ORIGINAL PAPER

Gene Expression of Joint Cartilage Preserved under Different Conditions and Treated with Growth Factors

C. Acosta[a], I. Izal[a], P. Ripalda[a] and F. Forriol[b]
[a]Experimental Orthopedics Laboratory. University of Navarre. [b] FREMAP Hospital. Majadahonda. Madrid.

Purpose. To analyze the gene expression of anabolic and catabolic factors in chondrocyte cultures obtained from cartilage preserved at different temperatures, using different preservation media and treated with growth factors.
Materials and methods. A culture was made of a young lamb's distal femoral cartilage preserved at 4 ºC without a cryopreservative agent, at 4 ºC with PBS, at 4 ºC with DMEM, at –80 ºC without a cryopreservative agent, at -80 ºC with 10% glycerol and –80 ºC with 10% DMSO. An additional culture was made of osteoarthritic and old cartilage. RT-PCR analyses were conducted of the cultured cells with 10 ng of RNA per reaction and 20 nmols of each of the two primers; the cells were also treated with growth factors (TGF-β, FGF-a, IGF-1, OP-1®). RT-PCR reactions were evaluated by means of electrophoresis in agarose gels.
Results. The best results were obtained for samples cultured at 4 ºC with PBS; in old cartilage a reduction was observed in the expression of all anabolic factors, except agrecan. Cartilage stored at -80 ºC, with glycerol or DMSO, expressed a higher amount of catabolic factors. On adding growth factors, an increase was registered in the expression of TGF-β while MMP-2 levels remained unchanged, at the same level as those of the control group. The best response was obtained with OP-1®, FGF-a and IGF-1.
Conclusion. Cartilage stored at -80 ºC expresses a higher amount of catabolic factors than cartilage preserved at 4 ºC.

Key words: *cartilage, osteoarthritis, chondrocytes, growth factors.*

Corresponding author:
F. Forriol.
Hospital FREMAP.
Ctra. de Pozuelo, 61.
28220 Majadahonda. Madrid.
E-mail: francisco_forriol@fremap.es

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Expresión génica de cartílago articular conservado en diferentes condiciones y tratado con factores de crecimiento

Objetivo. Analizar la expresión génica de factores anabólicos y catabólicos en cultivos de condrocitos obtenidos de cartílago, conservado a diferentes temperaturas, con distintos medios de conservación y tratados con diferentes factores de crecimiento.
Material y método. Se cultivaron condrocitos de cartílago femoral distal de cordero joven conservado: a 4 ºC sin criopreservador; 4 ºC con PBS; 4 ºC con DMEM; –80 ºC sin criopreservador; –80 ºC con glicerol al 10%; y –80 ºC con DMSO al 10%. También se cultivó cartílago artrósico y viejo.
Con las células cultivadas se efectuaron ensayos de RT-PCR con 10 ng de ARN por cada reacción y 20 nmoles de cada uno de los dos cebadores y fueron tratadas con diferentes factores de crecimiento (factor transformante de crecimiento β [TGF-β], factor de crecimiento fibroblástico [FGF-a], IGF-1, OP-1®). Las reacciones de RT-PCR fueron analizadas mediante electroforesis en geles de agarosa.
Resultados. Los mejores resultados fueron en muestras cultivadas a 4 ºC con PBS; en el cartílago viejo disminuyó la expresión de todos los factores anabólicos, excepto el agrecano. El cartílago almacenado a -80 ºC con glicerol o DMSO expresó mayor cantidad de factores catabólicos. Al añadir los factores de crecimiento aumentó la expresión de TGF-β y se mantuvieron niveles de MMP-2 semejantes al grupo control. La mejor respuesta se obtuvo con OP-1®, FGF-a e IGF-1.
Conclusión. El cartílago almacenado a -80 ºC expresa mayor cantidad de factores catabólicos que el cartílago conservado a 4 ºC.

Palabras clave: *cartílago, artrosis, condrocito, factores de crecimiento.*

The rate of joint defects in the general population is 5%[1], although in some groups of people who carry out intense work or sports activities this can be higher. Kaplan et al[2] used magnetic resonance to assess asymptomatic basketball players and found joint cartilage lesions in 47.5%, whereas Major and Helms[3] found cartilage lesions in 41% of young basketball players.

