Effects of low frequency electromagnetic fields on SHSY5Y cells - a neuroblast model.

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ABSTRACT

Electromagnetic fields (EMFs) are receiving increasing attention in basic research due to their ability to influence the behavior of cells and to evoke biological responses that may be important from a clinical point of view. In our study we analyzed the effects of exposure to low frequency EFM (2mT, 50Hz, 3 h) on a neuroblast model (SH-SY5Y cells).

These cells are a well-characterized model for studying in vitro differentiation towards a neuronal-like phenotype inducible by various exogenous agents. Viability and proliferation was assessed immediately after the treatment as well as after 24 h. No significant changes were observed in viability (>96%), while proliferation decreased (23%) after 24 h. Changes in cytoskeleton organization (analyzed by immufluorescence technique), in particular in actin microfilament, were also observed. These changes were accompanied by morphological modifications and the formation of cones and cytoplasmic extensions, or neurites and dendrites processes.

We finally monitored an increase in expression of markers typically expressed in neuronal differentiation: neurofilaments and NRF-1. In conclusion, our findings demonstrate that EMF exposure induces in SHSY5Y cells a biological response consisting in the remodelling and reorganization of the cytoskeleton and in the beginning of neuronal cell differentiation. A deeper knowledge of the mechanisms underlying the effects described above and a greater understanding of relationship between biological response and parameters variation could lead to concrete improvements in treatment protocols.

INTRODUCTION

Since many years, electromagnetic fields are widely used in medicine. Today, magnetic therapy is considered one of the most important physical therapies. It is experienced in many application and accepted by patients as a non invasive and easily to manage treatment. The main application in clinics are mostly addressed to the treatment of rheumatologic disease [1] and disorders characterized by bone loss, such as osteoporosis, and also to accelerate the healing of fractures [2,3]. In these fields, low frequency pulsed EMFs have shown to be the most effective

The molecular and cellular mechanisms underlying the systemic effects of EMsf are not completely understood, but recently many advancements have been made: it has been demonstrated that EMFs affect the permeability of the plasma membrane [4] by ion-channel or receptor redistribution, changes in activation kinetics of ion channels, reorientation of membrane phospholipids. Moreover, EMFs may modulate binding kinetics and ion bound or release from proteins [5].

In living tissues endogenous EMFs are generated by physiological activities such as the movements of the musculoskeletal system structures. Vibrations and contractions of human muscles induce in the underlying bone tissue low frequency EMFs which have an important role in maintaining bone mass. Bone cells are selectively sensitive to low frequencies: in the range 15-30 Hz, fields as low as 0.01mV/cm may affect the remodeling activity [6].

Different authors have shown that pulsed EMFs affect proliferation, differentiation and activity of osteoblasts, with stimulating or inhibitory effects depending on field type and exposure [7-9] expression of bone morphogenetic proteins [10,11], production of extracellular matrix [12].

Clinically, magnetic therapy has been also used to treat skin diseases [13]; chronic wounds in cancer patients undergoing radiation therapy [14], inflammation and edema [15]; the osteoarthritis of the knee [16,17]. Moreover, it has analgesic effect in fibromyalgia [18] and localized musculoskeletal pain [19].

The new medical frontiers are increasingly oriented toward complex and customized therapeutic protocols , in which physical medicine and biotechnology have a relevant role, whether used alone or combined with "traditional drug therapies".

Recently, the effectiveness of EMFs in the treatment of incontinence and rehabilitation of bladder and sexual function in radical prostatectomy [20] has been proved and new applications of EMFs to treat diseases that involve muscle and nervous tissue have been proposed.

The results of preliminary studies carried out in our laboratory suggested that pulsed low frequency EMFs, without altering cell viability, can influence the proliferation, differentiation and morphological characteristics of myoblasts and neuroblasts [21].
The present study was designed with the aim to confirm our preliminary findings on the effects of pulsed, low frequency EMFs on neuroblasts and to analyze how EMFs can interfere with the ability of neuroblasts to differentiate.

MATERIALS AND METHODS

Cell Culture

The human neuroblastoma cell line SH-SY5Y (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI medium supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and maintained at 37 °C in a humidified atmosphere (5% CO2/95% air). All the reagents for cell cultures were from Sigma Chemical Co. (St. Louis, MO, USA). Tissue plastic-ware was from Bibby Sterilin (Staffordshire, UK). For the exposure to EMFs, cells were seeded in 24-well plates.

