In vitro exposure of human chondrocytes to pulsed electromagnetic fields

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The effect of pulsed electromagnetic fields (PEMFs) on the proliferation and survival of matrix-induced autologous chondrocyte implantation (MACI®)-derived cells was studied to ascertain the healing potential of PEMFs. MACI-derived cells were taken from cartilage biopsies 6 months after surgery and cultured. No dedifferentiation towards the fibroblastic phenotype occurred, indicating the success of the surgical implantation. The MACI-derived cultured chondrocytes were exposed to 12 h/day (short term) or 4 h/day (long term) PEMFs exposure (magnetic field intensity, 2 mT; frequency, 75 Hz) and proliferation rate determined by flow cytometric analysis. The PEMFs exposure elicited a significant increase of cell number in the SG2M cell cycle phase. Moreover, cells isolated from MACI® scaffolds showed the presence of collagen type II, a typical marker of chondrocyte functionality. The results show that MACI® membranes represent an optimal bioengineering device to support chondrocyte growth and proliferation in surgical implants. The surgical implant of MACI® combined with physiotherapy is suggested as a promising approach for a faster and safer treatment of cartilage traumatic lesions.

In the past few years, the surgical treatment of chondral lesions has evolved towards techniques that promote the formation of new hyaline cartilage by implantation. In fact, articular cartilage in adults exhibits poor intrinsic ability to self-repair after a severe injury, due partly to the lack of innervation and blood supply (O'Driscoll, 1998). Articular damage can be caused by traumatic or mechanical stress or other, such as inflammation. Mechanical stress can be classified as direct (i.e. a violent hit), causing immediate damage to cartilage and subcondral bone, and indirect (i.e. weight bearing area alteration), resulting in transverseal force, or shear stress, damaging both cartilage and subcondral bone integrity. All stress conditions are associated with an inflammatory response, leading to an increase in inflammatory cytokine levels in the articular sinovial fluid. It has, in fact, been recently evidenced that IL-1 and TNF-α mediate the development of osteoarthritis, with subsequent degeneration of articular cartilage (Andjelkov, 2005; Goldring, 2004a), by stimulating prostaglandin E_2 and nitric oxide synthesis (Goldring, 2004b).

Acute injury and chronic inflammation generally lead to cartilage functional loss. Patients with significant chondral defects frequently have persistent joint line pain, swelling and catching in the knee (Gillobly, 1998); this ineffectiveness to restore a complete structural healing of the damaged cartilage leads to osteoarthritis in a high percentage of cases and, in worse cases, to subcondral bone exposure. Therefore, different experimental cartilage reconstruction techniques have been based on the induction of cartilage repair by extrinsic chondrogenic cell sources (Hendrickson, 1994; Silverman, 2000).

Methods aimed at rebuilding joint surface in patients with localized defects of the articular cartilage include autologous chondrocyte implantation (ACI) combined with a periosteal graft, first reported by Brittenberg *et al.* (1994). In contrast to bone

marrow stimulation treatment techniques, i.e. abrasion arthroplasty, drilling or microfracture, which populate the lesion with pluripotential stem cells, the use of cultured autologous chondrocytes fills the wound with cells programmed to develop hyaline-like cartilage. This hyaline-like cartilage more closely recreates the characteristic structure of normal hyaline cartilage than does the fibrous connective repair tissue (Brittberg, 2001). The primary goal of the initial *in vitro* chondrocyte culture was to increase the number of cells so as to provide a sufficient number to fill a focal defect of articular cartilage.

Chondrocytes were isolated from small slices of cartilage harvested arthroscopically from a non-weight-bearing area of injured knee. After removal of the extracellular matrix by enzymatic digestion, the cells were expanded in monolayer culture and then implanted into the damaged cartilage. Despite the promising profile of these surgical methods in cartilage repair, different studies evidenced that autologous cultured cells could alter during *in vitro* expansion and differentiate to fibroblastic cells which may cause the formation of fibrous connective tissue with failure of surgical treatment (Brittberg, 2001; Benya, 1982; Buschmann, 1992). Recently, an alternative approach has been proposed based on the introduction of three-dimensional supports that allow a better cell distribution within the implant, called Matrix Induced Autologous Chondrocyte Implantation, or MACI®, representing the transition from a concept of cellular therapy to one of tissue bioengineering. During the arthroscopic examination, the surgeon should ideally take a biopsy from the non-weight bearing area of the proximal part of the medial or lateral condyle. The biopsy of the cartilage is immediately placed in the nutrient medium tube and forwarded to the Verigen cell laboratory. The chondrocytes are separated from their matrix and cultured in Verigen's certified laboratories over a given period of time. The cells are then placed on a collagen membrane (MACI®) and returned in time for the proposed implantation date.

