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Validation of an *In Vitro* Model to Study Human Cartilage Responses to Compression

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ABSTRACT

The aim of this work was to develop an *in vitro* model to study mechanical compression effects on cartilage. A pressure-controlled compression device was used in this study. Cartilage explants obtained from human knee were compressed at 1MPa/1Hz for 7 hours (30 min ON, 30 min OFF) under normoxia (5% CO_2, 21% O_2) or hypoxia (5% CO_2, 5% O_2). Cell viability was analyzed while nitric oxide (NO) and glycosaminoglycans (GAG) release was assayed in culture media. Mechanical stimulation increased NO production and GAG release by human cartilage explants under normoxia and hypoxia culture. In normoxia and hypoxia conditions, mechanical stimulation alters human OA cartilage metabolism. There is also, an increase in matrix degradation after compression, as shown by levels of GAG found in culture media. This study put in evidence the importance of mechanical compression in the progression of the osteoarthritis and present and *in vitro* model for mechanobiological and pharmacological studies.

Keywords: Cartilage; Compression; *In Vitro* Model; Nitric Oxide; GAG

1. Introduction

Cartilage is an avascular tissue submitted *in vivo* to mechanical stimuli. These mechanical stimuli result from a complex combination of tension, shearing and compression forces, the latter being the most important within the cartilage [1]. The compressive forces exerted on the surface of the articular cartilages are variable according to the weight of the individual, his muscular tension and its physical-activity. Thus for example the average pressure being exerted on the hip is of 0.7 MPa but during physical exercises, it can reach 5 to 10 MPa. These mechanical forces affect the extracellular matrix as well as the chondrocyte metabolism [2-9]. During immobilization, the capacities of synthesis of chondrocytes as well as the thickness of the cartilage decrease. In the same way, in the zones subjected to maximal forces, the balance between the anabolism and the catabolism of the cartilage are disturbed.

It is know that physiological loading of articular cartilage is necessary to maintain normal joint function. Articular chondrocytes transform mechanical signals into biochemical ones to maintain the integrity of their extracellular matrix [10-12]. Several studies investigated the effect of mechanical stimulation on chondrocyte metabolism. In general, static compression decreases biosynthetic activity compared to unloaded tissue while dynamic compression has been found to stimulate, inhibit or have no effect on biosynthetic activity depending on the loading frequency and amplitude [13-15]. Other studies have shown that cyclic tensile strains of low magnitude (3–8% equibiaxial strain) and physiological levels of cyclic compressive forces (15% compression) elicit an anabolic response [16, 17], while strains of high magnitude (10–15% equibiaxial strain) initiate cartilage damage.

Most of the studies investigated the effects of mechanical compression on osteoarthritis (OA) development and use healthy cartilage principally from animal origin. In this work we aimed to investigate the response of human cartilage from osteoarthritic patients to dynamic unconfined compression. The aim of this work was to develop an *in vitro* model to study mechanical compression effects on cartilage and to define experimental protocols to be used in cartilage mechanobiology. This model could be used in pathophysiological or pharmacological studies of cartilage.

2. Materials and Methods

2.1. Cartilage Explants

Articular cartilage was obtained from preserved areas of femoral condyles of patients undergoing arthroplasty for OA at the Department of Orthopaedic Surgery, CHU Nancy, France. Samples from 10 patients (6 women and 4 men, 64 ± 7 years) were used. Cartilage was separated from the subchondral bone using a scalpel. Cylindrical explants (5 mm in diameter) were harvested using a sterile biopsy punch (Stiffel, France) and immediately incubated in culture medium (DMEM-F12) supplemented with 10% heat inactivated fetal bovine serum, 1% of antibiotics/antimycotic solution, and 2mM Glutamine at 37°C, 5% CO_2. Test and control explants were paired at harvest and originated from adjacent sites on the joint surface. All compression experiments were performed after allowing explants to equilibrate in culture for 72 hours after harvest.

2.2. Mechanical Stimulation

A FX-4000CTM Flexercell® Compression Plus TM System (Flexcell International, Hillsborough, NC) was used to apply dynamic compression. Explants were placed in one well of a

Biopress culture plates (Flexcell International) and mounted within the apparatus. The plates consist of a 6 well plates containing a flexible silicone rubber membrane at the bottom. The explant is putted on the plastic disc, into the Foam Sample Holder and the piston of the Stationary Platen is moved until it become in contact with the explants. A calibrated air pressure was applied to the membrane to obtain a compressive stress (σ); determined from the applied force (F) and the initial crosssectional area (A) of the explant using the equation σ = F/A (**Figure 1**). Two millilitres of culture medium were introduced into each well. Explants were subject to unconfined compression at compressive stress amplitude of 1 MPa at 1 Hz for 7 hours in an intermittent manner (30 min ON, 30 min OFF) in a humified incubator at 37°C, 5% CO_2. Unloaded (control) explants were incubated under the same conditions. Tests were performed in order to calculate the pressure applied to the sample by using a force sensor (XFL 205 R, FGP Sensors & Instrumentation) instead the sample and to be sure that all culture wells on each of the 4 compression plates of the device were subject to the same strain (**Figure 1**).