Cell viability and count

Cell viability after exposure to EMFs 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 cells were centrifuged and resuspended in a solution of phosphate buffered saline (PBS) and Trypan Blue (dilution factor: 2) and counted, after 5 min of incubation, using Neubauer hemocytometer.

Immunofluorescence microscopy

At the end of the experiments, cells were fixed for 5 min in cold acetone, then washed in PBS. After blocking unspecific binding with PBS containing 3% bovine serum albumin, cells were incubated overnight at 4°C with the specific antibodies: anti-actin, anti-tubulin, anti-vimentin, anti-$\alpha 5\beta 1$ integrin, anti-Nrf-1 and anti-neurofilament antibodies. Cells were then incubated with the FITC (fluorescein isothiocyanate) conjugated specific secondary antibodies (specifically: anti-mouse IgG for anti-tubulin, anti-$\alpha 5\beta 1$, anti-neurofilament; anti-rabbit IgG for anti-NRF-1; anti-mouse IgM for anti-vimentin). Cells incubated with anti-α 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). Image analysis was performed by extracting, for each cell image, the region of interest (ROI) by appropriate software (Image Pro Plus, Mediacybernetics, Inc., Silver Springs,Maryland). Then, the mean pixel value (16 bit, gray level) related to the mean fluorescence intensity and therefore to the specific epitope detection was calculated.

Statistics

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 ± SD. Statistical significance was determined using a Student's t test. A p value lower than 0.05 was considered statistically significant.

Elecromagnetic fields exposure

Cells were exposed to an EMFs produced by a pair of coils in the configuration of Helmholtz coils. The intensity in the central region of the system 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 such as to expose the cells to intensity of 2 mT and frequency of 50Hz. The experiment was carried out with a heating / hot air circulation to ensure that the temperature around the multiwell containing cells was stable at about 37 °C. The control cells are placed on the bench outside the coils, in an area where the field intensity produced by the coils is negligible.

Quantitative real-time RT-PCR for neuronal marker transcripts

Total RNA to be subjected to RT was extracted from SHSY5Y cells, exposed and not exposed to EMF. Non-exposed cells were taken as control. Total RNA isolation and cDNA synthesis were obtained as reported previously [22]. Primers and probes were TaqMan gene expression Assays ID Tau: Hs00902193_m1; SYP: Hs00300531_m1; MAP2: Hs00159041_m1 (Applied Biosystems, Foster City, CA, USA): The PCR mixture (25µL final volume) consisted of a 1 X final concentration of Assay-On-Demand mix, 1 X final concentration of Universal PCR master mix (Applied Biosystems) and 20 ng cDNA. Each measurement was carried out in triplicate. The mRNA quantification was based on a comparative Ct method according to the manufacturer' s instructions (Applied Biosystems) and data were normalized to ribosomal 18S RNA expression (assay ID Hs99999901_s1; Applied Biosystems). Results were expressed as the fold increase in mRNA untreated cells.

RESULTS

Viability and proliferation

In order to verify the effect of the EMF exposure on cell viability and proliferation, Trypan blue assays were carried out at 0 h and 24 h after 3h exposure to the EMF.
As shown in Fig. 1, in both cases, no significant differences were observed between treated samples and controls as regards cell viability, which resulted higher than 96% in all the samples.
Immediatly after EMF exposure, cell proliferation did not change significantly, but it showed a decrease of the ~ 22% after 24h (Fig. 2).

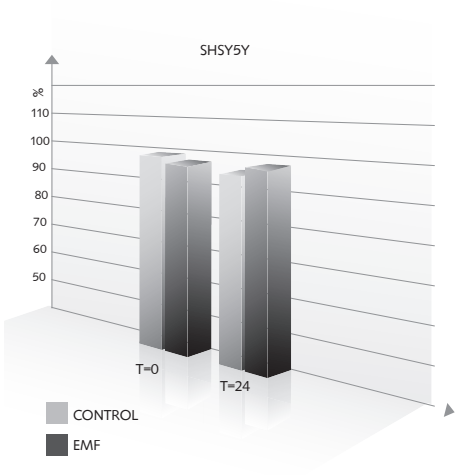

Fig. 1 Cell viability assessed at 0h and 24h after exposure to EMFs. Data were obtained by Trypan Blue assay.

