Effect of hydrostatic pressure of various magnitudes on osteoarthritic chondrocytes exposed to IL-1β

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Background & objectives: Several in vitro studies have shown the importance of mechanical compression or hydrostatic pressure (HP) as a modulator of cartilage metabolism. The present study was undertaken to evaluate the in vitro effects of cyclical low HP (1-5 MPa) and continuous high HP (24 MPa) applied in the presence or absence of interleukin (IL)-1β on human osteoarthritis (OA) chondrocytes.

Methods: Chondrocytes obtained from OA cartilage were cultivated for 48 h and then exposed to pressurization in the presence or absence of IL-1β. After pressurization, the culture medium was collected to detect the amount of proteoglycans (PG) and nitric oxide (NO) and the chondrocytes were immediately fixed for transmission electron microscopy (TEM) and processed for immunocytochemistry to localize the inducible nitric oxide synthase (iNOS).

Results: A significant increase in the level of PG and a small, non-significant, decrease in NO production were observed upon exposure to cyclical low HP. On the other hand, exposure to continuous high HP resulted in a significant decrease in the PG levels and a significant increase in NO production. The presence of IL-1β led to a significant decrease in PG levels as well as a significant increase in NO production. The cyclical low HP did not increase the PG levels significantly but caused a statistically significant decrease in NO production in cultures damaged with IL-1β. The continuous high HP in chondrocyte cultures stimulated with IL-1β did not significantly decrease PG production, but significantly increased NO production. The results concerning metabolic production were further confirmed by morphological findings obtained by TEM and immunocytochemical studies.

Interpretation & conclusion: The findings of this study confirmed that the response of chondrocytes varies with magnitude and frequency of HP. These findings are important to understand aetiopathogenetic mechanisms of OA and to find out which type of physical activity may be best suited for the prevention and therapy of OA.

Key words Chondrocytes - hydrostatic pressure - morphology- nitric oxide-proteoglycan

Osteoarthritis (OA) is a common rheumatic disease directly involving the articular cartilage. The structural breakdown of proteoglycans (PG) and collagen is believed to be the result of an increased catabolic activity by chondrocytes. Interleukin 1 beta (IL-1β) is a cytokine involved in cartilage degradation processes
and is a potent inhibitor of extracellular matrix (ECM) synthesis. In response to IL-1β chondrocytes secrete proinflammatory cytokines, chemokines, neutral metalloproteinases (MMPs) and nitric oxide (NO). NO is a highly reactive cytotoxic free radical that has been implicated in tissue injury in a variety of diseases. Increased nitrite concentrations in serum and synovial fluid have been reported in patients with rheumatoid arthritis and OA. NO promotes numerous effects on chondrocytes: inhibition of PG and collagen synthesis; activation of MMPs; induction of apoptosis; reduction of IL-1 R antagonist (IL-1 Ra) and reduction of transforming growth factor beta (TGFβ). Furthermore, inhibitors of NO synthesis have been shown to retard the development of clinical and histological signs in various models of experimental OA.

Several in vitro studies have shown the importance of mechanical compression or hydrostatic pressure (HP) as a modulator of cartilage metabolism. The biosynthetic response of chondrocytes to HP in vitro varies with the magnitude, frequency and duration of loading.

Chondrocyte cultures represent a valid and simplified biological model to test the effects of drugs and agents such as cytokines, hormones and growth factors. These can also be used to evaluate the influence of HP on chondrocyte morphology and metabolism. Our earlier in vitro studies have shown a positive effect of cyclical low HP (1-5 MPa) on OA chondrocyte metabolism and morphology.

The purpose of the present paper was to study the in vitro effects of cyclical low HP (1-5 MPa) and continuous high HP (24 MPa) in the presence or absence of IL-1β on human OA chondrocytes cultivated for 48 h in culture. Under these conditions, we evaluated the PG levels and NO production in culture medium and the morphology of chondrocytes by transmission electron microscopy (TEM). Immunocytochemical experiments were also performed to localize the inducible nitric oxide synthase (iNOS).