The complexity of hyaline cartilage repair has prompted several groups to investigate the possible applications of PEMFs on articular hyaline cartilage healing and regeneration, both in experimental and clinical studies (De Mattei, 2003; Fini, 2005). Several pathogenetic aspects were considered, such as chondrocyte proliferation, extracellular matrix synthesis, secretory activity and inflammation, with often conflicting results. Several *in vitro* studies reported differing effects on chondrocyte cultures due to different culture conditions (Liu, 1997; Hiraki, 1987; Pezzetti, 1999; Indouraine, 2001; Sakai, 1991; De Mattei, 2004), whereas little is known about the MACI®-related *in vitro* chondrocyte proliferation/survival effects of PEMFs.

Previous studies (Zheng, 2004; Narducci, 2005) have focussed on some morphological aspects of autologous implanted chondrocytes; in this report we evaluate the effects of pulsed electromagnetic fields (PEMFs) on autologous MACI®-derived chondrocyte replication and the bioptic follow-up of cartilage specimens obtained 6 months after transplantation in patients who underwent surgery after traumatic lesions.

Materials and Methods

MACI® membrane preparation Cartilage specimens were collected by arthroscopy from non-weight-bearing areas of the knee from 9 patients with traumatic lesions admitted to the Department of Orthopaedic Surgery, University of Trieste, Italy. The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and was approved by our ethics committee.

Bioptic specimens were placed in saline solution and digested with collagenase. The chondrocytes were then isolated and cells cultured in Verigen's (Genzyme Corporation) certified cleanroom laboratories. Cells were seeded on the rough surface of a natural resorbable bovine type I and type III collagen membranes in which differentiated chondrocytes produce autologous matrix to form hyaline cartilage (MACI® patented technique by Verigen Transplantation Services International, Leverkusen, Germany - Genzyme Corporation). Membranes were then returned to the Department of Orthopaedic Surgery, University of Trieste in time for the proposed surgical implantation date.

Cell cultures

Post-surgical MACI® membrane portions, obtained from 9 patients who underwent surgical MACI® implantation, were enzymatically digested in DMEM supplemented with 800 U/mL collagenase type I (Worthington Biochemical Corp, Freehold, NJ-USA), 100 U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL fungizone,

overnight at 37°C, in a 5% CO_2. The resulting cell suspensions were washed and seeded in Petri dishes in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL fungizone at a density of 2×10^5 cells/cm². The chondrocyte population was then expanded for one week so as to obtain a sufficient number of cells for experimental analyses.

Pulsed electromagnetic fields stimulation and viability assessment

Cells obtained from MACI® membrane were exposed to pulsed electromagnetic fields (PEMFs) exposure one week after isolation from the collagen scaffold. Cultured chondrocytes were split into an experimental group and a control group, in the same culture conditions described above, at a cell density of 5×10^4 cells/cm² in Petri dishes. In parallel, cells were seeded on glass coverslips and tested phenotypically before and after PEMFs stimulation, in order to exclude dedifferentiation of cells towards the fibroblastic lineage. The experimental cultures were placed on a plastic platform between two solenoids, i.e. Helmoltz coils, with 17 cm diameter, 12 cm apart, placed parallel to the cell growing surface (Biostim apparatus by Igea, Carpi, Italy). Petri dishes were fixed in two columns between the coils. The solenoids were powered by a Biostim SPT pulse generator (Igea, Carpi, Italy), a generator of PEMFs. The intensity of the magnetic field was 1.6 mT, signal frequency of 75 Hz, and pulse duration of about 1.3 ms (the intensity and the frequency of the exposure was chosen on the basis of the literature data (Fini et al., 2005). The magnetic field was measured with a Hall Effect transverse gaussmeter probe (catalog no. HTD61-0608-05-T, F.W. Bell line, Sypris Solutions, Inc.) and a gaussmeter (catalog no. DG-500, Laboratorio Elettrofisico, Milan, Italy), while the induced electric tension was measured with a standard coil probe and the temporal pattern of the electromagnetic signal was evaluated by a digital oscilloscope (catalog no. LT322, WaveRunner Series, LeCroy, Inc.). Chondrocytes were exposed to PEMFs under different experimental conditions: short term stimulation: 12 h on, 12 h off every 24 hh; and long term stimulation: 4 h/day for 10 days, to better simulate a protocol more suitable in the patient clinical applicable setting. Control cells were placed in a separate incubator under identical incubator conditions. Cells were then recovered at 12 h, in the first experiment, and at different time intervals (3, 5, 7, 10 days) after stimulation with PEMFs for 4h/day. In particular, samples containing 3¥105 cells were harvested by centrifugation at 200g for 10 min at 4°C, fixed with 70% cold ethanol for 1h at 4°C and treated as previously described. Propidium iodide (PI) fluorescence analysis was performed by an Argon laser equipped FACScan flow cytometer using Lysis II software (Becton Dickinson, San José, CA, USA). Cell cycle analysis was carried out as described in Zauli et al. (1994).

