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Anterior cruciate ligament transection of rabbits alters composition, structure and biomechanics of articular cartilage and chondrocyte deformation 2 weeks post-surgery in a site-specific manner

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Abstract

Anterior cruciate ligament (ACL) injury often leads to post-traumatic osteoarthritis (OA) and articular cartilage degradation, changing biomechanics of the tissue and chondrocytes, and altering the fixed charged density (FCD) and collagen network. However, changes in these properties are not known at a very early time point after ACL rupture, but recognizing early changes might be crucial for successful intervention. We investigated the effects of ACL transection (ACLT) in rabbits on the site-specific biomechanical properties of articular cartilage and chondrocytes, FCD content and collagen network organization, two weeks post-surgery.

Unilateral ACLT was performed in eight rabbits, and femoral condyles, tibial plateaus, femoral grooves and patellae were harvested from experimental and contralateral knee joints. An intact control group was used as a reference. We analyzed chondrocyte morphology under pre- and static loading, cartilage biomechanical properties, FCD content and collagen fibril orientation.

ACLT caused FCD loss in the lateral and medial femoral condyle, lateral tibial plateau, femoral groove and patellar cartilage ($p<0.05$). Minor changes in the collagen orientation occurred in the femoral groove and lateral and medial femoral condyle cartilage ($p<0.05$). Cartilage stiffness was reduced in the lateral and medial femoral condyles, and chondrocyte biomechanics was altered in the lateral femoral condyle and patellar cartilage ($p<0.05$).

We observed loss of FCD from articular cartilage two weeks after ACLT at several joint locations. These changes may have led to decreased cartilage stiffness and altered cell deformation behavior, especially in the femoral condyles.
Introduction

Articular cartilage is primarily composed of water, proteoglycans and collagen, which together determine the functional properties of the tissue. Cartilage structure, composition, function and chondrocyte (cartilage cell) biomechanics differ between the joint locations (Fick et al., 2015). The collagen fibril network is mainly responsible for the tensile and dynamic compressive stiffness of articular cartilage. Negatively charged glycosaminoglycans of proteoglycans result in a fixed charged density (FCD), which provides the primary compressive stiffness of cartilage at mechanical equilibrium. Chondrocytes maintain the structure and composition of articular cartilage. Chondrocyte activity varies according to the biomechanical stimuli and the articular cartilage condition (Korhonen and Herzog, 2008; Szafranski et al., 2004; Urban et al., 1993; Wong et al., 1997).

Osteoarthritis (OA) is the most common joint disease, which causes changes in all knee joint tissues leading to joint pain, functional impairment, immobility and reduced quality of life. Early OA is
associated with proteoglycan loss and collagen network degradation especially in the superficial
cartilage zone, which might lead to changes in chondrocyte morphology, biomechanics, and
subsequently, altered biosynthetic activity and reduced ability to maintain cartilage homeostasis
(Buschmann et al., 1996, 1995; Guilak et al., 1994).

Animal models can be used to investigate different subtypes and causes of OA (e.g. abnormal
loading, metabolic changes) in a controlled environment, within homogeneous groups, and without
other confounding factors typically present in human studies (e.g. different subtypes of OA, age,
gender, weight). Thus, they may be helpful when planning treatments and rehabilitation protocols
for certain subtypes of OA in humans as well, always considering the differences in joint anatomy
and structure, and the metabolic rates between humans and animal models of OA. Anterior cruciate
ligament transection (ACLT) causes knee joint instability and induces post-traumatic OA (Altman
and Dean, 1990; Galois, 2003; Hayami et al., 2006; O’connor et al., 1989; Sah et al., 1997; Setton
et al., 1999; Stockwell et al., 1983). We have earlier reported altered biomechanical behavior of
chondrocytes in patellar cartilage four and nine weeks after ACLT in rabbits (Han et al., 2010;
Turunen et al., 2013). At both of these time points, cell volume in the experimental group increased
under static loading, whereas cells in the control or contralateral group shrank (Han et al., 2010;
Turunen et al., 2013). Also, nine weeks post-surgery, local extracellular matrix strains were
increased (Han et al., 2010). Moreover, ACLT has been shown to cause proteoglycan content
reduction and changes in the collagen orientation angle of articular cartilage (Ehrlich et al., 1975;
Eyre et al., 1980; Han et al., 2010; Hayami et al., 2006; Marijnissen et al., 2002; McDevitt et al.,
1976; Sah et al., 1997; Turunen et al., 2013).
Site-specific alterations in articular cartilage structure and function have also been characterized earlier for the ACLT model of OA four weeks post-surgery or later (Han et al., 2010; Mäkelä et al., 2014; Turunen et al., 2013). Collagen network organization, amount of FCD, and stiffness of cartilage were altered, primarily in the femoral condyles (Mäkelä et al., 2014). However, it is not known if and how the aforementioned structural and functional properties of cartilage and cells change prior to the four-week time point evaluated to date. Furthermore, site-specific cell deformation behavior has not been characterized in any ACLT model of OA.

In the present study, we investigated the effects of ACLT on the structure, composition and biomechanical behavior of cartilage, and chondrocyte deformations two weeks after the surgery in a site-specific manner. Fick et al. (2016) suggested that at the earliest stages of OA caused by partial meniscectomy in rabbits, a loss of FCD in the superficial zone precedes collagen fibrillation of cartilage. This conclusion was based on digital densitometry and polarized light microscopy analyses of FCD and collagen fibril orientation, respectively (Fick et al., 2016). Therefore, we hypothesized that FCD loss will be the most substantial change at two weeks after ACLT, and that FCD loss is reflected in cartilage stiffness and chondrocyte deformations. Further, since the greatest changes in the FCD, collagen orientation angle and biomechanical properties of cartilage four weeks after ACLT were shown to occur in the femoral condyles of knees (Mäkelä et al., 2014), we hypothesized that the femoral condyles would also be the most sensitive to tissue- and cell-level changes at two weeks post-surgery.

