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Differentiation of Human Umbilical Cord-derived Mesenchymal Stem Cells, *WJ-MSCs*, into Chondrogenic Cells in the Presence of Pulsed Electromagnetic Fields

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Abstract. *During cartilage regeneration, proliferation and differentiation of new chondrocytes are required and towards this goal, in humans electromagnetic stimulation has been used in order to increase the spontaneous regenerative capacity of bone and cartilage tissue. In vivo tissue engineering has pointed out that the absence of an abundant source of cells accelerating the healing process is a limiting factor in the ability to repair articular cartilage. Considering that the umbilical cord is a viable alternative source of mesenchymal stem cells (MSC), our study evaluated the possibility of a combined use of Wharton's jelly - mesenchymal stem cells (WJ-MSCs) and pulsed electromagnetic field (PMEF). The first effect observed was that compared with the untreated cells, when the WJ-MSCs were treated with PMEF, there was an increase in the division of cells and a rapid increase in cell density and the morphological and biochemical data showed that the treatment with PMEF reduced the time to obtain chondrocyte cell differentiation and deposition of extracellular matrix. Taken together these data indicate the capacity of PEMF to induce early differentiation of WJ-MSCs cells towards cartilaginous tissue.*

One particular sector of tissue engineering involves the repair, replacement or regeneration of cartilage tissue. In fact, injured articular cartilage is not able to repair itself. *In vivo* tissue engineering has pointed out that the reason why the repair of articular cartilage fails is the necessity for an abundant source of cells to accelerate the healing process and need to identify factors that can promote host tissue integration (1).

The multipotent mesenchymal stromal cells, today better known as mesenchymal stem cells (MSCs), were isolated for the first time from the bone marrow of rats and guinea pigs by Friedenstein *et al.* and were initially described as precursors of fibroblasts due to their morphological similarity to these cells (2-4). Due to the same author by adhesion to plastic, the isolation of a population of cells morphologically similar to fibroblasts but able to form colonies (CFU-F, colony forming unit-fibroblast) was also achieved (2, 5).

During the past ten years, research has demonstrated that it is not easy to obtain a sufficient number of MSCs for therapeutic use after expansion *in vitro*. After thirty doublings, these cells undergo a phenomenon called replicative senescence, which slows down and blocks their numerical expansion.

For this reason, alternative sources to the bone marrow, such as the umbilical cord and the amniotic fluid, are currently being studied. In particular, the umbilical cord is an appreciable alternative source of MSCs thanks to its availability, eliminating the need to resort to invasive techniques (6).

Electromagnetic stimulation in humans has been studied in order to increase the spontaneous regenerative capacity of bone and cartilage tissue (7-10). *In vivo* study on cartilage has also established that electromagnetic stimulation suppresses inflammatory reaction (11, 12). Moreover, *in vitro* experiments on human tissues have shown that exposure to a pulsed electromagnetic field (PEMF) promotes proliferation of cells of the immune system, osteoblasts, chondroblasts and new angiogenesis in endothelial cell cultures (13-15).

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The effects are mainly attributable to the interaction of PEMFs with the cell membrane and in particular to the transduction processes, inside the cell, of the signals present on the surface of the cell itself.

Based on this background, we decided to isolate from human umbilical cord Wharton's jelly mesenchymal stem cells (MSCs) and explore the effects of PEMF on their differentiation into chondrogenic cells.

Materials and Methods

Isolation of MSCs from Wharton's jelly. Sections of umbilical cord were obtained, with authorization from the Department of Obstetrics and Gynecology Hospital of the Second University of Naples, following caesarean section, at the end of the gestation period.

MSCs were extracted from the Wharton's jelly according to the following protocol: sections of umbilical cord were washed with phosphate buffer saline (PBS) (Lonza, Verviers, Belgium) supplemented by antibiotics (penicillin-streptomycin; Gibco, Grand Island, NY, USA). After washing with PBS, the sections of umbilical cord were dissected in order to separate the gelatin from the rest of the cord. The gelatin, in small fragments, was immersed in an solution of collagenase 1 mg/ml (300 l of collagenase concentrated to 0.1 g/ml in 30 ml of Hanks' balanced salt solution) (HBSS; Gibco).