Immunohistochemistry

To determine chondrocyte phenotype, small aliquots of MACI® isolated chondrocytes were seeded on glass coverslips and kept in culturing medium. After 24 h of seeding, cells were fixed for 20 min with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) (Sigma) for 10 min and then incubated with the following primary antibodies: (1) mouse anti-collagen type I (1:50) (Sigma), (2) rabbit anti-collagen type II (1:50 (3) mouse anti-chondroitin sulphate (1:100), specific for glycosaminoglican portion of native chondroitin sulphate proteoglycan (Sigma). Coverslips were then incubated with the appropriate secondary antibody: anti-rabbit IgG FITC-conjugated (1:100) or anti-mouse IgG Cy3-conjugated (1:400) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Slides were reacted with 0.1mg/mL 4-6-diamidino-2 phenylindole diluted in 1% Tween 20 (DAPI, Sigma) to detect cell nuclei. The slides were washed three times in PBS (pH 7.4), dehydrated in a graded series of ethanol and finally mounted with a solution containing 2.3% 1,4-diazabicyclo [2.2.2] octane (DABCO; Sigma) to delay fading. Samples were photographed using a Zeiss Axiophot epifluorescence microscope. Digital images were obtained using a Photometric Cool SNAP camera (Roper Scientific, Duluth, GA, USA).

Ultrastructural analysis

Immediately after removal from culture medium, specimens of the residual collagen MACI® membrane, were fixed on the same day of surgery with 2.5% glutaraldehyde in 0.1 M PBS, pH 7.3 (in order to control membrane integrity and chondrocyte morphology. Samples were then rinsed in 0.1 M phosphate buffer, postfixed with 1% osmium

tetroxide, dehydrated in ethanol, then replaced by propylene oxide. Specimens were gradually included in resin (Durcupan ACM, Electron Microscopy Sciences) and polymerized at 60°C. Semithin or ultrathin sections of the cell monolayers were cut with a Reichert Ultracut OM ultramicrotome. Semithin sections were collected on slides, stained with 1% Toluidine blue in 0.5% sodium carbonate, dried, mounted with Canada balsam and photographed using a Zeiss Axiophot microscope.

For transmission electron microscopy (TEM) analysis the sections were counterstained with uranyl acetate for 15 min and with a lead citrate for 5 min and then examined with JEOL 100 S TEM operated at 80 kV.

For scanning electron microscopy (SEM) observation, membranes with cells were fixed with 2.5% glutaraldehyde in 0.1M buffered phosphate (pH 7.3) for 30 min at 4°C, then washed in phosphate buffer and postfixed with 1% OsO_4 in the same buffer for 1h at room temperature. Cells were then washed in distilled water, dehydrated in ethanol, and dried by the critical-point method with a CPD (Balzers Union). The cultures were then sputter-coated with gold and observed under a scanning electron microscope LEIKA Stereoscan 430i.

Statistical analysis

Statistical analysis was performed using the two-tailed Student's T-test.

Implant follow-up and histological analysis of bioptical samples

Six months after surgery, a small bioptic sample of articular cartilage (10 mm^2, 1mm thick) was arthroscopically obtained from the 9 transplanted patients at the site of the MACI® implant (the place where cells were proliferating and the scaffold was metabolised). Fragments were quickly fixed in 4% paraformaldehyde in PBS, and then processed for paraffin embedding; 10 μm thick sections were cut and mounted on slides, deparaffinized and pre-treated with 10mM citrate buffer, pH 6 at 90°C and then allowed to cool to room temperature. The blocking of endogenous streptavidin-biotin sites was performed using 0.3% H_2O_2 in water for 30 min at room temperature. Subsequently, slides were processed following the staining procedure for paraffin sections of Vectastain Universal Elite ABC KIT (Vector Laboratories, Inc. Burlingame, CA). We used as primary antibody a rabbit anti-collagen type II (diluted 1:50; Rockland, Gilbertsville, PA) and mouse anti-collagen type I (diluted 1:50; Sigma). Negative controls were achieved by omitting the primary antibody. Reactivity was detected by incubating sections in the diaminobenzidine (DAB) substrate system (Lab Vision Corporation, Freemont, CA). Slides were counterstained with haematoxylin, dehydrated, mounted with Canada balsam and samples observed under a light microscope.