**Material & Methods**

**Cell culture:** Between January and December 2007, OA human articular cartilage was obtained from the femoral heads of 6 consecutive patients (Department of Clinical Medicine and Immunological Sciences, Rheumatology Unit, University of Siena, 3 males and 3 females) with OA according to clinical and radiological criteria defined by the American College of Rheumatology (ACR), who were undergoing surgery for total hip prostheses. The mean age ± standard deviation of the group was 68 ± 6.7 yr (range 63-74 yr). Written consent was signed from each patient of this study. These samples were sufficient to obtain a large number of cell cultures to perform the study protocol.

Macroscopically, the cartilage was not altered, but histological study of representative samples showed typical OA changes, such as the presence of chondrocyte clusters, loss of methachromasia, and fibrillation. Immediately after surgery, the cartilage specimens were cut aseptically, minced into 2 mm3 pieces and sequentially digested by clostridial collagenase (SIGMA, Italy), 1 mg/ml in phosphate buffered saline (PBS) (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 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, 90-95 per cent of the recovered cells were alive.

Cells were cultured in 24-well microplates at a density of 5 X 10⁵ cells/well and overlaid with 1 ml of medium containing 10 per cent foetal calf serum (FCS), 200 U/ml penicillin, 200 µg/ml streptomycin, 2 mM glutamine in Dulbecco’s minimum essential medium (DMEM). Cells were maintained in an atmosphere of 5 per cent CO₂ in air at 37°C for 48 h.

**Pressurization system:** The pressurization system used has already been described in detail. Briefly, the pressure chamber system consisted of a hermetically sealed stainless steel cylinder with a height of 400 mm and 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 and 240 atm to be reached, while 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; DuPont, Italy) after expelling all air. Surlyn membrane is partially permeable to O$_2$ and CO$_2$ but not to water or other solutions and it is suitable for preserving a stable environment. The membrane was attached to the rim of the Petri dish with Jet Melt 3764 adhesive (3 M, Italy).

Pressurization procedures: Cells were subjected to two types of pressurization: some dishes were exposed to cyclic pressurization by sinusoidal waves (minimum pressure of 1 MPa and maximum pressure of 5 MPa) at 0.25 Hz frequency for a three hour period and some dishes were exposed to continuous pressure (24 MPa) for three hours. Control cultures were maintained under identical test conditions with the exception that HP was not applied.

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 processed for immunocytochemistry.

Biochemical assays: The quantity of PG in the culture medium was measured by an immuno-enzymatic method$^{14}$ performed on microplates (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 or samples containing PG react with capture monoclonal antibodies (MAbs 1) coated on the microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period that allows for the formation of a sandwich made up of coated MAbs1/PG/MAbs2-HRP, the wells were washed several times to remove any unbound labelled antibody. The amount of bound antibody was measured using 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. The results obtained for the different cultural supernatants were normalized to the related DNA content of each assayed culture. The DNA was extracted with a high pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) and measured with the high sensitive Qubit™ quantitation system (Invitrogen, USA).

Nitrite assay: The quantity of nitrates in the culture medium was measured by the Griess method$^{16}$. Equal volumes (100 µl) of supernatant and Griess reagent (1% sulphanilamide, 0.1% N-1 naphthylethenediamide dihydrochloride in 5% H$_3$PO$_4$) were incubated on microplates at room temperature for 15 min. The absorbance was measured with a spectrophotometer at 550 nm. The concentration of nitrates was calculated using a standard curve made by successive dilutions of a solution of sodium nitrate in water.

TEM: Cultures of human OA chondrocytes were fixed for 2 h at 4°C in cold Karnovsky fixative$^{16}$, rinsed overnight in 0.1 M pH 7.2 cacodylate buffer and post-fixed for 1 h at 4°C in 1 per cent buffered OsO$_4$, dehydrated in a graded series of ethanol and embedded in Epon-Araldite.

Ultra-thin sections cut with a Supernova ultramicrotome (Reickert Jung Vienna, Austria) were collected on copper grids, stained with uranyl acetate and lead citrate and then photographed using a Philips CM10 electron microscope (TEM; Philips Scientifics Eindhoven, The Netherlands). At least 100 cells from each group were examined.

