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Research Article

The initial repair response of articular cartilage after mechanically induced damage†

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Author contributions
All authors contributed to developing the study design and interpreting the results. EE van Haaften performed all experimental work, did the initial data analyses and drafted the manuscript. The manuscript was critically appraised, revised and approved for submission by all authors.

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Abstract

The regenerative potential of articular cartilage (AC) defects is limited and depends on defect size, biomechanical conditions, and age. Early events after overloading might be predictive for cartilage degeneration in the long term. Therefore, the present aim is to investigate the temporal response of cartilage to overloading at cell, matrix, and tissue level during the first period after mechanical overloading. In the present study, the effect of high loading (~8 MPa) at a high rate (~14 MPa/s) at day 0 during a 9 day period on collagen damage, gene expression, cell death, and biochemical composition in AC was investigated. A model system was developed which enabled culturing osteochondral explants after loading. Proteoglycan content was repeatedly monitored over time using μCT, whereas other evaluations required destructive measurements. Changes in matrix related gene expressions indicated a degenerative response during the first 6 h after loading. After 24 h, this was restored and data suggested an initial repair response. Cell death and microscopic damage increased after 24 h following loading. These degradative changes were not restored within the 9 day culture period, and were accompanied by a slight loss of proteoglycans at the articular surface that extended into the middle zones. The combined findings indicate that high magnitude loading of articular cartilage at a high rate induces an initial damage that later initiates a healing response that can probably not be retained due to loss of cell viability. Consequently, the matrix cannot be restored in the short term. This article is protected by copyright. All rights reserved

Keywords: articular cartilage; chondrocytes; in vitro; damage; repair
1. Introduction

Articular cartilage (AC) protects the underlying bone by providing a lubricated surface that absorbs shocks and distributes loads. This functionality is determined by its composition and the organization of its extracellular matrix (ECM). Pathological processes in cartilage can reduce this functionality. Unfortunately, the regenerative potential of AC is limited and defects commonly progress into osteoarthritis (OA), affecting the knee, hip, and hand in more than 35% of the population older than 60 years in only the US\textsuperscript{1}. The number of OA patients is likely to increase due to the aging population and higher prevalence of obesity.

It is commonly accepted that mechanical overloading can initiate AC damage, which is the onset of OA. In addition to mechanical loading, inflammatory conditions are important for the progression of cartilage damage\textsuperscript{5}. Full-thickness AC defects have limited regenerative potential and are therefore likely to develop into OA. Milder conditions are characterized by tissue softening\textsuperscript{2}, with initial collagen damage at or below the surface\textsuperscript{3}, and loss of superficial proteoglycan (PG) ~48 h after initiation of damage\textsuperscript{4}. In later stages, surface fibrillation occurs which often progresses into massive cartilage damage and eventually full erosion.

Although mechanical overloading can be destructive for cartilage, physiological mechanical loading is considered vital for healthy cartilage to maintain its load-bearing function. Indeed, dynamic compression can markedly stimulate matrix production\textsuperscript{7-10}. However, static compression inhibits the synthesis of PGs\textsuperscript{6,7} and dynamic stimulation above a threshold induces cartilage degeneration. Also, single impact loading leads to an increase in matrix loss\textsuperscript{11,12} and an initial decrease in matrix synthesis\textsuperscript{13-15}.

Thus, there is a narrow border between an anabolic and a catabolic response to mechanical loading. The premise is that if it were possible to modulate the early tissue response after an overloading event by modulating the prevailing mechanical conditions, we might use this to reverse the initial damage and ultimately prevent further cartilage degeneration in the longer term. Indeed, repair
activities in cartilage are recognized, for example by an eventual increase of matrix synthesis after a single impact\textsuperscript{16}.

To pursue these developments, the temporal responses to overloading at cell, matrix, and tissue level during the first period after mechanical overloading need to be better characterized. These earliest regenerative responses will form the basis for studying secondary, mid-long term regenerative responses in future studies. The present study hypothesizes that mild mechanical overloading would induce damage, quickly followed by an early regenerative response. The latter would become apparent at the expression of extracellular matrix-related genes, and would be followed within a week by enhanced protein synthesis and incorporation of extracellular matrix. The objective is to address this hypothesis in an osteochondral explant system, by monitoring gene expression, matrix synthesis and matrix composition for 10 days, after inducing early tissue damage through controlled mechanical overloading.

