

Low dose short duration pulsed electromagnetic field effects on cultured human chondrocytes

An experimental study

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ABSTRACT

Background: Pulsed electromagnetic field (PEMF) is used to treat bone and joint disorders for over 30 years. Recent studies demonstrate a significant effect of PEMF on bone and cartilage proliferation, differentiation, synthesis of extracellular matrix (ECM) and production of growth factors. The aim of this study is to assess if PEMF of low frequency, ultralow field strength and short time exposure have beneficial effects on *in-vitro* cultured human chondrocytes.

Materials and Methods: Primary human chondrocytes cultures were established using articular cartilage obtained from knee joint during joint replacement surgery. Post characterization, the cells were exposed to PEMF at frequencies ranging from 0.1 to 10 Hz and field intensities ranging from 0.65 to 1.95 μ T for 60 min/day for 3 consecutive days to analyze the viability, ECM component synthesis, proliferation and morphology related changes post exposure. Association between exposure doses and cellular effects were analyzed with paired 't' test.

Results: *In-vitro* PEMF exposure of 0.1 Hz frequency, 1.95 μ T and duration of 60 min/day for 3 consecutive days produced the most favorable response on chondrocytes viability ($P < 0.001$), ECM component production ($P < 0.001$) and multiplication. Exposure of identical chondrocyte cultures to PEMFs of 0.65 μ T field intensity at 1 Hz frequency resulted in less significant response. Exposure to 1.3 μ T PEMFs at 10 Hz frequency does not show any significant effects in different analytical parameters.

Conclusions: Short duration PEMF exposure may represent a new therapy for patients with Osteoarthritis (OA).

Key words: Human chondrocytes, osteoarthritis, pulsed electromagnetic fields

MeSH terms: Osteoarthritis, cartilage, articular, chondrocytes, electromagnetic fields

INTRODUCTION

Pulsed electromagnetic field (PEMF) has been used to treat bone and joint disorders for over 30 years.¹ Clinical use of PEMF preceded systematic research in its utility for bone and joint healing.² Later studies identified that PEMF is capable of producing significant cellular changes in bone and cartilage cells

by proliferation, differentiation, synthesis of extracellular matrix (ECM) and production of growth factors.³⁻¹⁰ A systematic review based on 3 clinical studies which assessed effect of PEMF therapy for osteoarthritis (OA) of knee, incorporating factors like pain, physical function, patient assessment, joint imaging, health related quality of life and physician global assessment indicates that electrical stimulation therapy may be useful in OA of knee, but stresses the need for confirmation in future studies.¹¹ Proteoglycan (PG) loss occurs in joint cartilage in OA and PEMF therapy has been shown to induce PG synthesis *in-vivo* and *in-vitro*.¹² PEMF has also demonstrated to have positive effect on cellular proliferation and DNA

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synthesis through opening of voltage sensitive calcium channels.¹³ Animal models have shown that PEMF therapy retards progression of OA.^{14,15}

Most studies employing PEMF have used frequencies of 6- 75 Hz and field strengths of 0.4- 2.3 milli Tesla (mT). We desired to enquire if low frequency (0.1- 10 Hz), low field strength of 0.65- 1.95 μ T and short duration exposure (60 min/day) of PEMF results in favorable effects on cultured human chondrocytes (synthesis of ECM; cell viability, proliferation and morphology). Further need for the study is to arrive at a minimal PEMF exposure protocol that is expected to decrease the concern related to unfavorable cellular changes and chromosomal aberrations that may result with high dose PEMF exposure.¹⁶