**Methods**
Animal model

A unilateral ACLT was performed in eight skeletally mature female New Zealand white rabbits (*Oryctolagus cuniculus*, age 12 months at the time of surgery, weight 4.44 ± 0.45 kg). To avoid bias, the operated knee joint was chosen randomly from each rabbit. Rabbits were euthanized two weeks post-surgery under anesthesia. Both experimental and intact contralateral (C-L) knee joints were collected. In addition, knee joints from healthy rabbits (CNTRL, *N* = 8, weight 4.57 ± 0.35 kg, one randomly selected knee joint from each rabbit) were used as a separate control group. All procedures were carried out according to the guidelines of the Canadian Council on Animal Care and were approved by the committee on Animal Ethics at the University of Calgary. Osteochondral samples from the lateral and medial femoral condyles and tibial plateaus, the lateral femoral groove, and the patella were prepared (Figure 1 a-d).

[Suggested position for Figure 1]

Biomechanical testing, microscopy and histology

All the methods used in this study have been introduced in earlier studies (Han et al., 2009, 2010; Király et al., 1996; Kiviranta et al., 1985; Mäkelä et al., 2014; Rieppo et al., 2008, 2009; Turunen et al., 2013). A brief summary of the study methods is presented here. More details of the methods are presented in the supplementary material.
Confocal microscopy imaging combined with biomechanical indentation testing (cylindrical glass indenter, diameter = 2 mm, relaxation time 20 min, 10µm/s ramp rate) (Han et al., 2009, 2010; Turunen et al., 2013) was used to evaluate the morphology of viable chondrocytes (~40 – 70 cells from each location of each group) from the superficial zone cartilage before and after loading (2 MPa stress followed by stress-relaxation) (Figure 1 e,f). Chondrocyte volume, surface area, height, width and depth, and their deformation as a result of loading were analyzed (Figure 1 g,h). Moreover, local extracellular matrix axial \((n = 4\) cell pairs/sample) and transversal engineering strains (major and minor, \(n = 4\) cell pairs/sample) of the superficial zone cartilage were determined using identified cell pairs.

Following the cell deformation analysis, biomechanical testing of the osteochondral samples (Mäkelä et al., 2014) was performed using a stress-relaxation protocol \((3 \times 5\% \text{ steps}, 100\%/s \text{ ramp rate, 1 mm indenter diameter})\) with a 15 min relaxation period after each step (Figure 2). Moreover, a sinusoidal dynamic test (amplitude of 2% of the remaining thickness, 1 Hz frequency, 4 cycles) was conducted. The Hayes-corrected equilibrium, dynamic, loss and storage moduli, and the phase difference between stress and strain were calculated (Hayes et al., 1972).

[Suggested position for Figure 2]

After indentation testing, the samples were fixed in formalin, decalcified with ethylenediaminetetraacetic, dehydrated and embedded in paraffin. After tissue processing, histological samples were prepared (2-3 sections/sample). The depth-wise optical density, and the depth-wise collagen orientation angle were determined using digital densitometry of Safranin-O stained sections (K Király et al., 1996; Kiviranta et al., 1985) and polarized light microscopy of
unstained sections (Rieppo et al., 2008, 2009), respectively. Safranin-O binds stoichiometrically to the negative charges, and thus, optical density from digital densitometry can be used to indirectly estimate FCD (K. Király et al., 1996; Kiviranta et al., 1985). Moreover, a gross FCD was calculated as an average throughout the cartilage depth from the depth-wise optical density values.

Statistical analysis

Linear Mixed Model analysis (McCulloch et al., 2008; Von Tress, 2003) with and without Bonferoni correction was used for the statistical comparisons between the groups. All statistical comparisons were performed with SPSS Ver. 26 (IBM Corp., Armonk, NY) software.

Results

Cell morphology and deformation

Before and after loading of cartilage, cell volume of the medial femoral condyle cartilage was greater in the ACLT (before: 569 µm$^3$, after: 464 µm$^3$) group joints compared with the control (before: 409 µm$^3$, $p=0.008$, after: 351 µm$^3$, $p=0.03$) and contralateral (before: 417 µm$^3$, $p=0.009$, after: 361 µm$^3$, $p=0.04$) group joints (Table 1). Also, for this same location, cell surface area was greater in the ACLT (before: 377 µm$^2$, after: 363 µm$^2$) group joints compared with the control group joints before and after loading (before: 310 µm$^2$, $p=0.007$, after: 300 µm$^2$, $p=0.017$), and was
also greater compared with the contralateral (318 µm², \( p=0.012 \)) group joints before loading (Table S-1). Change of the cell volume of the lateral femoral condyle cartilage between the loading states was smaller in the ACLT (-10%) compared with the control (-23.1%, \( p=0.05 \)) knee joints. Before and after loading, cell volume of the lateral femoral condyle cartilage was greater in the contralateral (before: 702 µm³, after: 586 µm³) compared with the control knees (before: 531 µm³, \( p=0.05 \), after: 406 µm³, \( p=0.02 \)). Further, change of the cell volume of the patellar cartilage was smaller in the ACLT (-4.0%) compared with the contralateral (-13.8%, \( p=0.03 \)) group joints. Change in the cell surface area of the lateral femoral condyle cartilage was smaller in the ACLT (-2.8%) compared with the control (-11.9%, \( p=0.021 \)) group (Table S-1).

