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Protective effects of polydeoxyribonucleotides on cartilage degradation in experimental cultures

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The capacity of cartilage self-regeneration is considered to be limited. Joint injuries often evolve in the development of chronic wounds on the cartilage surface. Such lesions are associated with articular cartilage degeneration and osteoarthritis. Re-establishing a correct micro/macroenvironment into damaged joints could stop or prevent the degenerative processes. This study investigated the effect of polydeoxyribonucleotides (PDRNs) on cartilage degradation in vitro and on cartilage extracted cells. The activities of matrix metalloproteinases 2 and 9 were measured in PDRN-treated cells and in controls at days 0 and 30 of culture. Human nasal cartilage explants were cultured, and the degree of proteoglycan degradation was assessed by measuring the amount of glycosaminoglycans released into the culture medium. The PDRN properties compared with controls were tested on cartilage tissues to evaluate deposition of extracellular matrix. Chondrocytes treated with PDRNs showed a physiological deposition of extracellular matrix (aggrecan and type II collagen: Western blot, IFA, fluorescence activated cell sorting, Alcian blue and safranin O staining). PDRNs were able to inhibit proteoglycan degradation in cartilage explants. The activities of matrix metalloproteinases 2 and 9 were reduced in all PDRN-treated samples. Our results indicate that PDRNs are suitable for a long-term cultivation of in vitro cartilage and have therapeutic effects on chondrocytes by protecting cartilage. Copyright © 2012 John Wiley & Sons, Ltd.
key words—polydeoxyribonucleotides (PDRNs); hyaluronic acid (HA); cartilage; connective tissue; tissue regeneration

INTRODUCTION

Several studies have emphasized the importance of extracellular nucleotides and nucleosides in cell growth[1,2,4]. However, it has been known for some years that oligodeoxynucleotides may have non-sequence-specific effects on cellular function[1,2]. These may result, at least in part, from their ability to bind cellular proteins. Phosphorothioate oligodeoxynucleotides appear to bind non-sequence specifically to rsCD4, gp120 and protein kinase C[1]. Oligonucleotides can bind to heparin-binding proteins, above all basic fibroblast growth factor (bFGF), with low nanomolar affinity. This binding has been recognized in a length- and concentration-dependent manner[1,2]. Polydeoxyribonucleotides (PDRNs), a mixture of deoxyribonucleotides polymers of different lengths (ranging between 50 and 2000 bp), have recently demonstrated to mobilize bFGF from their depot or storage sites on endothelial matrix and protect bFGF from digestion by trypsin and chymotrypsin and from air oxidation[3]. PDRNs can also induce in vitro fibroblast proliferation, collagen production and vascular endothelial growth factor expression[2,4]. Adenosine receptor stimulation can induce vascular endothelial growth factor expression in many types of cells, and this may be achieved by stimulating the A(2A) or A(2B) receptor or both[4,5]. Experimental models suggest that PDRNs may bind to the vascular endothelium, modulate platelet activity, promote fibrinolysis, decrease thrombin generation and reduce circulating levels of plasminogen activator inhibitor type 1 with enhancing endothelial cell survival[6,7]. It is known that PDRNs mobilize tissue-factor pathway inhibitors from endothelial cells and increase prostaglandin E_2 plasma concentrations, which inhibits platelet aggregation[8,9]. Only minimal anticoagulant activity, but potent antithrombotic and pro-fibrinolytic effects both in vitro and in vivo can be observed after PDRN administration[10–12]. Thus, these molecules are used in therapy as a tissue repair stimulating agents in diseases characterized by a loss of substance, in hind limb ischemia and in burn injury[13,14]. Yet, PDRNs potently down-regulated heparanase, which is overexpressed in multiple myeloma plasma cells and is also correlated with progression and metastasis in solid tumors[3]. Furthermore, PDRNs bind to collagen I with low nanomolar affinity and can promote cell mitogenesis and tubular morphogenesis in three-dimensional (3D) collagen-I gels[15]. As a result, PDRN treatment significantly can ameliorate clinical signs of arthritis and can reduce circulating levels of High Mobility Group Box-1 (HMGB-1), tumour necrosis factor α and cytokine

production from stimulated chondrocytes[16]. PDRNs could contribute to the restoration of a correct osmotic microenvironment in the articular cavity, but it is pharmacologically an extremely complicated agent, and its effects may be highly dependent on the extracellular or articular microenvironment[17]. Changes in the osmotic environment cause changes in volume of isolated cells and cells in tissue explants, and the osmotic environment becomes hypotonic in cartilage diseases such as osteoarthritis (OA)[18]. However, it is not known how cells respond to a hypotonic osmotic challenge when situated in the fully intact articular cartilage. [18] One fundamental key of the regeneration process can be the reconstitution of physic and physiological microenvironment in pathological context. Advances in cellular biotechnology may lead to in vitro restoration of the physiological tissue microenvironment.[4]

OA, also known as degenerative arthritis or degenerative joint disease, is a group of mechanical abnormalities involving degradation of joints, including articular cartilage and subchondral bone. When bone surfaces become less well protected by cartilage, bone may be exposed and damaged.

Pain may profoundly alter the articular movement. Treatments generally involve a combination of exercise, lifestyle modification and analgesics. If pain becomes debilitating, joint replacement surgery may be used to improve the quality of life. The fibro-cartilage, as repair tissue, has different biochemical and bioelastic characteristics from those of hyaline cartilage. The healing of the lesioned articular cartilage is a particularly challenging problem. The use of molecules capable of stimulating the proliferation and differentiation of chondroblast into chondrocytes is well known.[19,20] Such molecules are amino acids, vitamins, sugars, peptides or various proteins, growth factors, collagen, proteoglycans, glycosaminoglycans [GAGs; i.e. hyaluronan or hyaluronic acid (HA)].[21–24] To date, surgical treatments have attempted to induce growth at the site of the defect using chondroplasty/chondroabrasion techniques, with or without subchondral perforation or micro-fracture.[25–29] Results obtained by such methods provide a delay in the development of the progressive arthritic process.[30–35] The typical articular cartilage injury response often causes collateral damage, primarily an effect of inflammation, which results in the spread of lesions beyond the region where the initial injury occurs.[36] The diffusion of inflammatory cascade determines the spatial behaviour of injury response and lesion expansion. The concentration of pro-inflammatory cytokines compromises the densities of cartilage cells. The number of chondrocytes is a natural way to repair cartilage injury response[36] and for the dissemination of inflammatory reaction involving cartilage microenvironment with tissue degeneration and OA. Re-establishing a correct micro/macro-environment into damaged joints could stop or prevent the degenerative processes.

To better simulate the growth and possible repair of cartilage tissue, we have researched the principle of a correct biophysiological microenvironment. Normally, the joint microenvironment promotes the exchange of nutrients by diffusion in special conditions of osmotic pressure. This mechanism allows the secretion of synovial fluid viscous, transparent, lubricating and nourishing. In turn, when the synovial fluid present in the cavity is healthy, it allows to control and maintain stable some parameters such as the osmotic pressure, viscosity and nutrition of the articular cartilage. With PDRN formulation, we have attempted to recreate a physiological synovial fluid. First, the new fluid was tested on chondrocytes without extracellular matrix (ECM) deposition in vitro. Cell cultures treated with the new formulation have deposited new ECM and restored the conditions to support the development of healthy cartilage. In physiological conditions of microgravity and osmosis using the principle of selective viscosity on cell culture, we observed that this innovative PDRN formulation is suitable for a long-term cultivation of in vitro cartilage cell.

The in vitro models are useful to research, find and apply an appropriate microenvironment to cartilage recovery. This study was focused on the research of a proper microenvironment to re-induce the cell physiological functions in injured cartilage. Different culture media (PDRNs, HA, laboratory culture medium specific for cartilage and negative controls) were tested on explants of cartilage or derived cells. HA represents a positive control with clearly established in vivo efficacy. To date, it is the main device used in vivo to restore the appropriate intra-articular viscosity. Laboratory culture medium specific for cartilage represents an elective positive control for in vitro activity. It is generally used for in vitro cartilage cell culture. Negative controls were prepared consisting of non-specific cell culture media or cell culture media without supplements. PDRNs, positive controls and negative controls were compared to promote the eutrophy of chondrocytes and a correct ECM deposition.