Immunocytochemistry: OA chondrocytes, whether incubated or not with IL-1β, were mechanically detached from petri dishes, washed two times in PBS (4°C) and centrifuged at 700 g for 10 min at 4°C. The pellet was resuspended in 1 ml cold PBS. Cells were smeared on glasses slides, air dried, and fixed for 5 min in acetone at 4°C. The specimens were rehydrated in PBS, treated with 1.5 per cent normal horse serum (NHS) for 20 min in humid chamber to avoid non-specific staining. Slides were incubated with avidin (15 min room temperature), biotin (15 min room temperature) in humid chamber (Blocking kit, Vector Laboratories, Burlingame, CA).

After washes in PBS, chondrocytes were incubated in a murine monoclonal antibody against human iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at the concentration of 1.2 µg/ml in PBS for 30 min. After accurate rinses in PBS, slides were incubated for 1 h at room temperature in biotin-conjugated– rabbit
antimouse IgG used at a concentration of 0.5 per cent in 1 per cent-NHS.

A solution of 0.3 per cent of H$_2$O$_2$ in methanol for 10 min was used to block endogenous peroxidases. After rinse chondrocytes were treated with the complex avidin-biotin peroxidase for 30 min at room temperature. The presence of iNOS was detected by an incubation with diaminobenzidine (DAB) 2 per cent in PBS for 5 min. Ematossilene (Sigma Aldrich, Italy) was used to stain the cells.

Controls for secondary antibody were carried out by omitting the primary antibodies.

Slides were examined in a Leitz Aristoplan microscope (Wetzlar, Germany) and Olympus BH-2 optics (Center Valley, PA, USA). The same procedures were also performed on cultivated OA chondrocytes exposed to cyclical hydrostatic pressure. At least 100 chondrocytes from each group were evaluated.

The staining intensity was scored on a scale from absence, limited to intense$^{10}$.

**Statistical analysis:** The data were expressed as the mean ± SD of PG release into the culture medium per microgram of DNA and NO (ng/10$^6$ cells) release into the culture medium in the six tested cultures.

Mann-Whitney U test was used for the statistical analysis; $P<0.05$ was considered significant.

For morphometric studies, sections of three different blocks from each group were analysed. For standardization and comparison of the different groups, only medially sectioned chondrocytes were investigated; 100 chondrocytes were selected using the nucleous/cytoplasm ratio as the selection criterion. The analysis was based on an established method for ultrastructural quantitative evaluation of changes in chondrocyte function$^{17}$. For the functional activity of the chondrocytes, the number of mitochondria and Golgi bodies, expressed as the mean ± SD, were used as parameters.

Immunocytochemistry staining intensity was scored by the same researcher as either absent or from limited to intense; in each group, the scores were expressed as percentages of the total number of cells studied$^{10}$. The results of immunocytochemistry were analyzed by chi-square test.

**Results**

The total PG concentration in the culture medium during the 48 h at baseline conditions, in the presence of HP (cyclical 1-5 MPa and continuous 24 MPa) and in presence of damaging stimulus represented by IL-1β (5ng/ml) is shown in Fig. 1. In presence of cyclical low HP (1-5 MPa) there was a significant increase ($P<0.001$) in the level of PG in the culture medium in comparison to basal conditions. The presence of IL-1β resulted in significant decrease ($P<0.001$) in PG levels and the cyclical low HP pressurization did not significantly increase the PG levels in cells damaged with IL-1β.

The NO (ng/10$^6$ cells) production in the culture medium at baseline conditions, in the presence of IL-1β with or without pressurization is shown in Fig. 2. After cyclical low pressurization there is a small, insignificant decrease in the concentration of NO in comparison to basal conditions. The production of NO is significantly increased ($P<0.05$) by continuous high HP (24 MPa). The presence of IL-1β resulted in a significant increase ($P<0.001$) in NO production, but, when the cells are...
cultured in the presence of IL-1β and cyclical low HP, a statistically significant decrease ($P<0.05$) in NO production was observed (Fig. 2). When the cells were stimulated by IL-1β and subjected to continuous high HP (24 MPa) a significantly increase of NO productions ($P<0.05$) was observed in comparison to the cells cultivated only with IL-1β (Fig. 2).