To analyze the effect of oxygen tension in NO production and GAG release, experiments were performed in a humidified incubator at 37°C, 5%CO_2, 5%O_2. Explants were left overnight in hypoxia before each experiment in order to let cells to adapt their metabolism.

2.3. Viability Assay

Cell viability was determined in cartilage explants using the fluorescent probes Propidium Iodure (PI) and SYTO16 (both from Molecular Probes). The membrane-permeable SYTO16 labels live and dead cells to yield cytoplasmic and nuclei green fluorescence, whereas the membrane-impermeable propidium iodide labels nucleic acids of membrane-compromised cells with red fluorescence. After each experiment, the loaded and unloaded (control) explants were washed in DMEM W/O phenol red for 5 min and then sectioned perpendicular to the articular surface into 1-mm thick slices using parallel razor blades. The tissue sections were incubated in the SYTO16+PI solution for 5 min in a dark environment and then washed twice (5 min each) in DMEM W/O phenol red to remove free dye from the tissue matrix. The chondrocytes within the cartilage matrix were then viewed using a fluorescence confocal microscope (LEICA) in sequential mode (excitation/emission: 488 nm/ 520nm (1); 545/633 (2)) to simultaneously observe green and red fluorescence. Green cells are viable and yellow cells are dead. The percentage of dead cells was calculated by counting the total number of cells and the number of yellow cells in five random, non-contiguous fields.

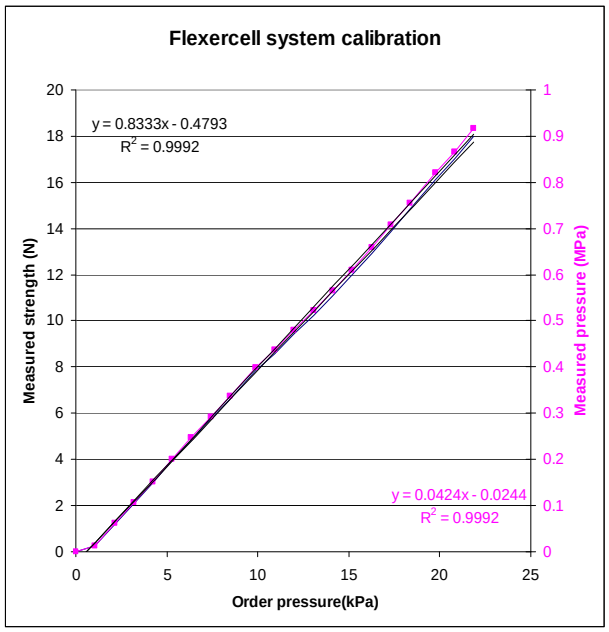

Figure 1. Calibration of the compression device using a force sensor.

2.4. Measurement of GAG Release

GAG levels in the culture medium were determined by the amount of polyanionic material reacting with DMMB (Polysciences, USA). Explants supernatants were removed and 125 µl were combined with 200 µl of DMMB solution. Samples were examined spectrophotometrically at 525 nm. For this assay, standards prepared with control media and chondroitin sulphate C (Sigma, France) were used. Results are reported as µg GAG per mg of wet weight of tissue.

2.5. Determination of NO

NO production was measured by estimating the stable NO metabolite, nitrite, in conditioned medium using a spectrophotometric method based on the Griess reaction (Griess Reagent Kit for Nitrite Determination, Molecular Probes). Following culture of the cartilage explants for the times indicated, 150 µl of the culture supernatants or sodium nitrite standard dilutions were mixed with 20 µl Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 5% H3PO4) and incubated for 30 min at room temperature. Nitrite concentrations were determined by measuring absorbance at 570 nm in a Microplate reader (BioRad, U.S.A.). Values are expressed as µM nitrite released per mg of wet weight of tissue.

3. Results

Cell viability in response to compression. Fluorescence staining indicated that cell death was confined to the cut edge and to the superficial zone in uncompressed control samples. In mechanically loaded explants, cell death was evident also in the intermediate region of the explants. When compared the superficial zone in unloaded and compressed samples, the percentage of dead cells is higher ($p < 0.05$) in compressed explants (59%) than in unloaded ones (23%). Similarly, the percentage of dead cells in the deep zone is higher ($p < 0.05$) in compressed samples (39%) than in unloaded samples (20%).

