Effect of continuous high hydrostatic pressure on the morphology and cytoskeleton of normal and osteoarthritic human chondrocytes cultivated in alginate gels

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Abstract

Objective

To investigate the effect of continuous high hydrostatic pressure on the cell ultrastructure and cytoskeleton of cultivated normal and osteoarthritic (OA) human chondrocytes

Methods

The effects of continuous hydrostatic pressure (24 MPa) for 3 hrs on normal and OA chondrocytes were assessed by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and immunofluorescence microscopy (IF).

Results

Structural differences at the nuclear, cytoplasmic and cytoskeletal levels were observed between normal and OA chondrocytes. Continuous high hydrostatic pressure severely altered normal chondrocytes that became similar in structural organization to OA chondrocytes and further reduced the number of cell organelles involved in the synthesis of collagen and proteoglycans. IF showed major changes in the distribution of actin and tubulin after pressurization in normal and OA chondrocytes.

Conclusions

The results confirm the major role of pressure on chondrocyte ultrastructure. Continuous high hydrostatic pressure caused structural alterations in normal chondrocytes, which obtained similar, if not identical, characteristics to those typical of osteoarthritic chondrocytes.

Key words

Chondrocytes, cytoskeleton, continuous high hydrostatic pressure, osteoarthritis.

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Introduction

The etiology of osteoarthritis (OA) is only partially understood, although joint loading is certainly involved. Intense use of joints and traumatic forces on joints may increase the risk of OA (1). Articular cartilage is a tissue specialized in supporting heavy and varying loads and in reducing friction between articular bony heads. Cartilage is constantly exposed to loads that depend on body weight and muscle tension, which vary with posture and physical activity. The ability of cartilage to withstand these stresses depends on the structure and composition of its matrix. The matrix is produced by chondrocytes, therefore it is fundamental that chondrocytes function adequately (2).

Several *in vitro* studies have shown the importance of mechanical compression or hydrostatic pressure (HP) as a cartilage metabolism modulator. The biosynthetic response of chondrocytes to HP *in vitro* varies with the magnitude, frequency and duration of loading (3-8). Chondrocytes respond to intermittent loading by increased PG synthesis, whereas continuous mechanical loading produces the opposite effect.

The cellular cytoskeleton plays a fundamental role in the control of chondrocyte phenotype and in the physical interactions between chondrocytes and their extracellular matrix and it may therefore be involved in the process of mechanical signal transduction in articular cartilage (9). The cytoskeleton of chondrocytes is composed of microfilaments formed by subunits of actin, tubulin microtubules and intermediate filaments consisting of different protein subunits (10). Actin filaments have a fundamental function in the control of cell shape and differentiation and in the formation and flow of vesicles. Tubulin microtubules play an important role in the distribution of cell organelles and they are involved in the synthesis of collagen and PG, and in the cell division process. Furthermore, cytoskeleton disruption in chondrocytes might be involved in OA pathogenesis (11).

Our previous *in vitro* studies confirmed the positive effect of cyclic low HP (1-5 MPa) on osteoarthritic chondrocyte metabolism (12, 13) and morphology (14), but this pressure did not modify the altered cytoskeletal organization of OA chondrocytes (14). The aim of this study was to investigate the effects of continuous high HP (24 MPa) on the morphology and cytoskeleton of normal and OA chondrocytes cultivated in alginate gels.

Methods

Cell culture

Normal human joint cartilage was obtained, with informed consent, from the femoral heads of 8 subjects with displaced femoral neck fractures (5 females and 3 males), who had no history of joint disease and who had macroscopically normal cartilage. Osteoarthritic cartilage was obtained, with informed consent, from the femoral heads of 8 patients with OA defined by clinical and radiological ACR criteria (15) undergoing surgery for total hip replacement. The mean age of the normal subjects was 63 years (range: 49-70) and that of the OA patients was 66 years (range: 52-74).

Normal chondrocytes were obtained from the middle layer of cartilage of the femoral heads. OA chondrocytes were obtained from the area adjacent to OA lesions.

