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DIRECCIÓN DE NUEVAS CREACIONES

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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 Folios 005

14686

Patente de Invención	☐	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☐	Cap. I ☐	Cap. II ☐

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 17 a 28	30/septiembre/2009
Reivindicaciones:	Folios 29 a 31	30/septiembre/2009
Prioridad:	MO 002554	03/octubre/2008

2. Análisis de la Prioridad

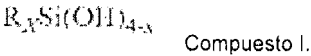
Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: “Complejo asociando un derivado orgánico del silicio con fragmentos calibrados de ácido hialurónico, para una acción preventiva y reparadora de las degradaciones cutáneas”.

El problema planteado en esta solicitud consiste en suministrar un complejo cosmético a base de fragmentos de ácido hialurónico y evitar el efecto adverso pro-inflamatorio de los pequeños de ácido hialurónico producidos por la acción catabólica de los tejidos cutáneos.

Para resolver este problema técnico la solicitud en estudio proporciona un complejo cosmético que asocia un derivado de silicio orgánico de formula I y fragmentos calibrados de ácido hialurónico.



El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 8/58 , 8/73 y A61Q 19/08.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de la reivindicación 11 no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

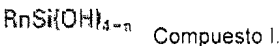
El uso de la reivindicación 10 no es patentable, de acuerdo con el Art 14.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: octubre 03 de 2008 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	GB 2159408	Produit hydratant	04/diciembre/1985
D2	OsteoArthritis and Cartilage (2005) 13, 688-696	Effect of hyaluronic acid (MW 500-730 kDa) on proteoglycan and nitric oxide production in human osteoarthritic chondrocyte cultures exposed to hydrostatic pressure	2005

D1:http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=19851004&CC=FR&NR=2561915A1&KC=A1 divulga una serie de composiciones cosméticas que comprenden un derivado del compuesto de fórmula I.



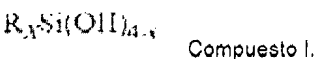
que de acuerdo con la descripción (Pág. 2, Ren. 52-57) y las realización preferidas corresponde a metilsilanotriol (Pág. 6, Ren 1-3) o fenilsalanetriol (Pág. 6, Ren 17-19) y ácido hialurónico (HA).

D2: divulga los resultados del efecto que tiene el ácido hialurónico de peso molecular entre 500-730 kDa al actuar sobre cultivos de condrocitos articulares humanos en presencia de la interleuquina IL-1β. De igual forma, indica (Pág. 1, Col. 1) que la interleuquina IL-1B es un potente inhibidor de proteoglicanos humanos y de la síntesis de colágeno y que (Pág. 2, Col. 1) el ácido hialurónico es un constituyente de los proteoglicanos humanos. Finalmente, concluye (Pág. 4, Col. 2) que la inhibición de la síntesis de proteoglicanos humanos en el modelo celular de condrocitos articulares humanos producida por la interleuquina IL-1β es restaurada por la presencia de ácido hialurónico y que esta depende de la concentración de HA.

7. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en un complejo a base de un derivado orgánico de silicio que se caracteriza en que dicho derivado se asocia con uno o varios fragmentos calibrados de ácido hialurónico con un peso molecular comprendido entre los 150 y 780 kDa, teniendo dicho derivado una fórmula general (I).



Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

SOLICITUD		D1	D2
Característica A	Complejo que comprende	Combinación que comprende	No refiere
Característica B	Un compuesto de fórmula I $R_nSi(OH)_{4-n}$, Compuesto I.	Un compuesto de fórmula I $R_nSi(OH)_{4-n}$ Compuesto I.	No refiere
Característica C	fragmentos calibrados de ácido hialurónico con un peso molecular comprendido entre los 150 y 780 kDa	ácido hialurónico	La interleuquina IL-1B es un potente inhibidor de proteoglicanos humanos y de la síntesis de colágeno y que esta es restaurada por el ácido hialurónico de peso molecular entre 500-730 kDa y es dependiente de su concentración.

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulgan una serie de combinaciones de componentes que comprenden derivados orgánico de silicio y ácido hialurónico.

La diferencia entre la invención, definida en la reivindicación 1 y la combinación de componentes definida en D1 es la característica C porque la invención define específicamente que el ácido hialurónico tiene un peso molecular que se encuentra entre los 150 y 780 kDa y la invención no refiere el peso molecular del ácido hialurónico empleado.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: “cómo seleccionar algunas combinaciones de los componentes divulgados en D1 para evitar el efecto adverso pro-inflamatorio de los pequeños fragmento de ácido hialurónico producidos por la acción catabólica de los tejidos cutáneos”

Sin embargo, dado que el documento D2 indica que la interleuquina IL-1B es un potente proinflamatorio e inhibidor de proteoglicanos humanos y de la síntesis de colágeno y que esta inhibición es restaurada por el ácido hialurónico de peso molecular entre 500-730 kDa.