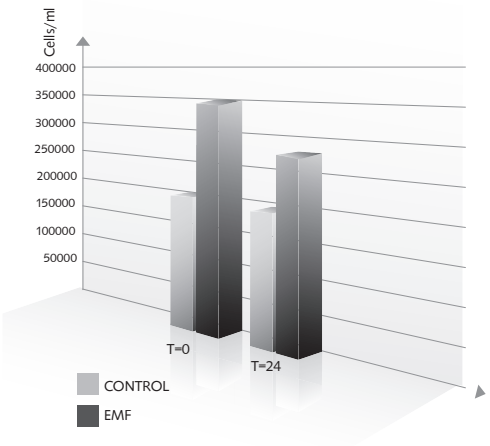

Fig. 2 Cell proliferation assessed at 0h and 24h after exposure to EMFs. Data were obtained by Trypan Blue assay.

Cytoskeleton

Cytoskeleton is an important structure for the cell since it allows both movement and shape modifications and has an important role in intracellular transport and signalling. Cytoskeleton is mainly composed of three elements: actin microfilaments, microtubules and intermediate filaments made of tubulin and vimentin, respectively. The distribution of actin, tubulin and vimentin in SHSY5Y exposed to EMF was studied by immunofluorescence microscopy and image processing.

The results of the analysis showed a reorganization of all three components of the cytoskeleton following exposure to EMF. In controls, actin microfilaments were distributed as expected: more thickened just below the plasma membrane and in the perinuclear area; stress fibers were arranged in parallel (Fig.3A). In cells exposed to EMF the concentration of actin increased, in particular in the peripheral region.

Stress fibers were organized to form cones, structures that precede the formation of neuritis and dendrites (Fig.3B).

In samples analyzed 24h after EMF exposure, cells showed branched extensions that allowed connections with neighboring cells, producing a network (Fig. 3C).

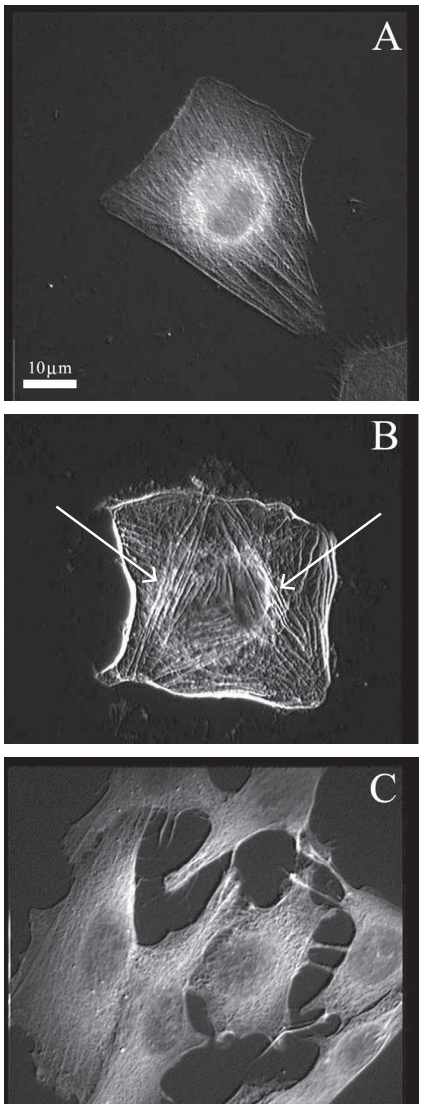

Fig. 3 Actin expression assessed by immunofluorescence microscopy. Control (A) 6h after exposure to EMFs (B) and 24 hours after exposure to EMF (C).

Specific staining for tubulin revealed that the exposure to EMF did not change significantly the expression of the protein, rather the distribution of the microtubules, which are formed by self-assembling of tubulin monomers. In controls, as expected, microtubules branched radially from the "organizing center" near the nucleus, while in treated samples the microtubules were organized into bundles that would go in the dendrites/neurites that were forming (Fig.4A, Fig.4B).