Results

We first selected the surgical portions of MACI® scaffold implanted in 9 patients with traumatic cartilage lesions to control membrane integrity and the morphological characteristics of MACI®-derived chondrocytes. Hence, in the first series of experiments, we examined 9 MACI® membranes on toluidine blue stained semithin sections (Figure 1A) by light microscopy, and ultrathin sections by TEM (Figure 1B) and SEM (Figure 1C). Of note, in all MACI® membranes, chondrocytes appeared elongated with one or more large nucleoli (Figure 1A) and were distributed in a regular bilayer at the apical sheet of collagen scaffold, without invading the matrix (Narducci, 2005).

Figure 1B shows a representative TEM analysis of an ultrathin section of an MACI® membrane. Most of the chondrocytes were flattened and slightly rounded in shape with abundant REG. Their nuclei showed abundant euchromatin, while heterochromatin was confined at the periphery of the nucleus and adherent to perinuclear membrane, a condition typical of active protein synthesis. Moreover, most of these cells were characterized by vacuoles formation, while native collagen fibrils were present in extracellular matrix.

These observations were confirmed by SEM analysis of MACI® membrane (Figure 1C), a technique that allowed us to appreciate the three dimensional morphology of the construct: the cells appeared to be regularly spread on the scaffold surface and undamaged. A higher magnification showeda homogenous distribution of new formation thin collagen fibers around the cells, evidencing that chondrocytes were undergoing of active metabolic synthesis.

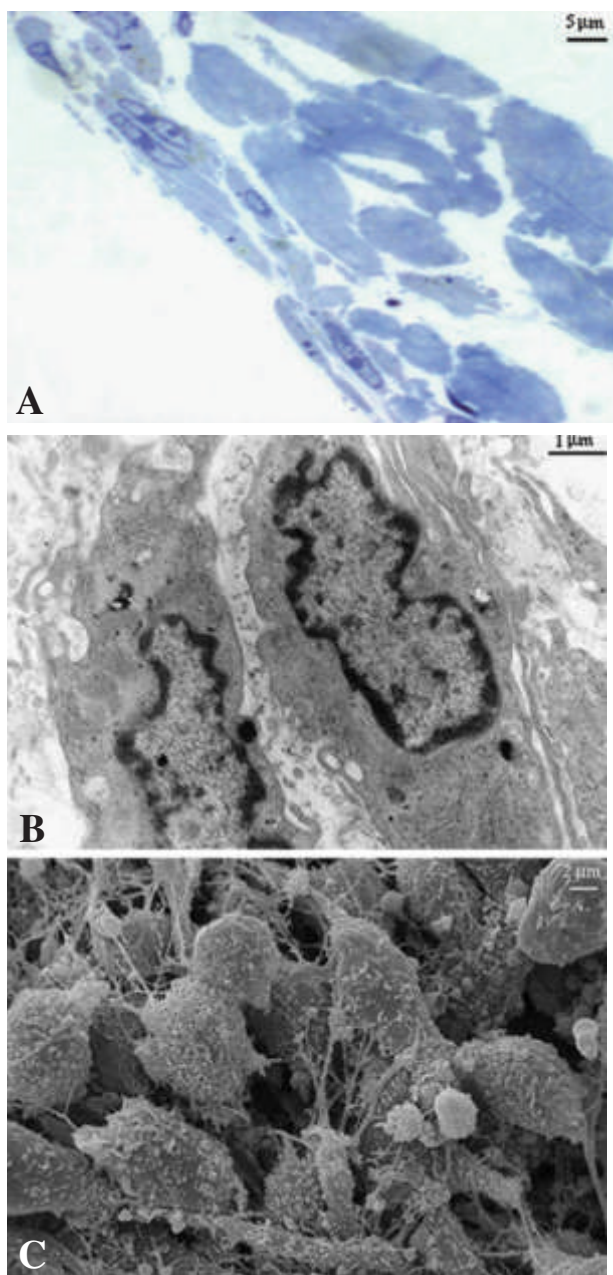Therefore, the morphological investigation on the