The use of cryopreserved osteochondral allografts may be indicated in young patients with functional limitations and single, completely localized lesions, although their use has also been indicated in post-trauma arthritis[4] and in osteochondral defects[5]. One of the advantages of this technique is the possibility of having available stored fragments of cartilage of the appropriate size and curvature from a load-bearing zone.

However, it has been pointed out that joint cartilage is difficult to preserve, as its cell viability decreases and the extracellular matrix undergoes alterations. Studies of chondrocyte gene expression in cartilage areas with arthritis show an increase in the expression of genes for transforming growth factor (TGF-β,) and type I collagen, which suggest an attempt to repair the lesion. Yagi et al[7], found, in patients with arthritis, a decrease of aggrecan, MMP-9 and –13 (metaloproteinases from the matrix), whereas the expression of collagen type II showed variations than can be explained by the severity of the disease, and the size and location of the lesion.

Our work hypothesis is that preservation conditions affect gene expression in chondrocytes, decreasing the expression of anabolic factors, and that normal gene expression is recovered by means of treatment with growth factors.

Since cryopreserved cartilage allografts are an option used for cartilage repair, we have analyzed their behavior studying gene expression of the anabolic and catabolic factors in chondrocyte cultures taken from young cartilage harvested from lambs preserved at different temperatures and with different preservation media, comparing it with old cartilage with arthritis from the same animals.

MATERIALS AND METHODS

We extracted joint cartilage from young sheep and we stored it at two different temperatures and with different preservation media to compare it with cartilage with arthritis and old cartilage. We carried out tests using RT-PCR and subsequently applied treatments with growth factors to all the groups of cells.

This study had the approval of the Ethics Committee for Experimental Studies in Animals of our center.

We used joint cartilage from the distal femur of 3 lambs of 3 months of age and a mean weight of 30-35 kg for the control group and cryopreserved tissue group, 2 lambs of 4 months of age for the group of cartilage with arthritis and 2 sheep of 6 years of age for the group of old cartilage. The tissue with arthritis was obtained by means of a total internal meniscectomy of each knee in the 2 young lambs, allowing 2 months evolution and subsequently killing the animals. The alteration of the cartilage by arthritis was checked histologically. The animals were killed by means of general anesthesia with sodium thiopental (Sodium Pentothal® 0.5 g Abbot) followed by an intravenous injection of 20 ml of KCl (2 meq/ml).

Once the distal femur was extracted, using a trocar we obtained an osteochondral cylinder of the load-bearing areas of the femur condyle. The samples were divided into 8 groups according to their characteristics, preservation and maintenance.

Group 0: control (fresh young cartilage); group 1: 4 °C without any cryopreservation media; group 2: 4 °C with PBS; group 3: 4 °C with DMEM; group 4: –80 °C without any cryopreservation media; group 5: –80 °C with glycerol at 10%; group 6: –80 °C with DMSO at 10%; group 7: cartilage with arthritis; group 8: old cartilage.

The samples were preserved under these conditions for a month before extracting the cells and culturing them. The cells extracted from the control group (group 0), the old cartilage (group 8) and the cartilage with arthritis (group 7) were cultured immediately.

Cell Extraction

The tissue was placed on culture plates and sliced with sterile blades. Collagenase (Sigma® 0.5 mg/ml) and dispase (Gibco-BRL®, 0.5 mg/ml) were placed in culture media (DMEM) supplemented with 10% bovine fetal serum, L-cisteine (1 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) as bactericidal agents, and hepes (10 mM) as pH buffer (Gibco-BRL®). Digestion was carried out during the night, at 37 °C and at 5% CO_2.

The contents of the plates was filtered with sterile gauze and centrifuged at 400 g and 4ºC for 5 minutes. The resulting cell precipitate was washed 3 times, centrifuged in the same way and resuspended in culture media. Finally, the resuspended precipitate, after the last wash, was seeded in 75cm^3 flasks. The cells were kept at 37ºC and 5%, the media was changed every 2-3 days.