Before loading of cartilage, cells of the medial femoral condyle cartilage were wider in the ACLT (15.0 µm) compared with the contralateral (13.5 µm, \( p=0.035 \)) group and cells of the patellar cartilage were narrower in the contralateral (14.4 µm) compared with the control group joints (17.5 µm, \( p=0.031 \)) (Table 2). After static loading, chondrocytes of the lateral and medial femoral condyle cartilages were wider in the ACLT (lateral: 16.1 µm, medial: 15.7 µm) compared with the control (lateral: 14.0 µm, \( p=0.023 \), medial: 14.1 µm, \( p=0.008 \)) group joints. After loading, cells of the medial femoral condyle cartilage were wider also in the ACLT (15.7 µm, \( p=0.008 \)) compared with the contralateral group (14.4 µm, \( p=0.008 \)). Moreover, after loading, cells of the lateral femoral condyle cartilage were wider in the contralateral (15.8 µm) compared with the control (14.0 µm, \( p=0.037 \)) group joints. During cartilage compression, cell width of the lateral femoral condyle cartilage increased more in the ACLT (1.2%, \( p=0.028 \)) and in the contralateral (2.7%, \( p=0.002 \)) compared with the control (-2.4%) group joints (Table 2). Change of the cell surface area due to
loading was different ($p=0.021$) between the ACLT (-2.8%) and control (-11.9%) group joints for the lateral femoral condyle cartilage (Table S-1). Cell depth and height did not alter (Tables S-2 and S-3).

[Suggested position for Table 2]

*Tissue biomechanics*

The cartilage equilibrium modulus was smaller in the ACLT (medial femur: 1.24 MPa, lateral femur: 1.12 MPa) compared with the control (medial femur: 1.63 MPa, $p=0.029$) group joints for the medial femoral condyle and also compared with the contralateral (lateral femur: 1.58 MPa, $p=0.023$) group joints for the lateral femoral condyle (Table 3). The dynamic modulus was smaller in the medial femoral condyle cartilage of the contralateral (4.08 MPa) compared with the control (6.18 MPa, $p=0.042$) group (Table 3). In the patellar cartilage, the phase angle of the contralateral (12.19°) group samples was smaller compared with the control (14.08°, $p=0.041$) group samples. The cartilage loss and storage moduli were similar between the groups (Tables 3 and S-4). The local transversal strain of the patellar cartilage was different in the ACLT (5.3%) group compared with the contralateral (12.9%, $p=0.032$) group (Table S-5).

[Suggested position for Table 3]
Cartilage thickness was greater in the lateral tibial plateau of the ACLT (628 µm) compared with the control group joints (528 µm, \(p=0.046\)) (Table S-6).

**Histological analysis**

FCD was smaller in the lateral (~1–3% of cartilage thickness) and medial (~8–23% of cartilage thickness) femoral condyle, lateral tibial plateau (~0–7% and ~32% of cartilage thickness), femoral groove (~23–28%, ~30–32% and ~35-36% of cartilage thickness) and patellar cartilage (~4–46% of cartilage thickness) in the ACLT compared with the control group samples \((p<0.05)\) (Figures 4 & S-1). FCD was also smaller in the ACLT compared with the contralateral group samples in the lateral (~1–23% of cartilage thickness) and medial (~8–23%, ~29–35%, ~54–62% and ~68–76% of cartilage thickness) femoral condyle, lateral (~4–7% of cartilage thickness) and medial (~2–32%, ~42–45% and ~79–91% of cartilage thickness) tibial plateau, femoral groove (~14–58% of cartilage thickness) and patellar cartilage (~0–65% and ~78–88% of cartilage thickness) \((p<0.05)\) (Figures 4 and S-1). FCD was higher in the contralateral compared with the control group animals in the medial tibial plateau cartilage (~1–2% and ~12–100% of cartilage thickness) \((p<0.05)\) (Figures 4 and S-1).

The gross FCD of the ACLT compared with the control group cartilage was smaller in the medial femoral condyle (ACLT: 1.52, control: 1.62, \(p=0.043\)) and patella (ACLT: 1.52, control: 1.62, \(p=0.009\)) (Table S-7). FCD was also smaller in the ACLT group compared with the contralateral
group cartilage in the medial femoral condyle (ACLT: 1.52, contralateral: 1.65, \( p = 0.009 \)), medial tibial plateau (ACLT: 1.63, contralateral: 1.78, \( p = 0.003 \)), femoral groove (ACLT: 1.30, contralateral: 1.56, \( p = 0.031 \)) and patella (ACLT: 1.35, contralateral: 1.62, \( p < 0.001 \)). Moreover, greater gross FCD in the contralateral group, compared with the control group, was observed in the medial tibial plateau cartilage (contralateral: 1.78, control: 1.58, \( p < 0.001 \)).