MATERIALS AND METHODS

Compositions

Cell culture medium compositions, as describe in Table 1.

PDRNs (20 $mgml^{-1}$; Condrotide™(MASTELLI SRL, Via Bussana Vecchia, 32 -18038 Sanremo (IM) Italy) were obtained from sperm trout by an extraction process.[37,38] In sample 1a, 2% PDRNs samples were treated with PDRNs (Condrotide™) at final concentration of 2% without diluents. In Sample 1b: 1% PDRNs samples were treated with PDRNs (Condrotide™) at final concentration of 1% (10 $mgml^{-1}$ PDRNs supplemented with culture medium).

HA(or poly(β-glucuronic-acid-[1→3]-β-N-acetylglucosamine - [1→4]) alternative; Hyaliaphase™, Roma, Italy) was obtained from bio-fermentation bacteria, molecular weight 1000 kDa and composition based on 20 mg ml^{-1}. [39,40] In the positive control 1a, 2% HA samples were exclusively treated with HA (Hyaliaphase™) at final concentration of 2% without diluents. In sample 1b, 1% HA samples were treated with HA (Hyaliaphase™) at final concentration of 1% (10 $mgml^{-1}$of HA supplemented with culture medium).
Positive control—two samples was exclusively treated with cartilage culture medium.
Negative controls—one sample was treated with common cell culture medium Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, Grand Island, NY) and supplements.
Negative controls—two samples were treated with common cell culture medium DMEM (Gibco) without foetal bovine serum.
Negative controls—three samples were treated with free culture medium DMEM (Gibco) without supplements.

Biopsies

Cartilage tissues were obtained during functional surgery from healthy adult volunteers. Twenty samples of cartilage were placed in 100-mm plates (Lab-Tek Chamber Slides, Nunc, Kamstrup, Denmark) with 10 ml of unsupplemented DMEM low glucose medium (Gibco). Using sterile forceps and scissors, the samples were cut into two big parts (approximately an inch square of overall tissue for each part): one part for tissue analysis at time zero of study and one part for cell studies at end time. Sections were washed twice inside the plates. Each part of biopsies was cut into sixteen small pieces (two 2% PDRN samples 1a, two 1% PDRNs samples 1b, two 2% HA positive controls 1a, two 1% HA positive controls 1b, two positive controls 2, two negative controls 1, two negative controls 2 and two negative controls three). Samples and controls were suspended in 10 ml of solutions as describe in Table 1 and placed into 60mm dishes (Nunc).
The biopsies were incubated for 30 days. All the controls and samples were placed into a Heraeus thermostatically controlled incubator at a temperature of 37°C in an atmosphere containing a constant flow of 5% CO_2 (v/v in air, CO_2 volume on air volume) with 6 ml of the culture medium being replaced every 5 days. After 30 days, one portion of biopsies for each condition studied was removed using sterile forceps and was transferred into a 100-mm plate (Nunc), with 10 ml of DMEM (Gibco) unsupplemented and 1$mgml^{-1}$ collagenase (Sigma, St. Louis, MO, USA). All samples were incubated for 2 h in a Heraeus thermostatically controlled incubator at a temperature of 37°C in an atmosphere containing a constant flow of 5% CO_2 (v/v in air). After incubation, 4 ml of foetal bovine serum was added to the samples to quench the collagenase activity. All the solution with the undissolved pieces and the cells in suspension were collected and centrifuged at 160 g for 10 min, at room temperature. The pellet was resuspended in unsupplemented DMEM low glucose (Gibco) and washed twice by centrifugation at 160 g for 10min. The cells were tested to verify the presence of aggrecan and type II collagen by using the fluorescence activated cell sorting (FACS) and Western blot analysis, as described in the following sections. Yet at day 30 of incubation, the second part of all biopsies were fixed (4% formalin) and tested to verify the presence of cartilage ECMusing colorimetric method with Alcian blue and safranin O, as described in the following sections.

Preliminary cell suspensions

The cells, obtained from tissue biopsies, were resuspended in 15 ml tubes at a final concentration of $5x10^5$ cells ml^{-1} for subsequent phenotypic analysis.

Trypan blue staining

The cell suspensions were incubated with 5% trypan blue (Sigma-Aldrich, Milan, Italy) for 5 min at room temperature to analyze cell viability (dead cells should be coloured by the blue dying). After incubation, 10 ml of the stained cell solution was counted in specific chamber with a light microscope at 20x and 40x sequential magnitudes.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) staining

Cells viability was also estimated with MTT assay. The treatment of the cells was performed in a 96-well plate (NUNC), assigning six wells for each experimental condition, at a density of 15,000 cells per well, in a final volume of 200 ml per well containing MTT at a final concentration of 0.5$mgml^{-1}$. The plate was placed into a thermostatically controlled Heraeus incubator at a temperature of 37°C under an atmosphere with a constant supply of 5% CO_2 (v/v in air) for 30min. Once the incubation was completed, the medium was removed from the wells by gentle suction with a multichannel pipette. After having ascertained that no traces of solution were still in the wells, 100 ml of dimethyl sulfoxide was added to each well, and the plate was

shaken for 10 min at room temperature to melt the formazan salt crystals.[41] The cell viability was quantified by reading the absorbance with a spectrophotometer for ELISA plates, at a 570-nm wavelength.
Each experiment was repeated at least three times, and the data were expressed as the percentage of the control, wherein the ability to reduce MTT was taken as 100% viability.

Western blot analysis

The cells were lysed in Laemmli buffer with two rounds of sonication and boiling. Extracted proteins were electrophoretically separated, transferred to poly-vinylidene-difluoride membranes immobilon P (Millipore, Bedford, MA, USA) and probed with different antibodies in PBS 0.1% Tween solution with 5% blocker no-fat dry milk (Bio-Rad Laboratories; Hercules, CA, USA): 1:1000 anti-type II collagen, anti-aggrecan and anti-actin (Santa Cruz Biotechnology). After overnight incubation, the membranes were washed for 1 h with PBS–Tween and subjected to a peroxidaseconjugated antimouse IgG antibody (Pierce, Rockford, IL, USA) in PBS 0.1% Tween solution with Blocker non-fat dry milk 5%. After washing, proteins were detected by autoradiography, using Super SignalWest Pico Chemiluminescent Substrate Kit (Pierce).

Fluorescence activated cell sorting

Cell suspensions obtained after collagenase digestion were transferred into 15ml tubes (Nunc, Kamstrup, Denmark) and fixed in a 4% paraformaldehyde solution for 30 min at room temperature. After washing three times with PBS (pH 7.4), the cells were permeabilized with 0.1% Triton for 15min at room temperature. The pellets were resuspended at a final concentration of $5x10^5$ cells/ml in PBS and incubated with the following primary antibodies (AbD SEROTEC, Kidlington-Oxford, UK): anti-type II collagen, anti-aggrecan, anti-CD 29, anti-CD 44, anti-CD 71, anti-CD 90 (THY-1), anti-CD 105 (endoglin) and anti-Stro-1. All the used antibodies were monoclonal (MAbs), conjugated to R-phycoerythrin or fluorescein-isothiocyanate. Specific controls with the corresponding isotypes were divided for each monoclonal antibody (AbD SEROTEC). The samples were subjected to quantitative analysis using a laser cytofluorimeter (Epics Profile II; Coulter, Hialeath, FL, USA) at 488nm and referred to the percentage of fluorescent cells as the geometric mean. Threshold values (gates) were established using control samples labelled with the corresponding isotypes. All values were analyzed with a minimum threshold of 15,000 cells.