Measurement of the RNA Concentration

Each group of cultured cells was treated with different growth factors. Treatments were also carried out in 75 cm^3 flasks at a concentration of 50 ng/ml in media without serum. Fibroblast growth factor (FGF-a) (Peprotech ®, Rozky Hill, NJ, USA), insulin-like growth factor (IGF-1) (Peprotech®), OP-1® (Stryker Biotech, Hopkinton, MA, USA.) and TGF-β (Peprotech®) were used. All cells underwent duplicate RT-PCR.

Table 1. Primers used in PCR reactions

Gene	Secc Fw (5'-3')	Secc Rv (3'-5')	T (ºC)
β-actin	tctacaacgagctgcgtgtg	Cgtgaggatcttcatgaggt	58
BMP-7	gaggcaggcatgtaagaagc	Tgaagtgtaccagcgtctgc	58
TGF-β	tggacaccaactactgcttc	Cagctgcacttgcaggagcg	59
Collagen type I	gatccgcaacatggagactggcga	caagaagcagacaggccctatgtccac	59
Collagen type I	cacaaggagtctgcatgtct	Gttcaccaggctcaccagca	59
Aggrecan	acgccatctgctacaca	Aaggctcctcaggttctgg	59
MMP-2	ccctgtgtcttccccttcac	Cctgtttgcagagctcagg	60
MMP-9	agtttggtgtcgcggagcac	Tacatgagcgcttccggcac	60
MMP-13	cagcaggttgaagcagagc	Tcatcatagctccagacttgg	58

BMP: Bone morphogenetic protein; MMP: Matrix metalloproteins; TGF-β: Transforming growth factor β; T: temperature.

The cells were collected from the flasks by means of treatment with tripsin/EDTA (Gibco-BRL®) and were centrifuged at 1.500 rpm for 5 minutes at 4 ºC. The precipitate was resuspended in 1 ml of Trizol® (Invitrogen®) reactant and RNA was extracted following the indications of the manufacturer. The final precipitate was resuspended in 20 ml of water treated with DEPC. RNA concentration was determined measuring the absorbance at 260 nm in a spectrophotometer (*Genequant II,* Amersham-Biosciences®). The measurement was carried out on dilutions 1:500 of the samples of water treated with DEPC. The ratio between the absorbance at 260 nm and 280 nm was used to estimate the degree of purity.

RT-PCR Tests (reverse transcription-polymerase chain reaction)

After the period of storage gene expression was determined with cell cultures extracted from each group in phase 0. The tests were carried out using the *Superscript Platinum RT-PCR System*® (Invitrogen®) with 10 ng of RNA for each reaction and 20 nmols of each of the two primers. Finally, a *Mastercycler Personal* (Eppendorf®) thermocycler was programmed to carry out the following reaction:

50 ºC, 30 minutes, 94 ºC, 2 minutes, 1 cycle.
94 ºC, 1 minute, *annealing* (Tm) temperature, 1 minute, 72 ºC, 1 minute, 30 cycles.
72 ºC, 10 minutes, 1 cycle.

The primers and temperatures used can be seen in Table 1. We used the ,-actin gene to standardize the samples.

DNA Electrophoresis with Horizontal Agarose Gels.

The results of the RT-PCR reactions were analyzed using electrophoresis in agarose gels. D-1 low-endosmosis gel agarose (Pronadisa®) was used at a concentration of 2% in TAE buffer. Electrophoresis was carried out with a constant voltage of 70 V in TAE buffer with ethidium bromide (100 µl at 1% per liter). The gels were observed and photographed in a Gel Doc (Bio-Rad® system. The intensity of each band was measured densitometrically.

RESULTS

Studies of Gene Expression in Untreated Chondrocytes

We used fresh cartilage (control, group 0) as a reference group to assess the status of the other groups.

Anabolic Factors

In the group stored at 4 ºC with PBS (group 2) there was an increase in the expression of OP-1®, collagen type II, aggrecan and TGF-,. The group stored at –80 ºC with DMSO (group 6) also showed and increase of the factors studied. However, the group of old cartilage (group 8) showed a lower level of expression in comparison with the group of fresh cartilage (Figure 1).