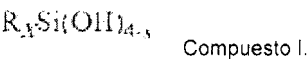
En consecuencia, la persona del oficio normalmente versada en la materia, tendría suficiente motivación para seleccionar las combinaciones de componentes que comprende el ácido hialurónico de peso molecular entre 500-730 kDa divulgadas en documento D1 de la manera como lo indica el documento D2 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:

La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

Dado que las características técnicas esenciales de la reivindicación 2-6 no refieren característica inventivas adicionales no se pueden considerar inventivas.

La reivindicación 7 consiste en un composición cosmética que comprende un derivado orgánico de silicio y uno o varios fragmentos calibrados de ácido hialurónico con un peso molecular comprendido entre los 150 y 780 kDa, teniendo dicho derivado una fórmula general (I).



Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

SOLICITUD		D1	D2
Característica A	Composición cosmética que comprende	Composición cosmética que comprende	No refiere
Característica B	Un compuesto de fórmula I $R_nSi(OH)_{4-n}$ Compuesto I.	Un compuesto de fórmula I $R_nSi(OH)_{4-n}$ Compuesto I.	No refiere
Característica C	fragmentos calibrados de ácido hialurónico con un peso molecular comprendido entre los 150 y 780 kDa	ácido hialurónico	La interleuquina IL-1B es un potente inhibidor de proteoglicanos humanos y de la síntesis de colágeno y que esta es restaurada por el ácido hialurónico de peso molecular entre 500-730 kDa y es dependiente de su concentración.

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 7 porque divulgan una serie de combinaciones cosméticas que comprende derivado orgánico de silicio y ácido hialurónico.

La diferencia entre la invención, definida en la reivindicación 1 y la composición cosmética definida en D1 es la característica C porque la invención define específicamente que el ácido hialurónico tiene un peso molecular que se encuentra entre los 150 y 780 kDa y la invención no refiere el peso molecular del ácido hialurónico utilizado.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: "cómo seleccionar algunas composiciones cosméticas divulgada en D1 para evitar el efecto adverso pro-inflamatorio de los pequeños fragmentos de ácido hialurónico producidos por la acción catabólica de los tejidos cutáneos"

Sin embargo, dado que el documento D2 indica que la interleuquina IL-1B es un potente proinflamatorio e inhibidor de proteoglicanos humanos y de la síntesis de colágeno y que esta inhibición es restaurada por el ácido hialurónico de peso molecular entre 500-730 kDa.

En consecuencia, la persona del oficio normalmente versada en la materia, tendría suficiente motivación para seleccionar las composiciones cosméticas que comprende ácido hialurónico de peso molecular entre 500-730 kDa divulgadas en documento D1 de la manera como lo indica el documento D2 para llegar así al objeto de la reivindicación 7 de la solicitud. Por lo cual se considera obvia.

Dado que las características técnicas esenciales de la reivindicación 8 y 9 no refieren característica inventivas adicionales no se pueden considerar inventivas.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	GB 2159408	1-9	Nivel inventivo
D2	OsteoArthritis and Cartilage (2005) 13, 688-696	1-9	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante (60 días) y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 29 AGO. 2012

Elaborado por: Lenin Roberto Guerrero.
Revisado Por: Consuelo Leguizamón Leguizamón.
Aprobado por: José Luis Salazar López.

Osteoarthritis and Cartilage



International
Cartilage
Repair
Society



Effect of hyaluronic acid (MW 500–730 kDa) on proteoglycan and nitric oxide production in human osteoarthritic chondrocyte cultures exposed to hydrostatic pressure

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Summary

Objective: This study investigated the *in vitro* effects of hyaluronic acid (HA) of molecular weight (MW) 500–730 kDa on human articular chondrocytes cultivated for 48 h in the presence of interleukin-1β (IL-1β) with and without hydrostatic cyclical pressure.