Figure 1. Ultrastructural analysis of a MACI membrane. Panel A: a toluidine blue stained semi-thin layer microphotograph of a MACI membrane. Cells appear to be localized on the surface of a scaffold of collagen fibers (Calibration bar: 5 μm). Panel B: transmission electron microscopy (TEM) analysis of chondrocytes of a MACI membrane fragment (Calibration bar: 1 μm). Chondrocytes show an elongated nucleus and a rich REG, typical of intense protein synthesis of matrix components. Panel C: scanning electron microscopy (SEM) analysis of chondrocytes of a MACI membrane fragment. Cells appear to be adherent on the collagen scaffold. New formation of collagen filaments are well evident, interposed with cellular extroflessions, confirming the matrix-formation of these cells (Calibration bar: 2 μm).

interaction between human chondrocytes and the scaffold showed that cells optimally proliferated on the construct surface. The ultrastructural analysis of undamaged cells and the evaluation of their functional integrity revealed that a good stability was supplied by scaffold, giving rise in the meantime to a favourable environment for proliferation of chondrocytes (Frenkel, 1997; Cherubino, 2003).

Effect of PEMFs on survival/proliferation of cultured chondrocytes

To elucidate whether the chondrocytes derived from scaffold may be stimulated to proliferate by PEMFs, we next explored the effect of this treatment on cells by classical flow cytometry.

After the overnight enzymatic digestion of MACI® scaffold, cells obtained form each membrane were cultured for a week in growing medium. The chondrocytes were seeded at low density (5×10^4) andthe effect of PEMFs on cellular proliferation/survival was assessed by propidium iodide flow cytometric analysis. We performed two different PEMFs stimulation approaches: a short term evaluation, based on previous observations (De Mattei, 2001) that chondrocytes need at least 12 h stimulation before evidencing a significant increase of proliferation rate. In this first experimental phase, samples were exposed to two 12 h/day PEMFs stimulation with a 12 h interval in between, and proliferation/apoptosis rate evaluated at different time intervals (12 and 36 h); a long term evaluation, aimed to mimic a protocol suitable with patient compliance. In this second experimental phase, samples were exposed to 4 h/day PEMFs stimulation up to ten days, and proliferation/apoptosis rate evaluated at different time intervals (3, 5, 7, 10 days) (Figure 2). In the first set of experiments, 12 h PEMFs treatment induced a significant increase in SG2M fractions only at 36 h (32±5% vs 19±4%), when compared to control groups ($p<0.05$), with a negligible variation of apoptotic index (Figure 2A).

In the second set of experiments, 4 h/day PEMFs treatment was able to elicit a significant increase in SG2M fractions (12±3% vs 5±2% at day 7 and 8±3% vs 2±1% at day 10) at days 7-10 after stimulation when compared to control groups ($p<0.01$). Moreover, these cell populations showed no significant changes in the apoptotic index. These data confirm that the positive effect of PEMFs on cell growth is lower especially from day 7 and 10 (the meaning of this sentence is not clear) (Figure 2B).