MATERIALS AND METHODS

Isolation and characterization of chondrocytes

Articular cartilage samples were obtained from knee joint during joint replacement surgery after obtaining informed consent from patients. The study protocol was approved by Institutional Ethics Committee. Cartilage tissue over the nonweight bearing portion of the joint was removed and minced in Dulbecco's modified eagle medium (DMEM) (Biogene technologies, India) supplemented with 10% FBS (Biogene technologies, India) and 1 ml Pen-strep (10000 units of penicillin and 10 mg of streptomycin, Invitrogen, India). Following this, the tissue was transferred into a conical flask and initially digested with pronase (1 mg/ml) (Biogene technologies, India) for 60 min, followed by type II collagenase (1 mg/1 ml) (Invitrogen) for 16- 18 hours at 37°C. The following day, cellular debris and undigested tissue were removed and cells were separated using a 100 micron cell strainer. Isolated cells were seeded into 25 cm² culture flasks (TPP, India) with DMEM complete medium and maintained at 37°C with 5% CO₂ levels. The cells were subcultured on attainment of 80% confluency. The attached cells were characterized by chondrocyte specific anti-Sox 9 transcription factor antibody staining (Abcam, India.). Chondrocytes that failed to form monolayer culture were not processed further. Post characterization, 4 × 10⁵ cells were seeded in each flask and used for PEMF exposure after first passage.

Pulsed electromagnetic field exposure

The PEMF coil system fashioned for exposure is a four member coil frames, two larger (inner) and two smaller (outer) coil frames. The coils are mounted coaxially and in a co-planar fashion to form an enclosure, where it delivers currents in milliamperes at desired waveforms, varying frequencies and magnetic field strength (Madras Institute of Magnetobiology, Chennai, India). This system designed

according to the parametrical equation of Fansleau and Brauenbeck and a modified version of the Helmholtz coil. A box is housed inside the coil in which a 100 W bulb with regulator was used to maintain the temperature at 37°C and water to maintain humidity. Instead of 5% CO₂, 20 mM HEPES was used as a buffering system. The chondrocytes were exposed to PEMF while monitoring field strength, frequency and temperature. The control (unexposed) cells were placed in the same environment and temperature but not exposed to PEMF.

Pulsed electromagnetic field treatment

The chondrocytes were seeded in 25 cm² culture flasks at concentrations of 6.5 × 10⁵ cells/ml after 20 h being plated the cells were washed with phosphate buffer saline (PBS), and given fresh medium and exposed to PEMF for the first three daily trials; media was not changed from this point onwards. PEMF at a frequency of 0.1, 1 and 10 Hz were applied with flux densities of 0.65, 1.3 and 1.95 μ T (peak-to-peak) for 60 min/day for 3 consecutive days. Whereas exposure to PEMFs at a repetition rate of 0.1 and 1 Hz with 1.95 and 0.65 μ T caused a significant increase in chondrocyte viability that was dependent on PEMF amplitude, PEMFs applied at a repetition rate of 10 Hz and 1.3 μ T did not produce any noticeable effects over cell viability and were not dealt with further in this manuscript. To test for effects of different exposure durations, cells were exposed to PEMFs of 1.95 and 0.65 μ T magnitude and at frequency of 0.1 and 1 Hz for 60 min/day for 3 days. Cells were analyzed on third day for further experimental studies.

Cell viability assessment

Chondrocytes were cultured in 96 well plates at a density of 5 × 10³ cells per well and exposed to PEMF in accordance to the exposure protocol mentioned. Twenty microliter of 0.5% 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) in phosphate buffered saline was added to each well after removal of medium and cells were incubated for 3 h at 37°C. Post incubation, 150 μ l dimethyl sulfoxide (Hi-media, India) was added to each well and absorbance values (optical density value) were noted at 570 nm and 695 nm in spectrophotometer.¹⁷

Quantitative measurement of extracellular matrix proteoglycan and glycosaminoglycan synthesis

Chondrocytes were cultured in 48 well plates at densities of 10⁴ cells per well and exposed to PEMF in accordance to the exposure protocol mentioned. Postexposure, glycosaminoglycan (GAG) synthesis was quantified by the dimethyl methylene blue (DMMB) assay. The DMMB reagent (Sigma, India) was prepared as detailed by Panin *et al.*¹⁸ and 200 μ L was added to each well after removal of culture medium. Subsequently, absorbance values at 525 nm were noted.

