Mechanical and structural characterization of articular cartilage of the bovine medial tibia plateau

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Chapter 1

Introduction

Articular cartilage is a thin layer of connective tissue that covers the ends of diarthrodial joints. The primary functions of articular cartilage are to minimize contact stresses, generated during joint loading, and to contribute to the lubrication mechanisms in the joint. Articular cartilage can be considered a composite organic solid matrix saturated with water (65-80% of the total weight), in which free ions are dissolved. The solid matrix consists of a collagen fibre network (on average 75% of the dry weight [1]), proteoglycans (20-30% of the dry weight [1]) and chondrocytes (rest). Collagen fibres extend perpendicular from the subchondral bone, and near the articular surface bend over to merge with the articular surface [2, 3]. Proteoglycans are large negative charged proteins that attract water, which creates an internal swelling pressure [1]. The viscoelastic collagen fibres provide resistance against this swelling pressure. Together these molecules provide a system capable of bearing relatively large loads. The cartilage thickness, composition and mechanical behavior vary between locations in the joints, between different joints, and between different animals and humans [4, 3]. The mechanical behavior and the composition of the articular cartilage are believed to correlate [5].

Osteoarthritis (OA), which involves the degeneration of articular cartilage leads to impairment of the load bearing and lubrication functions, which causes impaired joint motion, disability and pain. In OA the early signs of cartilage degeneration include an increase in water content, swelling, fibrillation of the cartilage surface, breakdown of the collagen network and depletion of proteoglycans (PGs) from the superficial layer [6]. It is expected that these changes influence the mechanical properties of articular cartilage.

The first goal of this study was to determine the correlation between the cartilage composition and structure, and the mechanical properties of the cartilage. The second goal of this study was to determine the mechanical and compositional changes of articular cartilage due to cartilage damage by impact loading, and the correlation between them.

The mechanical properties of cartilage were measured using indentation, confined compression, unconfined compression and 1-D swelling experiments. Digital densitometry (DD) was used to provide information about the proteoglycan (PG) content, Fourier transform infrared imaging (FTIRI) was used to measure the collagen content, and polarized light microscopy (PLM) was used to measure orientation and anisotropy of the collagen fibers. As a model for cartilage damage, the articular cartilage was damaged by mechanical overloading. To determine the influence of damage on the composition of the cartilage, the composition of the damaged cartilage samples was compared to the composition of the non-damaged cartilage.
samples. To monitor what happens to the mechanical properties of cartilage after damaging, unconfined compression tests were done before and after damaging.
To study the differences in the mechanical properties and the composition of cartilage between individual animals, and between different locations in the joint, samples were used from two different cows and three different locations on the medial tibia plateau.
Chapter 2

Materials and Methods

2.1 Sample preparation

Osteochondral plugs from three locations (fig 2.1) of two bovine medial tibia plateaus were used for the mechanical and microscopic analysis. Knees from two different cows (between 1 and 2 years old) were used.

From each location a plug was drilled with a diameter of 19.0 mm. The bone at the bottom side of the plugs was sawed parallel to the cartilage surface with a Buehler low speed saw. Two smaller plugs (6.5mm in diameter) were drilled from the large plugs (fig 2.1). One plug was separated from the larger plug with a Buehler low speed saw and appointed for the damage experiments. The other plug was used for the mechanical characterization of normal, healthy cartilage. Because the larger plug could be glued more easily to the holder, which was used for indentation, this plug was separated from the 19.0 mm plug after indentation. During preparation and mechanical testing samples were immersed in 0.154M PBS, a phosphate buffered saline containing enzyme inhibitors [7]. Between preparation and mechanical testing samples were stored at -18 °C.

![Figure 2.1: Preparation of the osteochondral plugs from the medial tibia plateau. Left: three plugs were drilled from each plateau. One plug was taken from the lateral side of the medial plateau (A), one from the peripheral edge (C) and one in the center (B). The A samples were not covered with the meniscus, the B and C locations were. Right: a 19mm plug with the locations of the two smaller plugs represented by the two dotted circles.](image)

For each plug all mechanical experiments were done on one day, after which the plugs were
cut into two halves and prepared for microscopy. The samples were fixed in 10% formalin, decalcified, dehydrated and embedded in paraffin as described by Kiraly et al [8]. For digital densiometry safranin-O (Fisher Scientific, Fair Lawn, N.J., USA) was added to reduce passive diffusion of PGs out of the tissue [9].

2.2 Mechanical experiments

Thickness measurement

The thickness (h) of the cartilage layer was measured with ultrasound, using a commercially available arthroscopic indentation instrument (Artscan 200, Artscan Oy, Helsinki, Finland). The instrument was modified by mounting a 10.5 MHz Panametrics XMS-310 (diameter 3 mm, frequency bandwidth 5.5 - 15.5 MHz (-6 dB), Panametrics Inc., Waltham, MA, USA) ultrasound transducer on the tip of the instrument rod. Software for the data acquisition and analysis was developed in the LabVIEW environment (version 6i, National Instruments, Austin, TX, USA). The ultrasound transducer was controlled with ULTRAPAC-system (Physical Acoustics Corporation, Princetown, NJ, USA) containing a 500 MHz AD-board and a 0.5-100 MHz pulser-receiver board [10]. The speed of sound in cartilage in the medial tibia plateau was assumed to be 1602 m/s [11]. The thickness was used to calculate the indenter movements needed for the applied strains in the mechanical experiments.

Indentation

For indentation the 19mm plug was glued onto a holder to prevent sliding of the sample. The holder was filled with 0.154M PBS. The measuring equipment consisted of a 1000 g load cell (Sensotec, Columbus, OH, USA), precision motion controller PM500-C (Newport, Irvine, CA, USA) and a computerized data acquisition system. A flat non-porous indenter with a diameter of 0.99333 mm was slowly moved towards the cartilage surface to make contact between the indenter and the surface. Contact was defined between a load of 0.5 and 1.1g. Three steps of 5% strain were applied with a velocity of 1 \( \mu \)m/s. After each step the sample was allowed to equilibrate for 20 minutes. The first step was used as a pre-strain to assure good contact. After equilibrium was reached in the third step, the dynamic response of the cartilage was measured. A sinusoidal movement was applied with an amplitude of 1% of the cartilage thickness after 15% strain. 10 cycles were applied with a frequency of 1.0Hz.