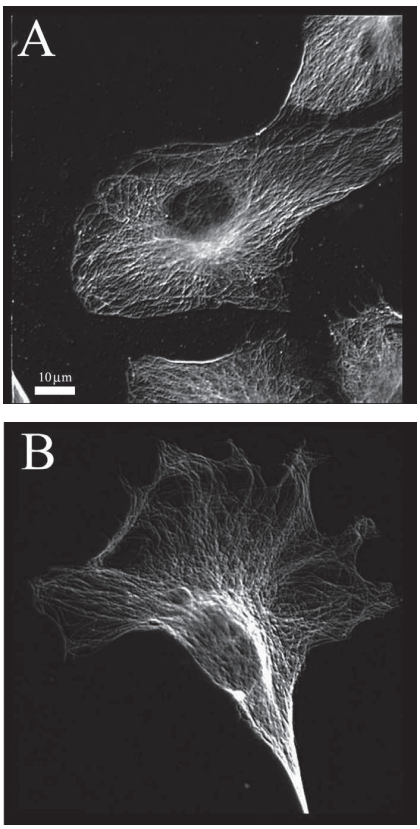

Fig. 4 Tubulin expression assessed by immunofluorescence microscopy. Control (A) and 24h after exposure to EMFs (B).

Vimentin is the major constituent of the intermediate filaments. In controls, they formed bundles mainly distributed in the perinuclear area (Fig. 5A). In treated samples the expression of the protein increased and we observed the formation of a dense ring around the nucleus, as shown in Fig. 5B.

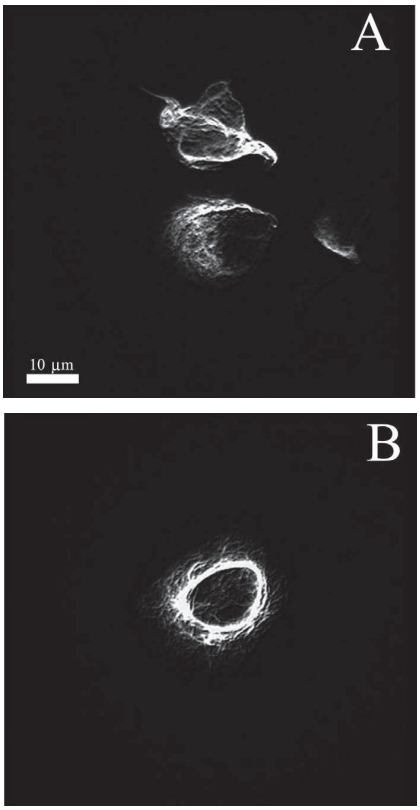

Fig. 5 Vimentin expression assessed by immunofluorescence microscopy. Control (A) and 24h after exposure to EMFs (B).

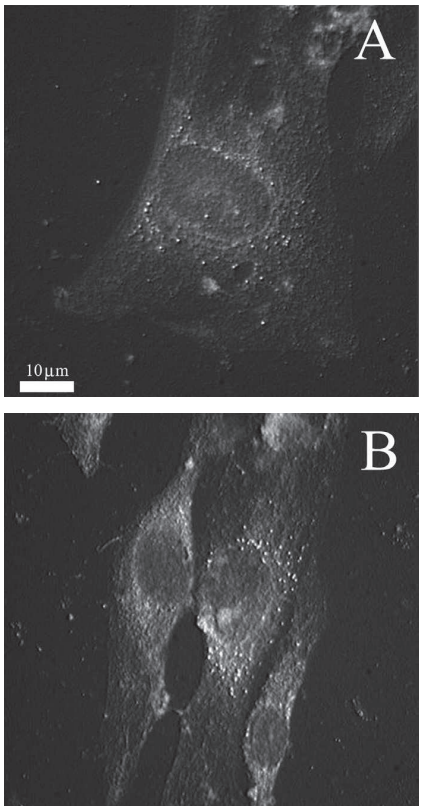

Fig. 6 Integrin expression assessed by immunofluorescence microscopy. Control (A) and 24h after exposure to EMFs (B).