Analysis of cell cycle by flow cytometry

Chondrocytes were cultured in 25 cm² culture flasks and exposed to PEMFs as mentioned earlier. After exposure, the cells were trypsinized, converted to single cell suspension in PBS and subjected to flow cytometry (FACS calibur, Becton Dickinson, Germany) according to the manufacturer's instruction (Invitrogen, India) as follows: The suspension was spun at 1000 rpm for 10 min and the cell pellet was fixed in 70% ice cold ethanol at 4°C overnight. The cells were washed with PBS, treated with 500 µl RNAse A (40 µg/ml) (Sigma, India.) for 30 min at 37°C and stained with 500 µl propidium iodide (40 µg/ml) for 15 min incubation at room temperature. Postincubation, cell distributions at distinct phases of the cell cycle were analyzed by flow cytometry.

Analysis of cell architecture and morphology

Cell architecture and morphology were analyzed by staining of actin filaments in chondrocytes. Chondrocytes were cultured on cover slips in 6 well culture plates and exposed to PEMFs as described earlier. Processing of cells was done according to the manufacturer's instructions (Invitrogen, India.). Briefly, the cells were fixed in 3.7% formaldehyde solution for 10 min after washing the slide with PBS and permeabilized in 0.1% Triton X-100 for 5 min. After washing with PBS, the cells were stained with 0.05 mg/ml Phalloidin solution at room temperature for 20-30 min, followed by counterstaining with 300 µl Propidium Iodide (500 nM). The coverslips were then rinsed in PBS, placed on a glass slide and cellular architecture and stress fiber formation was qualitatively analyzed by fluorescent confocal microscopy (LSM 510 META, Carl Zeiss, Germany).

Statistical analysis

Discrete variables were expressed as number (%) and continuous variables expressed as mean ± Standard Deviation. Association between field strengths (0.65, 1.3, and 1.95 µT) in variable frequencies (0.1, 1, and 10 Hz) and cellular effects (cell viability and ECM production,) was analyzed with paired 't' test. A $P < 0.05$ was considered as statistically significant. Analysis was done with Statistical Package for the social sciences (SPSS) software version 21.0. This software was released in 2012 and used to solve business and research problems by means of ad-hoc analysis, hypothesis testing and predictive analysis.

RESULTS

Isolation of chondrocytes

Healthy chondrocytes were observed in cultures by 3 days and these monolayers were 80% confluent by a week. The chondrocytes were spherical prior to attachment and later appeared polygonal in shape [Figure 1].

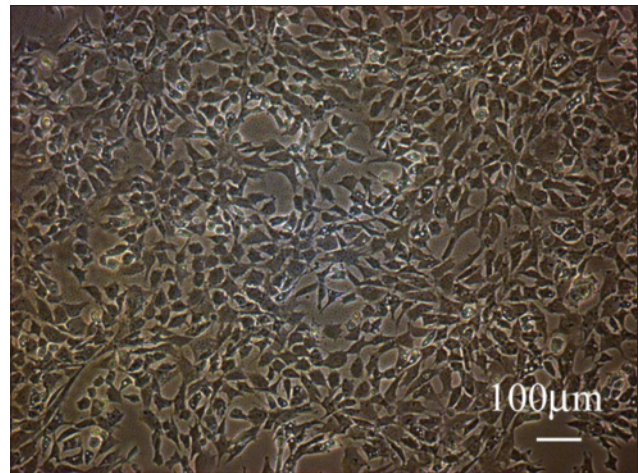


Figure 1: Primary human chondrocytes displaying typical polygonal conformation after attachment

Cell viability assessment

Viability of chondrocytes after PEMF exposure was quantified by the MTT assay to ascertain the effects PEMFs on chondrocytes which were exposed to PEMFs of field intensities between 1.95 and 0.65 µT at frequencies of 0.1 and 1 Hz for 60 min/day for 3 days. Following the third day exposure, samples were treated with MTT to quantify the cell viability and compared to control (unexposed) cultures. A highly significant viability of chondrocyte was observed in following field intensities and frequencies (1.95 µT-0.1Hz [$P < 0.001$], 1.95 µT -1Hz [$P < 0.001$] and 0.65 µT-0.1 Hz [$P < 0.001$]). Moderate favourable response was observed in other field intensities and frequencies [Table 1]. After 3 days of 60 min daily exposure to 1.95 µT PEMFs at a frequency of 0.1 Hz, the total number of cells in the culture increased, indicating heightened viability in response to PEMFs.