Confined and unconfined compression

For confined compression the 6.5mm plug was separated from the 19.0 mm plug and punched with a 3.7 mm punch. The 3.7mm plug was put directly into a non-porous confined compression chamber with 3.8 mm in diameter. The same measurement equipment was used as for indentation. A porous filter, which diameter corresponded with the chamber diameter, was used for compression. The sample holder was filled with 0.154M PBS. Contact was defined around a load of 15g. Three steps of 5% strain were applied with a velocity of 1 \( \mu \)m/s. After each step the sample was allowed to equilibrate for 30 minutes. The first step was used as pre-strain to assure good contact. After equilibrium was reached in the third step, the dynamic response of the cartilage was measured. A sinusoidal movement was applied with an amplitude of 1% of the cartilage thickness after 15% strain. 10 cycles were applied with a
frequency of 1.0Hz. After the confined compression test the samples were put into 0.154M PBS for 30 minutes, after which they were tested in unconfined compression. The sample was compressed between two smooth metallic plates. The same protocol was used as in confined compression.

Swelling

The swelling experiments were performed on the osteochondral plugs from knee 2. After confined compression the strain was removed and the solution changed to 0.005M PBS. After 20 minutes, again contact was made around a load of 15g and two steps of 5% strain were applied with a velocity of 1µm/s. After each step the samples were left to equilibrate for 30 minutes. To assure good contact the first step was used as pre-strain. After this, the solution was replaced with 0.154M and 1.0M PBS and the swelling response was measured for one hour in between solution changes.

2.3 Damage experiment

The samples appointed for the damage experiment were punched with a 6mm punch. The cartilage was damaged in unconfined compression with an Instron mechanical instrument (Instron 8874, Instron Corporation, Canton, MA). For the first knee the cartilage was compressed 60% with a speed of 5% strain/s. For the second knee the speed was increased to 30% strain/s. Before and after applying the damage load an unconfined compression test was performed to measure mechanical effects of the damaging. The unconfined compression experiment was performed as described before, but with only two steps of 5% strain and no dynamic loading. Before each unconfined compression experiment, the thickness of the cartilage was measured with ultrasound as described before, and the diameter was measured using light microscopy. After damaging, at least 30 minutes was waited before starting the second unconfined compression test.

2.4 Microscopy

Digital densitometry

A linear relation exists between measured optical density (OD) and the concentration of tissue PGs [9]. With digital densitometry (DD) the OD of a sample can be measured. Therefore DD was used to measure the PG content and distribution in articular cartilage [12]. 3 µm microscopic bone-cartilage sections were cut and stained with safarin O. The OD of the sections was measured with monochromatic light (wavelength 492 +/- 5nm) using a Photometrics CH 120 camera equipped with a 2.5x objective (adjusted for normal transmission) and a CCD camera. The system was calibrated between 0. and 3.6 absorbancy units ($r^2 > 0.99$) using neutral density filters (Schott, Mainz, Germany). Gray scale images (5.7 × 5.7 µm pixel size) of the cartilage were made and converted to OD. To reduce errors due to differences in section thickness, an average of a minimum of 3 samples was taken.
Polarized light microscopy

With polarized light microscopy (PLM), the local averaged orientation of collagen fibrils and their anisotropy were measured. An orientation of $90^\circ$ means that the collagen fibers lie perpendicular to the subchondral bone, and an orientation of $0^\circ$ means that the collagen fibers lie parallel to the articular surface. Anisotropy is a measure for the organization of collagen fibrils within one image pixel. In other words, anisotropy states how parallel the fibrils are running.

Anisotropy and orientation of collagen were determined from unstained 5 $\mu$m thick microscopic sections. PGs were removed from sections with hyaluronidase (18h, 37 °C, 1000 U/ml; Sigma Chemical, ST Louis, MO, USA) [8]. The PLM measurements were conducted using Leitz Ortholux BK-2 polarized light microscope (Leitz, Wetzlar, Germany) equipped with a 4× strain-free objective, a cooled 12-bit CCD camera (Photometrics, Tucson, Ariz., USA), monochromatic light source (wavelength 594 +/- 3nm) and crossed polarizers. Gray scale images (4.7 × 4.7 $\mu$m pixel size) were captured after six 15° stepwise rotations of the polarizers. 0°, 45°, 90° images and 90° images with a $\lambda$/4-retardation plate were used to solve Stoke’s parameters [13] and were converted to anisotropy and orientation. The measured orientation is depended on the plane of vertical sectioning. To minimize this effect the osteochondral plugs from each location were sectioned in two perpendicular orientations. In each orientation three microscopic samples were measured to reduce the effect of possible differences in section thickness. So for each plug six measurements were done and the average of these measurements was calculated.

Fourier transform infrared imaging

Fourier transform infrared imaging (FTIRI) is a microscopic tool for characterization of tissue composition. In this study it was used to determine the collagen distribution in the cartilage samples. The technique utilizes absorbance of the infrared energy by specific molecular bonds. Infrared absorption of FTIRI is directly proportional to the amount of absorbing material and thereby to the tissue thickness [14]. FTIRI measurements were conducted with a Perkin Elmer Spectrum Spotlight 300 FT-IR imaging system (Perkin Elmer, Shelton, CO), using transmission mode through 2mm ZnSe windows and air dried. 10 $\mu$m thick cryosections were evaluated with a 25 $\mu$m pixel resolution to determine the average absorption of the cartilage sample. The surface of the Amide I peak [15] in the absorption spectrum of the sample was used as a measure of the collagen content in the sample. Three sections were measured per sample and the average was taken.