The results concerning metabolic production were further confirmed by the morphological findings obtained by TEM (Fig. 3) indicating effects actuated by IL-1β and pressure on the metabolic functions of the chondrocytes. Cultured cells without pressurization (Fig. 3 A, B) showed under basal conditions (Fig. 3A) an euchromatic nucleus and a cytoplasm containing rough endoplasmic reticulum, Golgi bodies, and mitochondria. On the contrary, in the presence of IL-1β (Fig. 3B), OA chondrocytes displayed several vacuoles in the cytoplasm and a reduced number of mitochondria and Golgi bodies (Table I). The cyclical low HP partially restored many of characteristic cytoplasmic structures in OA chondrocytes at baseline conditions (Fig. 3C). When OA chondrocytes were subjected to continuous HP 24 MPa (Fig. 3D) the chromatin was marginated, the cytoplasm contained vacuoles and lacked its typical structures even if the plasma membrane was not broken. The number of mitochondria and Golgi bodies was significantly lower ($P<0.01$) (Table I). Cells presented almost picnotic nuclei, the absence of cytoplasmic organelles and a very high presence of vesicles.

![Fig. 3. TEM micrographs of OA chondrocytes. (A) Control. The cell shows an euchromatic nucleus (N) and a cytoplasm abundant in rough endoplasmic reticulum (RER), mitochondria (M). X, 12000; (B) Presence of IL-1β. The cytoplasm shows a high vacuolization (V), nucleus (N). X, 12000; (C) Cyclical low pressurization. The cell shows a good activity, abundant presence of RER, nucleus (N), mitochondria (M). X, 12000; (D) Continuous high pressurization. The cell shows an altered status of chromatin, the cytoplasm is devoid its typical structure. The plasma membrane is not broken, nucleus (N). X, 12000.](image)
Cytochemical examination of chondrocytes showed that the distribution of the iNOS has a clear localization of the signal inside the cytoplasm, the label was intense in 70 per cent of the OA cells at baseline conditions (Table II). In OA cells treated with IL-1 β the signal appeared to be strongest in the cytoplasm in 92 per cent of examined chondrocytes. After low cyclical HP the localization of iNOS in OA chondrocytes was reduced to 55 per cent of chondrocytes whereas in presence IL-1 β the percentage of cells showing an intense localization (75%) was the same as that observed in OA cells at baseline conditions (70%). After continuous high HP in the absence and presence of IL-1β the signal appeared to be strongest in the cytoplasm in 85 per cent of evaluated chondrocytes (Table II).

### Discussion

Under physiological conditions, articular cartilage is subject to cycles of loading which control the matrix through the metabolic activity of chondrocytes. These loads alter the extracellular physical environment of the chondrocyte in a complex manner.18

In our study, the applied pressures were within the physiological range of the human joint. In fact, pressure levels of 5 MPa are most often encountered in the knee joint during normal gait, while the pressure can rise to nearly 20 MPa during some activities.19 Further, we applied HP for a time as short as possible (3 h) to approximate physiological conditions of the human joint.

Chondrocytes undergoing pressurization according to sinusoidal waves with a minimum pressure of 1 MPa and a maximum pressure of 5 MPa and a frequency of 0.25 Hz presented a greater metabolic activity which was expressed in a significant increase of PG levels in the culture medium at basal conditions without IL-1β. The addition of IL-1β caused a diminished PG production, which could result from cytokine-induced inhibition of PG synthesis by the chondrocytes.2

The stimulating effect of the low cyclical HP was in agreement with that found by other authors.5,6,20,21 Previously our data demonstrated that exposure to low magnitude intermittent HP does not modify healthy chondrocytes. In the present study, however, the stimulating effect of this pressure was not enough to counterbalance the negative effect determined by the addition of IL-1β, which induces a serious metabolic and morphological imbalance.