Design: The effects of 10 and 100 µg/ml 1HA with and without IL-1β were assessed in the culture medium of cells exposed to pressurization cycles in the form of sinusoidal waves (minimum pressure 1 MPa, maximum pressure 3 MPa) at a frequency of 0.25 Hz for 3 h, by the immunocytochemical method on microplates for the quantitative measurement of human proteoglycans (PG) and by the Griess method for nitrites (NO). Morphological analyses were performed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Results: The presence of IL-1β determines a significant decrease in PG and a significant increase in NO concentrations measured in the culture medium. When the cells are cultured in the presence of IL-1β and HA at the two concentrations, a statistically significant restoration of PG and a decrease in NO levels are observed. Under pressurization conditions, we observed that the PG concentration in the medium of cells presented a very significant increase in all the conditions used in this study, except for IL-1β alone. NO production decreased very significantly in the presence of IL-1β + HA 10 and IL-1β + HA 100. The results of metabolic evaluation are confirmed by morphological findings obtained by TEM and SEM.

Conclusions: These *in vitro* studies confirm both the protective role of HA (MW 500–730 kDa), which counteracts the IL-1β-induced effects, and the importance of pressure on chondrocyte metabolism and morphology.

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Key words: Chondrocytes, Osteoarthritis, Hydrostatic pressure, Hyaluronic acid, Proteoglycan, Nitric oxide.

Introduction

The destruction of articular cartilage is a common feature in joint diseases such as osteoarthritis (OA). The structural breakdown of proteoglycans (PG) and collagen is believed to be the result of an increased catabolic activity by chondrocytes¹.

Interleukin-1β (IL-1β) is a cytokine involved in cartilage degradation processes². In response to IL-1β, chondrocytes secrete neutral metalloproteinases (MMPs), and nitric oxide (NO)^{3–5}. Moreover, IL-1β is a potent inhibitor of PG and collagen synthesis⁶.

NO is a highly reactive, cytotoxic, free radical that has been implicated in tissue injury in a variety of diseases. Increasing evidence indicates that NO may also contribute to the pathophysiology of OA. Increased nitrite concentrations in serum and synovial fluid from patients with rheumatoid arthritis and OA have been reported⁷. Moreover,

NO promotes numerous effects on chondrocytes: inhibition of matrix synthesis⁸, activation of MMPs⁹, induction of apoptosis¹⁰, reduction of IL-1 R antagonist (IL-1 Ra)¹¹.

The increased production of NO may be an additional factor contributing to the excessive production of prostaglandin E₂ (PGE₂) by OA tissue¹².

PGE₂ and NO are known to play a major role in nociception^{13,14}. NO in particular is an important neurotransmitter, responsible for central sensitisation of the pain pathway¹⁵.

Several *in vitro* studies have shown the importance of mechanical compression or hydrostatic pressure as a cartilage metabolism modulator. The biosynthetic response of chondrocytes to hydrostatic pressure *in vitro* varies with the magnitude, frequency and duration of loading^{16–21}. Chondrocytes respond to intermittent loading by increased PG synthesis, whereas continuous mechanical loading produces the opposite effect. Hydrostatic pressure affects proteins, the cytoskeleton and cell organelles²²; compression of cartilage results in the deformation of cells and the extracellular matrix, as well as physicochemical changes which include altered matrix water content, fixed charge density, mobile ion concentrations, and osmotic pressure²³.

Hyaluronic acid (HA) is a component of the PG of the cartilage matrix. It is synthesised by chondrocytes, fibroblasts and synovocytes and released into the extracellular matrix and synovial fluid. In the course of OA there is a decrease in the HA content of both the cartilage and synovial fluid²⁴.

Intra-articular injections of hyaluronan of molecular weight (MW) of 500–730 kDa (Hyalgan[®]) have been used for several years in OA treatment. Several clinical, controlled studies have shown that five intra-articular injections of this product, significantly reduce pain and ameliorate function in patients with knee OA, with an effect lasting several months^{25,26}. HA also has a structure-modifying potential in OA^{26–28}.

Chondrocyte cultures represent a valid and simplified biological model to test the effects of drugs and such agents as cytokines, hormones and growth factors. They can also be used to evaluate the influence of hydrostatic pressure on chondrocyte morphology and metabolism.

In this work, we studied the *in vitro* effects of HA (MW 500–730 kDa) on human articular chondrocytes cultivated in the presence or in the absence of IL-1β, during 48 h of culture with and without cyclic hydrostatic pressure of a magnitude and frequency close to those that presumably exist in articular cartilage^{29,30}. Under these conditions, we evaluated the effect of HA ± IL-1β through metabolic (PG levels and NO production in the culture medium) and morphological assessments carried out with a transmission electron microscope (TEM) and scanning electron microscope (SEM).