2.5 Data analysis

To compare the mechanical behavior of cartilage in the different samples and to determine the correlation between the mechanical behavior and composition of a sample, one single variable per test result was preferred. Therefore equilibrium moduli and dynamic moduli were calculated in indentation, confined compression, and unconfined compression, and approximations of the osmotic pressure was determined using the results of 1D-swelling.
Equilibrium moduli

For calculating the different moduli isotropic, homogeneous, linear material was assumed for cartilage. The bone was assumed to be rigid. First the axial stress $\sigma$ was calculated using

$$\sigma_{eq} = \frac{F}{A}, \quad (2.1)$$

with $F$ the measured reaction force, and $A$ the contact surface. For unconfined and confined compression $A$ was the total cartilage surface calculated with an assumed constant sample diameter of 3.8mm. For indentation the contact surface was the indenter surface (diameter 0.99333mm).

For confined compression the average aggregate modulus in equilibrium $H$ was calculated with

$$H = \frac{\sigma_{eq}}{\varepsilon_{eq}}, \quad (2.2)$$

with $\sigma_{eq}$ the axial equilibrium stress calculated by equation 2.1, and $\varepsilon_{eq}$ the axial equilibrium strain.

For unconfined compression, assuming a constant diameter, an estimated Young’s modulus $E$ in equilibrium was calculated with

$$E = \frac{\sigma_{eq}}{\varepsilon_{eq}}. \quad (2.3)$$

Note that no real unconfined compression was possible, due to the underlying bone. In indentation, the equilibrium Young’s modulus could be estimated by [16]

$$E_i = \frac{(1 - \nu^2) \pi a \sigma_{eq}}{2 \kappa h \varepsilon_{eq}}, \quad (2.4)$$

with $\nu = 0.4$ [17] the Poisson ratio, $a$ the radius of the indenter, $h$ the cartilage thickness and $\kappa$ the theoretical scaling factor, depending on $\nu$ and the $a/h$ ratio [16, 18].

Dynamic moduli

The dynamic modulus $E^*$ in unconfined compression was a complex modulus [19]:

$$E^*(\omega) = E'(\omega) + iE''(\omega), \quad (2.5)$$

with $E'(\omega)$ the storage or elastic modulus and $E''(\omega)$ the viscous or loss modulus and $\omega$ the frequency. The storage and loss modulus were given by [19]

$$E'(\omega) = \frac{\sigma_0}{\varepsilon_0} \cos \delta, \quad (2.6)$$

$$E''(\omega) = \frac{\sigma_0}{\varepsilon_0} \sin \delta, \quad (2.7)$$

with $\sigma_0$ the maximum axial stress, and $\varepsilon_0$ the maximum axial strain. The phase shift $\delta$ represents the ratio of the damped energy and the energy stored in the material. $\delta = 0^\circ$ means that the cartilage was a pure elastic material, and $\delta = 90^\circ$ means a pure viscous material.
The magnitude of the complex modulus $|E^*(\omega)|$ and $\delta$ were calculated by

$$|E^*(\omega)| = \sqrt{E''^2(\omega) + E'''^2(\omega)} = \frac{\sigma_0}{\varepsilon_0}$$

(2.8)

$$\delta = \tan^{-1}\frac{E''(\omega)}{E'(\omega)}.$$  

(2.9)

In the same manner the dynamic moduli ($H^\alpha$) for confined compression were calculated, in which $\alpha$ is *, ′ or ″ for the complex, storage, and loss moduli, respectively. In indentation the measured dynamic moduli ($E_{\alpha m}$) needed to be corrected in the same manner as for the equilibrium moduli (equation 2.4) by

$$E_{\alpha i}^i(\omega) = \frac{(1 - \nu^2)\pi a}{2kh} E_{\alpha m}^m(\omega).$$

(2.10)

**Swelling**

The equilibrium axial stress (calculated with equation 2.1) in 1-D swelling consists of the stress in the solid matrix $\sigma_s$, and the osmotic pressure $\Delta\Pi$:

$$\sigma_{eq} = \sigma_s - \Delta\Pi.$$  

(2.11)

Because the strain was constant during the swelling experiment, the solid stress was constant during the changing of the salt solutions. By assuming that at 1.0M the osmotic pressure was 0.0 [6, 5] the measured stress at 1.0M was equal to the stress in the solid matrix. Hence, the osmotic pressure gradient $\Delta\Pi$ in 0.005M and 0.154M can be given by

$$\Delta\Pi = \sigma - \sigma(1.0M).$$

(2.12)

**Microscopy**

As a measure for the PG and collagen contents, the averages were calculated of the OD and absorption values, respectively. Hence, for each sample a mean OD and mean absorption value give an indication of the amount of PGs and collagen in the cartilage. The orientation data was used to determine the relative thickness of the surface layer $h_s$ compared to the total normalized cartilage thickness. To calculate $h_s$ the mean collagen orientation in the deep zone was calculated for each sample by removing the data points of the upper and lower 20% of the cartilage thickness. The part of the data, which had an orientation value of this mean orientation minus 10° was defined as $h_s$.

**Correlation**

To determine the correlation between the mechanical behavior and the composition of cartilage the coefficient of determination ($R^2$) was determined. $R^2$ larger than 0.70 was defined as a significant correlation.
Chapter 3

Results

3.1 Indentation and confined, and unconfined compression

Thickness and equilibrium moduli

The cartilage thickness was largest for the A samples, and smallest for the C samples. In all locations the cartilage in knee 1 was thicker than in knee 2 (table 3.1).

In indentation and unconfined compression the moduli (table 3.1) were highest for the B samples. For knee 1 the indentation moduli of B were 1.8 and 4.5 times larger than A and C respectively. For knee 2 the B sample was 2.3 and 2.4 stiffer than the A en C samples, respectively. In unconfined compression sample B of knee 1 was 2.3 and 3.7 times stiffer than A and C, respectively. Modulus B of knee 2 was 1.8 and 1.4 times higher than for A and C, respectively. In confined compression no differences were found, except of the relative low aggregate modulus of sample 1C.

Table 3.1: Thicknesses \(h\) of cartilage samples and calculated moduli for indentation \(E_i\), confined compression \(H\), and unconfined \(E\) compression.