Immunofluorescence

Immunofluorescent labelling was performed on cells cultured on chamber slides. Cells were fixed in 4% paraformaldehyde solution for 1 h at room temperature and then permeabilized with 0.1% Triton for 30min. Unspecific binding sites were blocked with 20% normal goat serum (Sigma Chemical) for 1 h, followed by incubation for 30 min (min) with the following fluorescein-isothiocyanate-conjugated MAbs: anti–type II collagen, anti-aggrecan, anti-CD 29, anti-CD 44, anti-CD 71, anti-CD 90 (THY-1), anti-CD 105 (Endoglin receptor) and Stro-1 (Mesenchymal Progenitor Cells receptor) (AbD SEROTEC). Specific controls with the corresponding isotypes were prepared (AbD SEROTEC). Nuclei were stained with Hoechst solution (Sigma Chemical). Samples and controls were examined under a Leica DM-LA fluorescence microscope (Leica Microsystems, Heidelberg, Germany).

Tissue sections: histological processing and staining

After 30 days of incubation, tissue sections prepared for histology were fixed (4% formalin) before processing. All samples were processed to paraffin using a Leica TP1020 Tissue Processor and then embedded into paraffin blocks (FFPE) using a Sakura Tissue-Tek VIP-5 Embedding station (Sakura Finetek, Tokyo, Japan). Cartilaginous tissue was embedded to provide cross sections of all cartilage layers.
Two non-serial 3-mM sections per tissue block were cut using a Sakura Tissue-Tek SRM-200 (Sakura Finetek) microtome and mounted onto a single slide. All the samples and controls were dried overnight at 37° C before staining.
Sections of the biopsies were formalin fixed, paraffinembedded and stained with anti–type II collagen and antiaggrecan (Biocare, California, USA).

Tissue sections: haematoxylin and eosin staining

Tissue sections were stained with a haematoxylin and eosin kit (Sigma-Aldrich). One slide from each block was dewaxed, rehydrated through a descending series of alcohol concentrations, rinsed in water and then stained with haematoxylin and eosin using a Leica Autostainer XL (haematoxylin for 60 s and eosin for 90 s); sections were then dehydrated, cleared and mounted. Slides were viewed using a Zeiss Axiophot microscope and images taken using RT SPOT Imaging system.

Tissue sections: type II collagen and aggrecan staining

Tissue paraffin sections required deparaffinization. After deparaffinization, tissue partswere treated at room temperature with 0.4mgml^{-1} proteinase K for 5 min and washed with PBS for 10 min. Tissue sections were submitted to endogenous peroxidase blocking (0.3% hydrogen peroxide-containing methanol) for 30 min. Sections were washed with PBS (pH 7.4), and a non-specific block was performed using the blocking reagent (Blocker™ BSA in PBS; Takara bio Inc., Otsu, Shiga, Japan) for 30 min. The tissues were subsequently washed in PBS for 10 min and incubated with primary antibody (mouse MAbs anti-human-type II collagen and anti-human-aggrecan; Biocare) at room temperature for 60 min. Samples were rinsed with PBS and washed three times (5min for each washing). The secondary antibodies were added (peroxidase-labelled antimouse IgG antibody) onto the tissue sections. Biopsies were incubated at room temperature for 30 min. After the reaction, samples were rinsed with PBS and washed for 5 min 3 times. DAB substrate solution (Takara Bio Inc.) was placed on the tissue sections, and the samples were incubated at room temperature for 15 min (colour development). Sections were washed under running distilled water. Counter staining (methyl green) was performed for 5min; thereafter, sections were de-stained with absolute ethanol until reaching an appropriate colour. Samples were dehydrated, penetrated and mounted.

Alcian blue staining

Cells and tissue sections. For Alcian blue staining culture, flasks were rinsed with PBS and fixed in a 4% paraformaldehyde solution for 1 h at room temperature. After three 10-min washes at room temperature in PBS (pH 7.4), the samples were stained with 1% Alcian blue (Sigma Chemical) in a 3% acetic acid solution (pH 2.5). After 2 h of incubation at room temperature, this composition permanently stained acidic mucopolysaccharides and glycoproteins both sulfonated and carboxylated. Specific controls were set up for each sample. All samples were washed three times with PBS (pH 7.4) at room temperature for 5 min and then observed with light microscopy (Leica Microsystems).

Safranin O stain

Cells and tissue sections. All samples were washed for 10 min at room temperature in PBS and resuspended for 1 h at room temperature in a fixing solution containing 4% paraformaldehyde in RPMI 1640 medium. Samples and controls were treated with safraninOstaining (Sigma, St. Louis, MO, USA) for 15 min at room temperature. Specific controls were prepared for each sample. All samples were washed three times with PBS for 5 min at room temperature and then observed under light microscopy (Leica Microsystems).

GAG extraction and quantification.

In all the studied conditions, four washes were performed with saline: 15 ml buffer of 0.1M Na-acetate + 0.005M EDTA pH 7.5 + crystallized papain (1mgml^{-1}; Calbiochem-Merck Chemicals Ltd., Nottingham, UK) +N-acetylcysteine (3.2mgml^{-1} buffer) were added to sections. Samples and controls were incubated at 60 °C for 24 h. Then, a buffer of papain (1mgml^{-1}) and N-acetylcysteine (3.2mgml^{-1}) was added, and the tissues were again incubated at 60 °C for 24 h. Subsequently, the supernatant was brought to 50% per trichloroacetic acid (160 ml/ml) for 1 h at 4 °C. After centrifugation at 10,000 rpm for 15 min, the supernatants were collected and added into two volumes of absolute ethanol + 100% solution of potassium acetate (10 ml potassium acetate/ml ethanol). They were stored overnight at -20 °C.
All samples were centrifuged at 12,000 rpm for 20min at 4 °C. Pellets were suspended in 300 ml of distilled water, centrifuged at 12,000 rpm for 10min and resuspended in 50 ml of distilled water. The GAGs were analyzed quantitatively by the carbazole method, using glucuronolactone as a standard (40mg/100 ml solution in water saturated with benzoic acid). Three millilitres of sulfuric acid at 96% and 0.025M sodium tetraborate were placed in Pyrex tubes at -70°C. The samples were prepared on ice in 0.55ml of water saturated with benzoic acid. The samples were placed in boiling water for 10 min and allowed to cool to room temperature.
Added to all the samples was 0.1 ml of carbazole (0.125% carbazole in absolute ethanol), and the tubes were placed in boiling water for 15 min, again allowed to cool to room temperature and analyzed in a spectrophotometer at 530 nm.

Statistical analysis

All results were analyzed by multiway ANOVA and by oneway ANOVA followed by post hoc Tukey's test correction using Student's t-test, comparing the values of corresponding Markers, in samples and controls (negatives and positives) into the same experiment and among repeated experiments.

A level of $P<0.05$ was considered statistically significant.

RESULTS

Cell viability and mortality

Biopsies and cells were maintained in different culture media (Table 1) for 30 days. The viability of extracted cells was evaluated by trypan blue (Table 2) and MTT staining (Table 3). At day 0 of incubation, the viability and the morphology of cells were found equal both in all samples and in all controls. At the 30th day of incubation, the viability of chondrocytes was high in the 2% PDRN-treated samples 1a, 1% PDRN-treated samples 1b and positive control 1b (1% HA) and low in the positive controls 2; 80% of the positive control 1a (2% HA) and 90% of all negative control cells were detected dead.