Method

CELL CULTURE

OA human articular cartilage was obtained from the femoral heads of five patients with OA, defined by clinical and radiological american college of rheumatology (ACR) criteria³¹, who were undergoing surgery for total hip prostheses. The mean age of the group was 68 years (range 63–71).

Macroscopically, the cartilage was not altered, but histological study of representative samples showed typical osteoarthritic changes, such as the presence of chondrocyte clusters, loss of methadromatoma, and fibrillation. Immediately after surgery, the cartilage specimens were cut aseptically, minced into 2 mm² pieces and sequentially digested by clostridial collagenase (SIGMA, Italy), 1 mg/ml in phosphate buffered saline (in mM: NaCl 140, KCl 2.7, NaH₂PO₄ 8.1, K₂HPO₄ 1.5, pH 7.4), containing 200 U/ml of penicillin, 200 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. Collagenase digestion was carried out at 37°C for 18 h with moderate stirring. The chondrocytes obtained after collagenase digestion were rinsed twice in saline solution A (in mM: 10 2-(2-hydroxyethyl)-1-piperazine] ethanesulfonic acid (HEPES), 140 NaCl, 5 KCl, 5 Glucose, pH 7.4) and centrifuged for 10 min at 700 g. As shown by the Trypan blue viability test, 80–95% of the recovered cells were alive.

Cells were cultured in 24-well microplates at a density of 5 × 10⁵ cells/well and overlaid with 1 ml of medium containing 10% foetal calf serum, 200 U/ml penicillin, 200 µg/ml streptomycin, 2 mM glutamine (in Dulbecco's Minimum Essential Medium). Cells were maintained in an atmosphere of 5% CO₂ in the air, at 37°C for 48 h.

STUDY WITH HYALURONAN PREPARATIONS

The effects of HA (MW 500–730 kDa, Hyalgan[®]) in the presence of IL-1β were analysed on chondrocytes cultured

as described above. The HA (batch Nr. 056/00) at concentrations of 10 and 100 µg/ml, with or without human recombinant IL-1β (Boehringer Mannheim, Italy) 5 ng/ml was added to the culture medium throughout the test period. After 48 h, the medium was removed and stored at 70°C pending determination of its PG and NO content.

The production of NO and PG in samples of cell cultures at baseline and after incubation in the presence of IL-1β, alone or associated with the various concentrations of HA with or without pressurization, was evaluated in the culture medium removed after 48 h of incubation.

PRESSURIZATION SYSTEM

Our pressurization system has some special characteristics that have already been described in detail³². Briefly, this pressure chamber system consists of a hermetically sealed stainless steel cylinder, 400 mm high with an internal diameter of 90 mm.

The chamber, filled with distilled water and maintained at a temperature of 37°C by the thermostat system, is then pressurized using the hydraulic energy produced by an electro-power pack and applied through the transfer accumulator to the water in the chamber. By means of a data processing system installed in a personal computer and based on turbo-Pascal language, the operator can preset and modify the pressure inside the chamber for the entire duration of the experiment. This system permits pressure levels ranging between 0.24 Hpa to be reached in automatically carrying out the entire programme of the filling and thermostatic control steps of the pressure chamber and of pressurization according to preselected periodic functions. The loading and unloading periods for cyclic pressure can be freely selected.

Chondrocytes were cultivated on Petri dishes. After 48 h, the dishes were filled with culture medium and sealed with a covering of Surlyn 1801 Bynel CXA 3048 bilayer membrane (thickness 90 µm; Du Pont, Italy), after expelling all air. Surlyn membrane is partially permeable to O₂ and CO₂, but not to water or other solutions and it is suitable for preserving a stable environment³³. The membrane was attached to the rim of the Petri dish with Jet Melt 3764 adhesive (3 M, Italy). During the experiments, the dishes were immersed in preheated (37°C) distilled water.

In this study, the chondrocytes covered in culture medium were pressurized by applying sinusoidal waves with a minimum pressure of 1 MPa, a maximum pressure of 3 MPa and a frequency of 0.25 Hz for 3 h. Dishes cultivated in the loading chamber but without undergoing pressurization served as controls. After pressurization, the culture medium was collected and stored at 70°C for PG and NO determination and the chondrocytes were immediately fixed for TEM and SEM.

BIOCHEMICAL ASSAYS

The quantity of PG in the culture medium was measured by the immuno-enzymatic method³⁴ on microplates for the quantitative measurement of human PG (Medgenix Diagnostics, Belgium).

