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Osmotic Loading of in Situ Chondrocytes in Their Native Environment

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Abstract: Changes in the osmotic environment cause changes in volume of isolated cells and cells in tissue explants, and the osmotic environment becomes hypotonic in cartilage diseases such as osteoarthritis (OA). However, it is not known how cells respond to a hypotonic osmotic challenge when situated in the fully intact articular cartilage.

A confocal laser scanning microscope was used to image chondrocytes of intact rabbit patellae in an isotonic (300 mOsm) and hypotonic (172 mOsm) immersion medium. Cell volumes were calculated before and 5, 15, 60, 120 and 240 minutes after the change in saline concentration. Local tissue strains and swelling of the entire tissue were estimated from the relative movements of cells and displacements of single cells, respectively.

Cell volumes increased rapidly ($\leq$5 minutes, $p<0.05$) by $\sim$22%, after which they remained constant for an hour ($p>0.05$). However, two and four hours post the hypotonic challenge, cell volumes were statistically greater ($p<0.05$) than those at all earlier time points, and swelling of the entire tissue continued throughout the four hour loading period.

The results of our study suggest that osmotic loading induced volume changes of *in situ* chondrocytes in their native environment occur quickly and continue for hours. Understanding the behaviour of cells in their native environment provides novel insigth into the cell mechanics in osteartheritic joints and so may help understand the onset and progression of this disease.

Keywords: Articular cartilage, Chondrocytes, Cell-Matrix interaction, Confocal microscopy, Osmotic loading

1 Introduction

Articular cartilage is a highly specialized connective tissue that is composed of a solid matrix, a fluid phase, chondrocytes (cells), and small electrolytes (Na^+, Cl^-, etc.) which are dissolved in interstitial fluid. Chondrocytes synthesize solid matrix components, i.e. proteoglycans and collagens, and the balance between anabolic and catabolic activities of the cells determines cartilage health and the ability to withstand external loads. Proteoglycans contain negative charges that induce high swelling pressures in cartilage, which lead to extension and residual loading of the collagen network. Early osteoarthritis (OA) is characterized by a disruption of the superficial collagen network, loss of proteoglycans, increased interstitial fluid content and tissue swelling, decreased extracellular osmolarity, and increased chondrocyte volumes [1-6].

It has been found that small osmotic challenges affect volumes of *in situ* and isolated chondrocytes in a similar manner, suggesting that the extracellular matrix is not important for the mechanical behaviour of chondrocytes [7, 8]. However, when applying large osmotic challenges, volumes of isolated cells have been shown to increase more than those of *in situ* cells [3]. Also, the pericellular matrix has been shown to influence cell cross-sectional area following osmotic loading [9]. These results suggest that the peri- and extracellular matrix may affect volume changes of

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chondrocytes.

Cell volumes increase in a few minutes in response to a hypotonic osmotic shock [7, 10], from which regulatory volume decrease may [7] or may not [10] occur. If regulatory volume decreases were reported, a steady-state had been reached within 15-20 minutes [7]. However, it takes hours for the extracellular matrix of cartilage to reach steady-state following an ionic challenge [11, 12]. We speculate that in the intact joint, tissue swelling may contribute to cell volume and shape changes, thus, cell swelling *in vivo* may increase for hours following an osmotic challenge.

The purpose of this study was to measure volume changes of chondrocytes associated with a hypotonic challenge in fully intact articular cartilage that is attached to its native bone. Testing was performed using articular cartilage from the retropatellar surface of rabbits while exposing the tissue to an isotonic and then a hypotonic immersion medium. Volume changes of chondrocytes were measured for up to four hours using confocal microscopy. Understanding the behaviour of chondrocytes in their native environment might provide novel and unique insight into the biomechanical behaviour of articular cartilage chondrocytes and might provide insight to the onset and progression of osteoarthritis.