Cartilage specimens were stained with Safranin-O hystochemical stain to analyze proteoglycan distribution. OA specimens showed regions of macroscopic roughness, fissuring and eburnation, and sometimes mild fibrillation. Normal cartilage was characterized by a completely smooth glossy white surface and a healthy appearance without irregularities; no macroscopic nor microscopic roughness or fibrillation was observed.

Immediately after surgery, the cartilage specimens were cut aseptically, minced into 2-mm^2 pieces and digested sequentially with clostridial collagenase (Sigma, Italy) 1 mg/ml in phosphate buffered saline (PBS) (in mM: NaCl 140, KCl 2.7, NaH_2PO_4 8.1, K_2HPO_4 1.5, pH 7.4) containing 200 U/ml penicillin, 200 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Collagenase digestion was carried out at 37°C for 18 h with moderate stirring. Chondrocytes

thus obtained were rinsed twice in saline (A) (in mM: 10 HEPES, 140 NaCl, 5 KCl, 5 glucose, pH 7.4) and centrifuged for 10 min at 700 g. As shown by Trypan blue viable stain, 90-95% of the cells recovered were alive.
The cells were then mixed with alginate (Pronova LVG, Protan, Drammer, Norway) in saline solution (A) to a final density of 5×10^5 cells/ml and 0.75% alginate. Aliquots of alginate cell suspension were placed in 35-mm Petri dishes (Costar, Italy) containing a layer of polymerized alginate that prevented cells from sticking to the bottom of the dish. Polymerization was obtained by adding 2 ml $CaCl_2$ 50 mM in 10 mM HEPES, 0.1 NaCl, pH 7.4 which created a three-dimensional gel. After 5-10 minutes, when gel formation was complete, the excess solution was removed and the cell-containing gel washed twice with saline (A) and overlaid with a medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μg/ml ascorbate in Dulbecco's minimum essential medium (DMEM). The Petri dishes were maintained in an atmosphere of 5% CO_2 and air.

Pressurization
Our pressurization system has already been described in detail (16). Briefly, it 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, is pressurized by hydraulic energy produced by an electro-power pack and applied through the transfer accumulator to the water in the chamber. A data processing system based on turbo-Pascal language installed in a personal computer enables the operator to preset and modify the pressure inside the chamber for the duration of the experiment. Loading and unloading periods for cyclic pressure can be freely selected.
Chondrocytes were cultured on Petri dishes. After 48 hrs, the dishes were filled with culture medium and sealed with Surlyn 1801 Bynel CXA 3048 bilayer membrane (thickness 90 μm; Du Pont, Italy), after expelling all the air. Surlyn membrane is partially permeable to O_2 and CO_2, but not to water or to other solutions and it is suitable for preserving a stable environment (17). The membrane was attached to the rim of the Petri dish with Jet Melt 3764 adhesive (3M, Italy). During the experiments the dishes were immersed in preheated (37°C) distilled water. The experimental design permitted complete evacuation of air from the system so the application of pressure was purely hydrostatic.
A continuous pressure of 24 MPa was applied for 3 hrs. Dishes cultivated in the loading chamber at room pressure, but without undergoing pressurization, served as controls. After pressurization, the culture medium was removed and the cells in alginate gel were processed immediately for transmission (TEM), scanning (SEM) electron microscopy and immunofluorescence microscopy (IF).

Transmission electron microscopy
Cultures of human chondrocytes in alginate were fixed for 2 hrs at 4°C in cold Karnovsky fixative (18), rinsed overnight in 0.1 M pH 7.2 cacodylate buffer, postfixed for 1h at 4°C in 1% buffered OsO_4, dehydrated in a graded series of ethanol and then embedded in Epon-Araldite. Ultrathin sections cut with an LKB III ultramicrotome were mounted on copper grids, stained with uranyl acetate and lead citrate and then photographed with a Philips CM10 electron microscope. We observed at least 100 cells from each group.