After incubation for 20 h at 37˚C, 25 ml of sterile PBS were added to reduce the viscosity of the liquid; after sedimentation the supernatant was transferred to a new tube to be centrifuged at 1500 rcf ×*g* for 10 min, then the supernatant was discarded.

The pellet was then resuspended in 10 ml of sterile PBS and a second centrifugation was carried out at 1000 rcf ×*g* for 6 min. Finally, the supernatant was removed leaving only the pellet with 1.5 ml of liquid to which 5 ml of culture medium were subsequently added. The cells were then seeded in a 25 cm^2 flask and placed in moist incubator when 5% CO_2, at 37˚C.

The culture medium used for the isolated cells was Dulbecco's modified Eagle's medium (DMEM, Lonza Verviers, Belgium) to which 10% Fetal bovine serum (FBS; Hyclone, Logan, UT, USA) was added, with 50 mg/ml penicillin-streptomycin and 2 mM L-glutamine.

Characterization of WJ-MSCs by flow cytometric assay. To confirm that we isolated MSCs, flow-cytometric analysis was performed on the cells extracted. The data were acquired with a FACScan flow cytometer (Becton Dickinson, Milan, Italy) equipped with an argon laser with a wavelength of 488 nm. For the characterization, approximately $1{\times}10^6$ MSCs were detached, washed and incubated with the monoclonal antibodies against surface molecules: in particular against: CD44, CD105, CD45 (Sigma-Aldrich, Milan, Italy) and CD73 (Lifespan Bioscience, Seattle, Washington, USA). Fluorescein isothiocyanate (FITC) was used as fluorochrome, linked to secondary antibodies goat anti-mouse IgG. For each sample, a minimum of 10,000 events were collected .

PEMF treatment. PEMF treatment was performed as previously described (21). Briefly, the culture plates with WJ-MSCs were placed inside an incubator at equal distance from two solenoids arranged in parallel with one another and perpendicular to the support base, thus respecting the principle of focus signal in order to maintain constant physical characteristics of the stimulation signal. The external device produced an electromagnetic pulse signal of the square wave type with a frequency of 75Hz ±5% and an intensity of 18-30 Gauss.

The cells were cultured in six-well culture plates (BD Falcon TM, BD Biosciences, Mississauga, ON, Canada) and exposed to a PEMF continuously for 8 h each day during the 21 days of culture period. Control experiments were performed on the MSCs isolated from the same umbilical cord Wharton's jelly in a separate CO_2 incubator that had no PEMF device. The incubators were alternated for the treatment and control experiments.

The treatment was performed in double using BIOSTIM SPT (Igea, Carpi, Italy) and Osteoplus (Fisiokinetec, Naples, Italy). Results obtained from the two systems were not different.

Chondrogenic differentiation. MSCs isolated from the umbilical cord Wharton's jelly were induced in the following chondrogenic medium: DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml penicillin-streptomycin, 50 μg/ml ascorbic acid and 1 ng/ml human recombinant transforming growth factor β1 (TGF-β1).

Immunostaining. Immunostaining was performed as previously described (16, 17). Briefly, MSCs were fixed with a solution of 4% paraformaldehyde in PBS for 30 min, washed in PBS and then incubated with PBS containing 1% bovine serum albumin for 15 min. before the application of the primary antibody. Incubation with the primary antibody to collagen II (rabbit polyclonal) from Abcam (Cambridge, UK), was performed for 75 min at room temperature and the secondary antibody was applied for 1 h at room temperature. After incubation with both the primary and the secondary antibody, cells were washed three times in PBS. After the final washing step, cells were mounted with Glycergel Mounting Medium (C0563) (Dako, Milan, Italy) and then stored in the dark at 4˚C. Microscopic images were taken using a confocal microscope (SP1 UV CLSM; Leica, Wetzlar, Germany) and processed using Adobe Photoshop Software 7.0.