2 Materials and methods

A schematic diagram of the experimental protocol consisting of sample preparation, confocal laser scanning microscopy and image analysis is shown in figure 1. A detailed description of the materials and methods is presented in the following.

2.1 Sample preparation

Twelve knee joints of mature New Zealand white rabbits were prepared for osmotic loading experiments 30 minutes post mortem. Six additional joints were prepared for testing the validity of the intensity threshold for the analysis of cell volumes (n = 2), for determining the extent of tissue swelling (n = 2), and for the cell viability test (n = 2). Intact patellae (cartilage thickness $\approx$ 500μm) were harvested and incubated in a phosphate buffered saline (PBS) solution (isotonic, 300 mOsm) with 5 μM Calcein-AM (Molecular Probes, Eugene, OR, USA) for 30 minutes. After staining, samples were mounted on a Petri dish with dental cement, and then the dish was filled with the PBS.

2.2 Confocal laser scanning microscopy

A standard (single photon) confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss, Germany) was used for cell imaging. Images of the chondrocytes, located in the fully intact articular cartilage attached to its native bone, were obtained through the articular surface. The laser excitation wavelength was 488 nm and all experiments were performed at room temperature (21°C). Cell fluorescence (515 nm) was detected using a 500 – 550 nm band pass filter. A series of planar optical sections (x-y plane, 512×512 pixels) were obtained at a 0.5 μm spacing (z-axis) using a ×40, 0.8 N.A. water immersion objective (Zeiss, Carl Zeiss, Germany). Analyzed cells were down to 50 μm from the cartilage surface. Thus, based on light microscopic imaging of the histological sections of the samples, the cells were in the superficial zone of cartilage which was $\sim$10% of cartilage thickness. The pixel size in the x-y plane was 0.4×0.4 μm.

After imaging the samples in the isotonic PBS, the saline concentration was changed to hypotonic (172 mOsm) in $\sim$30 seconds, corresponding to the osmotic environment of chondrocytes used in other studies and potentially present in OA [3, 7, 10, 13]. Confocal images were taken at 5 - 240 minutes following solution exchange. Movements of the chondrocytes due to tissue swelling were accounted for so that the same set of cells were imaged over the four hour period.

In order to study the swelling of the entire tissue, two additional samples were tested by fixing the measurement stage of the microscope, and by capturing images of chondrocytes at 0, 5, 15, 60, 120 and 240 minutes after the change in saline concentration (figure 1). In order to validate the intensity threshold that was used for the analysis of cell volumes, two additional samples were im-