Scanning electron microscopy
Cultures of human chondrocytes in alginate were fixed for 2 hrs at 4°C in cold Karnovsky fixative (18), washed in cacodylate buffer 0.1 M pH 7.2 overnight, postfixed in 1% buffered OsO_4 in veronal acetate buffer for 2 hrs, washed briefly in cacodylate buffer 0.1M and then placed in citrate pH 7.4 to remove alginate. The chondrocytes were dehydrated in a graded series of ethanol, placed in tert-butanol, frozen at 0°C and then air dried by sublimation of tert-butanol in a vacuum chamber. The samples were sputter coated with gold and observed in a Philips SEM 505. We evaluated at least 200 cells from each group.

Immunofluorescence microscopy
Normal and OA chondrocytes cultivated in alginate and washed in PBS were smeared on glass slides, air dried and fixed for 15 min in methanol at –20°C. The specimens were rehydrated in PBS, treated with 5% normal goat serum (NGS), diluted to 1:20 in PBS-0.1% bovine serum albumin (BSA) to avoid a non-specific staining, and then incubated in antiactin monoclonal antibody (Sigma, Italy) diluted to 1:500 with PBS-0.1%BSA-1%NGS or in antitubulin monoclonal antibody (Sigma, Italy) diluted to 1:500 with PBS-0.1%BSA-1%NGS for 2-3 hrs at room temperature (or overnight at 4°C). After accurate rinses in PBS, they were incubated for 1 h at room temperature in FITC-conjugated–goat antimouse IgG diluted to 1:1000 in PBS 0.1%-BSA 1%-NGS.
Controls for secondary antibodies were carried out omitting the primary antibodies.
All slides were then washed for 30 min in PBS, mounted in PBS-glycerol 1:10 containing 5% propyl-gallate (to reduce the fading of fluorescence), and examined in under a Leitz Aristoplan microscope equipped with fluorescence and Olympus BH-2 optics. Photographs were taken with a Kodak TMAX 400. The same procedures were also performed on cultured chondrocytes exposed to continuous high HP. At least 100 chondrocytes from each group were evaluated.

Morphometric and statistical analysis
For morphometric studies, we analyzed sections of four different blocks from each group. Only medially sectioned chondrocytes were investigated for standardization and comparison of the different groups; 100 chondrocytes were selected using the nucleus/cytoplasm ratio as the selection criterion. Our analysis was based on an established method for ultrastructural quantitative evaluation of changes in chondrocytes (19). Mitochondria and Golgi bodies were counted and expressed as

mean ± SD. Because distribution was not normal in all cases, statistical analysis for differences between groups was performed with distribution-free non-parametric tests. Morphological parameters were tested by the Mann-Whitney U test. Values of $p<0.05$ were considered significantly different.
Actin and tubulin signals were scored by the same researcher as absent, limited or intense (20). In each group, scores were expressed as percentages of the total number of cells studied.

Results

Structural and morphological examination by TEM and SEM showed marked differences between normal and OA chondrocytes under basal conditions. Normal chondrocytes had bright euchromatic nuclei and cytoplasm with many organelles with well developed smooth and rough endoplasmic reticulum and regularly shaped mitochondria. The Golgi bodies were well organized in cisternae and surrounded by many vesicles (Fig. 1A). Some OA chondrocytes showed partially heterochromatic nuclei and cytoplasm containing few cell organelles compared to normal chondrocytes (Fig. 1B). The number of mitochondria and Golgi bodies in OA chondrocytes was significantly lower ($p < 0.01$) than in normal cells (Table I). SEM images showed abundant matrix fibers and secretion granules in normal chondrocytes (Fig. 1C), which were partially lost in OA chondrocytes (Fig. 1D).
Figure 2 shows a normal chondrocyte after pressurization. The nucleus contains marginated chromatin, typical of apoptotic cells. Golgi apparatus, rough and smooth endoplasmic reticulum, mitochondria and vesicles are completely absent. The plasma membrane is not broken (Fig. 2A). Figure 2B shows an OA chondrocyte after pressurization. The chromatin is marginated, the cytoplasm contains vacuoles and lacks its typical structures and the plasma membrane is not broken. After pressurization, the morphology of normal chondrocytes became quite similar to that of OA chondrocytes. The number of mitochondria and Golgi bodies was significantly lower ($p < 0.01$) in normal and OA chondocytes after pressurization. In normal chondrocytes, numbers became similar to those of OA chondrocytes under basal conditions (Table I).
SEM observations confirmed these findings: normal (Fig. 2C) and OA (Fig. 2D) chondrocytes did not appear particularly different from one another after pressurization. They both showed superficial changes and a complete loss of cytoplasmic processes.
Cytoskeletal examination of normal chondrocytes under basal condition by IF showed the polarized fluorescent signal of actin on the apical sides of the cytoplasm in 78% of cells, whereas in OA chondrocytes the actin signal was not well defined and it was present in only 3% of cells (Table II). The distribution of tubulin was also different in normal and OA chondrocytes, showing a uniform signal at the periphery of normal cells (82%) and a uniform intense peripheral signal in only 8% of the latter (Table II). After pressurization, the localization of actin and tubulin was similar in OA and normal chon-