Catabolic Factors

As to the expression of factors considered negative for joint cartilage, we saw an increase in the expression of collagen factors type I, MMP-13 and MMP-2 in the groups stored at –80 ºC with cryopreservatives, either glycerol (group 5) or DMSO at 10% (group 6) (Figure 2).

Study of the Effects of Growth Factors on Cultured Chondrocytes

Treatment with OP-1® (BMP-7)

The treatment with growth factor OP-1® (BMP-7) showed an increase in the expression of genes for TGF-?, MMP-2 and collagen type I in the 4 ºC group without cryopreservatives (group 1), and a decrease in the expression of the same genes in the 4 ºC group with PBS (group 2). In the 4ºC group in culture media (group 3), expression was similar to that seen in the control group (Figure 3A). In the group preserved at –80 ºC, the groups stored in glycerol and DMSO (groups 5 and 6) there was an increase in the expression of genes for collagen type II, aggrecan and, to a lesser degree, TGF-?, whereas the expression of MMP-2

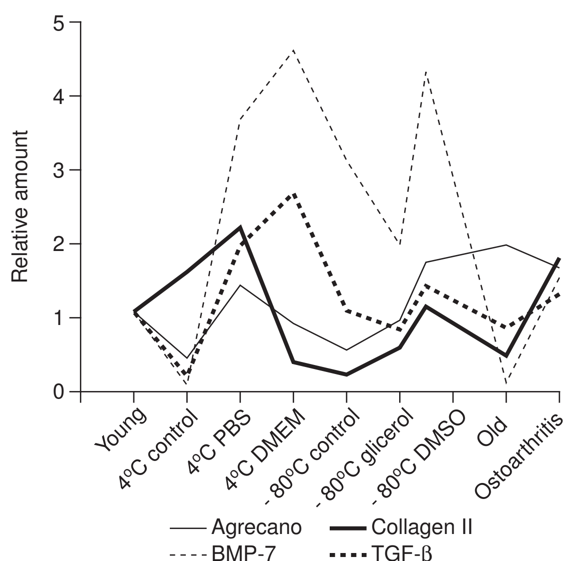


Figure 1. Using fresh young cartilage as a reference, relative quantities of mRNA for different anabolic factors studied in different groups. BMP-7 (OP-1): Bone morphogenetic protein 7; DMEM: Dulbeccos Modified Eagle Medium; DMSO: dimethyl sulphoxide; PBS: Phosphate buffer saline; TGF-β,: transforming growth factor β.

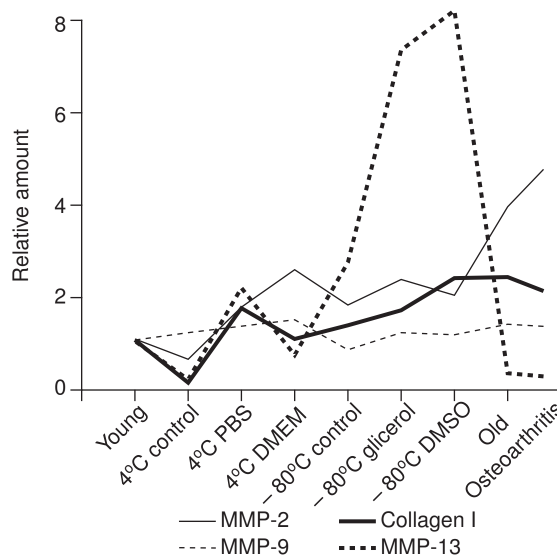
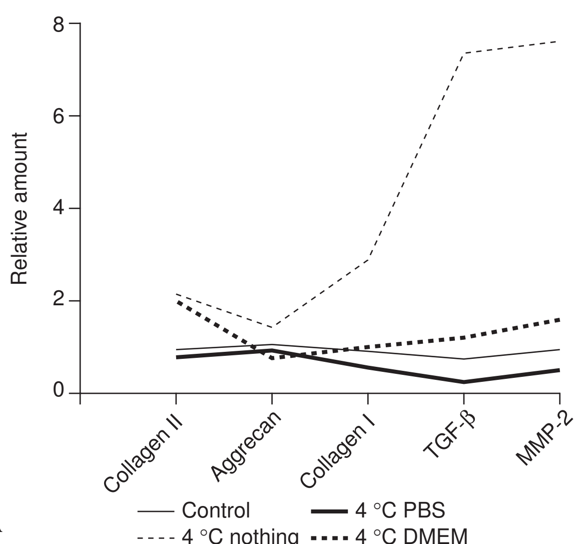