Quantitative measurement of proteoglycan glycosaminoglycan synthesis

Our spectrophotometric quantification of the ECM components such as GAG and PGs were assayed with identical PEMF parameters (field strengths, frequencies, and days of exposure and duration of exposure) as those used for MTT assay of cell viability with identical results. As compared with previously observed results, favorable responses to the production of ECM components were seen in following field strengths and frequencies (1.95 µT-0.1 Hz [$P < 0.001$], 1.95 µT -1 Hz [$P < 0.001$], 0.65 µT-0.1 Hz [$P < 0.001$], 0.65 µT-1 Hz [$P < 0.001$], 1.95 µT-10 Hz [$P = 0.001$] and 0.65 µT-10 Hz [$P = 0.001$]). Moderate favorable response was observed in other field intensities and frequencies [Table 2]. Our spectrophotometric quantification thus corroborates and strengthen our MTT assay results, indicating that exposure with 1.95 µT field intensity at frequency of 0.1 Hz for

Table 1: MTT assay for detection of viable cells after exposure to PEMFs for 3 consecutive days

0.1 Hz frequency			1 Hz frequency			10 Hz frequency		
Field strength	Mean±SD*	P	Field strength	Mean±SD*	P	Field strength	Mean±SD*	P
0.65 µT	0.3562±0.02235	<0.001	0.65 µT	0.3342±0.02178	0.001	0.65 µT	0.3010±0.02978	0.053
1.3 µT	0.2994±0.04127	0.094	1.3 µT	0.2976±0.01968	0.106	1.3 µT	0.2766±0.01976	0.954
1.95 µT	0.4187±0.03500	<0.001	1.95 µT	0.3689±0.01920	<0.001	1.95 µT	0.3332±0.02425	0.004

*Mean and SD of optical density values of cells exposed to PEMF. SD=Standard deviation, PEMFs=Pulsed electromagnetic fields

Table 2: DMMB assay for detection of ECM components after exposure to PEMFs for 3 consecutive days

0.1 Hz frequency			1 Hz frequency			10 Hz frequency		
Field strength	Mean±SD*	P	Field strength	Mean±SD*	P	Field strength	Mean±SD*	P
0.65 µT	0.4661±0.01124	<0.001	0.65 µT	0.449±0.00886	<0.001	0.65 µT	0.4346±0.00262	0.001
1.3 µT	0.4287±0.00773	0.003	1.3 µT	0.4247±0.01084	0.006	1.3 µT	0.4084±0.00496	0.042
1.95 µT	0.5026±0.01069	<0.001	1.95 µT	0.4777±0.01312	<0.001	1.95 µT	0.4357±0.00860	0.001

*Mean and SD of optical density values of cells exposed to PEMF. SD=Standard deviation, PEMFs=Pulsed electromagnetic fields, DMMB=Dimethyl methylene blue, ECM=Extracellular matrix

60 min/day was most effective in production of GAG and PG of chondrocytes.