Western blot

After 30 days of culture, the biopsies were dissolved. Cells were extracted and lysed. The expression of actin, mature type II collagen and aggrecan was evaluated. Densitometric analysis revealed that the protein bands were denser in PDRN samples than that in controls (Figure 1).
Fluorescence activated cell sorting Intracellular type II collagen, aggrecan, CD 29, 44, 71, 90 and 105 and Stro-1 expressions were evaluated in all controls and samples after 0 and 30 days of incubation. At time 0, FACS analysis showed moderate expression of type II collagen and aggrecan in all tested cell cultures. At the 30th day of incubation, type II collagen and aggrecan expressions (Figure 2) were significantly lower in negative controls 1, 2 and 3 with respect to positive controls 2 and positive Controls 1b (1% HA). On the other hand, all data (Table 4 and Figure 2) on positive controls 1a and 2 were extremely lower than those with the expression of type II collagen and aggrecan in all PDRN-treated samples and in positive controls 1b treated with 1% HA. The samples treated with all PDRN culture media showed high levels of the type II collagen and aggrecan expression (Table 4 and Figure 2) at day 30 of incubation. In contrast, the positive controls 1b treated with 1% HA Culture media showed high levels of CD 29, 44, 71, 90, 105 and Stro-1 expression (Table 4) at day 30 of incubation.
These results demonstrated that the cells from biopsies treated with both 2% PDRN culture media and 1% PDRN culture media had the ability to proliferate, differentiate and produce cartilage ECM proteins. However, the type II collagen and aggrecan expressions were significantly higher in samples 1% PDRNs than that in samples 2% PDRNs. Moreover, data showed that the cells from biopsies treated with 1% HA culture media had the ability to reproduce cartilage immature markers.
Flow cytometric analysis revealed that the CD 29, 44, 71, 90, 105 and Stro-1 were expressed at Time Zero in all samples and controls (Table 4).
After 30 days of incubation (Table 4), the same markers were
• not detected in negative controls 1, 2 and 3,
• expressed in the positive controls,
• much expressed in cells treated with 1% HA culture media, contemporary with a high positivity for type II collagen and aggrecan,
• not expressed in all PDRN-treated samples, contemporary whit a high positivity for type II collagen and aggrecan.

Immunofluorescence

At the 30th day of incubation, cells extracted from biopsies were examined. Immunocytochemical analysis of chondrocytes isolated from biopsies revealed that all the samples treated with 1% PDRN culture medium had the increased level of II collagen and aggrecan expression (Tables 5) compared with other samples and controls.

Tissue sections: haematoxylin and eosin staining

After 30 days of incubation, all samples and controls initially provided for histology were stained with haematoxylin and eosin. The negative controls 1, 2 and 3 and 2% HA-treated samples appeared to be in a degenerative state. The positive controls presented a morphology with a fibrous tissue containing many vesicle structures. Thereby, collagen fibres were still clearly found in positive controls, but most of the normal structures of the cartilage were vanished with the loss of ECM. The collagen fibres were clearly visible in the 1% HA samples and 2% PDRN samples. Only, the 1% PDRN samples showed the structures present in normal human hyaline cartilage. The results confirmed that all negative controls and 2% HA positive controls of cartilage explants were degenerated. The positive controls were really atrophied if compared with the

Samples treated with 1% PDRN Culture medium. All cells treated with 1% HA or with 2% PDRN culture media revealed a lower presence of mature cartilaginous structures when compared with 1% PDRN samples (Table 6 and Figure 3).

Tissue sections: type II collagen and aggrecan staining

After 30 days of incubation, all samples and controls initially provided for histology were stained with antibodies antihuman-type II collagen and anti-human aggrecan.
The negative controls 1, 2 and 3 and samples treated with 2% HA culture medium confirmed to be in a degenerative state. The positive controls showed weak staining only for type II collagen, revealing an initial atrophy of the tissues. Cells treated with 1% HA and with 2% PDRN culture media showed few, but clear, normal structures of human cartilage.
Morphologically, sections treated with 1% HA revealed a type of cartilage matrix with a sponge-like architecture, gaps between lacunae and collagen fibre bundles, as in the presence of fibrous cartilage.
All samples treated with 1% PDRN culture medium showed eutrophic state of healthy normal cartilage (Table 7). The morphology of cartilage biopsies treated with 1% PDRNs was characterized by a uniform matrix structure, glossy type tissue with regularly dispersed chondrocytes in lacunae and a large presence of protein (as collagen type II and aggrecan), as in the presence of hyaline cartilage.

Tissue sections: cartilage ECM staining with Alcian blue colorimetric method

Biopsies after the 30-day incubation were stained with Alcian blue. Only the 1% PDRN samples presented matrix deposition (positive Alcian blue staining) and growth in 3D overlapping layers (Table 1).

Alcian blue scores.

After an incubation of 2 h at room temperature, the Alcian solution bound acid mucopolysaccharides and glycoproteins with an indelibly blue staining of the ECM, specified as follows:
• cartilage tissue in vitro treated for 30 days with 2% PDRN culture medium= diffuse positive background staining (score = +++);
• cartilage tissue in vitro treated for 30 days with 1% PDRN culture medium= high positive background staining (score = +++++);
• cartilage tissue in vitro treated for 30 days with 2% HA culture medium (positive control 1a) = slightly positive (score = +);
• cartilage tissue in vitro treated for 30 days with 1% HA culture medium (positive control 1b) = diffuse positive background staining (score = +++);
• cartilage tissue in vitro treated for 30 days with cartilage laboratory medium (positive control 2) = diffuse positive background staining (score = +++);
• Negative control 1 = slightly positive (score = +);
• Negative control 2 = slightly positive (score = +);
• Negative control 3 = slightly positive (score = +).

A clear increase in cartilage ECM was detected, which turned blue, merely in the samples treated with 1% PDRN culture medium.

Tissue sections: cartilage ECM staining with safranin O colorimetric method

Biopsies after the 30-day incubation were stained with safranin O (Sigma) for 15 min at room temperature. Specific controls were prepared for each sample. The proteoglycans, mucopolysaccharides and type II collagen were stained in coral red colour. Only the chondrocytes treated for 30 days with 1% PDRN Culture medium presented the mucoid substance making up the matrix (positive safranin O staining) and growth in 3D overlapping layers.

Safranin O scores.

After an incubation of 15 min at room temperature, the safranin O solution bound the mucoid substance giving up the matrix an indelibly red staining (Table 8), specified as follows:
• Sample 1a treated with 2% PDRNs got clearly coloured with safranin O (score = +++);
•Sample 1b treated with 1% PDRNs: the cells in which a redeposition of mucopolysaccharides and glycoproteins was induced got clearly coloured with safranin O, with growth in layers one on top of the other (score = +++++);
• Positive control 1a treated with 2% HA had very bland staining (score = +);

• Positive control 1b treated with 1% HA got clearly coloured with safranin O (score = +++);
• Positive control 2 in light red (safranin O) was detected (score = ++);
• Negative control 1: very bland staining in light red (safranin O; score = +);
• Negative control 2: a very bland background staining (score = +);
• Negative control 3 treated: a very bland background staining (score = +).

A clear increase in ECM was detected, which turned red, in all samples treated with 1% and 2% of PDRN culture medium and positive control 1b (1% of HA culture medium) compared with all the controls.
GAGs extraction and quantification GAGs were detected by spectrophotometer at 530 nm. At day 0, data were not available. At day 30, the concentrations of GAGs detected in conditions treated with 1% or 2% of PDRNs or 1% of HA were less decreased than those in all other treatments. Table 9 showed the values of GAG concentrations expressed as micrograms per milligrams.