Figure 2. Using fresh young cartilage as a reference, relative quantities of mRNA for different catabolic factors studied in different groups. DMEM: Dulbeccos Modified Eagle Medium; DMSO: dimethyl sulphoxide; MMP: Matrix metalloproteinases; PBS: Phosphate buffer saline.

was similar to that seen in the control group. In the group without cryopreservatives (group 4) the expression of collagen type II decreased, and the expression of TGF-β increased (Figure. 3B).

Treatment with Transforming Growth Factor , (TGF-β)

The group preserved at 4 ºC without cryopreservatives increased its expression of TGF- β and MMP-2. The group stored with PBS (group 2) also increased, to a lesser degree, the expression of TGF-β and decreased its expression of MMP-2 in comparison with the control group. The group maintained at 4 ºC in culture media (group 3) had expression values similar to those of the control group (Figure. 4A). As to the –80 ºC without cryopreservatives (group 4), its expression of collagen type II and TGF-β increased; whereas its MMP-2 values were similar to the control group. This group showed a light improvement when preserved in glycerol



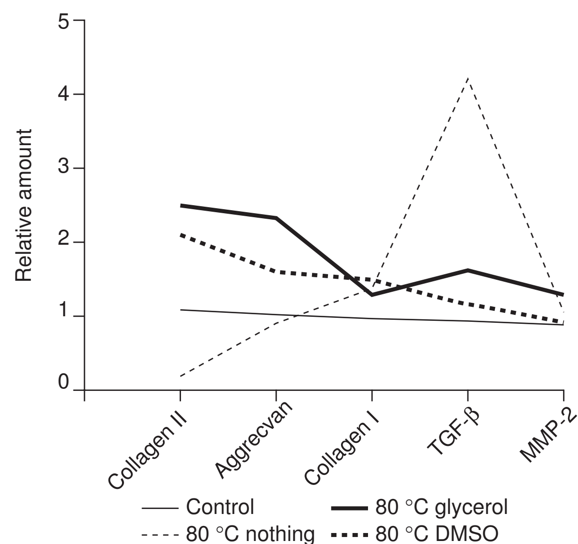


Figure 3. Gene expression of the genes studied treated with OP-1® (BMP-7) in cell cultures. (A) Preserved at 4 ºC. (B) A –80 ºC. DMEM: Dulbeccos Modified Eagle Medium; DMSO: dimethyl sulphoxide; MMP: Matrix metalloproteinases; PBS: Phosphate buffer saline; TGF-β,: transforming growth factor β.