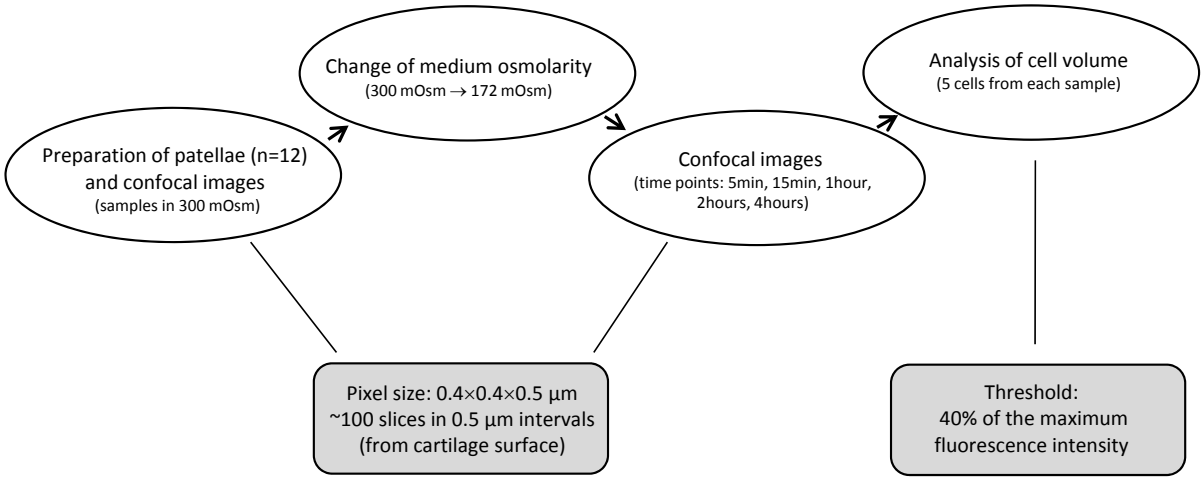

Figure 1: Diagram of the study protocol. Two additional samples were measured in 300 mOsm PBS for the validation of the fluorescence threshold (see text for details). Moreover, two samples were used for the entire tissue swelling experiments as well as cell viability tests (see text for details).

aged in a constant 300 mOsm PBS at 0, 5, 15, 60, 120 and 240 minutes.

Cell viability during the 4 hour measurement period (figure 1) was characterized for two additional samples using SYTOX® (Molecular Probes, Eugene, OR, USA). Sample processing was the same as with Calcein-AM, but the laser excitation wavelength and band pass filter were 543 nm and 565-615 nm in the measurements, respectively.

2.3 Data analysis

Image stacks were read with ImageJ (National Institutes of Health), and five cells in every sample were chosen for analysis. The Visualization Toolkit 5.0.1 (Kitware Inc.) was used to reconstruct 3D cell images, and a Python programming code with a threshold of 40% of the maximum fluorescence intensity, the same as used in Bush and Hall (2001), was used for cell volume calculations. Based on the relative 40% threshold, the absolute threshold value was calculated for every cell separately at every time point. For analysis of cell volumes, a Gaussian convolution of the original images was performed, followed by the generation of iso-surfaces of the cell volumes. Then, triangle polygons representing the iso-surfaces were created and cell volumes were calculated from the triangle meshes [14].

Local tissue strains (%) due to tissue swelling were estimated from the relative movements of cell centroids. Swelling of the entire tissue (μm) was estimated from the displacement of chondrocytes in a fixed coordinate system. Five cells from the samples were chosen, and their movements were followed during the 4 hour testing period, i.e. the distance between the original location of chodrocytes before the hypotonic challenge and the location of chondrocytes at every time point after the hypotonic challenge was determined. Local tissue strains were calculated from the same samples used for the analysis of cell volumes, while swelling of the entire tissue was estimated from two additional samples.

2.4 Statistical analysis

Data are presented as mean $\pm$ s.d. (n = number of patellae, five cells from each patellae were analyzed). Cell volumes at different time points were compared using the Wilcoxon signed ranks test (SPSS Inc., Chicago, Illinois, USA).

3 Results

3.1 Validation of fluorescence threshold

Cell volumes of the reference samples in a constant 300 mOsm osmolarity changed less than 4% during the four hour measurement period, and the change was random, indicating that the selected intensity threshold was adequate for the analysis of cell volumes. Further, cell locations did not change during the measurements, indicating negligible tissue swelling and microscope stage drift.

3.2 Cell volumes

Mean chondrocyte volume was 444 $\pm$ 59 μm^3 (n = 12) in the isotonic PBS. Chondrocyte volume increased rapidly (0 $\rightarrow$ 5 minutes, $p<0.05$) following the hypotonic challenge (figures 2, 3). From 5 to 60 minutes, average cell volumes did not change statistically (5 $\rightarrow$ 15, 60 minutes, 15 $\rightarrow$ 60 minutes, $p>0.05$) (figures 2, 3). At two and four hours post the hypotonic challenge, cell volumes were significantly greater than those at all earlier time points (5, 15, 60 $\rightarrow$ 120 minutes, 5, 15, 60, 120 $\rightarrow$ 240 minutes, $p<0.05$) (figures 2, 3).

