

EFFECTS OF ELECTROMAGNETIC FIELDS ON CHONDROCYTES CELLS OF HUMAN SEEDED ONTO 3D COLLAGEN-PLLA SCAFFOLDS AND CHONDRO-GIDE MEMBRANE

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ABSTRACT

Osteoarthritis is a common, chronic disorder of synovial joints with degenerative changes and locomotor limitations. In response to cartilage damage chondrocytes are forced to proliferate and increase matrix formation to replace the lost cartilage tissue. In this study, we aimed to investigate effects of pulsed electromagnetic field (PEMF) on SW-1353 chondrosarcoma human cells in various cell culture conditions using 3D collagen-PLLA scaffolds and Chondro-Gide membranes. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations were evaluated.

Keywords: Electromagnetic field, Chondrocytes cells, Collagen-PLLA scaffolds, Chondro-Gide membranes

1. INTRODUCTION

Osteoarthritis (OA) is a chronic disease which changes the balance between anabolic and catabolic processes in joints [1]. The tissue-specific mechanical properties of articular cartilage are dependent on the ECM structure and composition, which accounts for about 90% of cartilage wet weight, and is mainly composed of collagen type II, proteoglycans (in particular, aggrecan, hyaluronic acid), cations and water [2,3]. The distributions of these three main constituents by weight are as follows: 68–85% water, 10–20% type II collagen, and 5–10% proteoglycans. To develop a better understanding of PEMF effects on cartilage, studies have been performed on cultured human chondrocytes in vitro and shown that PEMF exposure increased both chondrocyte proliferation and proteoglycan synthesis. Interestingly, these studies utilized different magnetic field values (0.5–2.5 mT) and pulse frequencies (30–800 Hz) [4-5]. PEMF has several well-documented physiological effects on cells. More precisely, PEMFs were tested in human and animal monolayer chondrocyte cultures and tissue explants and their effects were investigated by different methodologies. Conflicting observations have been reported and some studies demonstrated a significant effect of PEMF in increasing articular chondrocyte proliferation [2,6-7].

The purpose of our study was to investigate whether required chondrocyte numbers could be acquired without causing any toxicity and dedifferentiation on 3D collagen-PLLA scaffolds and Chondro-Gide membranes in cell cultures by using PEMF.

2. MATERIALS AND METHODS

2.1. Scaffold and membrane characterization

In this study, type of scaffolds and membranes assessed were: (a) collagen-PLLA scaffold and (b) Chondro-Gide membrane (Geistlich Pharma AG, Switzerland) (Figure 1).

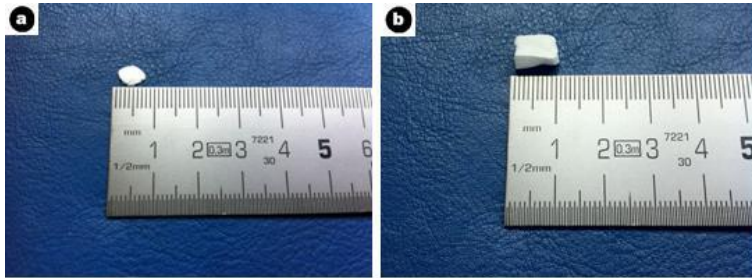


Figure 1: Photographs collagen-PLLA scaffold (a), Chondro-Gide membrane (b) Scaffold preparation and characterization

PLLA solution (4%) was prepared using PLLA (Purac Biochem by Gorinchem, Purasorb Poly-L-Lactide, MW: 800kDa, Holland), dissolved in chloroform to prepare collagen and PLLA scaffolds. Gelfix collagen (Gelfix® collagen, EURORESEARCH s.r.l., Italy) was soaked in pre-prepared PLLA solution and collagen fibrils were allowed to moisten.

Chondro-Gide membranes (Geistlich Pharma AG, Switzerland) were kind gifts from Dr. Pabbruwe (Department of Clinical Science at North Bristol, University of Bristol, UK). Collagen-PLLA scaffolds and Chondro-Gide membranes were sterilized with ethylene gas oxide before used in vitro.