This technique uses two monoclonal antibodies which are directed against keratan sulphate PG, and another monoclonal antibody which is directed against the bonding site of hyaluronic acid PG. Standards and samples containing PG react with the monoclonal antibody (MAb1) attached to the well of the microplate,

as well as with another monoclonal antibody (MAbs2) labeled with horseradish peroxidase (HRP). After an incubation period which allowed for the formation of a sandwich made up of coated MAbs1/PG/MAbs2-HRP, the wells were washed several times in order to remove the labelled antibody which had not bonded. The amount of bonded antibody was measured by adding and incubating a chromogen solution.

The reaction was then blocked by adding a stop solution. The microplate was read at 450 nm and the quantity of substratum was determined colorimetrically by measuring the absorbance, which is proportional to PG concentration. The assay sensitivity was 0.9 ng/ml.

NITRITE ASSAY

The quantity of nitrites in the culture medium was measured by the Griess method²⁵. Equal volumes (100 µl) of supernatant and Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthylethylenediamide dihydrochloride in 5% H₃PO₄) were incubated on microplates at room temperature for 15 min. The absorbance was measured with a spectrophotometer at 557 nm. The concentration of nitrites was calculated using a standard curve made by successive dilutions of a solution of sodium nitrite in water.

TEM

Cultures of human chondrocytes were fixed for 2 h at 4°C in cold Karnovsky fixative²⁶, rinsed overnight in 0.1 M pH 7.2 cacodylate buffer and post-fixed for 1 h at 4°C in 1% buffered OsO₄, dehydrated in a graded series of ethanol and embedded in Epon-Araldite.

Ultra thin sections cut with an LKB III ultramicrotome were collected on copper grids, stained with uranyl acetate and lead citrate and then photographed using a Philips CM10 electron microscope. We examined at least 100 cells from each group.

SEM

Cultures of human chondrocytes were fixed for 2 h at 4°C in cold Karnovsky fixative²⁶, washed in cacodylate buffer 0.1 M pH 7.2 overnight, post-fixed in 1% buffered OsO₄, in veronal acetate buffer for 2 h and washed in cacodylate buffer 0.1 M pH 7.2. The chondrocytes were dehydrated in a graded series of ethanol, placed in tetrahydrofuran and frozen at 0°C before drying by sublimation of the tetrathanol in a vacuum chamber. The samples were sputter coated with gold and observed in a Philips SEM 505. At least 200 chondrocytes from each group were evaluated.

STATISTICAL ANALYSIS

The data were expressed as the mean ± SD of PG release into the culture medium per 10⁶ cells and NO (ng/10⁶ cells) release into the culture medium in the five tested conditions.

For comparison of the two groups, a paired test of Wilcoxon was used; *P* < 0.05 was considered significant.

Results

The total PG concentration in the culture medium during the 48 h at baseline conditions, in the presence of a damaging stimulus represented by IL-1β at a concentration of 5 ng/ml and in the presence of IL-1β and of HA at the two concentrations used in the study (i.e., 10 and 100 µg/ml of culture medium, is shown in Fig. 1. The presence of IL-1β determines a very significant decrease (*P* < 0.001) in PG levels, but when the cells are cultured in the presence of IL-1β and HA at the two concentrations, a statistically significant restoration of PG production is observed (HA 10, *P* < 0.05; HA 100 *P* < 0.01). Data show that the restoration is dose-dependent (Fig. 1).

In Fig. 1 we compare the data obtained under pressurized conditions with those obtained without pressurization. There is a very significant increase (*P* < 0.001) in the level of PG in the culture medium in basal conditions, while pressurization does not significantly increase the PG levels in the cells

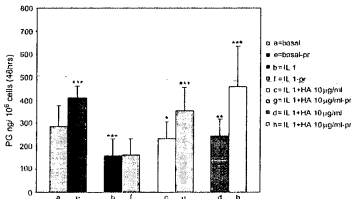


Fig. 1. Total amount of PG measured in culture medium (ng/10⁶ cells) during 48 h of culture (pooled data) in basal conditions, in the presence of IL-1β and in the presence of IL-1β and HA 10 and 100 µg/ml with and without pressurization conditions. - pr = cyclic pressurization minimum pressure 1 MPa and maximum pressure 5 MPa at 0.25 Hz frequency. Data are expressed as mean values ± SD. b vs a: *P* < 0.001; c vs b: *P* < 0.05; d vs b: *P* < 0.01; *P* < 0.05; *P* < 0.001. Data are expressed as mean values ± SD. c vs b: *P* < 0.001; d vs b: *P* < 0.001; *P* < 0.05; *P* < 0.01; *P* < 0.001.

damaged with IL-1β. In pressurized conditions, the addition of HA induces a very significant increase (*P* < 0.001) at both concentrations tested (10 and 100 µg/ml).