3.3 Tissue swelling

Local tissue strains caused by tissue swelling were less than 2% at every time point (figure 3b). Displacements of the superficial zone chondrocytes, caused by swelling of the extracellular matrix, were location-dependent (data not shown) and not necessarily perpendicular to the cartilage surface (figure 4a). The rate of entire tissue swelling was faster immediately following the hypotonic challenge and then slowed down but never stopped completely during the 4 hour test period (figure 4b).

4 Discussion

Confocal laser scanning microscopy was used to image *in situ* chondrocytes through cartilage surface of the intact tissue attached to its native bone in an isotonic and hypotonic saline solution. Changes in the superficial zone cell volumes, tissue swelling and local tissue strain were measured as a function of time post the osmotic challenge. Hypotonic osmotic loading resulted in a rapid cell volume increase, followed by a plateau with no cell volume change for the following hour. However, cell volumes at two and four hours post the hypotonic osmotic challenge were significantly greater than those at all earlier time points. Swelling of the entire tissue continued for the four hour experimental period, whereas local tissue strains were small throughout the entire testing protocol.

Earlier studies often focused on investigating cell volume changes within 5-30 minutes of an osmotic challenge, assuming that this was enough to reach steady state [7, 10, 15]. It has also been suggested that volumes of isolated cells do not change (for up to 20 hours) after this initial 5-30 minutes [7, 10, 15]. In contrast, our results suggest that cell volumes reach a pseudo steady state and remain relatively constant from 5 – 60 minutes, but then increase for up to four hours following a hypotonic challenge. The reason for these differences may be associated with the cell environment; intact in our study and non-intact or non-existing in the other studies. Our results support earlier studies on cell death [16-18] that suggest that cell mechanics and biology in tissue explants may differ from those observed in the intact tissue.

The rapid increase in cell volumes after the hypoosmotic challenge was likely due to the increased osmotic pressure and associated fluid flow into the chondrocytes. At five minutes post the hypotonic challenge, cell volumes had increased by 22% from original. This is consistent with other studies which report volume increases of 11-43% for cells in explant tissues (20-24% for the superficial zone cells, as was measured here) [3, 7, 10, 13]. Moreover, due to osmotic challenges, isolated cells have been shown to increase in volume in a similar manner as *in situ* cells of explant tissues, even though large osmotic challenges have been shown to increase volumes of isolated cells more than those of cells in explant tissues [3, 7, 10, 13]. Therefore, it appears that chondrocytes are not protected from vast volume increases by their matrix environment. However, it must be

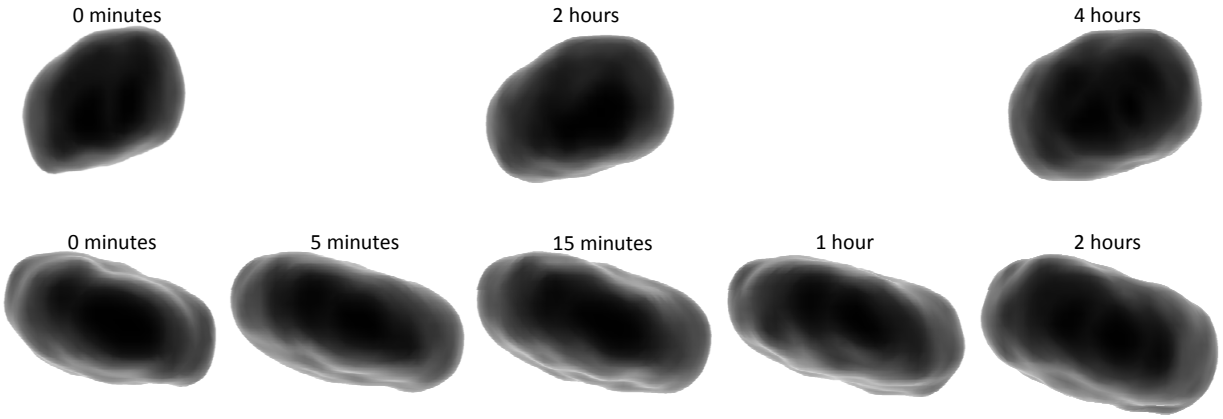

Figure 2: Examples of two cell shapes in an isotonic PBS (left column) and 5, 15, 60 and 120 minutes (lower row) or two and four hours (upper row) after changing the PBS osmolarity from 300 mOsm to 172 mOsm.