2.2 Chondrocyte harvest and culture

SW-1353 Chondrosarcoma Human Cell Line was obtained from ATCC (Manassas, VA 20108 USA) company (ATCC-CRL 1427 Lot number: 57840088). Cells were seeded and expanded in culture flasks in DMEM containing 10% FCS, 200mM L-Glutamine and antibiotics (100U/ml penicillin, 100µg/ml streptomycin, 5µg/ml amphoterycin B) at 37°C in a humidified 5% CO₂ atmosphere.

2.3 Cell seeding and cells/scaffold construct culturing

When confluent monolayer cultures were achieved, cells were trypsinized, centrifuged and supernatants were discarded. Scaffolds (8x6x3mm) and membranes (0.5x0.5mm) were placed in 24-well culture plates. Cell suspensions (3x10⁴cells/50 µl) in DMEM containing 10% FCS, 50µg/ml ascorbic acid and antibiotics (100U/ml penicillin, 100µg/ml streptomycin, 5µg/ml amphoterycin B and 50 µg/ml gentamycin) were seeded on each scaffold and membrane. In parallel, SW-1353 cells were also seeded in monolayer cultures in 24-well plates (3x10⁴cells/well) cultured to equilibrate for 48 hrs at 37°C in a humidified atmosphere of 5% CO₂/95% air.

2.4 Exposure to EMF

Following initial culturing period, chondrocytes were maintained on scaffolds, membranes or in monolayer cultures for 7 days. Some wells were exposed to pulsative EMF each day for 30 minutes/day. PEMF exposure apparatus consisted of a pair of Helmholtz coils placed opposite to each other and in a signal generator (Igea, Carpi, Italy). Multi-well plates were placed between Helmholtz coils so the plane of the coils perpendicular to the plates. The parameters of the pulsed signal were as follows: the pulse duration= 1.3 ms, intensity of magnetic field= 2.3 mT, induced electric field= 2mV, frequency= 75 Hz, yielding duty of cycle of 1/10.

2.5 SEM analysis

3D collagen-PLLA scaffolds and Chondro-Gide membranes were analysed by scanning electron microscope (SEM) prior to cell seeding. Cells adhering to the scaffolds and membranes were washed with PBS. Subsequently, the cells were fixed with 5% glutaraldehyde (pH 7.2), 7% sucrose and 2% osmium tetroxide in sodium cacodylate buffer (0.1M). The specimens were dehydrated using graded ethanol changes and gold splattered in vacuum (Polaron SC7620) at 10 kV and examined using SEM (JEOL JSM-6060).

2.6 TEM analysis

Cells were fixed in Karnovsky solution (2.5% glutaraldehyde, 2% paraformaldehyde, 2.5% glutaraldehyde) for overnight at 4°C. The next day the cells were post-fixed 0.1M sodium cacodylate buffer (pH 7.4). The cells were then dehydrated in acetone serial. The next day the cells were embedded in Epon Araldite for overnight at 4°C. The sections were then examined under a Philips (Eindhoven, The Netherlands) CM-12 transmission electron microscope.

2.7 Histochemical analysis

Histochemical staining was performed using haematoxylin and eosin (H&E) and periodic acid Schiff (PAS). PAS staining was used to localise the sulphated glycosaminoglycans (sGAG) depositions while H&E stained normal cartilage matrix light pink and the cytoplasm of chondrocytes blue.

2.8 Cell count and viability

After 7 days of culture monolayer culture medium were removed and the cells were detached by adding trypsin/EDTA for 2 min. The suspended cells were transferred into fresh culture medium and counted with Trypan blue staining that colours dead cells in blue. LDH test is a useful tool to show cell death and lysis. Extracellular LDH activity was measured with the help of an LDH detection kit (Roche Diagnostics, Mannheim, Germany, Catalogue no. 11644793001). Absorbance was measured at 490nm. Results were given as AU of LDH activity and normalized to protein content in medium.

2.9 Statistical analysis

Each experiment was repeated at least twice with similar results. Results are expressed as mean±SEM of triplicate determinations. Comparative studies of means were performed by using one-way ANOVA followed by post-hoc test (Fisher's projected least significant difference) with a statistical significance at $P < 0.05$.

3. RESULTS

3.1 SEM observations and cell morphology

SEM analysis showed that chondrocytes attached on meshlike collagen fibers on the collagen-PLLA scaffolds and the Chondro-Gide membranes (Figure 2).