Integrins are transmembrane proteins composed of two subunits α and β (there are 20 different integrins with different combinations of α and β). They bind to short sequences of aminoacids found in many extracellular matrix components, including collagen, fibronectin and laminin and also serve to anchor some components of the cytoskeleton. In all the samples the $\alpha 5\beta 1$ integrin was expressed at very low levels, in agreement with literature [20], which reports a down-regulation of this protein during the differentiation of early neuronal precursors. There were no significant differences between control (Fig. 6A) and treated (Fig. 6B).

Gene expression analysis by Real Time PCR

Evaluation of the expression of some genes involved in neurogenesis and development of synapses in nerve cells was assessed by the presence of transcribed mRNA of MAP2, TAU and synaptophysin.

The values shown in the graphs were calculated from the ratio between the amount of mRNA present in treated and control samples. Figure 7 shows MAP2 expression at 0h and 24h after EMF exposure. MAP2 gene encodes for a microtubule-associated protein (MAP2). The proteins of this family are involved in the construction of microtubules, important process in neurogenesis. In samples analyzed immediately after treatment, there was a marked decrease in the MAP2 transcript (approximately 90%) compared to the control. After 24h, the amount of mRNA in treated sample increased, returning to values comparable to the control. In controls the amount of transcript did not change at 24h from the treatment.

In controls the amount of transcript does not vary, both immediately after treatment and after 24 hours. This confirms that the cells did not undergo any stimuli that induce changes in the production of the transcript.

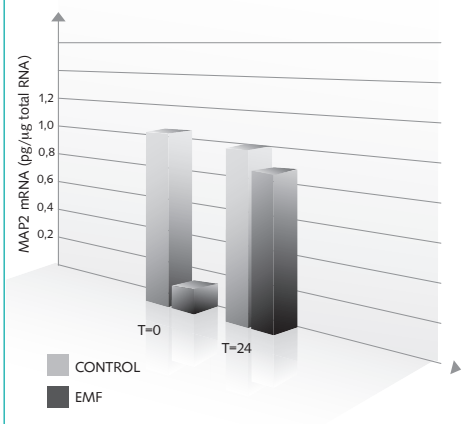

Fig. 7 Amount of MAP2 gene expression in SHSY5Y cells. * p<0.05

Figure 8 shows the amount of TAU transcript immediately after the treatment and measured after 24 h. The product of TAU is a protein (TAU) which promotes the elongation of microtubules by binding to the surface of neurites of protofilaments [23].

In samples analyzed at 0h after EMF exposure there is a 12-fold greater amount of transcript compared with control. After 24 h the amount of TAU transcript return at control values.

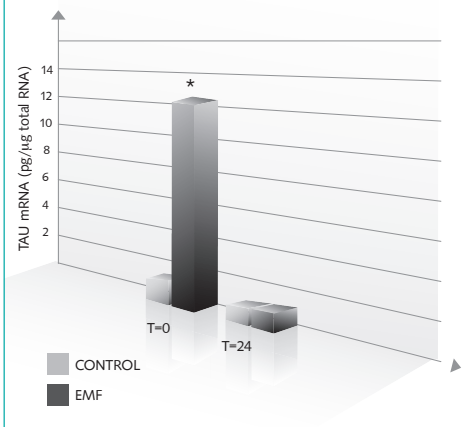

Fig. 8 Amount of TAU gene expression in SHSY5Y cells. * p<0.05

In Fig. 9 is reported the expression of SYP analyzed at 0h and 24h after EMFs exposure. Synaptophysin, the product of the gene SYP, is a membrane glycoprotein present in synaptic vesicles in neurons. The protein participates in the formation of a channel between the synaptic vesicle and presynaptic membrane through which flow the neurotransmitters [24].
Immediately after the treatment, SYP increased of about 6-7 times compared to control samples. After 24h from the treatment, the expression in treated samples returned similar to the control, as happened for TAU. Therefore we observed an increase in transcription of TAU and SYP immediately after EMF exposure, while transcription of MAP2 was inhibited. After 24h from EMF exposure the transcription of the genes returned at basal values.