<table>
<thead>
<tr>
<th>sample</th>
<th>(h [\text{mm}])</th>
<th>(E [\text{MPa}])</th>
<th>(H [\text{MPa}])</th>
<th>(E_i [\text{MPa}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1.97</td>
<td>0.116</td>
<td>0.225</td>
<td>0.262</td>
</tr>
<tr>
<td>1B</td>
<td>1.29</td>
<td>0.263</td>
<td>0.209</td>
<td>0.465</td>
</tr>
<tr>
<td>1C</td>
<td>0.65</td>
<td>0.071</td>
<td>0.072</td>
<td>0.104</td>
</tr>
<tr>
<td>2A</td>
<td>1.28</td>
<td>0.167</td>
<td>0.210</td>
<td>0.276</td>
</tr>
<tr>
<td>2B</td>
<td>1.16</td>
<td>0.294</td>
<td>0.211</td>
<td>0.632</td>
</tr>
<tr>
<td>2C</td>
<td>0.64</td>
<td>0.203</td>
<td>0.180</td>
<td>0.265</td>
</tr>
</tbody>
</table>

Dynamic moduli

For all samples the loss moduli were relatively small compared to the elastic moduli (tables 3.2, 3.3 and 3.4). Therefore the complex moduli were almost the same as the elastic moduli and the phase shifts were very small. In all samples the phase shift was largest in confined compression and smallest in indentation. Comparing the complex moduli of the A,B and C samples from knee 1 and 2 in all three methods, knee 2 had higher moduli.
In unconfined compression (table 3.2) the A samples had the lowest complex moduli and phase shifts. The phase shift of the C samples was highest, especially in knee 1. In confined compression (table 3.3) the C samples had higher complex moduli and phase shifts than the B samples. Sample 1A had a relative high complex modulus compared the other samples. In indentation (table 3.4) the A samples had lower complex moduli than the B and C samples.

Table 3.2: Loss moduli ($E''$), storage moduli ($E'$), complex moduli ($E^*$) and phase shift ($\delta_u$) of cartilage samples for unconfined compression

<table>
<thead>
<tr>
<th>name</th>
<th>$E''$ [MPa]</th>
<th>$E'$ [MPa]</th>
<th>$E^*$ [MPa]</th>
<th>$\delta_u$ [deg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0.034</td>
<td>1.353</td>
<td>1.353</td>
<td>1.437</td>
</tr>
<tr>
<td>1B</td>
<td>0.136</td>
<td>2.881</td>
<td>2.884</td>
<td>2.705</td>
</tr>
<tr>
<td>1C</td>
<td>0.367</td>
<td>2.282</td>
<td>2.311</td>
<td>9.142</td>
</tr>
<tr>
<td>2A</td>
<td>0.125</td>
<td>3.187</td>
<td>3.190</td>
<td>2.243</td>
</tr>
<tr>
<td>2B</td>
<td>0.496</td>
<td>4.585</td>
<td>4.612</td>
<td>6.177</td>
</tr>
<tr>
<td>2C</td>
<td>0.738</td>
<td>6.041</td>
<td>6.086</td>
<td>6.963</td>
</tr>
</tbody>
</table>

Table 3.3: Loss moduli ($H''$), storage moduli ($H'$), complex moduli ($H^*$) and phase shift ($\delta_c$) of cartilage samples for confined compression

<table>
<thead>
<tr>
<th>name</th>
<th>$H''$ [MPa]</th>
<th>$H'$ [MPa]</th>
<th>$H^*$ [MPa]</th>
<th>$\delta_c$ [deg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1.326</td>
<td>8.027</td>
<td>8.136</td>
<td>9.378</td>
</tr>
<tr>
<td>1B</td>
<td>0.310</td>
<td>3.039</td>
<td>3.055</td>
<td>5.826</td>
</tr>
<tr>
<td>1C</td>
<td>0.660</td>
<td>3.317</td>
<td>3.382</td>
<td>11.247</td>
</tr>
<tr>
<td>2A</td>
<td>0.414</td>
<td>3.580</td>
<td>3.604</td>
<td>6.593</td>
</tr>
<tr>
<td>2B</td>
<td>0.424</td>
<td>4.456</td>
<td>4.476</td>
<td>5.434</td>
</tr>
<tr>
<td>2C</td>
<td>0.823</td>
<td>5.259</td>
<td>5.324</td>
<td>8.899</td>
</tr>
</tbody>
</table>

Table 3.4: Loss moduli ($E''_i$), storage moduli ($E'_i$), complex moduli ($E^*_i$) and phase shift ($\delta_i$) of cartilage samples for indentation

<table>
<thead>
<tr>
<th>name</th>
<th>$E''_i$ [MPa]</th>
<th>$E'_i$ [MPa]</th>
<th>$E^*_i$ [MPa]</th>
<th>$\delta_i$ [deg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0.024</td>
<td>1.690</td>
<td>1.691</td>
<td>0.820</td>
</tr>
<tr>
<td>1B</td>
<td>0.005</td>
<td>3.421</td>
<td>3.421</td>
<td>0.088</td>
</tr>
<tr>
<td>1C</td>
<td>0.320</td>
<td>3.256</td>
<td>3.272</td>
<td>5.610</td>
</tr>
<tr>
<td>2A</td>
<td>0.127</td>
<td>3.054</td>
<td>3.055</td>
<td>2.377</td>
</tr>
<tr>
<td>2B</td>
<td>0.021</td>
<td>8.021</td>
<td>8.022</td>
<td>0.152</td>
</tr>
<tr>
<td>2C</td>
<td>0.112</td>
<td>8.175</td>
<td>8.175</td>
<td>0.783</td>
</tr>
</tbody>
</table>

3.2 Swelling

The calculated osmotic pressure was smallest for sample 2C and largest for 2A (table 3.5).
Table 3.5: Osmotic pressure gradients $\Delta \Pi$ for 0.005M and 0.154M for knee 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta \Pi$ [MPa] (0.005M)</th>
<th>$\Delta \Pi$ [MPa] (0.154M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>2.38e-2</td>
<td>1.32e-2</td>
</tr>
<tr>
<td>2B</td>
<td>2.55e-2</td>
<td>1.14e-2</td>
</tr>
<tr>
<td>2C</td>
<td>1.50e-2</td>
<td>0.53e-2</td>
</tr>
</tbody>
</table>

3.3 Microscopy

In the A samples the OD was low in the superficial zone, and gradually increased in the transitional and deep zone, with a maximum near the cartilage-bone interface, where the OD decreased again (fig 3.1). In the B samples the OD was low in the superficial zone and increased fast to a high level in the deep zone. At the cartilage-bone interface the OD decreased. The OD in 2C was constant. In 1C the OD reached a maximum value in the transitional zone. The mean OD values were highest in the B samples and lowest in the C samples (table 3.6).