Cell cycle analysis

Cells were analyzed to assess their distribution at different phases of the cell cycle by flow cytometry after staining of DNA with propidium iodide and recording of 10^6 events for each exposure parameter. The cells distribution in four distinct phases could be recognized in a proliferating cell population: G₁, S (DNA synthesis Phase), G₂ and M (Mitosis). As both G₂ and M phase have an identical DNA content, they could not be discriminated based on their differences in their DNA content. The percentage values were assigned to each population and also dot plot [Figure 2a and b] and histogram [Figure 2c and d] were used to denote the distribution of cells in distinct phases. PEMF at different field strengths and frequencies was found to promote cell cycle progression from the G₁ phase to the S and G₂-M phases. Cells present in G₂-M phase are in dividing state and show increased rate of proliferation. A shift to top of cell population (G₂-M) in dot plot shows great proliferation [Figure 2a and b]. Based on the percentage of cells distribution in G₂-M phase, proliferation effect was determined at different exposure parameters. Histogram indicates, cells exposed at 0.1 Hz frequency with 1.95 µT of PEMFs show 20.24% of their significant presence in G₂-M phase compared to other field strengths such as 0.65 (18.9%) and 1.3 µT (17.54%) [Figure 2c]. The cells exposed to 1.95 µT of PEMFs at 0.1 Hz frequency shows 20.24% of their significant presence in G₂-M phase compared to other frequencies such as 1 Hz (19.46%) and 10 Hz (17.83%) [Figure 2d].

Analysis of cell architecture and morphology

Actin filaments of the cytoplasm stained by Phalloidin and nucleus was counterstained with propidium iodide observed by confocal fluorescent microscopy showed a significant difference in morphological structure and formation of stress fibers between exposed chondrocytes at varying

frequencies (0.1, 1 and, 10 Hz) with specific field strength 1.95 µT and unexposed cells. Stress fiber formation was increased in chondrocytes exposed at frequency of 0.1 Hz with 1.95 µT compared to unexposed [Figure 3]. Stress fiber formation indicates that the cells stability, strength and their healthy attachment.

DISCUSSION

Our study observed that short term *in-vitro* chondrocyte exposure to PEMFs at frequency of 0.1 Hz and field strength of 1.95 µT for 60 min/day for 3 consecutive days have shown highly significant effects in different experimental parameters such as cell viability, ECM production, cell cycle progression and stress fiber formation. By contrast, exposure of identical chondrocyte cultures to PEMFs of 0.65 µT field intensity at 1 Hz frequency resulted in less significant levels of different parameters. On the other hand, exposure to 1.3 µT PEMFs at 10 Hz frequency does not shown any significant effects in different analytical parameters. These findings, apart from observing benefits of certain range of field strengths, also bring to light the ability of PEMF to inhibit cellular effects when used at certain field strengths and frequencies, a fact which has been observed earlier.

In our study design, we limited our experiments to within 3 days of exposure to PEMF to stay within the realm of better clinical applicability. For our analysis, we have chosen 3 days as an appropriate end point as it avoided the over confluence of chondrocytes and also it would minimize the contact inhibition that can induce changes in biochemical status and cause dedifferentiation. As the number of days of exposure to PEMFs increases, it may enhance the proliferative effects to the chondrocytes. The design of longer day exposure to PEMFs will be taken into future study. PEMF parameters used in this study such as frequency, field strength and duration of exposure could translate into the clinical application and will be innocuous