Scanning electron micrographs of scaffolds and membranes surface view after day 7 culturing are shown in Figure 2. Chondrocytes had both spherical and spread morphologies. Figure 2a and b show the morphology of scaffold porosity prior to cell seeding. Figure 2 c-f show the morphology of chondrocytes attached on the surface of porous scaffolds.

3.2 TEM analyses

TEM investigations established of cells around 3D collagen-PLLA scaffolds and Chondro-Gide membranes on day-7 (Figure 3).

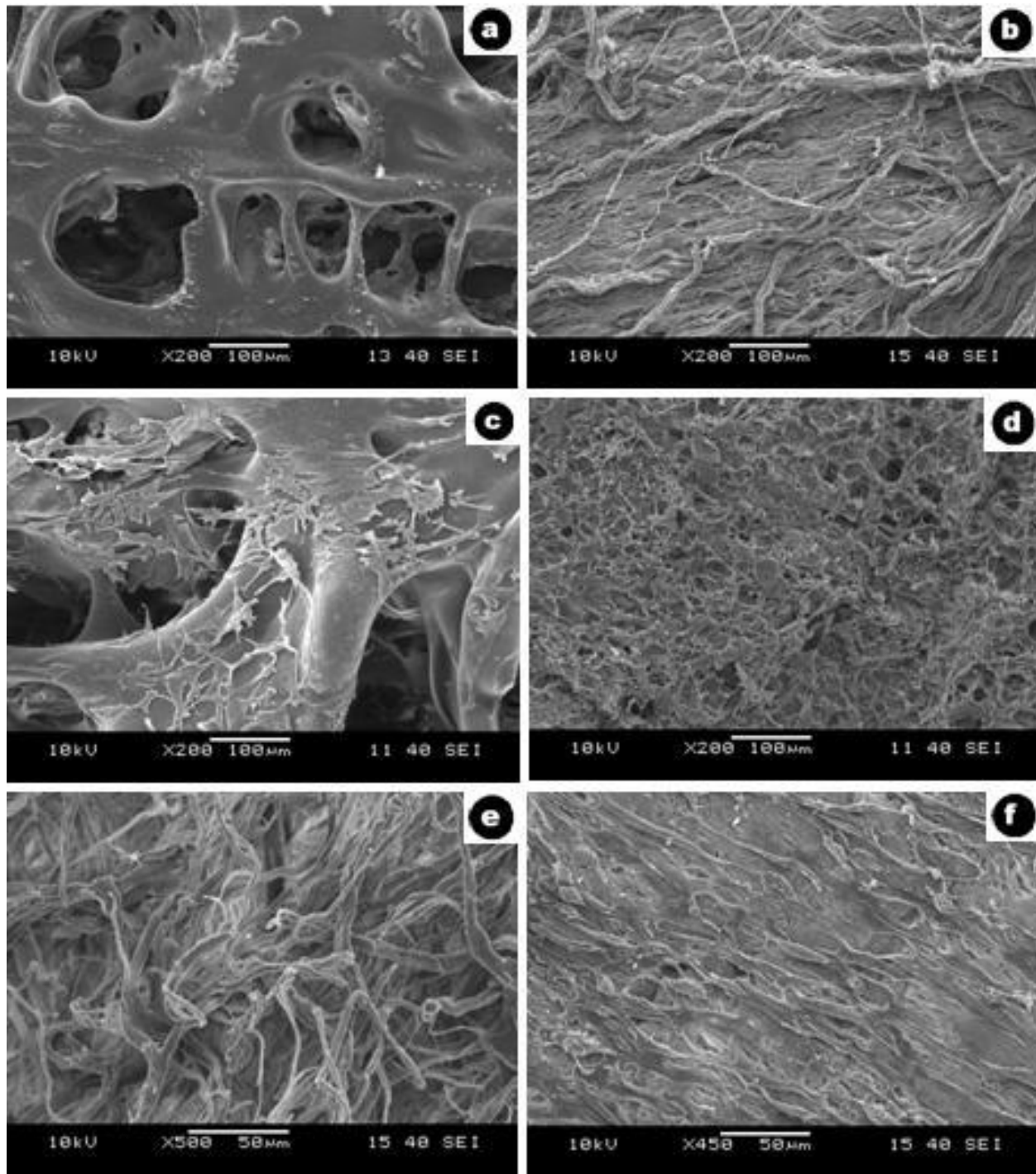


Fig. 2 SEM pictures of collagen-PLLA scaffold (a), Chondro-Gide membrane (b), SW-1353 Chondrosarcoma Human Cells on collagen-PLLA scaffold cultured for 7 days (unexposed to EMF) (c), SW-1353 Chondrosarcoma Human Cells on collagen-PLLA scaffolds after exposure