The NO (ng/10⁶ cells) production in the culture medium at baseline conditions, in the presence of IL-1β at a concentration of 5 ng/ml and in presence of IL-1β and of HA at the two concentrations used in the study, is shown in Fig. 2. The presence of IL-1β determines a very significant increase (*P* < 0.001) in NO production, but when the cells are cultured in the presence of IL-1β and HA, a very statistically significant decrease (*P* < 0.001) in NO production is observed at the two concentrations studied (Fig. 2).

In Fig. 2 we compare the data obtained under pressurized conditions with those obtained without pressurization. There is a slight, but not significant, decrease in the concentration of NO at basal conditions. As Fig. 2 shows, the physiological pressurization determines a significant decrease (*P* < 0.05) in the level of NO in the culture medium in the presence of IL-1β. The addition of HA induces a further significant decrease in NO in the culture medium in a dose-dependent manner at both concentrations used.

The results concerning metabolic production are further confirmed by the morphological findings obtained by TEM and SEM that indicate effects actuated by IL-1β, HA and pressure on the metabolic functions of the chondrocytes. Figure 3 shows cultured cells without pressurization, under basal conditions (A) the nucleus appears euchromatic, the cytoplasm contains rough endoplasmic reticulum (RER) and lipid droplets, and in the presence of IL-1β (B) the chondrocyte shows several vacuoles in the cytoplasm. Figure 3(C, D) shows cells in the presence of IL-1β + HA 10 (Fig. 3(C)) and IL-1β + HA 100 (Fig. 3(D)). In both cases the nuclei are euchromatic and the cytoplasm contains a reduced number of vacuoles and RER and lipid droplets are present.

Figure 4 shows cultured cells after pressurization: under basal conditions (A) the cell shows a cytoplasm rich in RER and mitochondria, in the presence of IL-1β (B) the chondrocyte shows RER and mitochondria limited to a part

of cytoplasm which also contains a number of vacuoles. Figure 4(C, D) shows cells in the presence of IL-1β + HA 10 (Fig. 4(C)) and IL-1β + HA 100 (Fig. 4(D)) and a recovery of the cell structures can be observed. SEM images confirm TEM data. Chondrocytes retain their spherical shape under basal conditions and some secretion granules and collagen fibrils are present (Fig. 5(A)). Conversely, cells cultured in the presence of only IL-1β show a loss of cytoplasmic processes and the presence of superficial alterations (Fig. 5(B)). After pressurization, in the presence of IL-1β + HA 10 or HA 100, chondrocytes present a restored surface morphology (Fig. 5(C)).

Discussion

In the present study, the effects of IL-1β and HA (MW 500–750 kDa) were tested in the presence and absence of cyclic hydrostatic pressure on the morphology of *in vitro* human osteoarthritic chondrocytes and their production of PG and NO. It is currently accepted that IL-1β is an important mediator in the cartilage destruction observed in OA²⁷. IL-1β is able to increase the synthesis of MMPs, to inhibit the synthesis of the major physiological inhibitors of these enzymes and to inhibit the synthesis of PG and collagen²⁸.

Our experiments have shown that the addition of IL-1β determines a reduction in the concentration of PG in the culture medium; this could be due to the fact that the cytokine IL-1β induces an inhibition of PG synthesis by the chondrocytes, as shown in the morphological studies.

The morphological aspects show signs of cellular suffering with the presence of vacuoles and a lack of the cellular organelles typically responsible for the synthesis of the matrix glycoproteins (Figs. 3(B), 5(B)). When the cells were cultivated in the presence of IL-1β and HA, a restoration of PG concentration in the culture medium was detected. The increase in PG levels depends upon the HA doses used (Fig. 1). This result is in accordance with data from previous studies proving the HA inhibiting effect of IL-1β in chondrocytes^{27–29}. Structural evaluation confirms these biochemical findings: chondrocytes exhibit

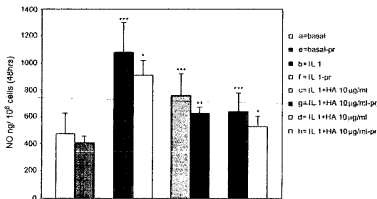


Fig. 2. Total amount of NO measured in culture medium (ng/10⁶ cells) during 48 h of culture (pooled data) in basal conditions, in the presence of IL-1β and in the presence of IL-1β and HA 10 and 100 µg/ml with and without pressurization conditions. - pr = cyclic pressurization minimum pressure 1 MPa and maximum pressure 5 MPa at 0.25 Hz frequency. Data are expressed as mean values ± SD. b vs a: *P* < 0.001; c vs b: *P* < 0.001; d vs b: *P* < 0.001; *P* < 0.05; *P* < 0.01; *P* < 0.001. Data are expressed as mean values ± SD. c vs b: *P* < 0.001; d vs b: *P* < 0.001; *P* < 0.05; *P* < 0.01; *P* < 0.001.