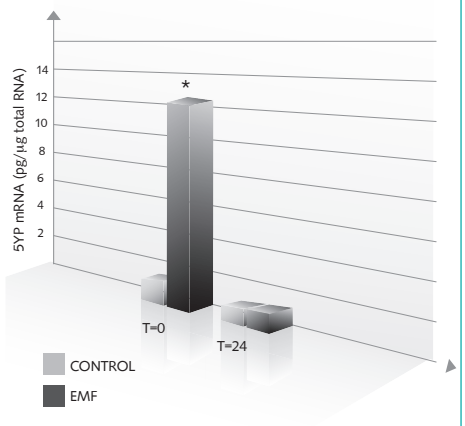

Fig. 9 Amount of SYP gene expression in SHSY5Y cells. * p<0.05

Neuronal marker expression assessed by immunofluorescence analysis

Since in our experiments we observed that the exposure to EMF induced in SHSY5Y cells morphological changes and increase in expression of genes involved in neurite growth, we decided to deeper investigate on possible role of EMF exposure in differentiation process. Therefore we assayed, by immunofluorescence microscopy and image analysis, the expression of two markers of neurogenesis: Nrf-1 and neurofilaments.
NRF-1 is a transcription factor associated with the regulation of neurite outgrowth, thus it is considered a marker of neuronal differentiation.
We found that Nrf-1 increased significantly in neuroblasts exposed to EMF compared with controls and the protein distribution appeared more punctiform (Fig. 10A and 10B).

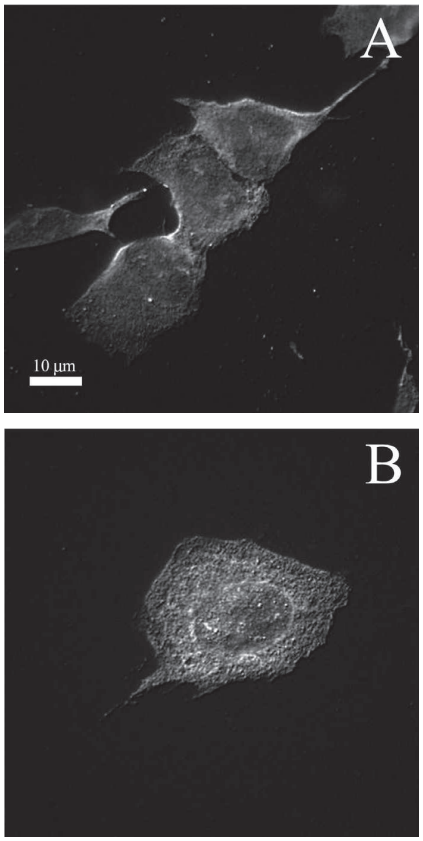

Fig. 10 NRF-1 expression assessed by immunofluorescence microscopy. Control (A) and after exposure to EMFs (B).

Also the expression of neurofilaments, in particular medium neurofilament (NF-M), significantly increased in cells exposed to EMF. Figs. 11B show that the protein accumulated in the perinuclear area. NF-M is a member of the intermediate filament family and an important component of neuronal cytoskeleton. Therefore it is considered a major marker of neuronal differentiation.

DISCUSSION

The long-term goal of our studies is to propose new clinical applications of magnetic therapy for the treatment of disorders involving muscle and nerve tissue. The aim of the experiments reported in this paper was, in particular, to investigate the effect of EMF on nerve cells and neuronal differentiation. Our experimental model was the cell line SH-SY5Y, a human neuronal model widely used for studies on neurogenesis. In fact SH-SY5Y cells can differentiate into neuronal direction in response to appropriate stimulation. The differentiation process is characterized by morphological changes and increase in expression of specific markers of neuronal differentiation. Cells form growth cones, and then long neuritic processes, through cytoskeleton rearrangement.