Collagen orientation angles were slightly different in the lateral (~0-3% and ~22-27% of cartilage thickness) and medial (~8-14% of cartilage thickness) femoral condyle, lateral tibial plateau (~1% of cartilage thickness) and femoral groove (~2% and ~6-7% of cartilage thickness) for the ACLT group compared with the control group animals (\( p < 0.05 \)) (Figures 5 and S-2). Moreover, the collagen fibril orientation angle differed between the ACLT group and contralateral group animals in the lateral (~39-46% of cartilage thickness) and medial (~7-13% of cartilage thickness) femoral condyle cartilage (\( p < 0.05 \)) (Figures 5 and S-2). The collagen orientation angle also differed in the narrow region in the lateral femoral condyle (~0-1% of cartilage thickness), lateral tibial plateau (~1% of cartilage thickness) and femoral groove cartilage (~7% of cartilage thickness) between the contralateral and the control group samples (\( p < 0.05 \)) (Figures 5 and S-2).

Discussion

We studied alterations in the biomechanical behavior of articular cartilage and chondrocytes, and depth-wise cartilage FCD distribution and collagen orientation at two weeks following ACLT. As
hypothesized, ACLT resulted in changes in cell morphology, cell deformation and the tissue equilibrium modulus that were primarily located in the femoral condyle cartilage. The greatest changes were observed in the depth-wise reduction of the FCD at several joint locations with ACLT. This observation was consistent with our hypothesis. Collagen fiber orientation angles barely changed with ACLT.

FCD was altered at all knee joint locations two weeks post-surgery. Consistent with well-accepted characteristics of early OA (Buckwalter et al., 2005), there was proteoglycan loss in the superficial cartilage. Previous studies reported significant reductions in FCD four weeks after ACLT in the lateral and medial femoral condyle (up to 20-30% of the tissue thickness), tibial plateau (up to 10% of the tissue thickness), femoral groove (20-30% of the tissue thickness) and patellar cartilage (Mäkelä et al., 2014; Turunen et al., 2013). These results are consistent with our findings. Interestingly, the gross FCD between the surgical and healthy rabbits did not differ at many of the sites. This is likely due to local FCD loss, shown in the depth-wise analysis, which does not appear anymore in the average FCD analysis. On the other hand, there might also have been spontaneous OA (Arzi et al., 2015) in the control rabbits. However, the gross FCD was smaller in the experimental group compared to the contralateral group in nearly all sites. This suggests that, for these animals and experiments, the contralateral joint of the operated rabbits might present a better control than the independent, non-operated control rabbits.

Compared with the four week time-point (Mäkelä et al., 2014; Turunen et al., 2013), we found a smaller FCD loss at the femoral condyle cartilage, and a greater loss at the medial tibial, femoral groove and patellar cartilage. Smaller FCD loss at two compared to four weeks might reflect a progressive degradation of femoral condyle cartilage due to continuous abnormal loading of the
knee. Greater FCD loss at two compared to four weeks might reflect early inflammation, which can partly recover due increased remodelling. Preliminary gene expression results for rabbits at two weeks post ACLT showed upregulated inflammatory responses in the operated knee joints compared with the healthy controls (Finnilä et al., 2017), which may have inhibited glycosaminoglycan production (Tsuchida et al., 2012). Recovery of the FCD closer to normal level at the four week time point may indicate that inflammation subsided and proteoglycan remodeling was restored. Analysis of low grade inflammation and tissue degeneration in respect to mechanotransduction and biosynthesis may provide more insight into the possible mechanisms of FCD homeostasis.

The collagen fiber orientation was affected only slightly by ACL surgery at two weeks following intervention. Previous studies found that collagen fibril orientation was altered mostly in the superficial zone of the lateral and medial femoral condyle, tibial plateau, femoral groove and patellar cartilage at four weeks post-ACLT (Mäkelä et al., 2014; Turunen et al., 2013). Collagen fibril orientation was also changed nine weeks following ACLT in patellar cartilage, which was the only location studied in that investigation (Han et al., 2017). The collagen re-orientation results, together with findings on FCD, strongly suggest that proteoglycan loss precedes alterations in the collagen network orientation in the ACLT animal model of OA. Consistent with earlier hypotheses (Rolauffs et al., 2010), proteoglycan loss and cartilage softening occurred before collagen network degradation in the superficial zone of cartilage (Rolauffs et al., 2010). This cartilage softening could lead to excessive shear and fibril strains, which may cause the observed damage of the collagen network (Wilson et al., 2006).
This is the first study in which local cell biomechanics was quantified as early as two weeks after ACLT at different knee joint locations. Cell morphology and cell deformation were altered due to ACLT in lateral femoral condyle cartilage. Cell surface area shrank less in the ACLT group compared to the control group samples, which is likely related to the increased lateral expansion of the chondrocytes. The reduced FCD at this location, and the associated softening of the local cell environment might have enabled greater cell expansion (Han et al., 2010, 2017; Turunen et al., 2013). A careful investigation of the pericellular matrix properties in the vicinity of cells should be considered (Alexopoulos et al., 2003; Korhonen and Herzog, 2008; Ojanen et al., 2018; Tanska et al., 2013) in the future. Artificial boundary effects in the tibial plateau and femoral groove samples due to the sample preparation might also affect to the chondrocyte biomechanics (see Supplementary material).

Chondrocyte resting volume and surface area were greater in the ACLT compared with the other groups in the medial femoral condyle cartilage. OA in humans has been associated with increased chondrocyte volume (Bush and Hall, 2003), which could alter the extracellular matrix metabolism (Bush and Hall, 2003, 2001; Schneiderman et al., 1986; Urban et al., 1993; Urban and Bayliss, 1989). An increased cell volume was also observed at nine weeks post-ACLT in rabbit patellar cartilage (Han et al., 2010). This result may be related to an increased water content (Buckwalter and Mankin, 1998), altered osmolarity observed in OA (Buckwalter et al., 2005) and a subsequent increase in osmotic pressure.