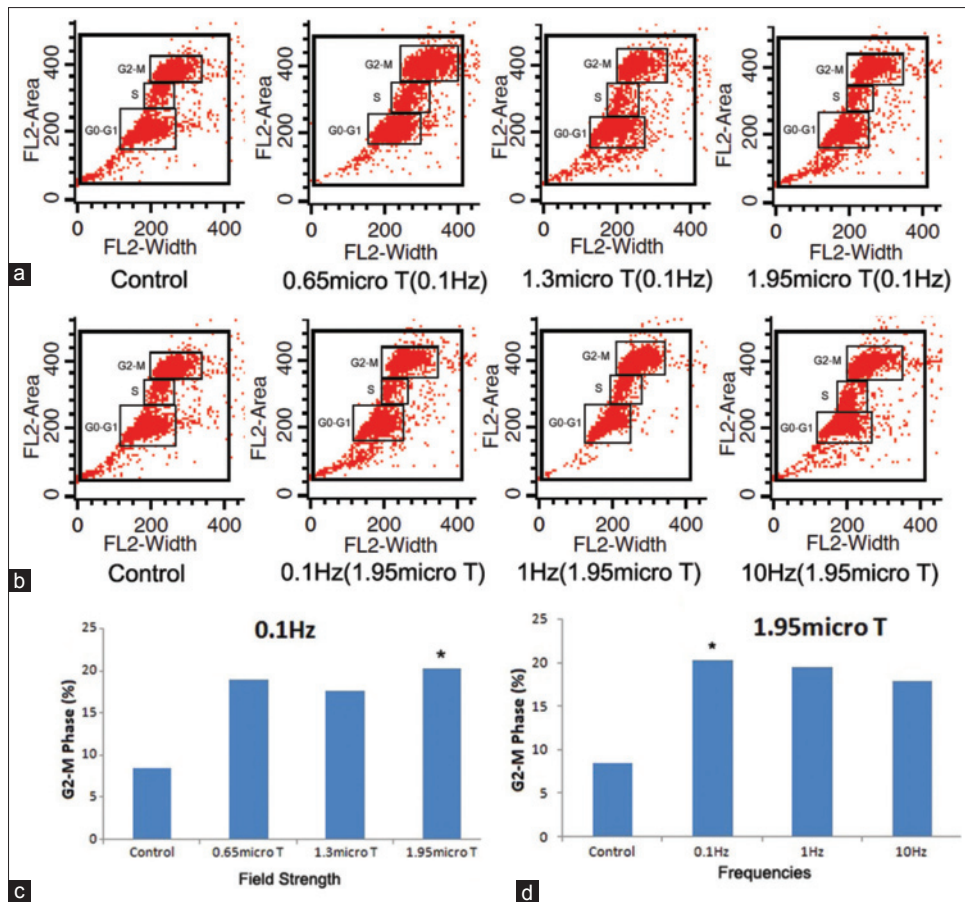


Figure 2: Cell cycle analysis by flow cytometer to determine the proliferative effect of chondrocytes in distinct cell cycle phases. Percentage of chondrocytes distribution in G2-M phase indicates cell proliferation effects as it has all mitotic cells. Significant cell distribution in G2-M was seen in samples exposed to 1.95 μT pulsed electromagnetic fields of all field strengths (a and c) and 0.1 Hz of all frequencies (b and d)

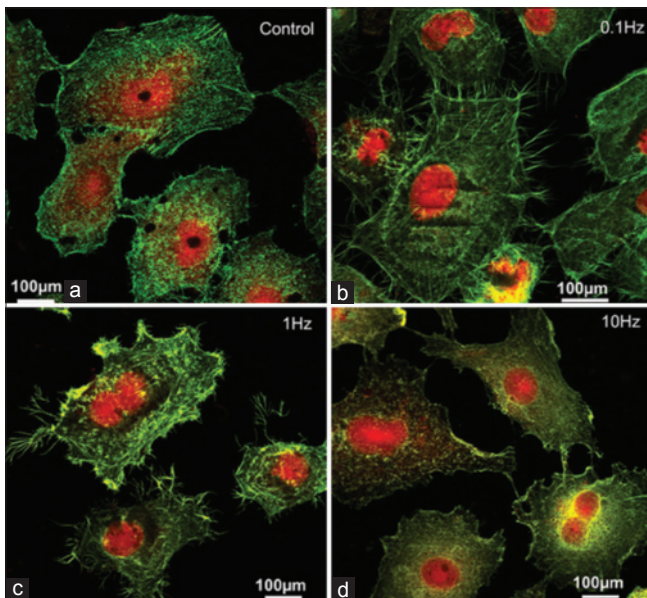


Figure 3: Human chondrocytes morphological structure was studied by staining with phalloidin and propidium iodide for visualizing stress fibers (green) and nuclear staining (red). (a) No stress fiber formation in chondrocytes unexposed to pulsed electromagnetic fields. (b) Cells exposed to 1.95 μT pulsed electromagnetic field at 0.1 Hz shows high stress fiber formation. Stress fiber formation was significantly decreased in cells exposed to 1.95 μT pulsed electromagnetic field at 1 and 10 Hz (c and d)

to the target tissue and their surrounding tissues which are exposed to PEMF during clinical therapy.