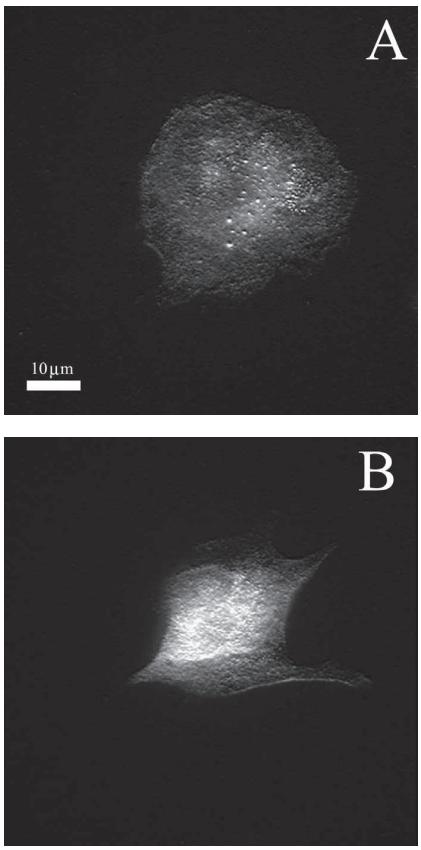

Fig.11 Neurofilament protein (NF-M) expression assessed by immunofluorescence microscopy Control (A) and after exposure to EMFs (B).

The results of our experiments showed that the EMF treatment did not produce changes in cell viability, evaluated immediately after exposure and after 24h. Cell proliferation did not change immediately after the treatment, while we observed a moderate but significant decrease after 24h. A decrease in cell proliferation associated with unchanged viability suggested the hypothesis that the exposure to EMF induced neuroblast differentiation.

Since it was shown that extremely low frequency EMF induces changes in cell morphology [19] and normally morphological changes occur also during differentiation, we studied the effect of EMF on cytoskeleton organization. Data, obtained by immunofluorescence microscopy, revealed that EMF exposure induced a significant rearrangement both in microtubles and in the network of actin microfilaments. In control samples, as expected, actin microfilaments were concentrated under the plasma membrane and in the perinuclear area, while after 6 h and even more after 24h from exposure to EMF we observed a reorganization of actin microfilaments with stress fibers which formed cones and then cytoplasmic extensions. These results agreed with the reports presented by other authors [16]. Also the microtubule network changed after exposure to EMF. In treated samples there was a significant increase in tubulin expression, especially in the cytoplasmic extensions. This findings are even more significant because they are associated with the increase in Tau expression. It is well known that, during early phases of neuronal differentiation, Tau is concentrated in the nascent axon and an intracellular redistribution of tubulin occurs [17].

The hypothesis that the exposure to EMF could promote, in our cell model, a differentiation process was further confirmed by the increase in expression of Nrf1 and NF-M, assessed by immunofluorescence analysis. As explained above, these proteins are considered major markers of neuroblasts differentiation and have a key role in the process of neuronal maturation.

Also the results of real time PCR supported the hypothesis that cells exposed to EMF began a differentiation process toward the neuronal line. In fact, after the treatment, we found downregulation of MAP2, overexpression of synaptophysin and TAU. MAP-2 function is not required when cell disassembles microtubules in the cell body to give rise to the formation of neurites, while TAU is required to add new subunits to microtubules which are forming in the neuritis [23]. Synaptophysin, as mentioned above, is involved in synapse formation and release of neurotrasmitter. [24]

In summary, rearrangement of microtubules and actin microfilaments, with formation of cones and cytoplasmatic extension, joined with increase in expression of neuronal markers and changes in expression of genes involved in cytoskeleton organization and neurite formation strongly agree with the hypothesis that exposure to EMF may induce neurogenic differentiation.

24h after exposure, the expression of synaptophysin, TAU and MAP2 returned to control value but the formation of cellular processes continued to progress, with the appearance of branched cytoplasmic extensions. This means that the modulation of protein expression and the morphological changes are part of a complex biological response that, once triggered by exposure to EMF, proceeds even after the cessation of the stimulus.

In conclusion, the data presented here indicate that the exposure to a 50-Hz and 2 mT EMF may significantly affect neuronal differentiation of SHSY5Y by upregulating the expression of genes involved in neurite formation and inducing cytoskeleton rearrangement.

The progress of the differentiation process is unequivocally proven by the increase in expression of specific markers of neurogenesis, such as NF-M and Nrf1, and the gradual growth of neurites.

These findings open perspectives for future application of EMFs in tissue engineering, tissue regeneration and repair. From the clinical point of view, the reported results indicate that there are bases to study EMFs application for recovering nerve tissue function. Additional studies are needed to further understand the cellular and molecular mechanism underlying the biological responses induced by exposure to EMF, since a better knowledge of these mechanisms can lead to significant improvements in the therapeutic protocols.

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