The FCD is the primary determinant of equilibrium stiffness of cartilage (Kiviranta et al., 2006; Korhonen et al., 2003). Loss of FCD, and consequent reduction in the cartilage compressive stiffness, are typical signs of early OA (Buckwalter et al., 2005; Buckwalter and Mankin, 1998). We
found altered FCD at virtually all knee joint locations, but the equilibrium modulus was changed only for the lateral and medial femoral condyles. The collagen network is known to contribute substantially to the mechanical response of cartilage in indentation testing, even at equilibrium (Korhonen et al., 2002, 2003). Thus, the only slightly altered collagen fibril orientation may explain why the tissue equilibrium modulus was not altered for most sites in the knee. The collagen network also contributes strongly to the dynamic modulus of cartilage (Bader et al., 1992; Bader and Kempson, 1994; Korhonen et al., 2003; Rieppo et al., 2003). Since there was not much difference in the collagen network orientation, the dynamic, storage and loss moduli, and the phase angle did not change due to ACLT.

Interestingly, the FCD was significantly higher for the medial tibial plateau in the contralateral compared with the control group samples. This finding is consistent with an earlier animal model study (Mäkelä et al., 2014). ACLT causes asymmetrical loading conditions between the operated and the contralateral knee joints (Bray et al., 1992), changing the external loading and the internal loading through muscles substantially, especially in the first few weeks following ACLT (Hasler et al., 1997; Herzog et al., 1998). Aggrecan concentration is often increased in cartilage which is habitually and dynamically loaded (Buckwalter et al., 2005). Therefore, abnormal, but not excessive loading, with a possible upregulation of cartilage FCD might explain the differences in FCD between the contralateral and control knees.

ACL injury has been reported to increase the risk of cartilage injury and OA in lateral and medial femoral condyles, lateral tibial plateau and patella (Potter et al., 2012). ACL deficiency shifts the load-bearing areas of the lateral and medial compartments of the knee joint (Li et al., 2006), which might change stress and strain distributions and magnitudes on the joint surfaces. This abnormal
loading might cause cartilage degradation and local defects, especially on the femoral condyles and tibial plateaus, and might explain the FCD loss and other alterations found in the present study.

This study included 16 animals (8 experimental, 8 controls), which might limit the power of some of the statistical comparisons. However, many significant differences were observed in primary outcome variables, indicating robust findings. Also, the number of animals was similar to many previous pre-clinical experiments of the same nature (Bi et al., 2007; Han et al., 2010, 2017; Hasler et al., 1997; Hasler and Herzog, 1997; Mäkelä et al., 2014; Setton et al., 1995; Turunen et al., 2013).

A linear mixed model was used for the statistical analysis with and without Bonferroni correction. Bonferroni correction is known as a conservative method and it might cause type two errors in the statistical analysis. On the other hand, the positives found with this correction can be trusted more reliably. For comparison, we also used the linear mixed model without Bonferroni correction to see whether there are differences between the analyses.

Even though alterations in the physical properties of the rabbit articular cartilage after ACLT are similar to those seen in humans (Sah et al., 1997), our findings cannot be directly compared to humans suffering from OA after acute ACL injury or other type of post-traumatic OA. For instance, cartilage biomechanics, the anatomical shape of the knee and the physiological range of knee motion, and the clean ACL injury in the rabbits vs. the uncontrolled injuries in humans provide many differences that need to be kept in mind.
This study provides novel information of structural and mechanical changes in cartilage and chondrocytes at a very early time point in a post-traumatic model of OA. ACLT-induced early OA is associated primarily with a loss of FCD from the superficial cartilage, resulting in a decrease in cartilage equilibrium modulus for the lateral and medial femoral condyles, thereby making chondrocytes vulnerable to the altered loading conditions caused by ACLT. These findings suggest that early treatments after an ACL injury should focus on the recovery or maintenance of cartilage FCD.
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Conflict of interest

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(A) Femoral condyle

Region of interest

Indentation point

Medial Lateral

1 cm

(B) Tibial plateau

Medial Lateral

1 cm

(C) Femoral groove

Medial Lateral

1 cm

(D) Patella

Medial Lateral

1 cm

(E) Scanning confocal microscope objective

Light transmittable indenter Ø = 2 mm

Sample holder

DMEM

Cartilage

Bone

Dental cement

(F) Scanning confocal microscope objective

(G) Before loading

width

depth

z-direction

(x, y)