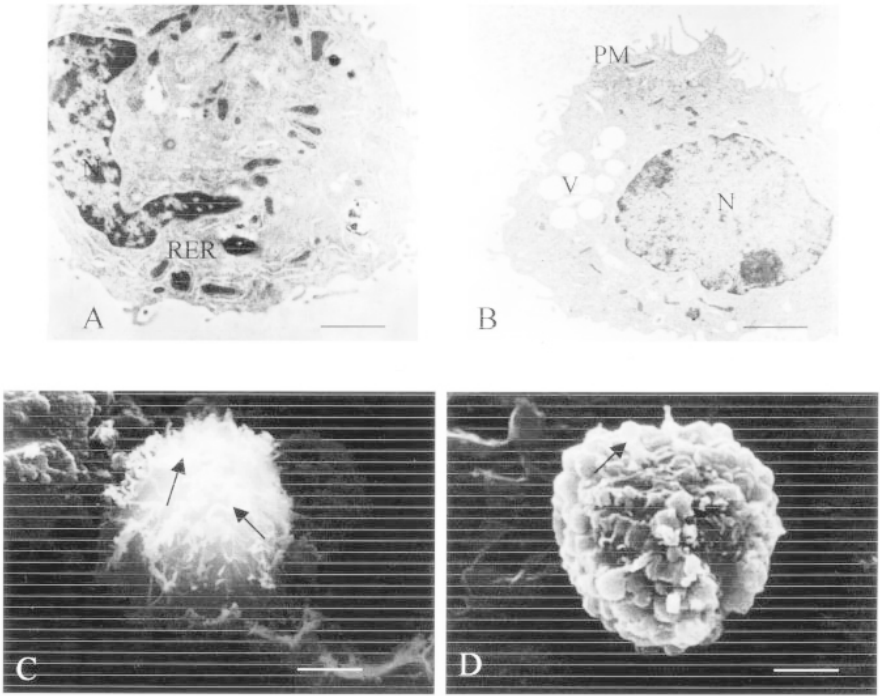

Fig. 1. Micrographs of cultured human chondrocytes.
(**A**) TEM micrograph of normal chondrocyte showing euchromatic nucleus (N) and cytoplasm with abundant rough endoplasmic reticulum (RER) (Bar 6.3 μm). (**B**) TEM micrograph of OA chondrocyte with many vacuoles (V) in the cytoplasm. The nucleus (N) contains normally condensed chromatin. The plasma membrane (PM) is not broken (Bar 4.5 μm).
(**C**) SEM micrograph of normal chondrocyte showing many superficial processes (arrows) and secretory granules (Bar 1.8 μm). (**D**) SEM micrograph of OA chondrocytes, spherical in shape, containing some secretory granules (arrows), but lacking superficial processes (Bar 2.5 μm).

Table I. Number of organelles in normal and OA chondrocytes under basal conditions and after pressurization.