The absorption was relative low in the superficial and transitional zone and increased towards the cartilage-bone interface in all samples (fig 3.1). Sample 1A showed a small peak in absorption in the superficial zone. Also in 1B, 2A, and 2B such peaks were found but smaller. In knee 1 and 2 the mean absorption of the A samples were highest, in knee 2 more than twice the value in B and C (table 3.6).

The orientation of the collagen fibers was between 65° and 80° in the deep zone in all samples, except sample 1C (fig 3.2). In 1C the orientation in the deeper zones was around 15°. In the superficial zones the measured orientation for all samples was between 20° and 45°.

The anisotropy of the collagen fibers showed for all samples an increasing anisotropy towards the surface of the cartilage (fig 3.2).

3.4 Correlation

The mean OD $\langle OD \rangle$, as a measure for the PG content, the mean absorption $\langle Abs \rangle$, as a measure for the collagen content, and the relative surface thickness $h_s$ (table 3.6) were calculated for correlation with the mechanical properties. Significant correlations of $\langle OD \rangle$ were found with $\Delta \Pi(0.005M)$ ($R^2=1.00$), $\Delta \Pi(0.0154)$ ($R^2=0.88$) and $\delta_c$ ($R^2=0.77$) (table 3.7). The relative thickness of the surface ($h_s$) was found to correlate with $E''_t$ ($R^2=0.86$). The osmotic pressure was found to correlate highly with the moduli and $\delta$ in confined compression (eight high $R^2$ values). For indentation no correlation with the osmotic pressure was found and for unconfined compression only a minor correlation (three high $R^2$ values) (table 3.7). For the collagen contents no significant correlations were found.
Figure 3.1: Proteoglycan contents: the left two pictures give the optical density (OD), a measure for the PG contents, for knee 1 and 2. Collagen contents: the right two pictures show the absorption of infrared light, a measure for collagen contents, for knee 1 and 2. The thickness is normalized for all samples; 0 is the bone-cartilage interface, 1 is the articular surface.

Figure 3.2: Orientation: the left two pictures give the orientation of the collagen fibers, for knee 1 and 2. Anisotropy: the right two pictures show the anisotropy of the collagen fibers, for knee 1 and 2. The thickness is normalized for all samples; 0 is the bone-cartilage interface, 1 is the articular surface.
Table 3.6: Microscopy: the mean OD $\langle OD \rangle$ is a measure for the PG content, the mean absorption $\langle \text{Abs} \rangle$ is a measure for the collagen content, and $h_s$ is the relative surface thickness.

<table>
<thead>
<tr>
<th>sample</th>
<th>$\langle OD \rangle$</th>
<th>$\langle \text{Abs} \rangle$</th>
<th>$h_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0.693</td>
<td>0.213</td>
<td>0.10</td>
</tr>
<tr>
<td>1B</td>
<td>1.294</td>
<td>0.184</td>
<td>0.13</td>
</tr>
<tr>
<td>1C</td>
<td>0.261</td>
<td>0.198</td>
<td>-</td>
</tr>
<tr>
<td>2A</td>
<td>0.962</td>
<td>0.353</td>
<td>0.20</td>
</tr>
<tr>
<td>2B</td>
<td>1.237</td>
<td>0.170</td>
<td>0.10</td>
</tr>
<tr>
<td>2C</td>
<td>0.140</td>
<td>0.153</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table 3.7: Coefficients of determination $R^2$, with $\langle OD \rangle$ the mean OD, $\langle \text{Abs} \rangle$ the mean absorption, $h_s$ the relative thickness of the surface layer, $\Delta \Pi$ the osmotic pressure gradients for 0.005M and 0.154M, $E$, $E'$, and $H$ the equilibrium moduli in unconfined compression, confined compression and indentation, respectively, $\delta$ the phase shift in unconfined compression ($u$), confined compression ($c$) and indentation ($i$), and $E''$, $H''$ $E''', H'''$, the loss moduli ($\alpha =''$), the storage moduli ($\alpha ='$), and complex moduli ($\alpha =*$) in unconfined compression, confined compression and indentation, respectively.