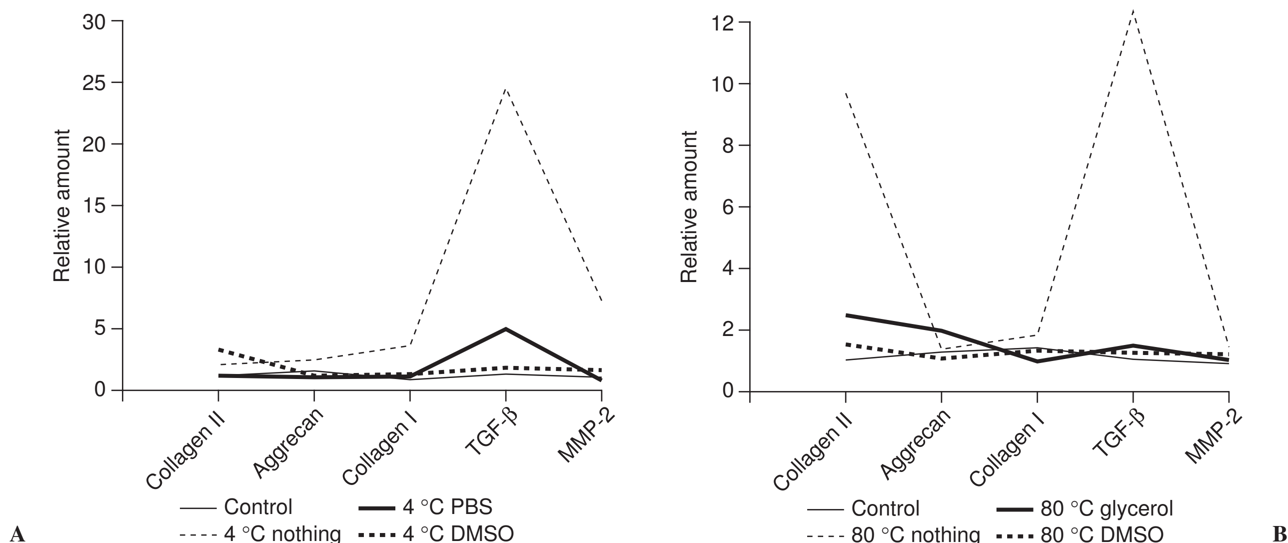


Figure 4. Gene expression of the genes studied treated with TGF-β in cell cultures. (A) Preserved at 4 ºC. (B) A –80 ºC. DMSO: dimethyl sulphoxide; MMP: Matrix metalloproteinases; PBS: Phosphate buffer saline; TGF-β: transforming growth factor β.

(group 5); whereas the group in DMSO (group 6) had values similar to the control group (Figure 4B).

Treatment with Fibroblast Growth Factor

The 4 ºC groups without cryopreservatives (group 1) and PBS (group 2) showed an increase in gene expression for collagen type II, aggrecan and TGF-β, this was highest in the 4 ºC group without cryopreservatives. Furthermore, in this last group the expression of collagen type I and MMP-2 also increased. The group with culture media (group 3) had less gene expression than the control group for genes: collagen type II, aggrecan and collagen type I, and an increase in gene expression for genes: TGF-β and MMP-2 (Figure 5A).

In the –80 ºC group, the 3 groups showed similar gene expression for collagen type II, aggrecan and collagen type I. Only the groups with cryopreservatives (group 4) and with glycerol (group 5) increased gene expression of TGF-β and