	Basal condition		Pressurization	
	N	OA	N	OA
Mitochondria:number	7.5 ± 2.3	3.4 ± 1.7**	3.7 ± 1.9**	1.9 ± 1.5**
Golgi bodies:number	3.0 ± 1.3	1.5 ± 1.1*	1.1 ± 0.8*	0.7 ± 0.6*

**$p < 0.01$ (Mann-Whitney U test) OA basal conditions versus normal basal conditions; OA pressure versus OA basal conditions, normal pressurization versus normal basal conditions.
*$p < 0.01$ (Mann-Whitney U test) OA basal conditions versus normal basal conditions; OA pressure versus OA basal conditions, normal pressurization versus normal basal conditions.

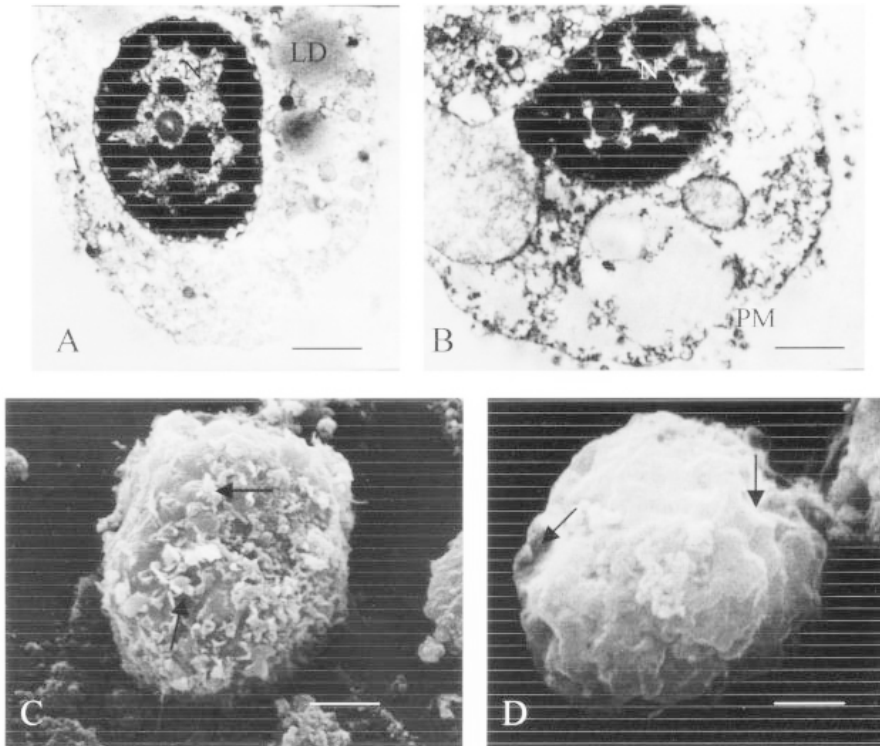

Fig. 2. Micrographs of cultured human chondrocytes after pressurization.
(**A**) TEM micrograph of normal chondrocyte showing nucleus (N) with marginated chromatin, cytoplasm devoid of organelles and full of vacuoles. Lipid droplets (LD) are present (Bar 7 μm). (**B**) TEM micrograph of OA chondrocyte structure became similar to that of normal chondrocytes. The nucleus (N). shows marginated chromatin and the cytoplasm is very vacuolated. The plasma membrane (PM) is not broken (Bar 2.5 μm).
(**C**) SEM micrograph of normal chondrocyte lacking collagen fibril processes and secretory granules (arrows) (Bar 1.8 μm). (**D**) SEM micrograph of OA chondrocyte with partially collapsed surface (arrows) and absence of superficial processes (Bar 2.5 μm).

Table II. Percentage of cells showing a regular disposition of filaments after incubation with actin (polarization signal) and tubulin (uniform peripheral signal) antibodies.