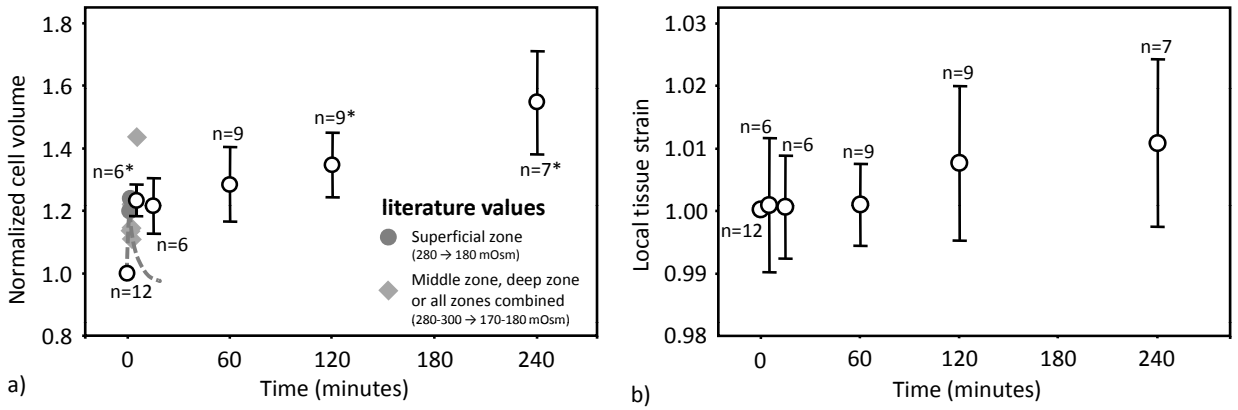

Figure 3: a) Normalized cell volume as a function of time, after inducing the hypotonic shock (300 mOsm → 172 mOsm). The number of samples is not the same at every time point, as images were not captured at all time points for every sample in order to reduce photobleaching effects. Literature values for the cells in explant tissues have been estimated from the other studies [3, 7, 10, 13]. A dashed line shows volume recovery for the superficial zone cells [3]. b) The effect of osmotic loading on local tissue strain. Strain was calculated from the relative movements of cell centroids, after changing the PBS osmolarity from isotonic to hypotonic (300 mOsm → 172 mOsm). *$p<0.05$ (see text for details)

kept in mind that cell isolation itself may induce cell swelling as the matrix environment is absent. Consequently, the absolute swelling of isolated cells may be larger than that of cells in their native environment, even though the relative swelling might be similar. The same phenomenon could occur in cells of tissue explants, as cells close to the cutting surface might not retain their original shape. The role of the matrix environment on the short-term cell swelling should be confirmed by testing isolated cells, cells of explants and cells in their native environment from the same cartilage samples.

From five minutes to one hour, cell volumes remained virtually constant, despite continuous swelling of the extracellular matrix. We speculate that during this phase, cell volumes may have been regulated through the activation of specific membrane transporters [7, 19, 20], and that the expected cell volume decrease [7] may have been restricted by the extracellular matrix. On the other hand, relatively low number of sam-