Glycosaminoglycan staining (GAG). To analyze the secretion of cartilage proteoglycans, Alcian blue staining was performed as previously described (1, 18). Briefly, the medium where MSCs were grown during the differentiating process, was removed and cells were then washed with PBS and fixed for 10 min in 4% paraformaldehyde in PBS. Cells were stained in 1% Alcian blue 8 GX reagent (Sigma, St. Louis, MO, USA) in 3% glacial acetic acid (pH 2.5) for 30 min at room temperature. Cells were washed before microscopic observation .

Reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was performed as previously described (16, 19, 20, 21). Briefly, total cellular RNA obtained from WJ-MSCs treated with or without PEMF using the RNeasy kit (Qiagen, Valencia, CA, USA) was converted to cDNA. The resulting cDNA was subjected to PCR amplification using rapid cycling in glass capillaries with a thermocycler (Light-cycler; Roche Molecular, Milan Italy). The reaction was performed in a total volume of 50 μl containing 2.5U Taq DNA polymerase, 50 pmole of each primer and 200 μM dNTPs in 1 × PCR buffer (10 mM Tris pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$). Specific oligonucleotide primers for type II collagen were used (sense: 5'-ATTTCAAGG CAATCCTGGTG-3', antisense: 5'-GGCCTGGATAACCTCTG TGA-3'). The following conditions

were used for PCR amplification: initial denaturation at 94˚C for 1 min, followed by 25-35 cycles of denaturation at 94˚C for 30 s, annealing at 56˚C for 50 s and extension at 72˚C for 30 s, followed by a final extension at 72˚C for 5 min. Each PCR was carried out three times. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Statistical analysis. Each experiment was performed in triplicate. The results are expressed as means±standard deviations (SD). Student's t-test was used to determine statistical differences between the means, and $p<0.05$ was considered a significant difference.

Results

The isolation of WJ-MSCs was obtained after digestion of sections of umbilical cord with collagenase for 24 h. The cells isolated in the pellet were plated in 25 cm^2 flasks. At the beginning of the culture, two cell types were detected, one with a fibroblastoid shape, being MSCs, and the other of a roundish shape, which disappeared in the early steps (data not shown).

At a confluence of 50-60%, the cells were washed twice in PBS, trypsinized using trypsin-EDTA 0.25% and then replated at a ratio of 1:4 under the same culture conditions. These steps were carried out five times before subsequent experiments.

To confirm that we had achieved isolation of MSCs, cells were diluted at 1×10^6 and incubated with a monoclonal antibody specific for surface molecules. The assay revealed that the isolated WJ-MSCs were positive for CD44, CD73, CD105, but were negative for CD34 and CD45 (data not shown).

The morphological changes in the WJ-MSCs were observed throughout the differentiation of untreated cells and cells treated with PEMF. As shown in Figure 1a-i and 1a-ii, seven days after the treatment, from an initial fibroblastoid shape with fusiform appearance, cells took on the typical morphology of cartilaginous cells. At day 14, the cells reduced their extensions, assuming a round shape and forming cell aggregates; from that moment on, an extracellular matrix was visible (Figure 1b-i and 1b-ii). At day 21, the cartilaginous elements already differentiated were numerous and well-differentiated (Figure 1c-i and 1c-ii). These morphological changes were more evident and earlier in cells treated with PEMF compared with the untreated ones.

Additionally we performed fluorescent staining for collagen II on cells collected at 21 days after chondrogenic differentiation. In Figure 1d-i and 1d-ii, the green signals represent the cellular distribution of collagen II in untreated cells and in cells treated with PEMF, respectively. The staining was higher in the treated cells (Figure 1d-ii) compared with the untreated (Figure 1d-i).

Furthermore, we evaluated the presence of GAG production. Alcian blue staining was used to evaluate GAG deposits by the cells in culture at day 21. In the cells treated with PMEF (Figure 1e-ii) the intensity of the blue colour around the cells was darker than the untreated cells (Figure 1e-i).

To further support the cell morphology observation, we performed a RT-PCR analysis using specific primers for Collagen II on treated and untreated WJ-MSC cells during their chondrogenic differentiation. As shown in figure 2 it was possible to observe that the expression of Collagen II constantly increased during the 21 days in treated and untreated cells with PMEF. However the expression of Collagen II was always higher in the cells treated with PMEF.