to EMF for 7days (d), SW-1353 Chondrosarcoma Human Cell on Chondro-Gide cultured for 7 days (unexposed to EMF) (e), SW-1353 Chondrosarcoma Human Cell on Chondro-Gide membrane after exposure to EMF for 7days (f).

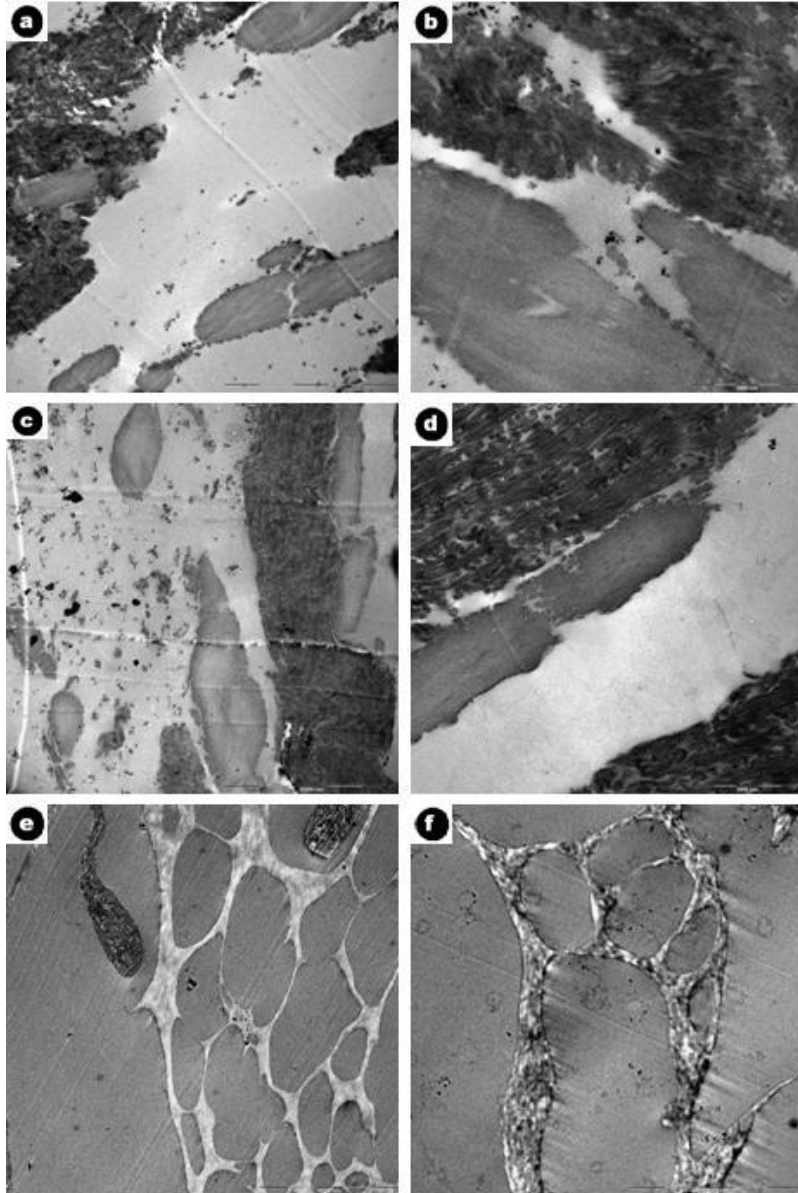


Fig. 3 TEM pictures of SW-1353 Chondrosarcoma Human Cell on Chondro-Gide membrane after exposure to EMF for 7days: Scale bar=10µm (a), Scale bar=2µm (b); Chondrosarcoma Human Cell on Chondro-Gide membrane (arrow) cultured for 7 days (unexposed to EMF): Scale bar =10µm (c), Scale bar =2µm (d); SW-1353 Chondrosarcoma Human Cell on collagen-PLLA scaffold (arrow) after exposure to EMF for 7days: Scale bar=10µm (e); SW-1353 Chondrosarcoma Human Cell on collagen-PLLA scaffold cultured for 7 days (unexposed to EMF): Scale bar=10µm (f).