<table>
<thead>
<tr>
<th></th>
<th>$\langle OD \rangle$</th>
<th>$\langle \text{Abs} \rangle$</th>
<th>$h_s$</th>
<th>$\Delta \Pi(0.005)$</th>
<th>$\Delta \Pi(0.154)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E'$</td>
<td>0.45</td>
<td>0.08</td>
<td>0.05</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td>$E''$</td>
<td>0.23</td>
<td>0.32</td>
<td>0.07</td>
<td>0.49</td>
<td>0.83</td>
</tr>
<tr>
<td>$\delta_u$</td>
<td>0.32</td>
<td>0.24</td>
<td>0.02</td>
<td>0.25</td>
<td>0.61</td>
</tr>
<tr>
<td>$E^*$</td>
<td>0.03</td>
<td>0.12</td>
<td>0.20</td>
<td>0.62</td>
<td>0.92</td>
</tr>
<tr>
<td>$H'$</td>
<td>0.39</td>
<td>0.03</td>
<td>0.42</td>
<td>0.98</td>
<td>0.94</td>
</tr>
<tr>
<td>$H''$</td>
<td>0.06</td>
<td>0.03</td>
<td>0.16</td>
<td>0.58</td>
<td>0.90</td>
</tr>
<tr>
<td>$H'''$</td>
<td>0.29</td>
<td>0.03</td>
<td>0.06</td>
<td>0.97</td>
<td>0.96</td>
</tr>
<tr>
<td>$\delta_c$</td>
<td>0.77</td>
<td>0.02</td>
<td>0.02</td>
<td>0.97</td>
<td>0.72</td>
</tr>
<tr>
<td>$H^*$</td>
<td>0.06</td>
<td>0.03</td>
<td>0.15</td>
<td>0.60</td>
<td>0.91</td>
</tr>
<tr>
<td>$E_i$</td>
<td>0.64</td>
<td>0.07</td>
<td>0.33</td>
<td>0.42</td>
<td>0.11</td>
</tr>
<tr>
<td>$E_i'$</td>
<td>0.01</td>
<td>0.27</td>
<td>0.02</td>
<td>0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>$E_i''$</td>
<td>0.45</td>
<td>0.02</td>
<td>0.86</td>
<td>0.27</td>
<td>0.03</td>
</tr>
<tr>
<td>$\delta_i$</td>
<td>0.29</td>
<td>0.07</td>
<td>0.49</td>
<td>0.01</td>
<td>0.20</td>
</tr>
<tr>
<td>$E_i^*$</td>
<td>0.01</td>
<td>0.27</td>
<td>0.02</td>
<td>0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>$\Delta \Pi(0.005)$</td>
<td>1.00</td>
<td>0.19</td>
<td>0.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\Delta \Pi(0.154)$</td>
<td>0.88</td>
<td>0.53</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.5 Damage experiment

During damaging of the samples, a drop in the axial compressive stress (fig 3.3) was defined as a point where the cartilage was damaged. For sample 1A a drop in the axial stress was seen at 65% strain. For sample 2A a drop in the axial stress was found at 55% and at 63% strain and for sample 2B at 55% strain. For sample 1B, 1C, and 2C no drop in the axial stress was seen.

The moduli of samples 1A, 2A and 2B were lower during the test after damaging. The second moduli in 1B was higher (table 3.8). The moduli for the C samples did not differ. The thickness measured before the second measurement was smaller for 1A, 2A and 2B, and approximately the same for the other samples (table 3.8). The diameters of both the A and B samples were larger after damaging (table 3.8).

**Figure 3.3:** An example of the compressive stress during unconfined compression with an Instron mechanical instrument. The arrows indicate a drop in the compressive stress, which indicates damage initiation in the cartilage.

**Table 3.8:** Moduli (E), thicknesses (h) and diameters (D) measured for the unconfined compression test before (1) and after (2) damaging the cartilage

<table>
<thead>
<tr>
<th>Sample</th>
<th>1st E [MPa]</th>
<th>2nd E [MPa]</th>
<th>h1 [mm]</th>
<th>h2 [mm]</th>
<th>D1 [mm]</th>
<th>D2 [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0.141</td>
<td>0.071</td>
<td>2.32</td>
<td>2.12</td>
<td>5.91</td>
<td>6.59</td>
</tr>
<tr>
<td>1B</td>
<td>0.478</td>
<td>0.687</td>
<td>1.27</td>
<td>1.27</td>
<td>5.95</td>
<td>6.29</td>
</tr>
<tr>
<td>1C</td>
<td>0.036</td>
<td>0.037</td>
<td>0.67</td>
<td>0.65</td>
<td>6.04</td>
<td>6.11</td>
</tr>
<tr>
<td>2A</td>
<td>0.144</td>
<td>0.081</td>
<td>2.21</td>
<td>1.97</td>
<td>6.18</td>
<td>6.76</td>
</tr>
<tr>
<td>2B</td>
<td>0.145</td>
<td>0.074</td>
<td>1.97</td>
<td>1.60</td>
<td>6.01</td>
<td>6.81</td>
</tr>
<tr>
<td>2C</td>
<td>0.037</td>
<td>0.031</td>
<td>0.53</td>
<td>0.49</td>
<td>6.02</td>
<td>6.02</td>
</tr>
</tbody>
</table>

3.6 Composition of undamaged and damaged samples

The distribution of PGs (OD values in fig 3.4) and collagen (absorption values in fig 3.5) of damaged and undamaged samples was comparable, except for the samples 2B and 2C.
Table 3.9: The mean OD \( \langle OD \rangle \) and mean absorption \( \langle Abs \rangle \) values for undamaged(N) and damaged(D) samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \langle OD \rangle_N )</th>
<th>( \langle OD \rangle_D )</th>
<th>( \langle Abs \rangle_N )</th>
<th>( \langle Abs \rangle_D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0.69</td>
<td>1.15</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td>1B</td>
<td>1.29</td>
<td>1.19</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>1C</td>
<td>0.26</td>
<td>0.27</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td>2A</td>
<td>0.96</td>
<td>0.90</td>
<td>0.35</td>
<td>0.18</td>
</tr>
<tr>
<td>2B</td>
<td>1.24</td>
<td>1.02</td>
<td>0.17</td>
<td>0.18</td>
</tr>
<tr>
<td>2C</td>
<td>0.14</td>
<td>0.16</td>
<td>0.15</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Figure 3.4: Proteoglycan distribution - optical density(OD), a measure for the PG contents, for knee 1 and 2 in the undamaged(N) and damaged samples(D). The thickness is normalized for all samples; 0 is the bone-cartilage interface, 1 is the articular surface.

For 2B and 2C the OD values for the damaged samples were lower than for the undamaged samples in the upper half of the distribution curve, and higher in the deeper zone (fig 3.4). Also the mean OD value of 2B was lower in the damaged sample (table 3.9). Compared to the undamaged samples, the damaged 1A sample had an almost doubled mean OD value, the damaged 2A sample had half of the mean absorption value, and the damaged 2C sample had also an increase in mean absorption.