(H) After loading

width

depth

z-direction

(x, y)
Figure & table legends

Figure 1. Samples were prepared from both lateral and medial femoral condyles (A), tibial plateaus (B) and from the lateral side of femoral groove (C) and the center of patella (D). Region of interest from each site was defined as the primary load bearing area (on a sagittal plane): the highest point of the femoral condyles, the center of the tibial plateaus, the center of the femoral groove and patella. Histological sections were prepared perpendicular to the cartilage surface and parallel to the long axis of each sample site. Confocal microscopic imaging (E) of the Dextran and Propidium iodine stained cartilage samples under static loading were performed through a light transmittable glass indenter (d = 2 mm) (F). Imaging was performed before and after static loading and the cell volume, surface area and morphology were analyzed (G, H).
Figure 2. Stress-relaxation test (3×5% steps, ramp rate 100%/s, 350 ± 150 µm/s, 10 g pre-strain, indenter diameter 1 mm) was performed to evaluate the biomechanical properties of the cartilage tissue at equilibrium. Further, dynamic testing was conducted, from which tissue dynamic modulus and the phase difference between the stress and strain were analyzed.
Figure 3. Histological analysis was made to evaluate the amount of FCD (Safranin-O staining) and collagen orientation angle in a depth-wise manner. Red, blue and black lines represent the depth-wise mean values of a 150 µm wide section of the ACLT, contralateral and control groups, respectively. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; A.U., Absorption Unit.
Figure 4. Depth-wise optical density profiles to evaluate the FCD content from all analyzed locations: lateral and medial femoral condyle and tibial plateau and from lateral femoral groove and patella. Red, blue and black lines represent means of operated, contralateral and control groups, respectively. Shaded areas around the colored lines represents the confidence intervals (95% CI) and the two colored dashed lines statistical difference (p < 0.05) between the color-coded groups. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, Confidence Interval; A.U., Absorption Unit. Analyses are done with a linear mixed model with Bonferroni correction.
Figure 5. Depth-wise orientation angles from all analyzed locations: lateral and medial femoral condyle and tibial plateau and from lateral femoral groove and patella. Red, blue and black lines represent means of operated, contralateral and control groups, respectively. Shaded areas around the colored lines represent the confidence intervals (95% CI) and the two colored dashed lines statistical difference ($p < 0.05$) between the color-coded groups. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, Confidence Interval. Analyses are done with a linear mixed model with Bonferroni correction.
Figure S-1. Depth-wise optical density profiles to evaluate the FCD content from all analyzed locations: lateral and medial femoral condyle and tibial plateau and from lateral femoral groove and patella. Red, blue and black lines represent means of operated, contralateral and control groups, respectively. Shaded areas around the colored lines represents the confidence intervals (95% CI) and the two colored dashed lines statistical difference (p < 0.05) between the color-coded groups. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, Confidence Interval; A.U., Absorption Unit. Analyses are done with a linear mixed model without Bonferroni correction.
Figure S-2. Depth-wise orientation angles from all analyzed locations: lateral and medial femoral condyle and tibial plateau and from lateral femoral groove and patella. Red, blue and black lines represent means of operated, contralateral and control groups, respectively. Shaded areas around the colored lines represents the confidence intervals (95% CI) and the two colored dashed lines statistical difference (p < 0.05) between the color-coded groups. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, Confidence Interval. Analyses are done with a linear mixed model without Bonferroni correction.
Table 1. Chondrocyte volume (µm$^3$ and 95% confidence interval, CI) before and after the loading state as well as the deformation between the states from all analyzed locations: lateral and medial femoral condyle, tibial plateau, lateral femoral groove and patella. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI Confidence Interval.
Table 2. Chondrocyte width (x-direction; µm and 95% confidence interval, CI) before and after the loading state as well as the deformation between the states from all analyzed locations: lateral and medial femoral condyle, tibial plateau, lateral femoral groove and patella. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, Confidence Interval.
Table 3. Tissue equilibrium modulus (MPa and 95% confidence interval, CI), dynamic modulus (MPa and 95% confidence interval) and phase angle (° and 95% confidence interval) between the dynamic stress and strain from all analyzed locations: lateral and medial femoral condyle, tibial plateau, lateral femoral groove and patella. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, Confidence Interval.
Table 1. Chondrocyte volume (µm$^3$ and 95% confidence interval, CI) before and after the loading state as well as the deformation between the states from all analyzed locations: lateral and medial femoral condyle, tibial plateau, lateral femoral groove and patella. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, confidence interval.

| Location          | Before loading | After loading | Change         | ACLT (CI 95%)          | C-L (CI 95%)          | CNTRL (CI 95%)          |
|-------------------|----------------|--------------|----------------|------------------------|------------------------|------------------------|
| **Femoral condyle** |                |              |                |                        |                        |                        |
| Lateral           |                |              |                | 532.50 (412.36 - 652.64) | 702.4 (582.40 - 822.40) | 531.14 (399.38 - 662.89) |
|                   | 472.75 (359.89 - 585.60) | 586.1 (483.27 - 688.93) | -0.101 (-0.197 to -0.005) | 405.96 (303.01 - 508.91) | -0.231 (-0.319 to -0.143) |
|                   | -0.153 (-0.240 to -0.064) | -0.231 (-0.319 to -0.143) |                        |                        |                        |
| Medial            |                |              |                | 568.69 (492.07 - 645.31) | 417.18 (340.96 - 493.39) | 409.41 (327.76 - 491.06) |
|                   | 463.49 (392.89 - 534.00) | 360.63 (290.43 - 430.83) | -0.171 (-0.253 to -0.089) | 350.6 (275.42 - 425.78) | -0.136 (-0.224 to -0.049) |
|                   | -0.122 (-0.304 to 0.040) | -0.136 (-0.224 to -0.049) |                        |                        |                        |
| **Tibial plateau** |                |              |                | 507.29 (368.88 - 645.70) | 535.93 (405.67 - 666.20) | 442.10 (303.75 - 580.44) |
| Lateral           | 490.42 (361.89 - 619.95) | 506.94 (386.94 - 627.94) | -0.03 (-0.123 to -0.063) | 399.52 (271.05 - 527.98) | -0.098 (-0.191 to -0.006) |
|                   | -0.029 (-0.117 - 0.058) | -0.098 (-0.191 to -0.006) |                        |                        |                        |
| Medial            | 414.51 (285.69 - 543.33) | 476.21 (348.14 - 600.14) | 450.87 (323.86 - 577.83) | 361.93 (233.75 - 490.11) | -0.081 (-0.152 to -0.010) |
|                   | -0.086 (-0.157 to -0.015) | -0.081 (-0.152 to -0.010) |                        |                        |                        |
| **Femoral groove** |                |              |                | 720 (599.64 - 840.36)   | 769.00 (651.89 - 891.75) | 726.21 (607.35 - 846.71) |
|                   | 601.04 (492.36 - 709.72) | 678.53 (570.23 - 786.82) | -0.142 (-0.243 to -0.042) | 650.11 (542.03 - 758.18) | -0.096 (-0.197 to -0.004) |
|                   | -0.124 (-0.224 to -0.023) | -0.096 (-0.197 to -0.004) |                        |                        |                        |
| **Patella**       |                |              |                | 462.36 (309.20 - 615.15) | 532.83 (379.70 - 685.95) | 652.66 (476.97 - 828.36) |
|                   | 440.61 (299.96 - 581.26) | 460.01 (319.39 - 600.63) | -0.040 (-0.102 to -0.022) | 591.39 (430.08 - 752.70) | -0.091 (-0.161 to -0.020) |
|                   | -0.138 (-0.201 to -0.076) | -0.091 (-0.161 to -0.020) |                        |                        |                        |