Discussion

The scientists are working on the development of human tissue, in fact in literature several projects and protocols to obtain skin, muscle, bone, cartilage exist. Some limiting factors in obtaining these tissues are the absence of an abundant source of cells to accelerate the healing process and the unknown identification of factors that can promote host tissue integration. The use of umbilical cord is a possible alternative source of MSCs since it is a not invasive techniques which gives the possibility to obtain an abundant quantity of cells.

During cartilage regeneration, proliferation and differentiation of new chondrocytes are required and towards this goal electromagnetic stimulation in humans has been used in order to increase the spontaneous regenerative capacity of bone and cartilage tissue (22). Moreover *in vivo* studies on cartilage has also established that electromagnetic stimulation is able to suppress the inflammatory reaction (11). Our study evaluated the possibility of a combined use of WJ-MSCs and PMEF.

In our experimental model, flow cytometry showed that the cell population isolated from the cord Wharton's jelly is composed of cells with a mesenchymal profile (23), which do not express hematopoietic or leukocyte markers and that, in particular, are positive for the CD44 receptor of hyaluronic acid.

The data obtained from the flow-cytometric assay, showed that when the WJ-MSCs were treated with PMEF compared with the untreated cells, there was an increase in the division of cells with a rapid increase in cell density. These results demonstrate that exposure to PEMF influences cell division, allowing for cells to reach an appropriate cell density for the subsequent differentiation into a shorter time. These observations suggest that treatment with electromagnetic pulses acts on cells producing a shortening of the latency period.

The morphological data showed that the treatment with PMEF reduced the time to obtain cells with a typical morphology of chondrocyte with a deposit of an extracellular matrix, as shown with the Alcian blue staning for GAG. Moreover as a further evidence of the increase of