3.3 Histochemical analysis

Monolayer SW1353 cell cultures were analyzed with histochemical analysis after 7 days of culture. H & E staining was used to show cell morphology. Purple represented the cell nuclear and red/pink color represented cytoplasm (Figure 4A to 4D). This finding contradicted previous data showing PEMF to increase primary chondrocyte numbers in monolayer cultures. Cells were more hypertrophic and spindle like in PEMF group (Figure 4C&D). To determine the effect of PEMF on monolayer SW1353 cell cultures on glycosaminoglycans (GAG) production, we performed PAS staining of SW1353 cells after 7 days of culture. Cells were stained strongly with PAS stain. However we did not detect any PAS staining in extracellular matrix (Figure 4E&F).

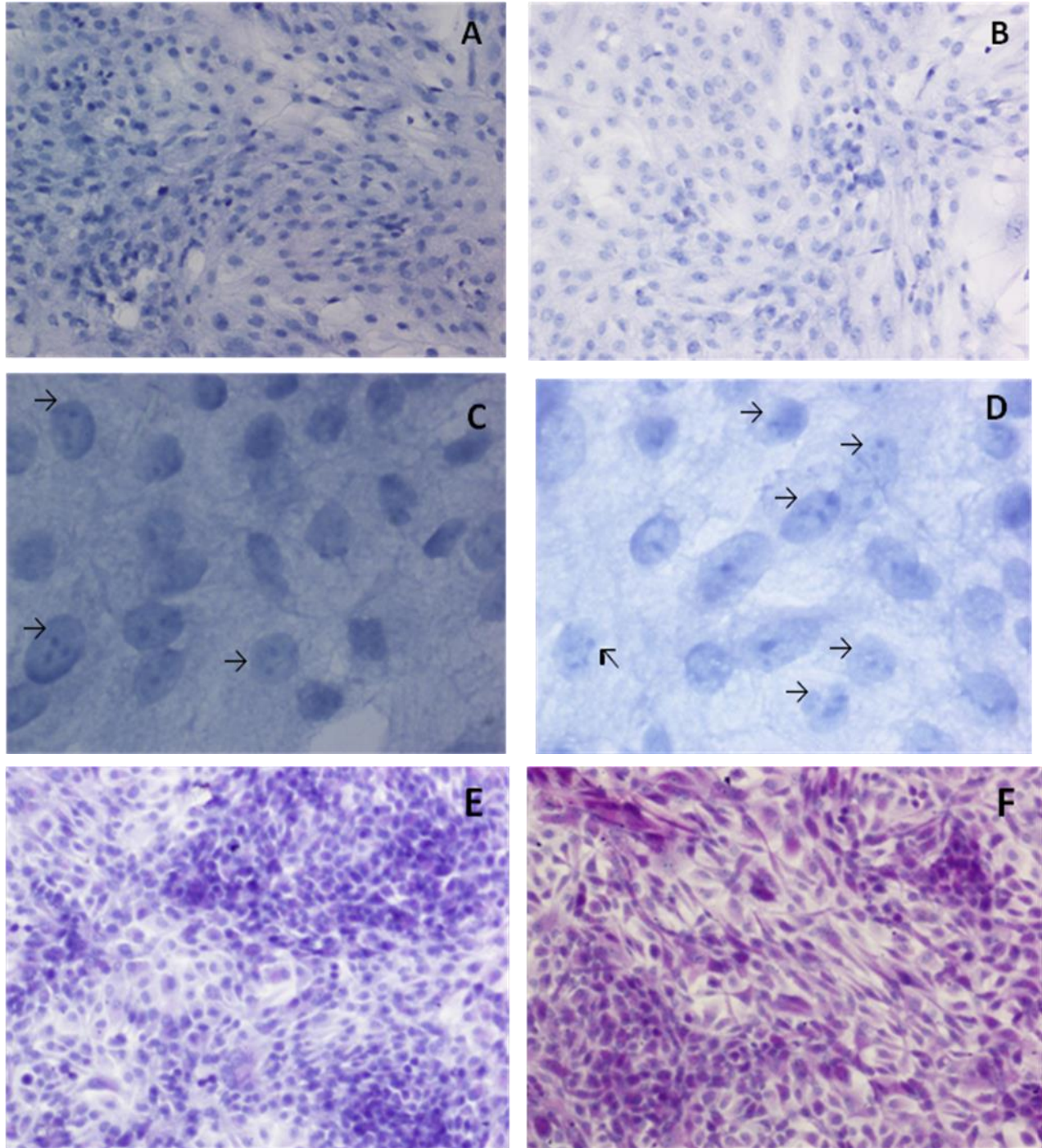


Fig. 4 Histology of monolayer cells stained with Hematoxylin and eosin (H&E) staining (A–D) and periodic acid Schiff (PAS) staining (E–F). Control: the cultures without EMF exposure for 7 days; PEMF: the cultures with EMF exposure for 30minutes/day hours for 7 days. → points multinucleated chondrocytes.

