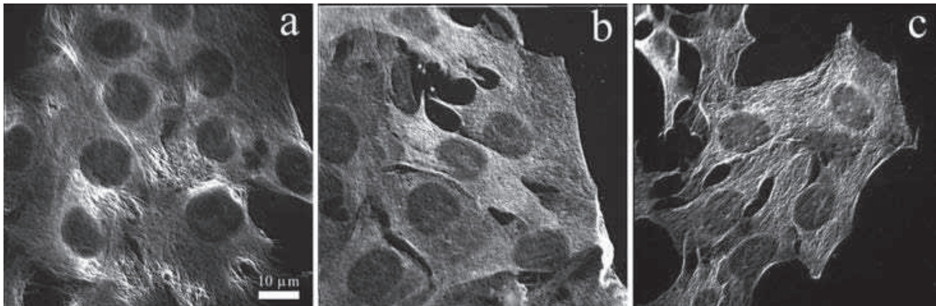

Figure 6: Tubulin expression assessed by immunofluorescence microscopy. Control (a) at 72h (b) and 6days (c) after 3h of exposure to EMFs.

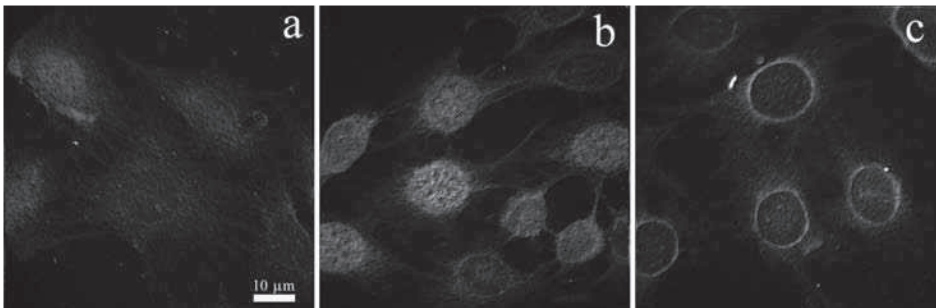

Figure 7: MyoD expression assessed by immunofluorescence microscopy. Control (a) at 24h (b) and 72h (c) after 15 min of exposure to EMFs.

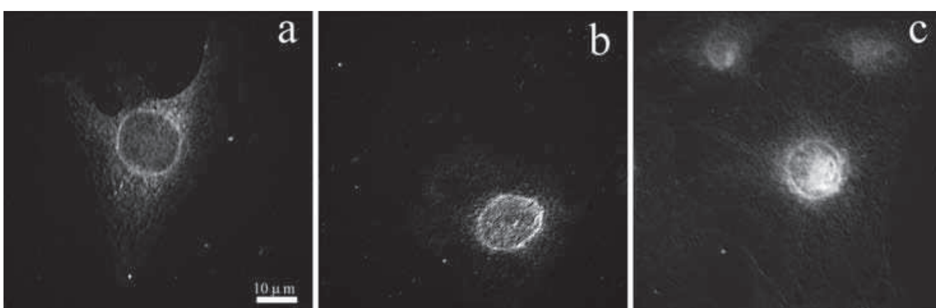

Figure 8: MyoD expression assessed by immunofluorescence microscopy. Control (a) at 24h (b) and 72h (c) after 3h of exposure to EMFs.

after 24 h and the protein remained highly expressed also after 72 h and 6 days, while the microtubule organizing centre disappeared.(Fig.6).

Expression and subcellular localization of MyoD

MyoD is a member of the Muscle Regulatory Factors family. It is a transcription factor and plays a pivotal role in the complex mechanism of skeletal muscle cell differentiation. The effects of ELF-PEMFs on its expression and intracellular distribution were analyzed by immunofluorescence microscopy.

In control samples (Fig.7a) MyoD showed a very low expression and cytoplasmic localization. While, 24 h after a 15 min treatment, MyoD expression increased in myoblasts and showed a nuclear localization (Fig.7b). The effect was reversed 72 h after the treatment (Fig.7c): MyoD did not show nuclear localization but again appeared distributed in the cytoplasm surrounding the nucleus.

In cells exposed to the ELF-PEMFs longer treatment (3 h), MyoD increased and presented a nuclear localization, the expression was particularly significant after 24 h and 3 days after treatment. After 6 days the expression decreased (Fig.8)..