Our study observed correlation between critical cell characteristics (cell viability and promotion in cell multiplication) of exposed samples and induction of extracellular components which include GAG and PG. This raises the question on the validity of using changes in ECM components as a marker of chondrocyte healing in studies using *in-vitro* models.

The earliest *in-vitro* study with bovine articular chondrocytes exposed using Helmholtz coils found no significant effect of PEMF on ECM component synthesis.¹⁹ Sakai and colleagues studied the effect of 0.4 mT field strength at 6.4 Hz delivered over a period of 5 days on rabbit growth cartilage and human articular cartilage and observed that PEMF stimulated cell proliferation and GAG synthesis in growth cartilage cells but resulted in only cell proliferation with no increase in GAG content in articular cartilage cells.²⁰ The latter finding of our observation on extracellular components (GAG and PG) synthesis is comparable with earlier studies observation.

De Mattei *et al.* exposed chondrocytes from healthy patients to PEMF to varying duration of exposure (1- 18 h and 1- 6 days) using a field strength of 2.3 mT at 75 Hz. The study observed that short duration of exposure (1 and 6 h) did not result in increased DNA synthesis, while longer duration of exposure (9 and 18 h) increased DNA synthesis.²¹ Chang *et al.*, exposed porcine chondrocytes to a field of 1.8- 3 mT at a frequency of 75 Hz for 2 h/day for 3 weeks and observed that long term 3 weeks PEMF exposure was beneficial over the short term 1 week exposure.²² However, our observations contradict these findings and reports the better efficacy of even short term PEMF exposures. Though our study observed the efficacy of a daily PEMF exposure of 60 min for only 3 days, benefits of exposure should be expected to enhance with daily exposures exceeding 3 days. We could not observe the benefits beyond day 3, since confluent chondrocyte cultures de-differentiated due to contact inhibition beyond this period in two-dimensional cultures.

Our observation on promotion of cell cycle from G1 phase to G2-M phase with certain field strengths is comparable with the findings of Nicolin *et al.* which observed similar results with field strength of 2 mT at 75 Hz with an exposure time of 4 h or 12 h/day.²³ The striking observation of similar findings in our study with much lower field strength for exposure duration of 60 min has better clinical applicability.

A recent *in-vivo* animal study exposed rabbits with experimental osteochondral defect to PEMF for a period of 60 min/day for 6 weeks and observed a better total histological score in the study group to conclude that PEMF is beneficial for hyaline cartilage formation.²⁴ The only *in-vitro* study on human chondrocytes harvested from OA knee reports no effect on PG production using field strength of 2mT at 50 Hz for 14 days.²⁵ However both studies did not evaluate fine cellular effects (cell viability and cell cycle promotion).

Based on our data, the study informs that the future *in-vitro* studies on the topic should probably use exposure duration not more than 60 min/day but we can increase more number of days to PEMFs at 0.1 and 1 Hz frequencies and 1.95 and 0.65 μ T field intensities. However, future studies should aim to utilize collagen matrix in three-dimensional (3D) cultures and focus more on exposure for more number of days to overcome the limitation of dedifferentiation and contact inhibition due to over confluent in 3D model and also focus on the effect of PEMF on chondrocyte cytoskeleton (observed as stress fibers in Phalloidin staining). It would of interest to investigate the strength of the chondrocyte cytoskeleton between exposed and control cells. Though it may be argued that occurrence of stress

fiber formation observed with PEMF exposure is a result of heating effect due to Helmholtz system, the low dose of PEMF is less likely to have produced a heating effect which may happen with higher doses.

To conclude, our study observed that short duration (60 min/day) low frequency (0.1 Hz) low field strength (1.95 μ T) PEMFs have beneficial effects on chondrocyte viability, ECM production, multiplication and probably cytoskeleton even for a short period of 3 days. Short duration PEMF exposure for patients with OA has the potential to produce favorable clinical effects. However, the results of the study have to be confirmed with a methodology incorporating assessment of both mass and strength of PEMF exposed chondrocytes.

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Conflicts of interest

There are no conflicts of interest.

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