DISCUSSION

Cartilage diseases is the most common form of arthritis, affecting millions of people worldwide.[42,43] OA is the most common form of arthritis and is a public health problem throughout the world. There are a large number of active research and new drug programs projected to reduce joint destruction as well as existing drug therapies to diminish symptoms. None of these advances modified the progression of cartilage disease.[44] To date, several methods are used to restore the tropism of damaged articular cartilage.[34,45] The principal goals for management of the symptomatic chondral defects are to reduce symptoms, to improve joint congruence by restoring the cartilage surface with the best possible tissue and to prevent additional cartilage deterioration.[46–48] All used drugs, surgery and methods do not take into account the reconstruction of cartilage physiological microenvironment.[49,50]
In every case, the formed fibro-cartilage repair tissue has different biochemical and bioelastic characteristics compared with those of the hyaline cartilage.[51] Pathological local conditions probably correspond to origin and effect of the cartilage and matrix degeneration.
The restoration of the physiological microenvironment can lead to the reconstitution of a normal chondrocyte proliferation with physiological deposition of cartilage matrix. PDRNs were been tested to induce the restoration of a proper microenvironment for the development of healthy cartilages.
PDRN formulations were able to stimulate the growth of cartilage tissue through direct action on chondrocytes with the recovery of the atrophic tissues, re-establishing a correct physiological microenvironment of healthy cartilages.
All the atrophic cartilage tissue biopsies treated in vitro reacquired the normal functions of collagen type II and aggrecan deposition and showed active matrix deposition, which was previously lacking. PDRNs showed protective effects in vitro to prevent the deterioration of tissues or cells derived from biopsies of cartilage. Our results illustrate two key points.
First, both PDRN formulations had the most significant effect to stimulate the proliferation of cartilage cellularity and ECM deposition compared with all HA solutions.
Second, PDRNs compositions were more effective than HA solutions in inducing functional effects through a correct microenvironment stimulation, preserving cellular normal functions of deposition of ECM typical of hyaline cartilage.
In fact, osmotic measurements reveal that the pressure of HA on the cells and the specific interactions between HA and cells depend on the HA weight and hydrophilic properties of HA.[52] Changes in the osmotic environment of joints can cause changes in volume of isolated cells and can induce dystrophy or acute atrophy of treated cartilages. Our data confirm these observations. Biopsies treated with a solution of 1% HA (1000MW KD) showed a mortality rate much lower (30%) compared with biopsies treated with 2% solution of HA (1000MW KD) after 30 days of incubation.
On the other hand, HA hydrophilicity serve to retain fluid in the joint cavity during articulation.[53] All these physicochemical and biological properties of HA may be dependent on molecular weight. HA treatment seems beneficial in OA patients with decreased pain and increased range of motion.[54] In addition, HA can form a peri-cellular coat around cells and bind to cell receptors, such as cluster determinant CD 44 and receptor for hyaluronate-mediated motility, where it modulates cell proliferation, migration and gene expression.[53] CD 44, a multivariate trans-membrane glycoprotein known to be the main cell surface receptor for HA,[55] is present in the synovium[56] and synovial fluide[57] of OA patients, and levels correlate with the degree of inflammation but not Kellgren grade.[58,59]
OA is characterized by cartilage deterioration, as evidenced by quantitative and qualitative modification of proteoglycans and collagen. An imbalance between the biosynthesis and the degradation of matrix components leads to a progressive destruction of the tissue, resulting in extensive articular damage.[42] Non-steroidal anti-inflammatory drugs and COX-2 inhibitors were extensively used to afford symptomatic relief, but the increased risk of ischemia associated with these drugs led to the recall of some products from the market and warnings concerning their use.[60] As alternatives to non-steroidal anti-inflammatory drugs and

COX-2 inhibitors, other symptom-modifying drugs currently in clinical trials for OA include nitric oxide-releasing analgesics, bradykinin B2 receptor antagonists and capsaicin analogues.[61] One recent alternative therapy is nutraceutical treatment with glucosamine.[62,63]

Other treatments for OA can include intra-articular injection with long-acting corticosteroids or hyaluronan to provide symptomatic relief.[60] Recent study demonstrated the lack of lubricating ability of HA in solution on in vitro cartilage.[64]

HA does not have necessary mechanism to adhere to the cartilage surfaces; thus, it is readily squeezed out of the contact interface and can little contribute to mediate either friction or wear. Large and highly variable friction forces were measured between hydrophobic surfaces in the presence of HA.[64] The cartilage surface is topographically and chemically heterogeneous.

On the native cartilage tissue, HA may have specific binding affinity to components of the cartilage surface, which can be lost on the in vitro model surfaces.[64] Some studies have found that HA and lubricin together lubricate more effectively than either molecule on its own.[65,66] It was observed in a latex–glass interface under high contact pressure (180 kPa)[66] and in a cartilage–cartilage interface under similar pressure,[65] and this may be due to either specific or non-specific interactions.

The high contact pressure is not conducive to the reconstruction of a cartilaginous physiological microenvironment.

The deposition of fibrous matrix can be reactive to pathological microenvironments.[67] Although the mechanisms of HA action remain unclear, several studies have shown the efficacy of HA for the treatment of knee.[68]

Instead, no previous study has examined the in vitro effects of PDRNs in restoring human cartilage and in preserving chondrocyte survival with correct functions of ECM deposition, especially if compared with HA effects on the same bioptic tissues.

After 30 days of in vitro treatments, 20 cartilage biopsies were analyzed to assess the preservation of chondrocyte phenotypes and correct ECM deposition. In this regard, cells extracted from cartilage biopsies showed normally functioning ECM deposition when cultured with 1% PDRN solution. The immunohistochemical analysis of samples treated with 1% PDRNs demonstrated that the deposition of ECM was similar to hyaline cartilage, whereas in the samples treated with 1% HA, the deposition of ECM was similar to fibrous cartilage.

Cartilage has limited repair capabilities: because chondrocytes are bound in lacunae, they cannot migrate to damaged areas. Also, hyaline cartilage does not have a blood supply, the deposition of new matrix is slow. Damaged hyaline cartilage is usually replaced by fibro-cartilage scar tissue.

The type of protein fibre embedded within the matrix of cartilage determines the cartilage type. Fibro-cartilage (fibrous) is a type of cartilage that contains a large number of collagen type X fibres. In contrast to the very uniform appearance of hyaline cartilage, fibro-cartilage possesses a more open or sponge-like architecture with gaps between lacunae and collagen fibre bundles, as in the previously mentioned case about cartilaginous biopsies treated with HA solution. In hyaline cartilage, protein fibres are large and predominantly made of collagen type II. The optical density of these fibres is the same as the ground substance surrounding them, and as a result, they are not visible within the ECM. Hyaline cartilage subsequently appears as a very uniform, glossy type tissue with evenly dispersed chondrocytes in lacunae. The same homogeneous structure depicts the morphology of cartilage biopsies treated with 1% PDRNs, as mentioned earlier.

Furthermore, cell vitality was significantly higher in all the biopsies treated with the 2% PDRN solution than that in those treated with 2% HA solution ($P < 0.001$). These results reveal that the state of cartilage atrophy that occurs in degenerative processes is reversible.

Normally, the joint microenvironment promotes the exchange of nutrients by diffusion in special conditions of osmotic pressure. This mechanism allows the secretion of synovial fluid viscous, transparent, lubricating and nourishing.

In turn when the synovial fluid present in the cavity is healthy, it allows to control and maintain stable some parameters such as the osmotic pressure, viscosity and the nutrition of the articular cartilage. With PDRN formulations, we have attempted to recreate a physiological synovial fluid.

The new fluid was tested on chondrocyte without ECM in vitro. Cell cultures treated with the new formulation have deposited new ECM and restored the conditions to support the development of healthy cartilage. The in vitro model is useful to clarify the pathogenesis of OA and apply correct osmotic and nutritional factors to repair cartilage. In physiological conditions of microgravity and osmosis, using the principle of selective viscosity on cell culture, we observed that this innovative formulation is suitable for a long-term cultivation of vitro cartilage. This cultivation device that we developed is still in a exploration stage, but it certainly provides an microenvironment similar to the physiological condition of healthy articular cartilage. The development of a device intra-articular injecting in vivo is ongoing.