This protocol thus evidenced a positive effect of PEMFs on chondrocytes proliferation, suggesting
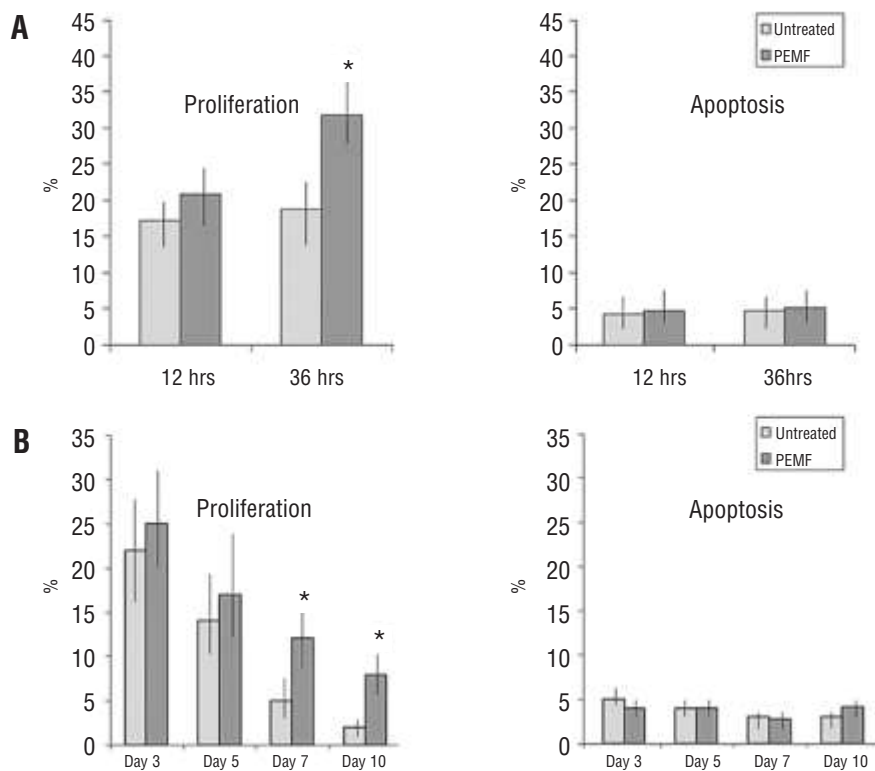
Figure 2. SG$_2$M cells and apoptotic fractions of chondrocyte cultures exposed to a PEMFs stimulation of 2G. Cells were stimulated for i) 12 h on and 12 h off every 24 h (panel A); and ii) 4 h/day for 10 days (panel B). In Panel A, cell cycle was assessed by flow cytometric analysis after 12 h and 36 h after stimulation: in Panel B cells were collected at 3, 5, 7 and 10 days of culture. Data are expressed as means ± standard deviation (SD) of all samples performed in duplicate.

that treatment with PEMFs can be a promising clinical setting for faster cartilage healing and reassembly.

Immunocytochemical characterization of chondrocytes

In order to rule out the possibility of a de-differentiation of MACI®-derived chondrocytes to a fibroblastic type after a week in monolayer culture and PEMFs treatment, small aliquots of MACI® isolated chondrocytes were seeded on glass coverslips, divided into experimental and control groups and analyzed phenotypically before and after 10 days of PEMFs exposure (long term conditions) by indirect immunofluorescence assay (Figure 3).

A week after isolation from MACI® membrane, cultured cells synthesized chondroitin sulfates type A and C, and the specific chondrocyte marker type II collagen but not collagen type I (*not shown*), which is a typical marker for fibroblasts. No cell staining was observed when the analysis was carried out with unspecific isotype antibodies (not shown). In particular, chondroitin sulfate types A and C, and type II collagen immunofluorescence staining was prevalent in cell cytoplasm associated with a punctuated distribution in the extracellular matrix, confirming the synthetic condition of these

cells. In detail, chondroitin sulfate types A and C, classically exclusively Golgi-derived, showed a punctuated distribution, probably in vesicles, both in the cytoplasm and the extracellular matrix. After 10 days of PEMFs treatment, the cell phenotype was maintained both in the experimental and in control group, as shown in Figure 3.

Follow-up analysis of implanted tissue

In order to evaluate MACI® long-term efficacy on hyaline cartilage reconstruction, we performed a bioptic follow-up analysis of specimens of cartilage obtained from patients, 6 months after surgical transplantation. Cartilage samples were fixed in paraffin and section analyzed for collagen type I (Figure 3G-H) and type II (Figure 3I-J) by immunocytochemistry assay. The samples have the histological appearance of hyaline cartilage, with the presence of typical isogenic groups dispersed in a well-organized extracellular matrix.