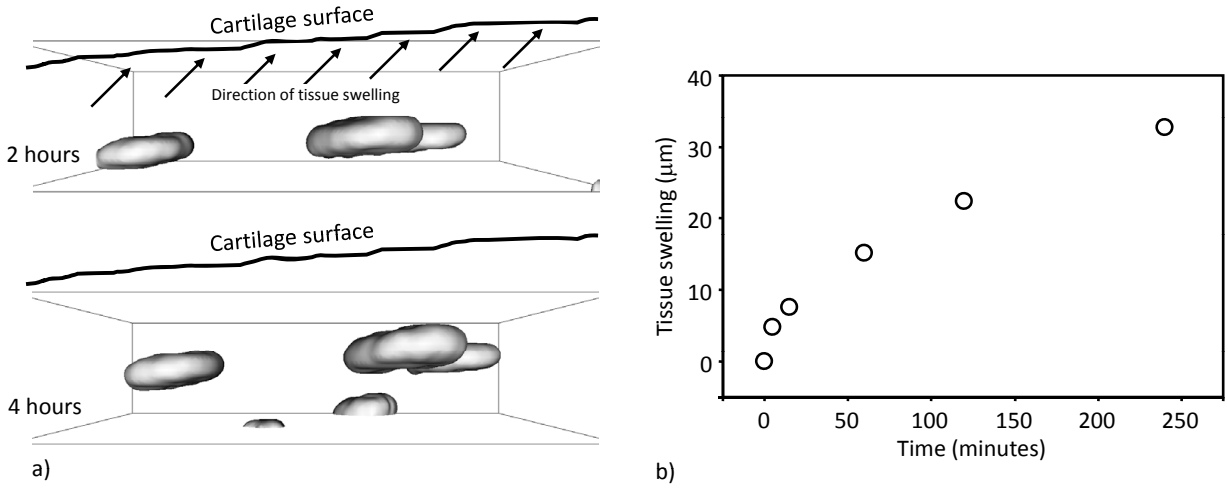

Figure 4: The effect of osmotic loading on tissue swelling. a) Cell locations are visualized two and four hours after changing the PBS osmolarity (300 mOsm → 172 mOsm). b) Displacements of the superficial zone chondrocytes (estimate of tissue swelling), after changing the PBS osmolarity from isotonic to hypotonic (300 mOsm → 172 mOsm). Cartilage swelling continued for the four hour loading protocol likely because of the negatively charged proteoglycans and the slow fluid flow into the tissue through the articular surface. Standard deviations have not been presented, as only two samples with consistent behaviour were measured for this test.