However, our data need to be confirmed by the in vivo clinical studies to include all biomechanical and physiopathological intra-articular variables for better understanding the potential effectiveness of PDNR and the mechanism on the base of their biological activity.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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Table 1. Culture medium compositions

Substance	Concentration
2% Condrotide™ culture medium (as 2% PDRN sample 1a)	
PDRNs	20 mg ml^{-1}
F12 culture medium	20 μl ml^{-1}
MEM, non-essential amino acid solution	40 μl ml^{-1}
Hanks salt solution	4 μl ml^{-1}
DMEM medium with high glucose	q.s. per ml of solution
1% Condrotide™ culture medium (as 1% PDRN sample 1b)	
PDRNs	10 mg ml^{-1}
F12 culture medium	20 μl ml^{-1}
MEM, non-essential amino acid solution	40 μl ml^{-1}
Hanks salt solution	4 μl ml^{-1}
DMEM medium with high glucose	q.s. per ml of solution
2% Hyaliaphase™ culture medium (as 2% HA, positive control 1a)	
HA (molecular weight, 1000 kDa)	20 mg ml^{-1}
F12 culture medium	20 μl ml^{-1}
MEM, non-essential amino acid solution	40 μl ml^{-1}
Hanks salt solution	4 μl ml^{-1}
DMEM medium with high glucose	q.s. per ml of solution
1% Hyaliaphase™ culture medium (as 1% HA, positive control 1b)	
HA (molecular weight, 1000 kDa)	10 mg ml^{-1}
F12 culture medium	20 μl ml^{-1}
MEM, non-essential amino acid solution	40 μl ml^{-1}
Hanks salt solution	4 μl ml^{-1}
DMEM medium with high glucose	q.s. per ml of solution
Cartilage laboratory medium (positive control 2)	
Ascorbic acid	100 μg ml^{-1}
Transforming growth factor β	20 ng ml^{-1}
Foetal bovine serum	100 μl ml^{-1}
F12 culture medium	20 μl ml^{-1}
MEM, non-essential amino acid solution	40 μl ml^{-1}
Hanks salt solution	4 μl ml^{-1}
DMEM medium with high glucose	q.s. per ml of solution
Supplemented common culture medium (as negative control 1)	
Foetal bovine serum	100 μl ml^{-1}
F12 culture medium	20 μl ml^{-1}
MEM, non-essential amino acid solution	40 μl ml^{-1}
Hanks salt solution	4 μl ml^{-1}
DMEM medium with high glucose	q.s. per ml of solution
Unsupplemented common culture medium (as negative control 2)	
F12 culture medium	20 μl ml^{-1}
MEM, non-essential amino acid solution	40 μl ml^{-1}
Hanks salt solution	4 μl ml^{-1}
DMEM medium with high glucose	q.s. per ml of solution
Free culture medium (as negative control 3)	
DMEM medium with high glucose	q.s. per ml of solution

Table 2. Quantification by trypan blue of the cell viability between samples *versus* controls

Trypan blue staining	Incubation (days)	
Origin	Mortality (%)	
Biopsy-derived cells	30 days	*P*
Sample 1a	30%	<0.05
2% PDRN culture medium	±5	
Sample 1b	8%	<0.001
1% PDRN culture medium	±2%	
Positive control 1a	82%	<0.001
2% HA culture medium	±3%	
Positive Control 1b	58%	<0.03
1% HA culture medium	±8%	
Positive control 2	50%	<0.02
Cartilage culture medium	±5%	
Negative control 1 (Table 6)	62%	<0.01
Supplemented cell culture medium	±7%	
Negative control 2 (Table 7)	66%	<0.01
Unsupplemented cell culture medium	±6%	
Negative Control 3 (Table 8)	70%	<0.01
Free cell culture medium	±9%	

After 30 days of incubation, sections of biopsies were disaggregated in collagenase (Sigma-Aldrich), and the pellets were analyzed. Trypan blue is a dye capable of selectively staining dead cells. Dead cells will appear intensely coloured in blue; the living cells will not be coloured in blue.
The results related to viability and mortality in samples and relevant controls have been expressed as a scale of percentage values.
P values resulted from the one-way ANOVA with post hoc Tukey's test correction using Student's *t*-test. Each data point represents the mean and standard error of four independent replicates.

Table 3. Quantification by MTT of the cell viability between samples versus controls

MTT staining	Incubation (days)	
Origin	Viability (%)	
Biopsy-derived cells	30 days	*P*
Sample 1a	72%	<0.05
2% PDRN culture medium	±4%	
Sample 1b	92%	<0.001
1% PDRN culture medium	±3%	
Positive control 1a	20%	<0.001
2% HA culture medium	±6%	
Positive control 1b	45%	<0.03
1% HA culture medium	±5%	
Positive control 2	57%	<0.02
Cartilage culture medium	±7%	
Negative control 1 (Table 6)	35%	<0.01
Supplemented cell culture medium	±5%	
Negative control 2 (Table 7)	32%	<0.01
Unsupplemented cell culture medium	±6%	
Negative control 3 (Table 8)	28%	<0.01
Free cell culture medium	±8%	

After 30 days of incubation, sections of biopsies were disaggregated in collagenase (Sigma-Aldrich), and the pellets were analyzed. The results related to viability and mortality in samples and relevant controls have been expressed as a scale of percentage values compared with the control wherein the ability to reduce MTT was taken as 100% viability. Statistical analysis was performed using the one-way ANOVA with post hoc Tukey's test correction using Student's *t*-test. Each data point represents the mean and standard error of four independent experiments.

Table 4. FACS data

Day 0

Markers	All samples and controls
Type II collagen	44.0%–48.5%
Aggrecan	40.1%–42.0%
CD 29	20.0%–28.8%
CD 44	33.0%–35.8%
CD 71	16.0%–18.5%
CD 90	1.8%–2.0%
CD 105	18.1%–22.4%
Stro-1	5.0%–7.0%

Day 15

Markers	Sample 1a	Sample 1b	Positive control 1a	Positive control 1b	Positive control 2	Negative control 1	Negative control 2	Negative control 3	*P*
	2% PDRNs	1% PDRNs	2% HA	1% HA					
Type II collagen	48.0%–50.9%	52.2%–55.1%	18.0%–14.9%	42.2%–45. 5%	34.2%–38.0%	32.5%–30.0%	25.1%–22.5%	20.1%–19.5%	<0.01
Aggrecan	44.8%–46.3%	48.8%–49.0%	20.0%–21.3%	41.8%–42.8%	40.0%–41.7%	33.0%–30.3%	27.4%–28.0%	20.0%–21.8%	<0.01
CD 29	25.0%–28.0%	20.8%–24.0%	19.5%–20.0%	32.7%–34.0%	32.0%–33.0%	32.0%–35.0%	20.0%–21.5%	15.9%–17.8%	NS
CD 44	32.0%–34.8%	34.0%–35.2%	30.0%–35.7%	40.0%–45.8%	31.0%–32.8%	28.4%–29.2%	25.0%–26.5%	27.8%–28.9%	<0.05
CD 71	20.8%–23..3%	21.0%–22.5%	12.0%–13..5%	16.0%–17..5%	21.0%–23.1%	27.2%–29.0%	28.0%–29.5%	30.0%–32.4%	<0.05
CD 90	1.5%–2.0%	1.0%–1.5%	1.7%–2.5%	1.3%–2.8%	1.4%–2.5%	0.9%–2.9%	0.7%–1.5%	0.5%–1.3%	NS
CD 105	23.0%–24.1%	22.8%–23.2%	22.1%–23.0%	21.0%–24.0%	23.1%–25.9%	24.9%–26.0%	15.1%–18.9%	10.0%–13..2%	<0.05
Stro-1	0.4%–1..2%	1.9%–2..5%	0.9%–1.7%	5.9%–6.8%	6.5%–7..2%	3.4%–4..9%	2.8%–3.7%	0.5%–1.0%	<0.05