On the other hand, the continuous high HP (24 MPa) used in our study inhibited the PG synthesis as demonstrated by others.4,22,23 The modifications of anabolic activity in chondrocytes subjected to different HP were confirmed by morphological changes detectable by TEM and by morphometric analysis. The same structural alterations were already observed on normal chondrocytes. In particular, this is in line with reports of the packing of Golgi bodies in bovine chondrocytes subjected to continuous high HP.22
Recent findings showed that NO is a potent mediator of the cartilage damage in many joints and *in vitro* studies demonstrated that NO and its products can accelerate cartilage catabolism and reduce its anabolism. In the present study, a significant increase in the production of NO was shown as reported earlier. When cells were cultivated under cyclical pressure (1-5 MPa), a reduced production was recorded compared to those at basal conditions and this pressure also significantly decreased IL-1β induced NO production. These results confirm the protective effect of cyclical HP on the release of NO by chondrocytes when stimulated by various factors. The continuous high HP significantly increased NO production at basal condition and in the IL-1β stimulated cells. These results were confirmed by immunocytochemical study on expression of the iNOS. Since NO can modify PG synthesis and breakdown, HP-induced increase in NO could contribute to the previously reported changes in PG synthesis in cells subjected to high continuous HP.

HP appears to modulate aggrecan biosynthesis through membrane-mediated pathways, such as the transport of cations, aminoacids and macromolecules. HP may also alter the action of the membrane Na+/K+ pump, thus altering intracellular K+ concentrations. Also, cyclic AMP (cAMP), which has been identified as an important mediator of PG synthesis and cartilage growth, seems to play a direct role in the mechanical stimulation of matrix biosynthesis. Constant HP that inhibits PG synthesis also inhibits cAMP accumulation through a calcium-mediated process. Conversely, intermittent HP in chondrocyte cell culture systems results in concurrent increases in both cAMP and PG synthesis rates.

In chondrocyte-like cells 1-5 MPa HP has been shown to increase the synthesis of transforming growth factor-β (TGFβ) which is one of the most potent stimulators of the ECM synthesis and a high continuous HP increases the expression of IL-6 and tumor necrosis factor α (TNFα). It is shown that TNFα promotes the inhibition of ECM synthesis, the stimulation of NO production and also induces chondrocyte apoptosis. Different groups observed that HP influences the cytoskeleton structure and ECM synthesis rates. We earlier confirmed that high continuous HP modified the cytoskeletal structure in normal chondrocytes, while low cyclic HP did not cause any cytoskeletal changes in normal human chondrocytes. The cytoskeleton plays a fundamental role in the cell differentiation and proliferation, formation and flux of vesicles, synthesis of the ECM and cell migration.

In conclusion, our study confirmed the importance of HP on chondrocyte metabolism and morphology and showed that the response of chondrocytes was dependent on the magnitude and frequency of HP. Cylindrical low HP used in this study (1-5 MPa) stimulated PG production and counteracted IL-1β induced NO release, whereas continuous high HP (24 MPa) produced opposite effects. Structural alterations such as margined chromatin and presence of vacuolization that are characteristic features of cell apoptosis, were observed in chondrocytes subjected to a continuous high HP confirming previous demonstration that this pressurization may induce chondrocyte apoptosis.

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 ECM in signal transduction and in transmitting the external load to the cell and intracellular components. However, studies using chondrocyte cultures under pressure represent the most suitable experimental models for understanding both the aetiopathogenetic mechanisms of OA and which type of physical activity may be best suited for the prevention of OA and for its therapy.

Our study demonstrated that a cyclical low HP (1-5 MPa) corresponding to the physiological pressure in joints under light to moderate activities seemed to have protective effects on the chondrocyte. It is, therefore, possible to hypothesise that a moderate physical activity could produce both preventive and therapeutic effects in OA patients. More sophisticated experiments will be necessary to analyze the effects of HP of various magnitude and frequency on chondrocytes metabolism and morphology and to understand the role of the mechanical factors in the aetiopathogenesis of OA and to translate this information on the therapeutic programme of OA patients.

References


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