Gene expression analysis by Real Time PCR

Myogenesis consists of numerous ordered steps that require a wide variety of transcription factors which control proliferation and differentiation. The expression of genes involved in myogenesis was assessed by RT-PCR analysis. MyoD is an early-differentiation marker involved in the commitment of precursor cells to a myogenic fate, whereas myogenin and MHC (Myosin Heavy Chain) expression is associated with terminal differentiation.

In samples analyzed 24h after treatments (Fig.9), there were no differences between controls and treated samples, except for

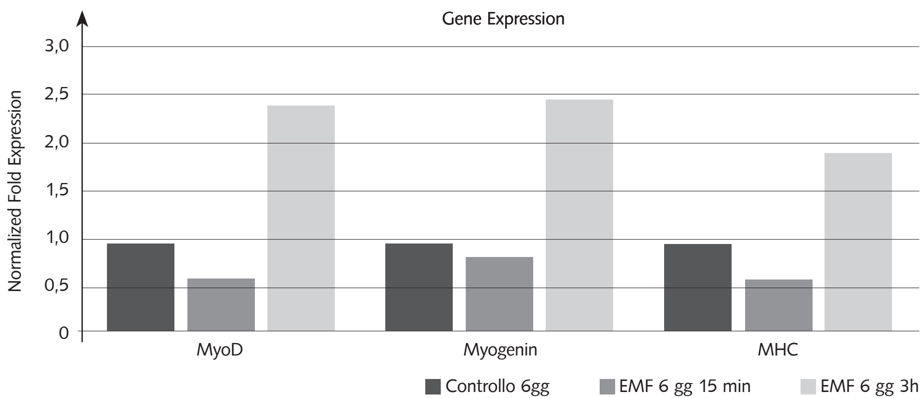

Figure 9: MyoD, Myogenin, and MHC expression 24h after 15 min of exposure to EMFs

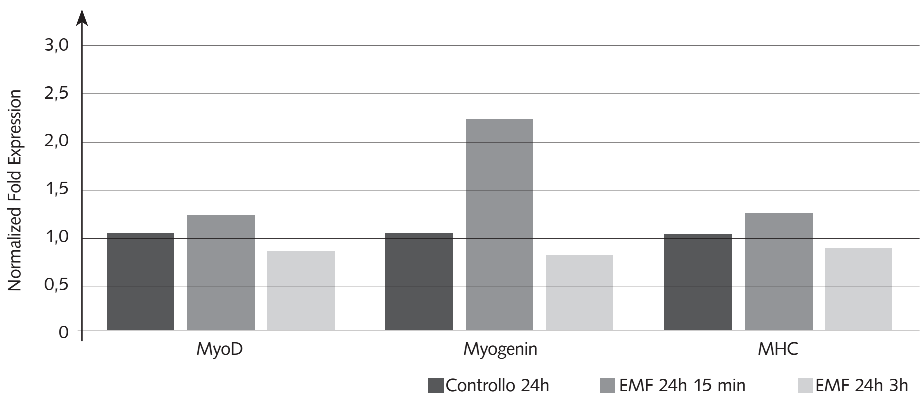

Figure 10: MyoD, Myogenin, and MHC expression 24h after 3h of exposure to EMFs

myogenin mRNA transcript. We observed a marked increase in myogenin mRNA transcript (more than 2 fold) in C2C12 cells after 15min of exposure to ELF-PEMFs, compared to the control.

In Fig.10 is reported the expression of mRNA transcripts 6d after the ELF-PEMFs treatments. In cells exposed for 3 h MyoD, myogenin and MHC expression increased at least 2 fold, compared to the control. Otherwise, in cells treated for 15 min, MyoD and MHC transcripts decreased 0.5 fold.

DISCUSSION

The goal of this study was to analyze the behavior of myoblasts exposed to ELF-PEMFs in order to open possible future perspectives of clinical application for treating muscle disorders after accurate evaluation of suitable parameters for effective stimulation. Our experimental model was the C2C12 cell line, a mouse myoblast model widely used for studies on myogenesis and repair mechanisms in muscle tissue.

The findings of this research show that the exposure to ELF-PEMFs did not affect cell viability but, depending on exposure time and elapsed time between exposure and implementation of the proliferation assay, was able to mildly inhibit cell proliferation. However, the magnitude of the effect did not result statistically significant, except for the proliferation assay performed immediately after the short treatment (15 min), where the decrease in proliferation was definitely significant.