Day 30

Markers	Sample 1a	Sample 1b	Positive control 1a	Positive control 1b	Positive control 2	Negative control 1	Negative control 2	Negative control 3	*P*
	2% PDRNs	1% PDRNs	2% HA	1% HA					
Type II Collagen	67.0%–69.9%	88.2%–89.1%	10.9%–11.9%	31.0%–33. 1%	20.2%–23.5%	11.5%–13.3%	10.0%–11..5%	8.5%–11.7%	<0.01
Aggrecan	67.8%–68.3%	82.8%–83.9%	10.0%–11.3%	30.9%–31.8%	28.7%–31.0%	10.0%–11.3%	8.4%–10.8%	7.0%–9.3%	<0.01
CD 29	33.0%–34.3%	28.1%–29.0%	10.5%–10.0%	29.3%–30.0%	31.5%–32.3%	12.0%–15.0%	11.0%–11.9%	13.9%–14.8%	NS
CD 44	22.0%–23.3%	19.0%–21.0%	10.0%–10.9%	42.7%–44.9%	25.0%–28.8%	18.1%–21.5%	15.0%–16.5%	11.0%–12.0%	<0.05
CD 71	22.3%–23.5%	28.7%–30.0%	22.1%–23.0%	27.0%–29..9%	24.0%–25.1%	20.0%–20.9%	18.0%–19.1%	10.0%–11.5%	<0.05
CD 90	0.5%–1.5%	1.9%–2.9%	0.1%–0.5%	4.3%–5.8%	7.4%–12.5%	0.5%–0.9%	0.1%–0.5%	0.1%–0.3%	NS
CD 105	20.0%–21.8%	22.8%–23.2%	8.1%–10.0%	21.0%–24.0%	23.1%–25.9%	4.9%–6.0%	5.1%–8.9%	9.0%–11..2%	<0.05
Stro-1	0.4%–1..2%	0.9%–1..5%	2.5%–3.7%	10.2%–11.8%	1.5%–3..2%	5.4%–6..9%	4.8%–5.7%	3.5%–4.0%	<0.05

Characterization of the cartilage tissues treated with Condrotide culture medium versus controls. At days 0, 15 and 30 of incubation, biopsy extracted cells had been subjected to quantitative analysis using a laser cytofluorimeter (Epics Profile II; Coulter) at 488 nm. Threshold values (gates) have been established using control samples labelled with the corresponding isotypes. All values have been analyzed with a minimum threshold of 15,000 cells. The results relating to the expression of type II collagen, aggrecan and CD 29, 44, 71, 90, 105 and Stro-1 were referred to the percentage ranges of fluorescent cells in four independent replicates. It is noted that there were not significant differences among multiple experiments. Statistical analysis was performed using the one-way ANOVA, followed by post hoc Tukey's test correction using Student's *t*-test. NS, not significant.

Table 5. Immunofluorescence: characterization of the chondrocytes from cartilage tissue treated with Condrotide culture medium versus controls

	Day 30							
	Sample 1a	Sample 1b	Positive control 1a	Positive control 1b				
Markers	2% PDRNs	1% PDRNs	2% HA	1% HA	Positive control 2	Negative control 1	Negative control 2	Negative control 3
Type II collagen	++++	+++++	++	+++	++	–/+	–/+	–––––
Aggrecan	++++	+++++	++	+++	++	–/+	––––	–––––
CD 29	++	+	++	++	++	+	+	+
CD 44	++	+	++	++	++	+	++	+
CD 71	––––	––––	++	++	++	+	––––	+
CD 90	––––	––––	++	+++	+++	+	+++	+
CD 105	––––	––––	++	+++	+++	+	+	+
Stro-1	––––	––––	++	++	+	+	+	+

After 30 days of incubation, all biopsy extracted cells were examined under a Leica DM LA fluorescence microscope. The results relating to the expression of type II collagen, aggrecan, CD 29, CD 44, CD 71, CD 90, CD 105 and Stro-1 were expressed on a median values in an observational scale and presented as mean of four independent experiments. There were not appraisable differences among replicates. Magnification x 40.

----, no fluorescence (no fluorescent cells); -/+, very low fluorescence for optical field (1–2 fluorescent cells/field); +, low fluorescence for optical field (3–5 fluorescent cells/field); ++, medium fluorescence for optical field (6–10 fluorescent cells/field); +++, sharp fluorescence for optical field (11–25 fluorescent cells/field); ++++, high fluorescence for optical field (26–40 fluorescent cells/field); +++++, very high fluorescence for optical field (>40 fluorescent cells/field).

Table 6. Tissue sections. haematoxylin and eosin staining

	Day 30							
Structures and colours	Samples 2% PDRNs	Samples 1% PDRNs	Samples 2% HA	Samples 1% HA	Positive controls	Negative control 1	Negative control 2	Negative control 3
Eosin	Positive = eutrophic cells	Positive = eutrophic cells	Low positive = dystrophic cells	Positive = eutrophic cells	Positive = eutrophic cells	Low positive = atrophic cells	Negative = atrophic cells	Negative = necrotic cells
Haematoxylin	Positive = eutrophic cells	Positive = eutrophic cells	Low positive = dystrophic cells	Positive = eutrophic cells	Positive = eutrophic cells	Low positive = atrophic cells	Negative = atrophic cells	Negative = necrotic cells

After 30 days of incubation, all biopsies were examined under a Zeiss Axiophot microscope. The results relating to haematoxylin and eosin staining were expressed on a median values in an observational scale and presented as mean of four independent experiments.

Table 7. Tissue sections: type II collagen and aggrecan staining

	Day 30							
	Sample 1a	Sample 1b	Positive control 1a	Positive control 1b				
Markers	2% PDRNs	1% PDRNs	2% HA	1% HA	Positive control 2	Negative control 1	Negative control 2	Negative control 3
Type II collagen	++++	+++++	++	+++	+++	++	+	+
Aggrecan	++++	+++++	++	+++	+++	++	+	+

After 30 days of incubation, all biopsies were examined under a Zeiss Axiophot microscope. The results relating to type II collagen and aggrecan were expressed on a median values in an observational scale and presented as mean of four independent experiments. Magnification x 40.

----, no colour for optical field; -/+, very low colour for optical field; +, low colour for optical field; ++, medium colour for optical field; +++, elevated colour for optical field; ++++, high colour for optical field; +++++, very high colour for optical field.

Table 8. Tissue sections: Alcian blue and safranin O staining

	Day 30							
	Sample 1a	Sample 1b	Positive control 1a	Positive control 1b				
Colorimetric method	2% PDRNs	1% PDRNs	2% HA	1% HA	Positive control 2	Negative control 1	Negative control 2	Negative control 3
Alcian blue	+++	+++++	+	+++	+++	+	+	+
Safranin O	+++	+++++	+	+++	++	+	+	+

After 30 days of incubation, all biopsies were examined under a Zeiss Axiophot microscope. The results relating to Alcian blue and safranin O were expressed on a median values in an observational scale and presented as mean of four independent experiments. There were not appraisable differences among replicates. Magnification x 40.
----, no colour for optical field; -/+, very low colour for optical field; +, low colour for optical field; ++, medium colour for optical field; +++, elevated colour for optical field; ++++, high colour for optical field; +++++, very high colour for optical field.

Table 9. GAGs extraction and quantification

	Day 30							
	Sample 1a	Sample 1b	Positive control 1a	Positive control 1b				
GAG concentrations	2% PDRNs	1% PDRNs	2% HA	1% HA	Positive control 2	Negative control 1	Negative control 2	Negative control 3
Total GAGs ($\mu g\ mg^{-1}$)	2.594 ±0.032	2.832 ±0.028	1.820 ±0.039	2.598 ±0.030	2.641 ±0.035	2.270 ±0.029	1.900 +/0.057	1.890 +/0.055
*P**	<0.05	<0.03	<0.01	<0.05	<0.05	<0.02	<0.01	<0.01

The concentration ($\mu g\ mg^{-1}$) of GAGs was detected by spectrophotometer at 530 nm. The results were expressed in median values.
*Data obtained by the one-way ANOVA test.