		Basal conditions		Pressurization	
		N	OA	N	OA
Actin	absent	7%	72%	76%	91%
	limited	15%	25%	23%	9%
	intense	78%	3%	1%	0%
Tubulin	absent	7%	67%	87%	97%
	limited	11%	25%	12%	3%
	intense	82%	8%	1%	1%

drocytes (Fig. 3). The fluorescent signal was diffuse (Figs 3A, 3B), and faint (Fig. 3C) with both antibodies. The percentage of cells showing intense polarity after incubation with anti-actin antibody and intense peripheral localization after incubation with anti-tubulin antibody was close to 0-1% in normal and OA chondrocytes.

Discussion

HP has multiple effects on chondrocyte behaviour but, in order to study the *in vitro* effect of pressure, it is also important to use a culture system that maintains the phenotypic stability of the chondrocytes. We used alginate as support for culturing human chondrocytes after considering the advantages and drawbacks of most currently-used techniques. Alginate gel is a valid alternative to other culture techniques since it enables chondrocytes to retain their 3D-structure and cell shape, while preventing dedifferentiation (21-23). Chondrocytes cultivated in alginate gel are in a condition that is as similar, as is currently possible, to the *in vivo* situa-

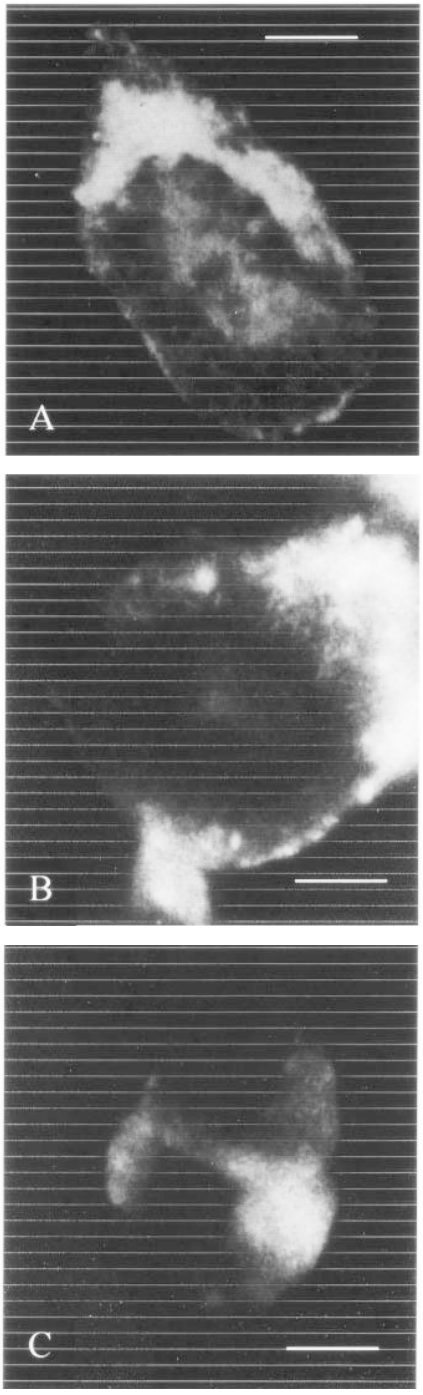

Fig. 3. UV micrographs of cultured human chondrocytes after pressurization: (**A**) Normal and (**B**) OA chondrocytes incubated with anti-actin showing no clear localization of the polarization signal and diffuse fluorescent staining at the cell periphery. (**C**) Normal chondrocyte incubated with antitubulin showing faint fluorescent signal in a thin layer at the periphery of the cell, immediately under the plasma membrane. (A-C: Bar 3 μm)

tion which is necessary for making reliable morphological and metabolic assessments (22, 23).

We found significant nuclear and cytoplasmic structural differences between normal and OA chondrocytes cultivated *in vitro* under basal conditions, normal cells were rich in synthetic and secretory cell organelles, while the cytoplasm of OA cells was poor in these organelles and it contained many vacuoles and glycogen deposits. Other authors have recently shown altered mitochondrial respiratory activity in OA chondrocytes (24). These changes represent an intracellular biochemical-enzymatic imbalance affecting chondrocytes during the OA process (25). HP is considered to have a major effect on chondrocyte metabolic activity and morphology (3-8, 14, 26-28).