For the collagen orientation (table 3.6) and collagen anisotropy (table 3.7) hardly any differences were found. Only in 2A and 2B a small increase in orientation in the superficial layer was found for the damaged samples. In sample 2C, the orientation of the collagen fibers was around 20 degrees for the damaged sample, while it was around 70 in the undamaged sample. Because of the small number of damaged samples, no correlations were found between changes in the mechanical properties and the composition.
Figure 3.5: Collagen distribution - Absorption of Infrared light, a measure for collagen contents, for knee 1 and 2 in in the undamaged(N) and damaged samples(D). The thickness is normalized for all samples; 0 is the bone-cartilage interface, 1 is the articular surface.

Figure 3.6: Orientation of collagen fibers for knee 1 and 2 in in the undamaged(N) and damaged samples(D). The thickness is normalized for all samples; 0 is the bone-cartilage interface, 1 is the articular surface.

Figure 3.7: Anisotropy of collagen fibers for knee 1 and 2 in in the undamaged(N) and damaged samples(D). The thickness is normalized for all samples; 0 is the bone-cartilage interface, 1 is the articular surface.
Chapter 4

Discussion

The first goal of this study was to determine the correlation between the bovine tibial cartilage composition and structure, and the mechanical properties of the cartilage. The second goal was to determine the mechanical and compositional changes of bovine tibial cartilage due to damage initiation by impact loading, and the correlation between them.

Mechanical and compositional properties of articular cartilage from three different locations on two bovine medial tibia plateaus were determined. Indentation, confined compression, and unconfined compression tests were used to determine equilibrium and dynamic moduli, and a swelling experiment was used to calculate the osmotic pressure of the samples. With DD the optical density (OD) was measured as a measure for the PG content, the measured absorption from FTIRI was used as a measure for the collagen content and with PLM the collagen orientation and anisotropy were determined. Correlation factors between mechanical properties and the composition of articular cartilage were calculated to determine how mechanical behavior and composition relate. Also the effect of impact loading on the equilibrium moduli in unconfined compression and the composition of cartilage was determined to find the consequences of cartilage damage on mechanical behavior and composition.

Composition

The thickness of the cartilage influences the total amount of collagen en PGs in the cartilage. The thickness of the cartilage in knee 2 was smaller compared to knee 1. The cartilage was thickest in the A locations, and thinnest in the C samples. Hence, it was important to calculate the mean OD and mean absorption values for calculating the correlation with the mechanical properties, without the influence of the thickness.

In general the PG content was high in the deep zone of the cartilage and lower near the surface as measured before by Laasanen et al.\textsuperscript{[10]}. A low PG contents was also found near the cartilage bone interface, which indicates that also calcified cartilage was measured. The mean PG contents was highest in the B locations. In C samples PG contents was lowest.

PLM results showed a fiber orientation for A and B samples which were in agreement with reports from Clark [4, 3] and Benninghoff [2] and with birefringence measurements by Laasanen et al.\textsuperscript{[10]}. The collagen orientation value in the superficial zone was higher, and in the deep zone lower, than expected from literature, because the mean value of several measurements was taken. Nevertheless, the difference in high and low orientation in the deep and superficial zone, respectively, was still clear.

Striking were the differences in orientation in the four C samples. Compared to the A and
B samples, the undamaged 2C sample showed the same collagen orientation throughout the cartilage, while the other three C samples showed lower values for orientation in the deep zone (fig 3.6). An explanation might be that the undamaged 2C sample was located more in the middle of the tibia plateau and the other three samples more to the periphery. Hence, the orientation in the C samples indicated that the main part of the collagen fibers at the periphery of the tibia plateau had an orientation parallel to the articular surface, which was reported earlier by Clark [4, 3].

**Mechanical properties and correlations**

The equilibrium and dynamic moduli for knee 2 were higher than for knee 1 in all locations, which means that knee 2 was generally stiffer. The equilibrium moduli as determined from unconfined compression and indentation tests, were highest at the center of the medial tibia plateau (B samples), as was the PG contents. However, no correlation was found between the mean optical density (PG content) and the equilibrium moduli in unconfined compression and indentation. The aggregate modulus H was highly correlated with the osmotic pressure. Also the PG contents was highly correlated with the osmotic pressure, but not with H. Hence, other factors than the PG contents also influenced the osmotic pressure and H.

The dynamic indentation moduli were highest in B and C samples. This indicated that cartilage covered by the meniscus (B and C samples) had a stiffer superficial layer, compared to the part not covered by the meniscus (samples A). High correlations were also found between the osmotic pressure and the dynamic moduli and phase shift in confined compression, but not in indentation and unconfined compression. This was expected because in confined compression, the cartilage was confined in the radial direction, which results in a direct volumetric change, when applying an axial strain. A greater volume decrease gives an increased fixed charge density and therefore a higher osmotic pressure gradient in confined compression.

The phase shift was relatively low in all geometries. Hence, the cartilage samples have a dominantly elastic response under dynamic loading. The phase shift as determined from the confined compression tests was larger for all locations compared to indentation and unconfined compression. This indicates that the viscous behavior of cartilage was most obvious in dynamic confined compression.

The PG contents also correlated well with the phase shift in confined compression; the higher the mean OD the lower the phase shift. This means that the larger the PG contents, the smaller the viscous part of the dynamic modulus of the cartilage. This might be explained by a lower outflow of water, because the water was attracted stronger by the high amounts of PGs. Taking into account the short time the cartilage is compressed, more PGs might lead to a more elastic dominated dynamic response.

Another correlation was found between the cartilage surface thickness and the loss modulus in indentation. The larger the relative thickness of the surface layer the larger was the loss modulus. The dynamic response of the surface layer was mainly dependent on the collagen fibers. Hence, in a thicker layer the viscous part of the collagen fibers was more important than in a thin layer.
It was expected that the mean absorption, that is the collagen content, would correlate with mechanical properties, because the collagen network is known to restrain swelling and therefore keeping osmotic pressure high, influencing the compressive properties. Hence, it is probable that not the collagen contents, but the distribution and orientation of the fibers is most important in the mechanical behavior of cartilage. For example, in sample 1C hardly any fibers were found with an orientation perpendicular to the cartilage bone interface, which means that the swelling was not constrained. This results in a lower osmotic pressure and a lower H.