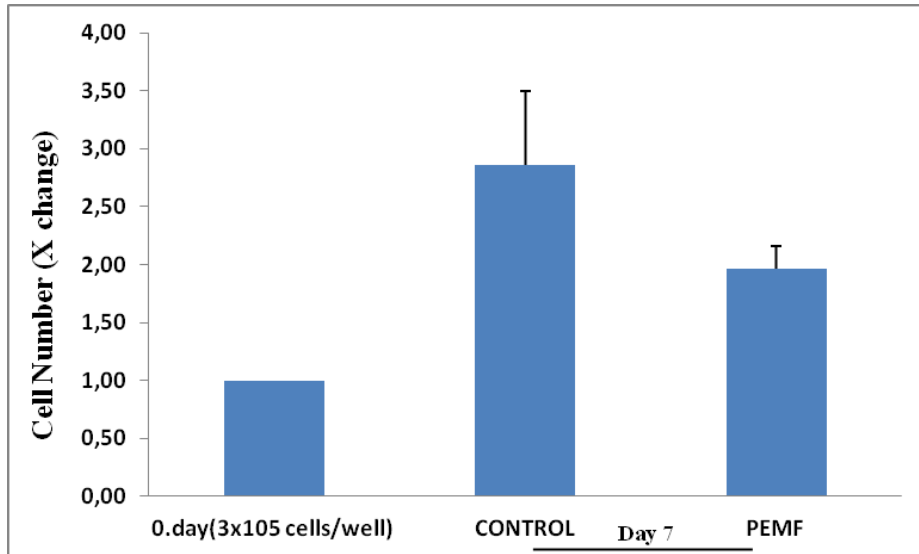


Figure 5: Quantification of cell proliferation rate was determined by counting cells in each well. Control: the cultures without EMF exposure for 7 days; PEMF: the cultures with EMF exposure for 30minutes/day hours for 7 days. Change in cell number was expressed percent increase compared to cell number seeded into wells at day “0”(3x10⁴cells/50 μ l).

3.4 Cell number and viability

Cell proliferation was evaluated by Trypan blue exclusion dye experiments in conditions as described above (Figure 5). Although there was a slight decrease in cell number in wells exposed to PEMF, this decrease was not statistically significant. LDH activity was analysed in the supernatant of each cell culture. Compared to controls no significant increase in LDH activity was observed in chondrocytes subjected to PEMF (Figure 6).