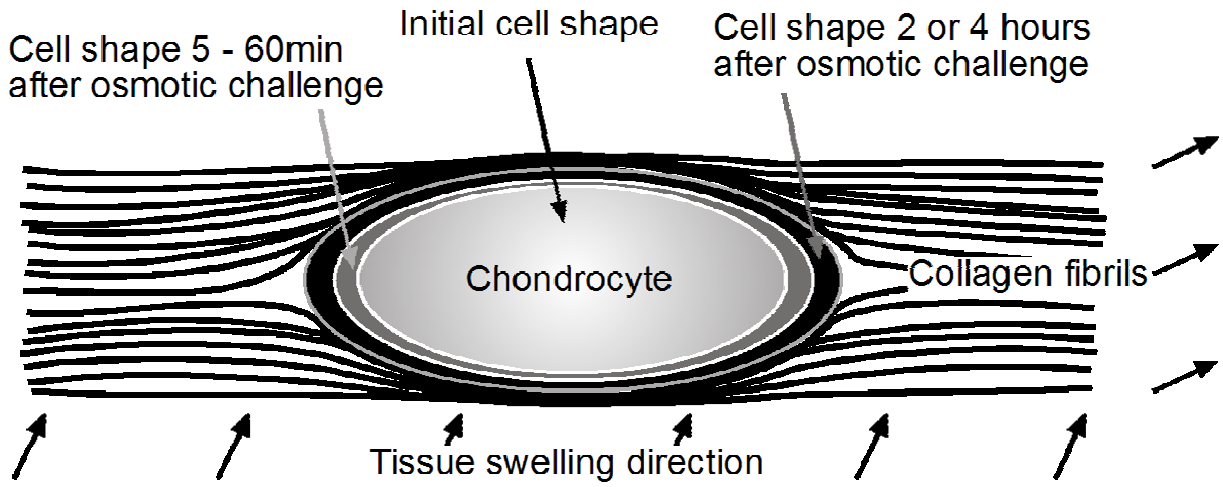

Figure 5: Schematic side view of an *in situ* chondrocyte, and a possible mechanism for its mechanical behaviour upon a hypotonic osmotic challenge. 5 minutes after an osmotic challenge (300 → 172 mOsm media osmolarity), the cell has expanded because of the osmotic pressure change (light grey vs. dark grey), while at the same time it may be protected from excessive expansion by the peri- and extracellular matrix, since its volume only increased by 22% (figure 3) compared to up to 60% increases for isolated chondrocytes [3]. Tissue swelling continues between 15 – 60 minutes (figure 4) but cell volumes remain constant (dark grey), which suggests that cells regulate their volumes actively [7]. Tissue swelling continues between 2 – 4 hours (figure 4) and cell volumes increase (black), suggesting that the extracellular matrix gives extra space for the cells to swell, or that cells are pulled "passively" by the extracellular matrix. Arrows indicate the possible direction of tissue swelling due to the osmotic pressure (see figure 4).

ples and low power may have contributed to the lack of statistically significant volume increase at one hour time point. Between one and four hours post the hypotonic challenge, tissue swelling continued throughout, and chondrocyte volumes increased, which might be associated with cartilage matrix that allowed the cells to swell freely, or pulled the cells passively. Thus, we speculate that the extracellular and pericellular matrix may play an important role in modulating cell volume changes for long-term hypotonic conditions. Figure 5 illustrates a hypothesis how cell volumes might be modulated by the extracellular matrix. These issues should be clarified by measuring isolated cells, cells of explant tissues and cells in their native environment of the same joints[1].

The measurements were conducted at room temperature for practical reasons. This may have inhibited the regulatory volume decrease of the cells. As stated also in Bush and Hall (2001) [7], the volume regulation mechanism is faster at the physiological temperature (37˚C) than at the room temperature (21˚C). Change of the immersion medium from isotonic to hypotonic, focusing the image to the prefererred cells, and data acquisition would not have been possible in such a short period of time ($t_{1/2} \sim 100$ s for regulatory volume decrease [21]). As the room temperature only slowed down the regulatory volume decrease in Bush and Hall (2001) [7], but did not entirely inhibit it, we preferred to use the same temperature here. In the long-term, active trasporters in chondrocytes, particularly Na^+/K^+ pump, may have been compromised at the room temperature, contributing to cell swelling. However, as cell volumes increased almost 60% in long-term as a result of the osmotic loading, while the volume increase in the short-term was only 22%, tissue swelling was likely the main cause for the long-term cell swelling behavior (see 1).

Narmoneva et al. (1999) and Flahiff et al. (2002) measured cartilage swelling using tissue explants. They suggested that four hours was enough to achieve equilibrium conditions following a hypotonic challenge. Our results suggest that four hours is not sufficient time to reach equilibrium. This difference might be due to species differences (dogs/pigs vs. rabbits), sample locations (femoral condyle/tibial plateau vs. patella), material properties of the samples (e.g. permeability) or sample preparation. In these earlier studies, the edges of the femoral condyles were cut, thereby allowing fluid flow through the cut surfaces. In our study, fluid could enter the tissue only through the cartilage surface, thereby possibly increasing diffusion and equilibrium times. Sample thickness do not seem to explain the difference as Narmoneva et al. (1999) and Flahiff et al. (2002) investigated samples of 900 μm and 400 μm in thickness, respectively, while our samples were 500 μm thick.