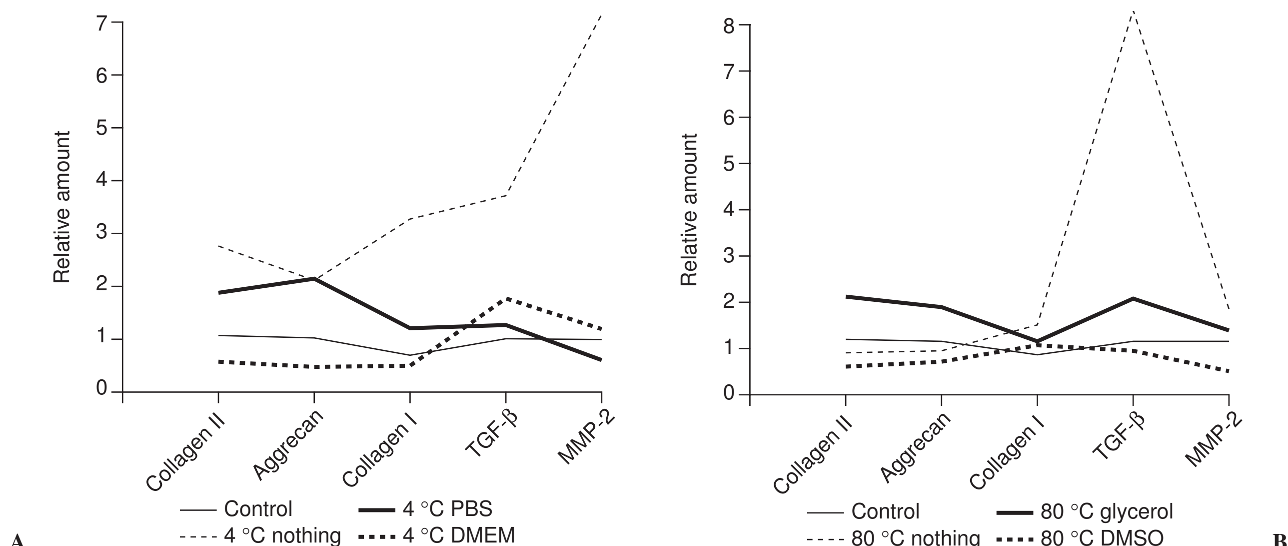


Figure 5. Gene expression of the genes studied treated with FGF-a in cell cultures. (A) Preserved at 4 ºC. (B) A –80 ºC. DMEM: Dulbeccos Modified Eagle Medium; DMSO: dimethyl sulphoxide; MMP: Matrix metalloproteinases; PBS: Phosphate buffer saline; TGF-β: transforming growth factor β.

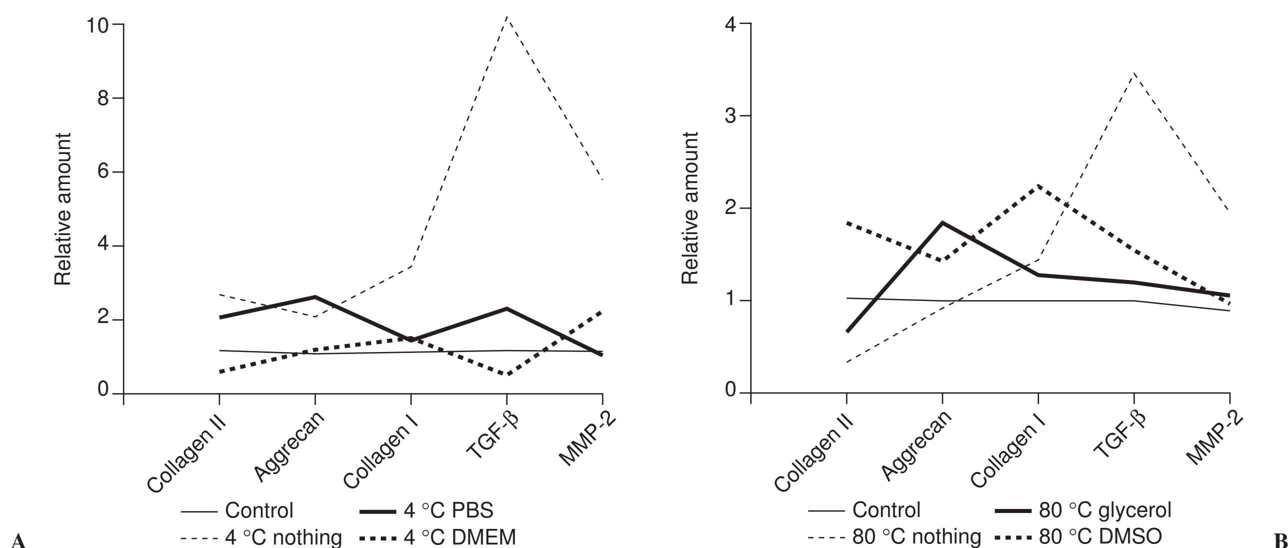


Figure 6. *Gene expression of the genes studied treated with IGF-1 in cell cultures. (A) Preserved at 4 ºC. (B) A –80 ºC. DMEM: Dulbeccos Modified Eagle Medium; DMSO: dimethyl sulphoxide; MMP: Matrix metalloproteinases; PBS: Phosphate buffer saline; TGF-β,: transforming growth factor β.*

MMP-2, whereas this decreased slightly in the group with DMSO (group 6) (Figure 5B).

Treatment with IGF-1 (insulin-like growth factor-1)

The 4 ºC group without cryopreservatives (group 1) showed a significant increase in expression of TGF-β, collagen type 1 and MMP-2, and less expression of collagen type II and aggrecan. The 4 ºC group in PBS (group 2) increased the expression of collagen type II, aggrecan and TGF-β, whereas the expression of MMP-2 was similar to the control group. In the group stored with culture media (group 3) the expression of collagen type II and TGF-β decreased, whereas the rest of its gene expression was similar to the control group (Figure 6A). In the cells stored at –80 ºC, only the group stored in DMSO (group 6) showed and increase in the expression of collagen type II, collagen type I and, slightly, TGF-β. The group without cryopreservatives (group 4) showed an increase in the expression of TGFβ whereas the group preserved in glycerol (group 5) only increased its expression of aggrecan (Figure 6B).