Discussion

Cartilage is a tissue characterized by a limited regenerative and healing capacity. As a result, an articular lesion often leads to the formation of fibrocartilagenous repair tissue lacking the structural and biomechanical properties of hyaline carti-
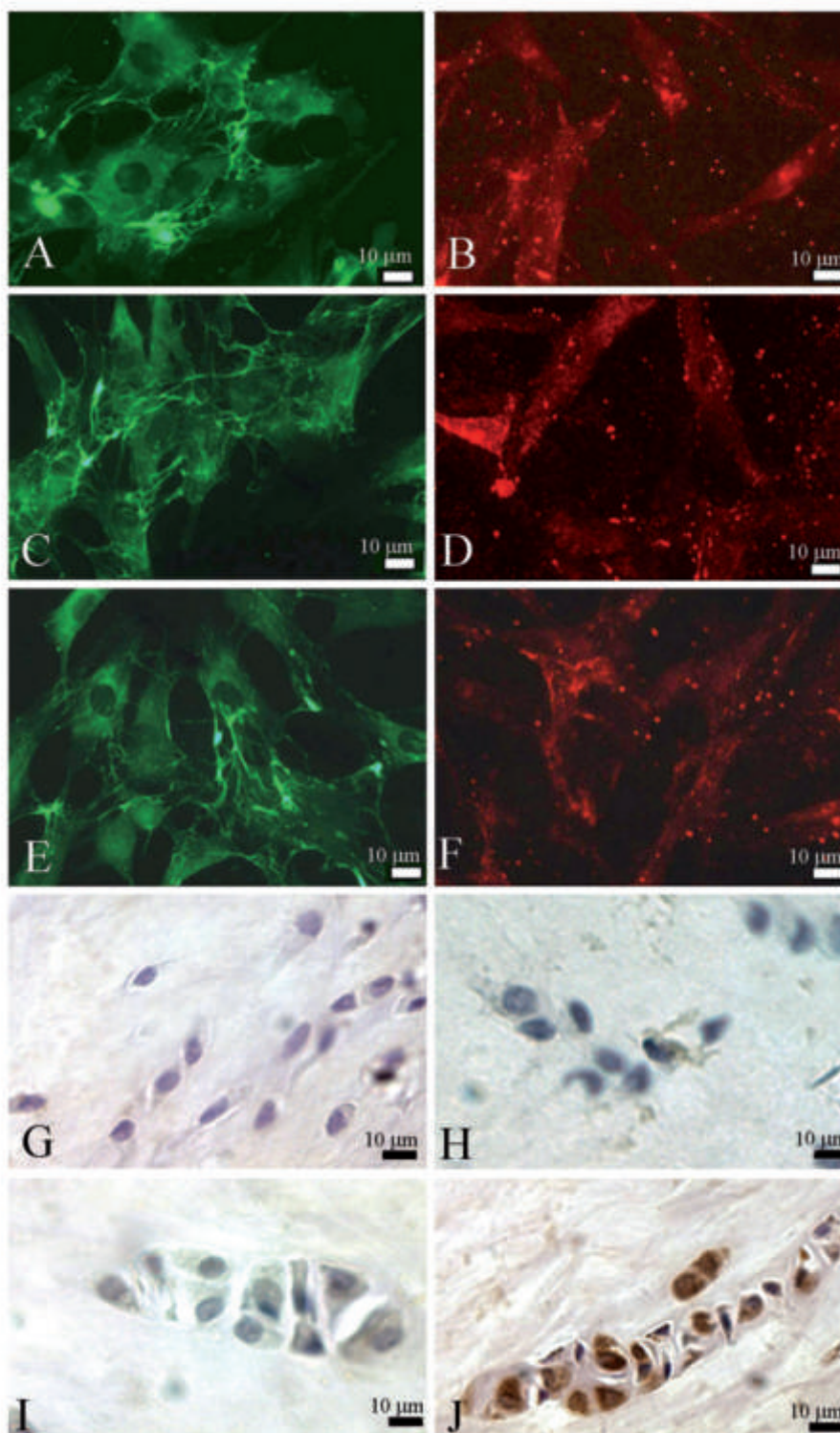
Figure 3. A-F) Immunocytochemical characterization of chondrocytes following PEMFs treatment: MACI-derived cells were cultured for a week and then analyzed by immunocytochemistry before (day 0) and after PEMFS exposure (Day 10) under long-term study conditions (4hrs/day for 10 days). Cells confirmed to synthesize chondroitin sulfates and a specific chondrocyte marker such as type II collagen all treatment long, both in experimental (+PEMFs) and control group (-PEMFs). For immunofluorescence analysis, cells were stained with anti-type II collagen and anti-type A, C chondroitin sulfates primary antibodies. Secondary antibodies were FITC-conjugated (green fluorescence) and Cy3-conjugated (red fluorescence), respectively. G-J) Follow-up analysis of implanted tissue: Cartilage sample were obtained from patients transplanted with MACI membrane 6 months after surgery. Cartilage samples were fixed in paraffin and section analyzed for Collagen type I and type II by immunocytochemistry. Figure shows a representative bioptical sample. Panel G-I : negative controls. Panel H: The reaction to collagen type I is negative showing the absence of connective cells. In panel J: The reaction to collagen type II, marker of chondrocytes, result to be positive in the cells of typical isogenyc groups that are condrocytes.

lage, signifying that the implantation of autologous chondrocytes is pivotal to overcome the limitations of traditional surgical techniques. Indeed, the theoretical expectations have been supported by good clinical and functional results in patients. The implantation technique gives stable and effective long-term results in 84-90% of patients with different types of lesions (Steinwachs, 1999).