* significant difference to CNTRL p<0.05 with Bonferroni correction
** significant difference between the ACLT and C-L groups p<0.05 Bonferroni correction
# significant difference to CNTRL p<0.05 without Bonferroni correction
### significant difference between the ACLT and C-L groups p<0.05 without Bonferroni correction
Table 2. Chondrocyte width (x-direction; µm and 95% confidence interval, CI) before and after the loading state as well as the deformation between the states from all analyzed locations: lateral and medial femoral condyle, tibial plateau, lateral femoral groove and patella. ACLT, Anterior Cruciate Ligament Transection; C-L, Contralateral; CNTRL, Control; CI, confidence interval.

| Cell width (µm)         | ACLT (CI 95%) | C-L (CI 95%) | CNTRL (CI 95%) |
|-------------------------|---------------|--------------|----------------|
| **Femoral condyle**     |               |              |                |
| **Lateral**             |               |              |                |
| Before loading          | 15.88 (14.50 - 17.27) | 15.37 (14.11 - 16.26) | 14.34 (13.08 - 15.60) |
| After loading           | 16.06 (14.76 - 17.35)† | 15.76 (15.76 - 16.93)† | 13.98 (12.81 - 15.16) |
| Change                  | 0.012 (-0.012 - 0.037)† | 0.027 (0.006 - 0.048)† | -0.024 (-0.045 to -0.003) |
| **Medial**              |               |              |                |
| Before loading          | 14.96 (14.03 - 15.89)‡‡ | 13.54 (12.61 - 14.46) | 13.78 (12.79 - 14.77) |
| After loading           | 15.65 (14.87 - 16.43)*, **, №, ‡‡ | 14.11 (13.34 - 14.88) | 14.05 (13.23 - 14.88) |
| Change                  | 0.051 (-0.002 - 0.104) | 0.047 (-0.006 - 0.100) | 0.023 (-0.034 - 0.080) |
| **Tibial plateau**      |               |              |                |
| **Lateral**             |               |              |                |
| Before loading          | 13.54 (12.01 - 15.07) | 13.97 (12.52 - 15.41) | 14.09 (12.56 - 15.62) |
| After loading           | 14.18 (12.78 - 15.59) | 14.61 (13.27 - 15.93) | 14.55 (13.14 - 15.96) |
| Change                  | 0.052 (-0.000 - 0.103) | 0.049 (0.001 - 0.096) | 0.038 (-0.014 - 0.090) |
| **Medial**              |               |              |                |
| Before loading          | 13.63 (12.16 - 15.10) | 13 (11.54 - 14.46) | 12.38 (10.91 - 13.85) |
| After loading           | 14.32 (13.03 - 15.60) | 13.58 (12.31 - 14.86) | 13.37 (12.09 - 14.66) |
| Change                  | 0.062 (-0.006 - 0.117) | 0.048 (-0.0067 - 0.104) | 0.085 (0.030 - 0.141) |
| **Femoral groove**      |               |              |                |
| Before loading          | 16.86 (15.96 - 17.75) | 16.19 (14.50 - 17.88) | 16.93 (15.24 - 18.62) |
| After loading           | 17.09 (16.16 - 18.02) | 16.89 (15.96 - 17.81) | 16.41 (15.49 - 17.33) |
| Change                  | 0.015 (-0.011 - 0.041) | 0.029 (0.003 - 0.054) | 0.032 (0.007 - 0.058) |
| **Patella**             |               |              |                |
| Before loading          | 14.87 (13.07 - 16.67) | 14.44 (12.65 - 16.24)† | 17.48 (15.42 - 19.53) |
| After loading           | 16.04 (14.48 - 17.60) | 15.66 (14.10 - 17.23) | 17.41 (15.63 - 19.19) |
| Change                  | 0.085 (-0.016 - 0.155) | 0.095 (0.026 - 0.165) | 0.001 (-0.079 - 0.081) |

* significant difference to CNTRL group \( p < 0.05 \) with Bonferroni correction
** significant difference between the ACLT and C-L groups \( p < 0.05 \) with Bonferroni correction
† significant difference to CNTRL \( p < 0.05 \) without Bonferroni correction
‡‡ significant difference between the ACLT and C-L groups \( p < 0.05 \) without Bonferroni correction
Table 3. Tissue equilibrium modulus (MPa and 95% confidence interval, CI), dynamic modulus
(MPa and 95% confidence interval) and phase angle (° and 95% confidence interval) between the
dynamic stress and strain from all analyzed locations: lateral and medial femoral condyle, tibial
plateau, lateral femoral groove and patella. ACLT, Anterior Cruciate Ligament Transection; C-L,
Contralateral; CNTRL, Control; CI, Confidence Interval.