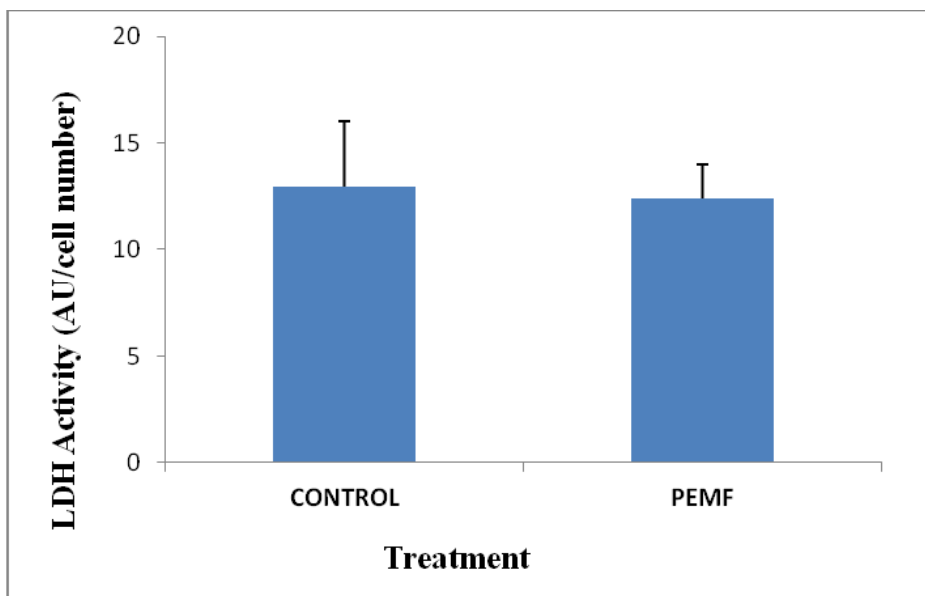


Fig. 6 Cell viability was determined by biochemical LDH assay after 7 days of culture. LDH activity was expressed as AU of LDH activity and normalized to cell number. Control: the cultures without EMF exposure for 7 days; PEMF: the cultures with EMF exposure for 30minutes/day hours for 7 days.

4. DISCUSSION

Articular chondrocytes are responsive to biophysical stimuli such as ultrasound, mechanical stresses and electromagnetic fields (EMF). Applications of different electromagnetic pulses and continuous waves of different shapes have been described in the scientific literature for cartilage and chondrocytes. The majority of scientists use pulsed magnetic fields with frequencies of the bursts between 1.5 and 75 Hz and a peak magnetic flux density between 0.1 and 2.5 mT because in contrast to sinusoidal magnetic fields, magnetic pulses of the same amplitude induce higher electrical field peaks within the tissue [8-9]. It has been reported that PEMF plays a regulatory role in cartilage metabolism by increasing the chondrocyte proliferation rate and synthesis of extracellular matrix components, and reducing the matrix degradation. In vitro studies clearly show that chondrocyte proliferation and matrix synthesis are significantly enhanced by PEMF stimulation [2, 6].

The structure of 3D collagen-PLLA scaffolds and Chondro-Gide membranes were analyzed by SEM and the pore size of the scaffolds were in the range of 50-200 μm . The results from SEM analysis of scaffolds seeded with SW1353 chondrocytes demonstrated that cells were localized within pores and that cells adhered to the surface of the scaffold especially when the surface was somewhat uneven, allowing cells to attach SEM investigation pointed that SW1353 cells do attach to scaffold surface by the end of 7day cultures. In addition, in some areas cells observed to enter deeper into the scaffolds and tended to cluster within the pore (Figure 2). However, our data TEM shows that by the end of 7 day cultures cells are able to enter deeper into the scaffolds even in the presence of PEMF suggesting a longer time frame is required for cells to move into deeper zones.

Monolayer cultures of SW1353 showed that PEMF on its own did not have a significant effect on chondrocyte proliferation. However, PEMF exposure induced a change in chondrocyte morphology. Although comparison of the sections revealed increased numbers of multinucleated cells in PEMF group as compared to controls suggesting an increased mitotic activity in PEMF applied cells (Figure 4C&D) the increase in number of PEMF exposed cells was lower compared to the controls (Figure 5). Cells were hypertrophic and more spindle like in PEMF group compared to the controls after 7 days of PEMF exposure. The cell vitality staining (trypan blue) and the LDH activity test showed that PEMF do not have any toxic effect on SW1353 cells at the frequency and strength used.

In conclusion, cheap, clean and non-invasive method in the treatment of osteoarthritis of the EMF which was launched recently with the use of tissue engineering techniques will help to create more economical and more rapid cartilage transplants. PEMF can be used as a chondroprotective agent for the treatment of chronic diseases such as osteoarthritis which involves progressive cartilage damage.

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