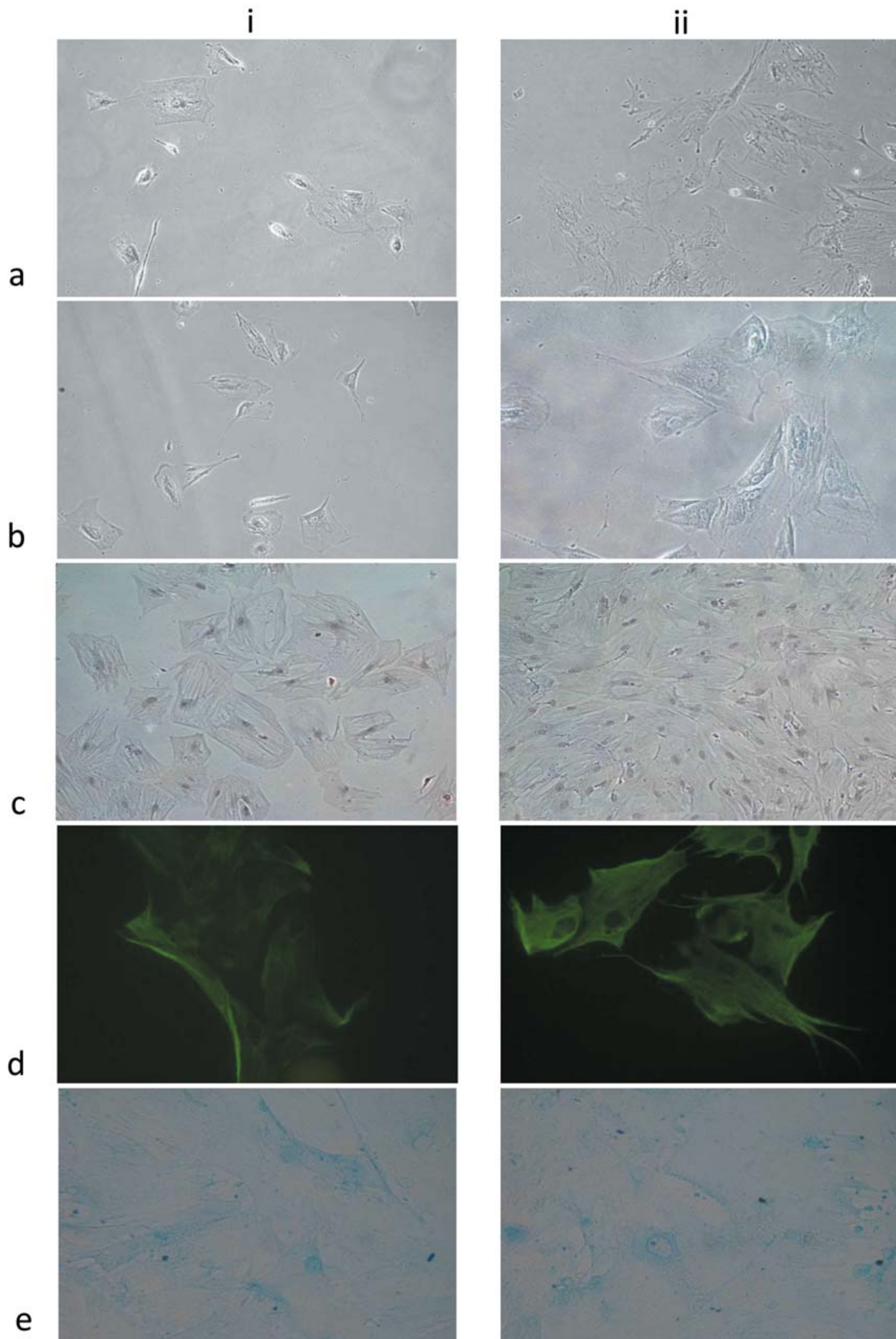

Figure 1. *Untreated (i) and pulsed electromagnetic field-treated (ii) Wharthon's jelly mesenchymal stem cells. Morphology of cells at day 7 (a), 14 (b) and 21 (c) of experiment. Fluorescent staining for collagen II at day 21 (d). Alcian blue staining for glycosaminoglycan at day 21 (e).*

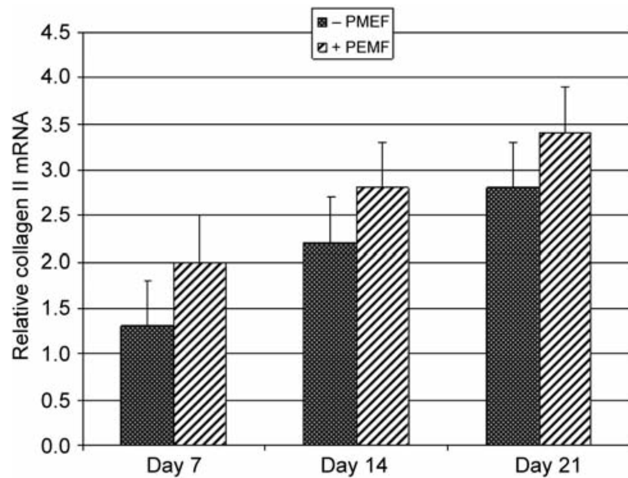

Figure 2. *Real-time analysis using specific primers for collagen II. Total RNA of Wharthon's jelly mesenchymal stem cells treated and not treated with pulsed electromagnetic field was extracted at 7, 14 and 21 day from the start of chondrogenic differentiation.*

differentiation towards cartilaginous tissue, we observed an increased expression of collagen II, by immunofluorescence and also by RT-PCR.

Taken together these data indicate the greater capacity of PEMF to induce early differentiation of WJ-MSCs towards cartilaginous tissue compared to the standard laboratory methods.

In conclusion, the use of non-invasive methods for cell differentiation could be a new therapeutic approach to the repair of cartilage injuries. This method also determines a reduction in the time of regeneration, with a consequent reduction of costs per patient.

It is easy to appreciate the enormous possibilities of treatment that a combined approach of cell therapy and physical therapy with electromagnetic fields may be offering. However, further studies are required to better understand how PEMF influences the kinetics of growth and the differentiation potential of adult MSCs and to evaluate whether this differentiation is stable *in vivo* or not.

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