| Location        | Equilibrium modulus (MPa) | ACLT (CI 95%)         | C-L (CI 95%)          | CNTRL (CI 95%)         |
|-----------------|---------------------------|-----------------------|-----------------------|-----------------------|
|                 |                            | 1.12 (0.84 - 1.40)##  | 1.53 (1.28 - 1.79)    | 1.45 (1.31 - 1.60)    |
| Femoral condyle | Lateral                   | 1.24 (1.07 - 1.41)#   | 1.58 (1.33 - 1.83)    | 1.63 (1.37 - 1.89)    |
|                 | Medial                    | 1.34 (0.86 - 1.82)    | 1.28 (1.05 - 1.52)    | 1.15 (0.86 - 1.45)    |
|                 |                            | 1.04 (0.81 - 1.26)    | 0.98 (0.66 - 1.30)    | 0.92 (0.68 - 1.17)    |
| Tibial plateau  | Lateral                   | 1.10 (0.90 - 1.30)    | 1.09 (0.89 - 1.30)    | 1.20 (1.00 - 1.40)    |
|                 | Medial                    | 0.72 (0.56 - 0.88)    | 1.07 (0.78 - 1.36)    | 0.99 (0.76 - 1.23)    |
| Patella         |                            | 7.38 (5.56 - 9.19)    | 6.32 (5.18 - 7.47)    | 5.80 (4.90 - 6.71)    |
|                 |                            | 4.43 (3.56 - 5.30)    | 4.08 (3.36 - 4.79)#   | 6.18 (4.13 - 8.22)    |
| Dynamic modulus (MPa) | ACLT (CI 95%)         | 7.00 (3.50 - 10.51)   | 4.85 (3.90 - 5.80)    | 5.59 (4.21 - 6.97)    |
|                 |                            | 2.85 (2.10 - 3.60)    | 2.46 (1.62 - 3.31)    | 2.14 (1.65 - 2.63)    |
|                 |                            | 6.36 (4.60 - 8.11)    | 8.15 (5.47 - 10.83)   | 7.75 (6.24 - 9.26)    |
|                 |                            | 4.09 (3.40 - 4.78)    | 4.21 (3.30 - 5.11)    | 4.44 (2.98 - 5.89)    |
| Phase angle (°) | ACLT (CI 95%)         | 13.37 (12.11 - 14.63) | 12.94 (11.68 - 14.20) | 12.20 (10.94 - 13.45) |
|                 |                            | 13.01 (12.12 - 13.90) | 11.90 (11.01 - 12.78) | 11.88 (10.99 - 12.77) |
|                 |                            | 12.62 (11.76 - 13.49) | 11.50 (10.63 - 12.37) | 12.35 (11.48 - 12.21) |
|                 |                            | 11.53 (10.38 - 12.69) | 11.13 (9.97 - 12.28)  | 11.80 (10.65 - 12.96) |
|                 |                            | 12.31 (9.98 - 14.65)  | 11.51 (9.01 - 14.01)  | 12.82 (10.48 - 15.16) |
| Patella         |                            | 12.98 (11.71 - 14.25) | 12.20 (10.93 - 13.47)# | 14.09 (12.81 - 15.36) |

* significant difference to CNTRL p<0.05 with Bonferroni correction
** significant difference between the ACLT and C-L groups p<0.05 with Bonferroni correction
# significant difference to CNTRL p<0.05 without Bonferroni correction
## significant difference between the ACLT and C-L groups p<0.05 without Bonferroni correction

| Location        | Dynamic modulus (MPa) | ACLT (CI 95%)         | C-L (CI 95%)          | CNTRL (CI 95%)         |
|-----------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Femoral condyle | Lateral               | 5.80 (4.90 - 6.71)    | 5.80 (4.90 - 6.71)    | 5.80 (4.90 - 6.71)    |
|                 | Medial                 | 6.18 (4.13 - 8.22)    | 6.18 (4.13 - 8.22)    | 6.18 (4.13 - 8.22)    |
| Tibial plateau  | Lateral               | 5.59 (4.21 - 6.97)    | 5.59 (4.21 - 6.97)    | 5.59 (4.21 - 6.97)    |
|                 | Medial                 | 2.14 (1.65 - 2.63)    | 2.14 (1.65 - 2.63)    | 2.14 (1.65 - 2.63)    |
| Femoral groove  | Lateral               | 7.75 (6.24 - 9.26)    | 7.75 (6.24 - 9.26)    | 7.75 (6.24 - 9.26)    |
|                 | Medial                 | 4.44 (2.98 - 5.89)    | 4.44 (2.98 - 5.89)    | 4.44 (2.98 - 5.89)    |
| Patella         | Lateral               | 12.20 (10.93 - 13.47)# | 12.20 (10.93 - 13.47)# | 12.20 (10.93 - 13.47)# |
|                 | Medial                 | 14.09 (12.81 - 15.36) | 14.09 (12.81 - 15.36) | 14.09 (12.81 - 15.36) |

* significant difference to CNTRL p<0.05 with Bonferroni correction
** significant difference between the ACLT and C-L groups p<0.05 with Bonferroni correction
# significant difference to CNTRL p<0.05 without Bonferroni correction
## significant difference between the ACLT and C-L groups p<0.05 without Bonferroni correction