The most significant stage in the development of these autologous chondrocyte implantation methods is represented by MACI®, a bioengineering tissue technique in which a resorbable three-dimensional scaffold of type I-III collagen is used to improve the structural and biological properties of the implant: a better stability is guaranteed, giving rise at the same time to a favourable environment for the proliferation and differentiation of chondrocytes (Grande, 1999). Of note is that the collagen membrane for autologous chondrocytes culture offers the advantage of being applied using exclusively fibrin glue without having to suture. This allows the surgeon to expose the chondral lesions through minimal invasive approaches, where suturing is virtually impossible.

An important limit of autologous implantations has been pinpointed by different studies which showed that, during *in vitro* expansion, autologous cultured cells can de-differentiate to fibroblastic cells, causing the formation of fibrous connective tissue with failure of surgery (Brittberg, 2001; Benya, 1982, Buschmann, 1992). For this purpose, Zheng *et al.* (2004) focused on the assessment of the phenotype of dedifferentiated autologous chondrocyte phenotype used in ACI implants and confirmed the maintenance of the chondrocytic markers of the implanted cells. Since de-differentiation maintenance is a delicate balance of biological factors, we investigated whether the introduction of a three-dimensional collagen scaffold could influence the chondrocytic phenotype. The data reported here demonstrated that in patients who underwent surgery, the MACI®-derived cells showed all of the proliferating chondrocytes morphology and lineage markers as analyzed by ultrastructural TEM and SEM analysis of membrane portions and immunocytochemical analyses. Moreover, we did not detect any de-differentiated cells such as fibroblasts, that would elicit an ineffective fibrotic synthesis with failure of the MACI approach. Chondrocytes isolated from the scaffold maintained their phenotype, even after a week in culture followed by long-term

PEMFs treatment (10 days).

In the last years, several studies reported often contradictory effects of PEMFs stimulation on chondrocyte proliferation *in vitro* (Liu, 1997; Hiraki, 1987; Pezzetti, 1999; Indouraine, 2001; Sakai, 1991; De Mattei, 2004), which depended on several factors. De Mattei *et al.* (2004) reported the dependence of the PEMFs-induced proliferative effects on cell culture density while Pezzetti et al. (1999) indicated a pivotal role of the serum concentration in the culture medium, suggesting a serum growth factor-dependant proliferation. Another factor related to PEMFs, proliferative effect is the contemporary administration of anabolic (IGF-I) and pro-inflammatory (IL-1,) cytokines (Fini, 2005; De Mattei, 2003; Fioravanti, 2002). Moreover, with respect to PEMFs stimulation conditions, proliferation depended on exposure time, the frequencies of the magnetic fields and the duration of the experiment. Other reports showed that PEMFs stimulation improved articular functionality in patients with chronic joint diseases (Jacobson, 2001; Nicolakis, 2002; Trock, 1003).

It has been demonstrated that biophysical stimulation induced human chondrocyte proliferation (Pezzetti, 1999) and anabolic activities in bovine cartilage explants (De Mattei, 2003). As reviewed in Fini (2005), different clinical studies emphasized the efficacy of PEMFs in provoking significant improvement of joint motion and tenderness, with a decrease in the pain level. However, to our knowledge there are no available data regarding PEMFs stimulation on chondrocytes isolated from MACI. Therefore, we investigated whether the introduction of a three-dimensional collagen scaffold could influence not only differentiation, but also cell proliferating activity and therefore affects possible therapeutic treatments. We tested chondrocytes isolated from collagen membranes after surgical intervention of patients undergoing MACI® implant subsequent to traumatic cartilage lesions. The data showed a significant increase of SG2M cell number in PEMFs treated cells i) in a short term study: at 36 h (with 12 h PEMFs stimulation every 24 h), and ii) in a long term study: at days 7 and 10 of 4 h/day PEMFs treatment.

In addition, PEMFs stimulation did not elicit cell toxicity, the apoptosis rate being negligible. Of note, there was a clear time-dependent decrease in SG_2M of the cells and the positive effect of PEMFs exposure was observed when the cell growth was lower

(day 7 and 10). It is also worth noting that, the induction of cell proliferation by 4 h/day stimulation may be suggested as a promising clinical application for articular healing from the 7th day of treatment onwards.

In conclusion, the MACI® technique can be considered as the most promising and favourable technique in articular cartilage damage repair, and PEMFs stimulation represents a positive physiotherapeutic application after surgery for faster healing and collagen matrix reconstruction.

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