The small local tissue strains in the superficial zone, while significant swelling of the entire tissue, may be explained by the inhomogeneous structure and composition of cartilage [12]. The horizontally aligned collagen fibrils in the superficial zone may have limited the relative movements of chondrocytes, thereby preventing large local tissue strains. This hypothesis is further supported by the observation that cells increased mainly in width, while changes in height were small (data not shown). On the other hand, swelling of the entire tissue might have been caused primarily by the deep zone of cartilage which is known to contain high fixed charge density [22]. Neighbouring cells in the superficial zone likely moved then in the same direction as a result of tissue swelling, minimizing local tissue strain. The horizontally oriented collagen fibrils in the superficial zone may also help explain the direction of tissue swelling (figure 4a).

We imaged chondrocytes through the articular surface. This procedure has the advantage that the articular cartilage can be kept fully intact, allowing a native and natural environment for the chondrocytes. The limitation in this approach is that cells in the deep zone could not be included. If the dye, e.g. Calcium-AM or Dextran (Molec-

[1] Our preliminary results on bovine cartilage showed a difference in the long-term cell volume changes between the cells below the cut surface of the cartilage explants and the cells in their native environment. Further, regulatory volume decrease was noticed for the cells of explants (but not for the cells in their native environment), as has also been shown earlier at room temperature [7].

ular Probes, Eugene, OR, USA), would penetrate the entire tissue ($\sim$500 μm in our case), dual photon microscopy might be applicable to be used to quantify cell behaviour throughout the tissue [23].

Fluorescence intensity diminished slightly as a function of time. The decrease in intensity was mainly caused by photobleaching, and not the dilution of the dye due to osmotic loading. This was ensured by imaging additional set of cells at different locations in the long-term time points. Those measurements showed that the fluorescence intensity had not been reduced much, a remaining intensity being more than 150 (maximum was 255) for the cells. To minimize the photobleaching effects on cell volume calculations, a percentual threshold of 40% of the maximum intensity, the same as in [7], was used in the analysis (absolute threshold value was calculated individually for each cell in every time point), and was found to be appropriate for this application, as cell volumes in an iso-osmotic solution changed randomly and less than 4% over a four hour period. Moreover, in order to reduce photobleaching effects, images were not captured at all time points for every sample (see figure 3).

Cell death increased during the 4 hour measurement period, especially in the most superficial layer of cartilage ($\sim$0-20μm), while the analyzed cells were located mostly below that layer. Cell death and photobleaching were the primary reasons why only five viable cells from each sample were analyzed. Since the same cells were analyzed at different time points, we don't believe that relatively low number of analyzed cells had a significant effect on the results.

Recently, (an)isotropic and (in)homogeneous biphasic mathematical models of cartilage with chondrocytes have been developed [24-27]. Some models also include triphasic or swelling properties of cartilage and cells [26, 27], and cells embedded in the matrix have been modelled as ideal osmometers, where the cell membrane is only permeable to fluid but not to ions [28, 29]. Moreover, some models of chondrocytes have been able to predict cell volume regulation following osmotic challenge [30-33]. Our results can be used for validation of such theoretical predictions, and finite element models may then be used to study biophysical processes in cartilage and chondrocytes which cannot be measured at present.

It has been suggested that hypotonicity contributes to decreased cartilage biosynthesis [34-36] and increased cell death of mechanically tested cartilage [37], and that these are linked to increased cell volumes. Cell volumes have also been shown to increase in osteoarthritis [2]. However, these studies were done for isolated cells or cells in explant tissues. The relationships between cell volumes, cartilage biosynthesis and cell death in an intact tissue using different osmolarities and loading types (static, dynamic, impact) might provide novel information on biomechanical and biological responses of chondrocytes in changing mechanical environments, as observed in osteoarthritis.

In conclusion, volumes of cells that were fully embedded in their native mechanical environment increased within minutes post the osmotic challenge and continued for hours, and tissue swelling continued throughout the four hour testing period. Therefore, we suggest that the intact extracellular tissue affects cell volume changes post the osmotic challenge. Understanding the behaviour of cells in their native environment provides novel insigth into the cell mechanics in ostearthritic joints and so may help understand the onset and progression of this disease.

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