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Fig. 3. Sections of OA chondrocytes cultured under basal conditions. A) Control. The cell shows an euchromatic nucleus (N) and a cytoplasm abundant in RER. B) Presence of IL-1 β . The cell presents a very vacuolated cytoplasm (arrow); nucleus (N). C, D) Presence of IL-1 β + HA 10 (C) and IL-1 β + HA 100 (D). The cells recover their good state of health. The cytoplasm contains RER; nucleus (N), mitochondria (M), vacuole (V). A) 30,000 $\times$; B) 10,000 $\times$; C) 13,000 $\times$; D) 10,000 $\times$.

a good cytoplasmic organization [Fig. 3(C, D)] after the addition of HA. In a study on PG synthesis in bovine articular cartilage explants both in the presence and absence of IL-1 β , it was also reported that HA penetrated the cartilage and accumulated in the pericellular matrix of chondrocytes, thereby maintaining synthesis, which was suppressed by IL-1 β in the absence of HA²⁹. The articular cartilage tissue is constantly subjected to loading, which varies according to body weight, muscular tension, posture and physical activity. Hydrostatic pressure is one of the several factors which operate in the articular cartilage subjected to loading³⁰⁻³². In our study, the applied pressure was within the physiological range of the human joint. In fact, pressure peaks of up to 18 MPa were measured in human hip joints;

however, pressure levels of 5 MPa are most often encountered in the knee joint during normal walking^{33,36}. Chondrocytes undergoing physiological pressurization according to sinusoidal waves with a minimum pressure of 1 MPa, a maximum pressure of 5 MPa and a frequency of 0.25 Hz, presented a greater metabolic activity which was expressed in a very significant increase of PG levels in the culture medium at basal conditions (Fig. 4). This was also shown in the morphological analysis by TEM [Fig. 4(A)] and SEM; chondrocytes presented a significant improvement in cellular features confirming this shift towards a more anabolic activity. In our opinion, the increase of PG in the culture medium which was also observed in basal conditions, could be determined by a stimulation of cellular activity induced by

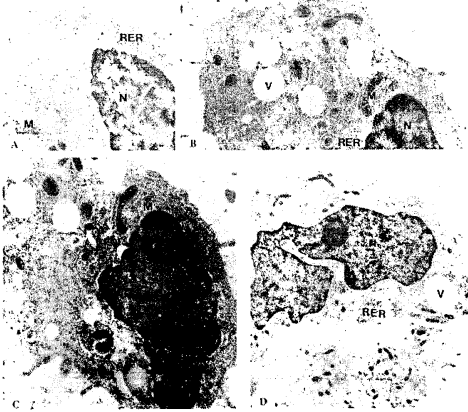


Fig. 4. Sections of OA chondrocytes cultured under pressurization. A) Control. The cell shows an euchromatic nucleus (N) and a cytoplasm abundant in RER, mitochondria (M). B) Presence of IL-1 β . The cytoplasm shows a partial vacuolization (V); also a fair amount of RER is present, nucleus (N), mitochondria (M). C, D) Presence of IL-1 β + HA 10 (C) and IL-1 β + HA 100 (D). The cells show a good activity (abundant presence of RER) and reduced number of vacuoles (V). nucleus (N). A) 17,500 $\times$; B) 16,000 $\times$; C) 13,000 $\times$; D) 18,000 $\times$.