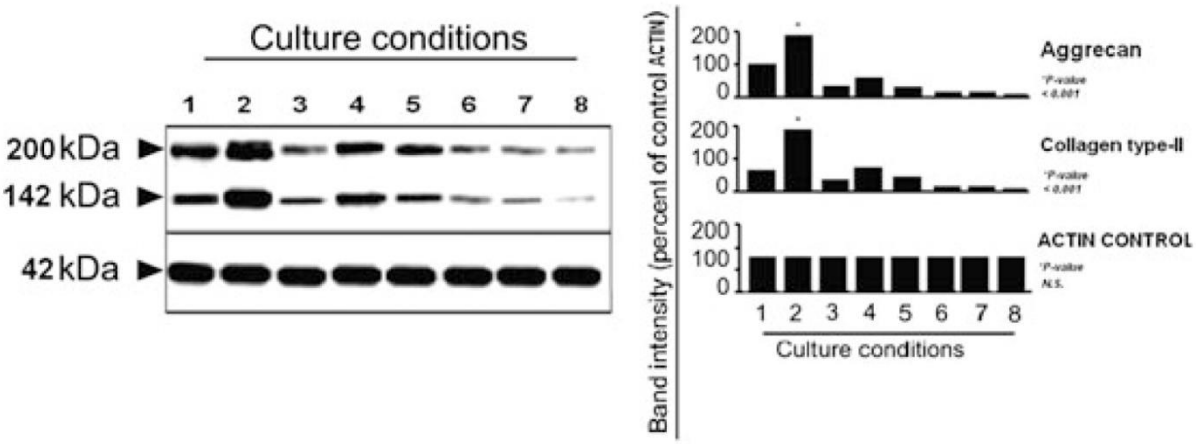

Figure 1. Western blot results. In vitro, different treatments (1–8) of cell extracted from cartilage explants demonstrated culture medium-dependent effects on controls and samples, after 30 days of incubation. The cells cultured under different conditions were analyzed by Western blot. The controls and the samples were treated as described in the following: (1) with 2% PDRN culture medium; (2) with 1% PDRN culture medium; (3) with 2% HA culture medium; (4) with 1% HA culture medium; (5) with cartilage laboratory medium, as positive control; (6) with supplemented common culture medium, as negative control 1; (7) with unsupplemented common culture medium X, as Negative control 2; (8) with free culture medium, as negative control 3. The results were confirmed by four independent replicates. There were not significant differences among replicates.Multiple experiments were compared using the densitometry. The different test conditions were pooled and the means compared. *P* values resulted from the one-way ANOVA with post hoc Tukey's test correction using Student's *t*-test. The culture condition 2 showed the most significant *P* values in respect of all other conditions tested for each marker analyzed, except the actin case. About the actin marker, all conditions expressed equal concentration without significant differences (*P* = not significant)

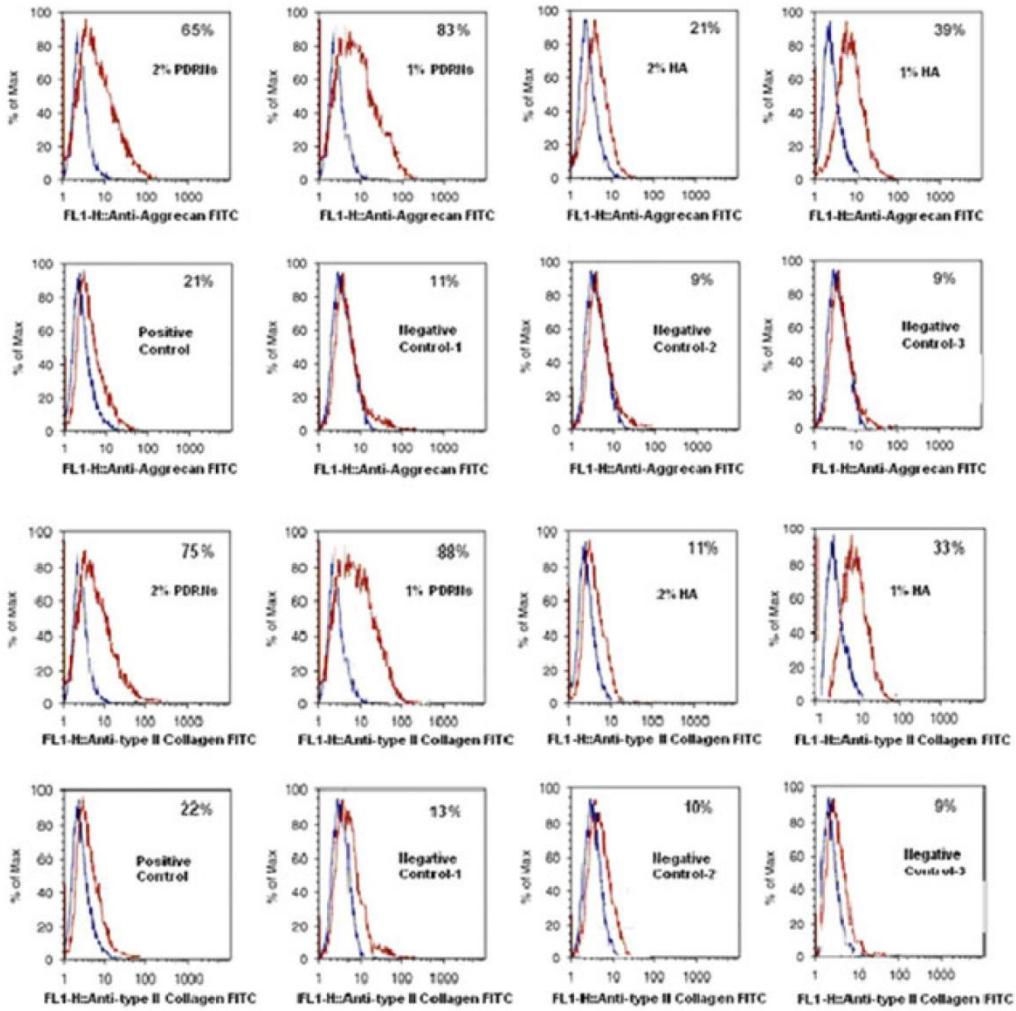

Figure 2. Aggrecan and type II collagen, FACS data. Intracellular flow cytofluorimetric analysis (FACS) shows the expression of aggrecan and type II collagen in cultures of cartilage biopsies treated with different media (1–8), after 30 days of incubation. The samples and controls were treated as follows: (1) with 2% PDRN culture medium; (2) with 1% PDRN culture medium; (3) with 2% HA culture medium; (4) with 1% HA culture medium; (5) with cartilage laboratory medium, as positive control; (6) with supplemented common culture medium, as negative control 1; (7) with unsupplemented common culture medium X, as negative control 2; (8) with free culture medium, negative control 3.

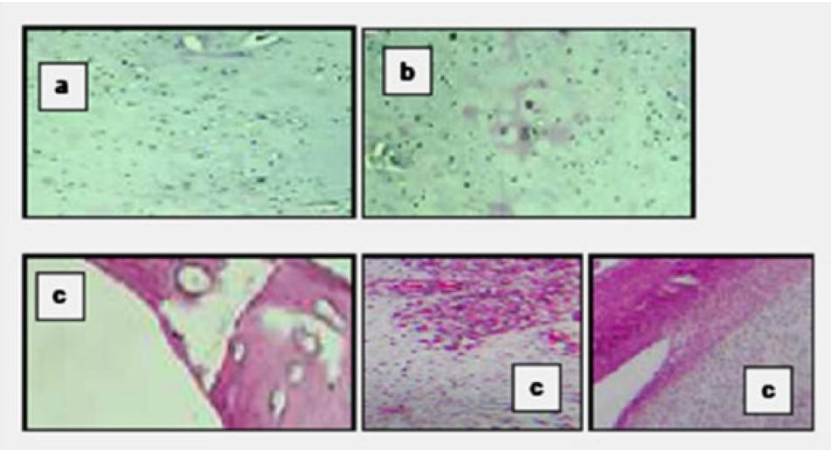

Figure 3. Haematoxylin and eosin stain. (a–c) In vitro, different treatments of cartilage explants demonstrated culture medium-dependent effects on controls and samples, after 30 days of incubation. Shown are haematoxylin and eosin staining of human skin biopsies treated as described in the following: (a) with cartilage laboratory medium called positive control (x 20, the fragment of articular cartilage was decalcified, clusters of chondrocytes were embedded in the ECM, as in healthy cartilage); (b) with 2% PDRN culture medium (x20, the fragment of articular cartilage was decalcified, chondrocyte clusters were immersed in the matrix, which shows a faint pink eosin colour, as typical of hyaline cartilage); (c) with 2% HA culture medium (x20, the fragment and slices of articular cartilage were decalcified, the presence of lamellar bone and the occurrence of vascularmany shortcomings can be seen, normal cartilage matrix is absent)