DISCUSSION

Cartilage cells can constantly synthesize and degrade the components of the extracellular matrix. Alterations in their culture conditions cause significant changes in their gene expression. Age and different pathological conditions also cause modifications. It is important to know about the existence of these phenomena so as to prevent or treat them, with the help of interleukin[8] growth factors that stimulate or inhibit the synthesis of matrix components[9-11], modulating the action of molecules such as proteases (MMP) or their tissue inhibitors (TIMP)[12,13].

Adult joint cartilage is a complex structure, formed by a heterogeneous population of cells that express collagen type I in the surface layer, collagen type II in the middle layer and collagen type X in the deepest or mineralized[14] layer. On the other hand, cartilage suffering from arthritis synthesizes collagen type I during the final phases of degeneration[15]: this causes the transformation of cartilage into fibrocartilage.

Mitotic and synthetic activity of human chondrocytes decreases with age[16,17], and responds less to anabolic stimuli. It would not be something strange if from the fifth decade of age on the prevalence of joint degeneration were responsible for an increase in arthritis with each decade and that the capacity for repair should decrease. In our study we saw that in the groups of old cartilage and cartilage with arthritis, there was an increase in the expression of factors such as MMP-2 and collagen type I, although the expression of MMP-13 was less than in the control group.

The response of chondrocytes to TGF-??increases in arthritis, and this stimulates the synthesis of proteoglycans in normal cartilage and in cultures of human cartilage with arthritis[18,19]. BMP-2, 4 and 7 are potent chondrocyte and osteoblast differentiation factors in vivo, they induce ectopic formation of cartilage and bone and help to the differentiation of mesenchymal cells into mature bone or cartilage cells[20].

Furthermore OP-1® (BMP-7) inhibits processes of degradation[21] and has an effect on the metabolism of chondrocytes, stimulating the synthesis, organization and reten-

tion of matrix molecules. In concrete terms, in human and bovine cultures it induces the synthesis of proteoglycans and collagen type II better than TGF-,[22].

Chondrocytes in joint cartilage express receptors for IGF-1, basically in the middle and deep zones[23]. Age affects both circulating levels of IGF-1[21] as also the sensitivity of chondrocytes to IGF- 1[24]. The capacity of chondrocytes to respond to IGF-1 decreases 30% with age[21].

Matrix metalloproteinases (MMP), especially MMP-1 and MMP-13, are enzymes that degrade components of the extracellular matrix; specifically interstitial collagens (types I, II and III)[25,26]. The MMP appear as a response to cytokines and growth factors in inflamed joints. Hsieh et al[27] studied serum samples of patients with knee arthritis and demonstrated the presence of significant higher levels of MMP-2 and 9. MMP-13 degrades cartilage and hydrolizes collagen type II[28,29].

In our study, we used the level of expression of anabolic factors in fresh cartilage as a reference to determine the relative quantities of mRNA codified by anabolic factors. In cartilage stored at 4 ºC with PBS there was an increase in the expression of collagen type II, TGF-??and BMP-7. BMP-7 also increased its expression in the 4 ºC groups in culture media without additives, in the three groups maintained at –80 ºC (without cryopreservatives, with glycerol and with DMSO) and in old cartilage. On the other hand, the greatest gene expression of cartilage catabolic factors (collagen type I and MMP-2 and 13) was seen in the group of cartilage stored at –80 ºC with glycerol or DMSO.

Likewise, the chondrocytes stored at different temperatures without cryopreservatives and treated with growth factors increase TGF–,'s gene expression, which makes us think about a possible proliferation stage or matrix synthesis.

The results obtained show that cartilage stored at –80 ºC expresses a greater quantity of catabolic factors (MMP-13, MMP-2 and collagen type I). Treatments with growth factors improve gene expression of anabolic factors in chondrocytes. It is necessary to plan new studies using different less aggressive freezing techniques and applying growth factors that improve cell viability and the characteristics of the extracellular matrix of cryopreserved cartilage.

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Conflict of interests

The authors have declared that they have no competing interests.