The literature is rich of reports describing the effect of PEMFs on proliferation. The results are controversial: some authors describe an increase [15] and some others a decrease [16] in cell proliferation induced by PEMFs, thus suggesting that the effect strongly depends on the exposure parameters (frequency, intensity and exposure time) used. These are often different, therefore the various studies are difficult to compare.

The analysis of major cytoskeleton components, actin microfilaments and microtubules, by immunofluorescence microscopy demonstrated that the exposure to ELF-PEMFs strongly affected cell morphology and cytoskeleton organization.

Actin has a crucial role in cytoskeleton organization, cell motility and contraction and is also considered a marker of myogenic differentiation. After exposure to ELF-PEMFs myoblasts generally showed an increase in actin expression, disappearance of the actin ring under the plasmamembrane, increase in filopodia, appearance of stress fibers. Sometimes the cells were aligned and tended to merge to form tube-like or syncytia-like structures.

These data confirm results we obtained on previous studies both on neuroblasts [11] and agree with reports by other authors [17].

It is known that ELF-PEMFs, and in general physical stimuli, cause the redistribution of focal adhesion on the plasmamembrane to create new contacts between cell and extracellular matrix and stress fibers have a key role in the reorganization of focal adhesions [18].

Also the microtubule network changed after exposure to PEMFs-ELFs. In treated samples tubulin expression generally

increased, the classical radial distribution of the microtubules disappeared and sometimes also the microtubule organizing centre became indistinguishable, microtubules formed a dense network and sometimes they appeared even fragmented.
The outcomes of our observations indicate that ELF-PEMFs may strongly alter cytoskeleton organization and, consequently, cell morphology, interaction with the extracellular microenvironment and motility. It is known that microtubules, throughout their assembly and disassembly, and stress fibers represent the mold [19] and the scaffold Sanger et al. [20], respectively, to build new myofibrils.

These effects on cytoskeleton are even more significant since they are associated to changes in MyoD expression and distribution, as shown by immunofluorescence analysis. Some aspects of cytoskeletal rearrangement and morphological changes that occur during differentiation are mediated by transcriptional and translational induction of regulators of the process. MyoD, in myoblasts induces not only the expression of muscle-specific genes, in particular myogenin, but also elongation and fusion into multinucleated myotubes [21].
Taken together, the data on cytoskeleton components and MyoD expression suggest that ELF-PEMFs can trigger myoblasts differentiation in a time dependent manner.

The results of the RT-PCR assay further support this hypothesis because in myoblasts exposed to the shorter treatment (15 min) we observed, after 24 h, a significant upregulation of myogenin and weak upregulation of MyoD and MHC, while in cells exposed to the longer treatment (3 h), after 6 days, an upregolation of MyoD, myogenin and MHC was found. As mentioned above, these myogenic regulatory factors transactivate skeletal muscle specific differentiation genes that contain an E-box motif, a DNA binding site with general consensus sequence CANNTG. For example MyoD and myogenin can bind to E-boxes in the regulatory region of mouse desmin gene and transactivarte desmin gene in vitro [22].

In summary rearrangement of microfilaments and microtubules, over-expression and nuclear localization of MyoD, formation of stress-fibers and syncytia-like structures, increase in expression of genes involved in myogenesis regulation are consistent with the hypothesis that exposure to ELF-PEMFs can induce myogenic differentiation.

In conclusion the ELF-PEMFs are physical stimuli recognized and elaborated by cells. The outcomes of this study clearly show that ELF-PEMFs treatment induces morphological and functional changes that ELF-PEMF treatment induces morphological and functional changes that could underlie the early stages of a differentiation process toward myogenesis. The exposure time seems to play a fundamental role in establishing the effect: the genes encoding for factors which regulate myogenesis are differently expressed when comparing the two exposure times considered. Further studies are needed to better understand the relationship between duration of treatment and modulation of the involved pathways.
However, these findings open perspectives of future application of ELF-PEMFs in order to favour myogenesis and muscle tissue repair.

CONCLUSIONS

In conclusion, this study revealed that a short-period application of HILT biostimulating protocol is more effective in pain reduction and in functional ability improvement than no treatment in patients with symptomatic knee OA. Thus, HILT can be an important instrument in pain control contributing to the long-term management of chronic painful knee. The study confirms the safety of the technique.

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