cyclical pressurization, as demonstrated by other authors^{34,35,37}. Hydrostatic pressure appears to modulate aggrecan biosynthesis through membrane-mediated pathways, such as the transport of cations, amino acids and macromolecules. It has also been suggested that hydrostatic pressure may alter the action of the membrane Na⁺/K⁺ pump, thus altering intracellular K⁺ concentrations³⁴. Also, cyclic adenosine monophosphate (cAMP), which has been identified as an important mediator of PG synthesis and cartilage growth³⁸, seems to play a direct role in the mechanical stimulation of matrix biosynthesis. Constant hydrostatic pressures that inhibit PG synthesis also inhibit cAMP accumulation through a calcium-mediated process³⁹. Conversely, intermittent hydrostatic pressures in chondrocyte cell culture systems result in concurrent increases in both cAMP and PG synthesis rates⁴⁰. The stimulating effect of the physiological hydrostatic pressure which we have shown is in agreement with that found by other authors who have also utilized human osteoarthritic cartilage^{41,42}. Some studies have shown that OA chondrocytes are more sensitive to physiological hydrostatic pressure than normal chondrocytes⁴²⁻⁴⁵. In our

study, however, the stimulating effect of pressure was not enough to counterbalance the negative effect determined by the addition of IL-1 β , which does indeed induce a serious metabolic and morphological imbalance [Fig. 4(B)]. However, the addition of HA provided protection from the effects of IL-1 β under the pressurization conditions used in the study. The simultaneous use of pressurization with the presence of HA, in fact determined a highly significant increase in PG concentration, as if a synergism existed between the drug and the mechanical factor regarding IL-1 β . Biochemical data were also supported by the morphological analysis (Figs. 4(C, D) and 5(C)). The morphological features we highlighted by TEM match those reported in other studies on man^{46,47}. In particular, the treatment with HA (MW 500–730 kDa) in patients with knee OA determined a significant increase in the superficial cartilage layer thickness, with an increase in chondrocyte density and a restoration of the characteristic structures controlling chondrocyte anabolism⁴⁸. Recent findings showed that NO is a potent mediator of cartilage damage in OA. Several *in vitro* studies demonstrated that NO and its products can accelerate cartilage catabolism and reduce its anabolism⁴⁹⁻⁵¹. Moreover,



Fig. 5. SEM micrographs of OA chondrocytes. A) Basal conditions. The cell exhibits a spherical shape; granules and collagenic fibrils are present (arrows). B) Presence of IL-1 β . The cell morphology is altered, moreover it is largely devoid of granules and fibrils (arrows). C) Pressurization - 8-16 h HA 100. The cell shows a spherical shape; granules and fibrils are present (arrows). A) B) 5000 $\times$, C) 6000 $\times$.

a selective inhibition of inducible nitric oxide synthase, can reduce the progression of experimental OA *in vivo*¹⁰.

In the present study, we demonstrated that, as reported, IL-1 β significantly increases the production of NO and that HA decreases in a very significant manner the production of NO at both concentrations used (Fig. 2).

These results tally with those recently published by Manero *et al.*¹¹ When cells were cultivated under pressure, a reduced production was already recorded under basal conditions (Fig. 2); moreover, physiological pressure significantly decreased IL-1 β -induced NO production. These results confirm the protective effect of cyclic hydrostatic pressure on the release of NO by chondrocytes when stimulated by various factors^{11,12}.

The addition of HA considerably increased the effect of hydrostatic pressure on NO production (Fig. 2). The exact mechanism of action of HA (MW 500-730 kDa) on chondrocytes has not yet been fully elucidated. Some of the biological activities of HA may be mediated by linking its specific receptors (cluster determinant (CD) 44, intracellular adhesion molecule (ICAM)-1, receptor for hyaluronate-mediated motility) that are expressed by a wide range of cells, including those implicated in the pathology of OA (e.g., inflammatory cells, synovocytes and chondrocytes)^{13,15}.

Recently, it was reported that the anti-apoptotic effect of HA in OA human chondrocytes was significantly decreased by the addition of both anti-CD-44 and anti-ICAM-1 antibodies to the cultures¹⁶. Other authors have shown that preincubating human cartilage explants with anti-CD-44 antibody reduces the inhibitory effect of HA on IL-1 β -induced MMP production¹⁷.

In conclusion, our study confirms the importance of hydrostatic pressure on chondrocyte metabolism. The use of isolated chondrocytes in the study of metabolic response to loading must be approached with caution, because of the important role of the natural extracellular matrix in signal transduction and in transmitting the external load to the cell and intracellular components. Cultured chondrocytes can be used, however, to study the mechanism by which pressure effects are mediated in the cells.

Furthermore, our study points out the stimulating action of HA on PG production and metabolic chondrocyte activation (TEM and SEM analysis) as well as its inhibiting effect on NO production.

These results confirm *in vitro* the data obtained in clinical studies on patients with OA that demonstrated a potentially structure-modifying action of HA (MW 500-730 kDa)¹⁸⁻²⁰. HA's effect is boosted *in vitro* by physiological pressurization; therefore, drug efficacy in man may be improved by appropriate physical exercise.

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