The goal of this study was to measure the properties of the cartilage only. However, it was not possible to remove the underlying bone, because this resulted in misshapen swollen cartilage samples due to damaging of the collagen fibers. Hence, the bone was not removed and was assumed rigid in all experiments. In the unconfined compression test the underlying bone restricted the radial swelling of the cartilage, so no real unconfined compression was possible. In calculating the estimated compressive modulus in unconfined compression, the diameter of the cartilage was assumed constant.

For the calculations of the compressive moduli cartilage was also assumed homogeneous, isotropic and linear elastic. In the microscopic results it was clearly shown that cartilage is an anisotropic and non-homogeneous material. For indentation, the estimation of the moduli was not correct because the same Poisson ratio, measured by Laasanen et al. [17] in the center of the medial tibia plateau, was used for all samples. It was not possible to perfectly align the cartilage surface and the indenter in the confined and unconfined compression experiments. Due to this applied displacements were not uniformly distributed over the articular surface.

In the confined compression test the used punch had a diameter of 3.7 mm, while the confining chamber had a diameter of 3.8 mm. Due to this cartilage was allowed to increase in diameter, which leads to an under estimation of the aggregate modulus. This effect was bigger because the edges could also swell, due to the damaging by the punching of the samples.

In spite of the limitation mentioned above, we believe that the calculated moduli gave a good indication of the stiffness of the bovine tibial cartilage and a comparison could be made between the different locations and knees. The correlations between composition and mechanical properties were calculated for a maximum of only six samples. Hence, no reliable p-values could be determined, and the significance of the coefficient of determination was unsure. Nevertheless, we believe that the coefficients of determination gave a good indication of the relations between the composition and the mechanical properties of the cartilage.

In 1-D swelling it was assumed that the swelling pressure was zero at 1 M. However, this may not be correct. A non-zero osmotic pressure would mean a lower solid stress, which would have lead to higher osmotic pressure gradients.

The edges of the cartilage were damaged by the punching which might have caused extra swelling pressure at the edges. This effect would have been most prominent at 0.005 M. After the confined compression tests in 0.154 M, the samples were left to swell in 0.005 M for only 20 minutes before a new strain was applied. This was probably too short for the samples to reach a new equilibrium. Hence, during the application of the 10% strain, the swelling pressure gradient was too high.
Hence, although the results were influenced by several problems, it could be concluded that the C sample showed a much smaller osmotic pressure in 0.005M and 0.154M, compared to the A and B samples.

**Damage experiment**

The samples 1A, 2A and 2B, were believed to be damaged by impact loading. After damaging, the moduli of these three samples had only half of the value as before damaging, and lower thicknesses and larger diameters. For 2A lower mean absorption values were measured compared to the undamaged samples, but no difference in mean OD. For 1A a higher mean OD value and for 2B a lower mean OD, and a different distribution was measured compared to the undamaged samples, but no change in mean absorption. Hence, no overall consequence of damage in the composition of all three samples could be found. Because the differences in composition were determined with two different samples, and no correlations were found between the calculated moduli and the compositional changes, it is questionable whether the differences are due to damaging or due to local differences in mechanical properties and composition. That these local differences are present was clear from differences found between the undamaged A, B and C samples.

Hence, from mechanics it could be concluded that the samples were damaged, but this was not confirmed by a change in composition. To see changes in composition, it would probably have been better to give more time for outflow of PGs and degeneration of collagen fibers by waiting between damaging en the unconfined compression test after damaging and before preparing the samples for microscopy.

**Conclusions**

In conclusion, differences were found between the mechanical properties and composition of bovine tibial articular cartilage from two knees from different animals. Also local differences were found in the mechanical properties and composition of articular cartilage from the medial tibia plateau. The cartilage at the lateral side of the tibia plateau was thickest and at the periphery thinnest. In equilibrium the cartilage from the center of the tibia plateau was most stiff in unconfined compression and indentation. In the dynamic response the center and periphery were most stiff. The collagen orientation were found to be different at the periphery compared to the rest of the plateau, and also PG content differed along the tibia plateau. However, no high correlation factors were found between the mechanical properties and the collagen and PG content. Good correlations were found between the PG content and the osmotic pressure and also between the osmotic pressure and the mechanical behavior in confined compression.

By impact loading of the cartilage three samples were damaged, resulting in a change of mechanical properties by a decrease of the modulus in unconfined compression. However, no correlations were found with a change in cartilage composition. Hence, the consequences of damage for the composition of articular cartilage could not be determined.

More experiments are needed to calculate more reliable and significant correlations between mechanics and composition and to obtain compositional changes after damage. Nevertheless, more insight was obtained in the variations of mechanical properties and composition of the articular cartilage of the bovine medial tibia plateau.
Bibliography


Appendix A: Reaction force in stress-relaxation experiments

**Figure 1:** Reaction force [N] during unconfined compression for knee 1 (left) and knee 2 (right)

**Figure 2:** Reaction force [N] during confined compression for knee 1 (left) and knee 2 (right)

**Figure 3:** Reaction force [N] during indentation for knee 1 (left) and knee 2 (right)
Appendix B: Reaction force in dynamic experiments

Figure 4: Reaction force [N] during dynamic unconfined compression (top), confined compression (middle) and indentation (bottom).
Appendix C: Reaction force during 1-D swelling

Figure 5: Reaction force during swelling for knee 2. At the start the external concentration was 0.005M. In the first step the concentration was increased to 0.154M and in the second step to 1.0M.
Appendix D: Compressive stress during impact loading

Figure 6: Compressive stress, during impact loading with an Instron mechanical instrument. For knee 1 the cartilage was compressed 60% with a speed of 5% strain/s. For knee 2 the speed was increased to 30% strain/s. Sample 2C was compressed six times. This sixth time the total strain was increased to 200%. Hence, probably the bone was compressed. Samples 1A, 2A and 2B show a drop in the stress, indicated damage initiation.
Appendix E: Reaction force during unconfined compression for damaged samples.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{Reaction force during the unconfined compression tests before (1) and after (2) damaging of the samples.}
\end{figure}