In the present study, we used a continuous HP of 24 MPa which is beyond the range of physiological HP measured in human hip joints (29). We applied HP for as short a time as possible (3 hrs) to approximate physiological conditions. This pressure caused significant morphological changes detectable by TEM in normal chondrocytes, namely nuclear modifications, disorganisation of cytoplasmic organelles and an increased abundance of vesicles, typical features of OA chondrocytes. SEM also showed changes in the three-dimensional appearance of chondrocytes. Morphometric analysis of normal and OA chondrocytes showed a statistically significant reduction in the number of mitochondria and Golgi bodies compared to basal conditions. This is in line with reports of the packing of Golgi bodies and inhibition of PG synthesis in bovine chondrocytes subjected to continuous high HP (30, 31).

The results of IF examination confirmed the data from our previous study regarding the different localization of the two cytoskeletal proteins in chondrocytes (14). This localization depends on the different roles of actin and tubulin in the cell. Apical polarization of actin is fundamental for maintaining the form of the cell membrane and interactions with the extracellular matrix. The peripheral integrity of microtubulins ensures the correct localization and distribution of the cytoplasmic organelles (9, 10, 32). The different organization of the cytoskeletal structure of OA cells compared to normal cells shows that the regular cytoskeletal arrangement of actin and tubulin is altered during the course of OA (11, 14). After exposure to HP in our study, normal chondrocytes also showed diffuse and extremely faint fluorescent signals with both antibodies, similar to those observed in OA chondrocytes under basal conditions. The percentage of cells with regular intense localization of fluorescence after incubation with anti-actin and anti-tubulin was pratically zero in both normal and OA chondrocytes (Table II).

The results of the present study are similar to those reported by Parkkinen *et al*. (33) which demonstrated that short-term high continuous HP (30 MPa) modified the cytoskeletal structure of normal cultured bovine chondrocytes. On the other hand, low cyclic HP did not cause any cytoskeletal changes in normal human chondrocytes, as also found in our previous study (14).

The alteration of actin and tubulin distribution in chondrocytes affects a series of phenomena, including cell differentiation, the formation and flux of vesicles, and the synthesis of extracellular matrix molecules (9, 10, 32, 34, 35). The cytoskeleton also plays a fundamental role in the migration and proliferation of chondrocytes; these processes are known to be essential for cartilage repair (36).

The alteration of actin filaments may be responsible for morphological changes induced by mechanical stress, as observed by various authors (26-28). Disruption of the actin cytoskeleton also causes apoptosis in different cells (37-39) and could therefore be responsible for pressure-related induction of apoptosis in chondrocytes as observed by Islam *et al*. (40). The changes in tubulin that we observed may explain the effects of continuous high HP that were found in organelles and the reduced production of PG observed by others (4, 5, 30). It is not known what triggers cytoskeletal alterations induced by HP, but nitric oxide may be a mediator (34, 36). TNF-α increased NO synthesis by chondrocytes (41) and high HP increases the expression of IL-6 and TNF-α in cultures of a chondrocyte-like cell line (42).

In conclusion, our results confirm the existence of major nuclear, cytoplasmic and cytoskeletal differences between normal and OA chondrocytes. The continuous high HP used by us caused severe ultrastructural damage in normal and OA chondrocytes, while, after pressurization, normal chondrocytes acquired similar, if not identical, characteristics to OA chondrocytes, in contrast with the effects of low cyclic HP demonstrated in our previous study (14).

The present results suggest that mechanical stimulation may be used for *in vitro* and *in vivo* approaches to cartilage engineering. More sophisticated experiments are necessary to analyse the factors responsible for cytoskeletal damage in both OA and normal chondrocytes after high continuous HP and to determine whether pharmacological and/or physical treatment could prevent and/or reverse cytoskeletal alterations.

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