NO production and GAG release after compression. The mechanical compression used in this *in vitro* study affects the NO production and GAG release from human cartilage explants. As shown in **Figure 2**, NO increases in culture medium of compressed explants after 7 hours of intermittent compression when compared to uncompressed explants. In parallel, conditioned medium from compressed and uncompressed cartilage

explants was analyzed for sulphated GAG content after 7 hours. The results presented in **Figure 3** showed that mechanical stimulation increase GAG release in culture medium of compressed explants when compared to uncompressed ones. We next investigated the effect of oxygen tension in the level of NO production and GAG release in response to mechanical compression. Under static conditions, NO production increased under hypoxia when compared to normoxia conditions (increase of 202 ± 51 %, p<0,05). Moreover, mechanical compression significantly increased NO production (**Figure 2**) under hypoxia. Furthermore, under static conditions GAG release under hypoxia did not change when compared to normoxia conditions. Mechanical compression significantly increased GAG release (**Figure 3**) under hypoxia although the increase is less important than under normoxia.

4. Discussion

In this study, the effect of unconfined compression on cartilage explants from human osteoarthritic knee was studies. Results showed that in this in vitro model, mechanical compression increased NO production and GAG release under normoxia and hypoxia conditions.

Under physiological conditions, large forces which are the result of normal joint movements are applied to articular cartilage. Mechanical load has been demonstrated in many *in vivo* and *in vitro* investigations to be an important factor affecting the health of articular cartilage and consequently the function of the diarthrodial joint and the progression of joint degeneration [18,19]. The effect of the stimulation is strongly dependent on magnitude and frequency of the applied load. In the in vitro model used in this study, unconfined compression was used. Under this condition, the construct is free to expand laterally and is subject to both compressive strains (along the axial direction) and tensile strains (along the radial and circumferential directions). This loading regime represents a physiologic loading environment and produces a more uniform mechanical signal throughout the thickness of a cylindrical cartilage sample than that of confined compression (where radial expansion is prevented).

In cartilage, chondrocytes exhibit a predominantly anaerobic metabolism because they are in hypoxia under physiological conditions. The hypoxic conditions are further enhanced during OA because the oxygen consumption of the synovium from OA patients is elevated and the synovial fluid O_2 tension is decreased compared with that of normal synovial fluid. In conclusion, the standard cell culture conditions with 21% oxygen tension do not mimic the physiological situation within cartilage. In this study, mechanical compression increased NO production and GAG release when experiences were performed at 5% O_2 but we noted that the increase is lower than at 21% O_2.

In conclusion, this study shows that high levels of compression increase NO production and GAG release by human OA under normoxia and hypoxia. In an in vitro model we observed that OA cartilage is sensitive to mechanical stimulation and show the importance of mechanical stimulation in the progression of the osteoarthritis.

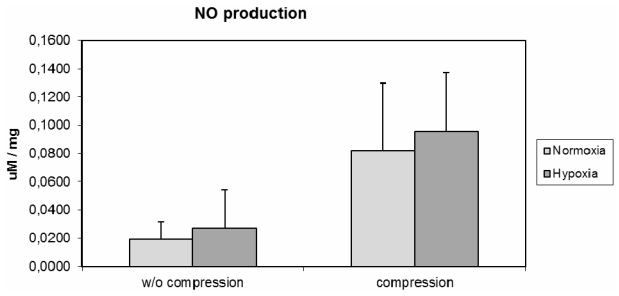

Figure 2. Production of Nitrite (µM/mg wet weight) by articular cartilage explants compressed at 1 MPa,1 Hz for 7h (30 min on, 30 min off) under normoxia conditions (21% O_2) or under hypoxia conditions (5% O_2). Data are presented as mean ± S.D. of 3 independent experiments with n = 3/group/experiment, p <0.05 : w/o compression vs compression.

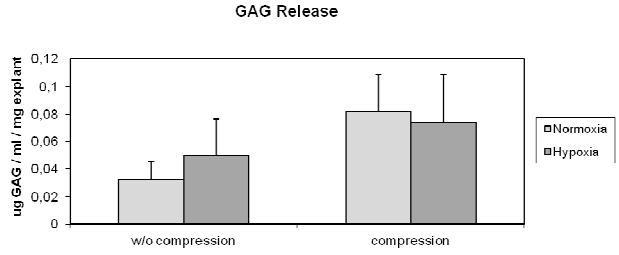

Figure 3. GAG released (µg/mg wet weight) in culture medium of articular cartilage explants compressed at 1 MPa, 1 Hz for 7h (30 min on, 30 min off) under normoxia (21% O_2) or hypoxia (5% O_2). Data are presented as mean ± S.D. of 3 independent experiments with n = 3/group/experiment, p <0.05 (w/o compression vs compression).

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