2. Methods

2.1 Construct Preparation

Twelve porcine knee joints (5-7 months) were obtained from a local slaughterhouse. Under sterile conditions, six cylindrical osteochondral explants (\(\varnothing 8\) mm, bone thickness 4 mm) were drilled from each knee perpendicular to the articular surface. Relatively flat samples from the femoral condyles were used for mechanical loading (3 per knee, 1.66±0.31 mm thick); more curved patellofemoral groove samples served as unloaded controls (3 per knee, 1.74±0.47 mm thick), which were similar in thickness (\(p=0.57\)). The explants were transferred to custom-made inserts with separated bone and cartilage compartments (LifeTec Group, Eindhoven, The Netherlands) and cultured in custom-made chambers at 37 °C and 5% CO\(_2\) (figure 2). The bone compartment was cultured in 3 ml osteogenic medium (standard culture medium with 10% fetal bovine serum (Greiner, Alphen aan den Rijn, The Netherlands) and 10 mM β-glycerophosphate (Merck, Amsterdam, The Netherlands, 35675)) and the cartilage compartment in 2.5 ml chondrogenic medium (standard culture medium...
with 100 nM Dexamethasone (Sigma, Zwijndrecht, The Netherlands, D1756), 1% ITS™ Premix, 40 μg/ml L-proline (Sigma, P5607), and 1 mM sodium pyruvate (Gibco, Carlsbad, CA)). Standard culture medium contained DMEM, 4.5 g/l glucose (Gibco), with 100 nM Dexamethasone, 50 μg/ml L-ascorbic acid-2-phosphate (Sigma, A8960), 1% Amphotericin B (Invitrogen, Carlsbad, CA, 15290-018), and 1% Penicillin Streptomycin (Lonza, Basel, Switzerland, DE17-602E). Medium was refreshed 3 times per week.

2.2 Experimental design

After 4 days of initial culture, mechanical damage was induced by indentation loading (25 N, 5 cycles at 120 mm/min in 20 s) using a round indenter tip (⌀2 mm) resulting in average peak stresses of ~8 MPa at ~14 MPa/s. After loading (day 0), the constructs were kept in culture for an additional 9 days for subsequent analyses (figure 1). This culture protocol was repeated 3 times, each time with 12 static controls and 12 loaded constructs resulting in 72 samples in total. For each repeated batch and condition, 2 explants were prepared for histology at 0 h and 48 h. In the first batch, the 0 h explants were obtained for measurements of gene expression. At 6 h, and 24 h, 2 explants were obtained for analysis of gene expression. Finally, 2 explants were monitored for PG content and 2 explants for PG loss. At the end of the culture period, these explants were used for histology as well.

[Figure 1 around here]

2.3 Analyses

2.3.1. Collagen damage
The antibody Col2-3/4m was used to visualize type II collagen disruption. Cryosections (7 μm) were fixed in 3.7% 0.1 M phosphate buffered formaldehyde (5 min) and washed in PBS (15 min). To enhance permeability of the ECM, sections were incubated with 1% hyaluronidase (Sigma, H3506) in PBS at 37°C (30 min). Nonspecific staining was blocked by incubation with 10% normal

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horse serum. Sections were incubated overnight with the Col2-3/4m antibody (1:100) at 4°C in a humidified chamber. Biotin-labeled horse anti-mouse antibody (1:400, Vector Laboraties, Burlingame, CA) was used as secondary antibody (1 h at room temperature). The biotin was detected using Streptavidin 555 (1:500, Invitrogen) for 30 min. After washing in PBS (15 min), sections were counterstained with DAPI to visualize cell nuclei. An inverted epi-fluorescence microscope (Zeiss Axiovert 200M, 1 pixel=1.063 μm with a 10×/0.3 NA EC Plan-Neofluar lens) was used to identify sites of collagen damage (λex = 510-560 nm, λem = 590 nm) and DAPI stained nuclei (λex = 365 nm, λem = 420-470 nm).

2.3.2. Viability
Viability of the cartilage constructs was studied by measuring lactate dehydrogenase (LDH) activity, which becomes inactive 24 h after cell death. Cryosections were stained for viability according to a protocol adopted from Stoddart et al.\textsuperscript{18}. In short, an LDH solution was prepared freshly on the day of testing by adding 60 mM lactic acid (Sigma, L1875) and 1.75 mg/ml β-nicotinamide adenine dinucleotide (Sigma, 43410) to a base solution (40% polypep (Sigma) and 50 mM Gly-Gly (Sigma, G3915)). The pH was adjusted to 8.0 with NaOH and 3 mg/ml Nitroblue Tetrazolium (Sigma, N5514) was added immediately prior to use. After thawing, sections were incubated in reaction medium for 4 h at 37°C in a humidified chamber protected from light. Sections were then rinsed with warm (50°C) water and PBS, and fixed with 70% EtOH. Sections were stained with DAPI to visualize cell nuclei.

2.3.3. Gene expression
Liquid nitrogen frozen cartilage specimens from the center (⌀3 mm, tissue wet weight 8.5±2.5 mg) of the loaded samples (n = 6) and static controls (n = 3) were reduced to a fine powder in a mikro-dismembrator (Sartorius-Stedim Biotech, Nieuwegein, The Netherlands). Total RNA was extracted from the homogenized samples according to a protocol described by Ruettger et al.\textsuperscript{19} using the
RNAqueous Midi™ kit (Life Technologies, Bleiswijk, The Netherlands). Concentration and purity of the RNA was measured by a NanoDrop spectrophotometer (Isogen, De Meern, The Netherlands) at 260 nm and 280 nm. RNA concentration was 39.2±10.0 ng/μl with typical 260/280 values of ~1.72 and 260/230 values of ~0.82. The SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) was used for synthesis of template cDNA of 200 ng of total RNA. 7 genes related to cartilage damage were measured in the extracted RNA using quantitative real-time PCR (qPCR): COL1, COL2, ACAN (cartilage matrix); MMP3, MMP13, ADAMTS5 (matrix proteases); and TIMP1 (protease inhibitor) (table 1). Primer pairs for qPCR were adopted from McCulloch et al.²⁰,²¹ and Nygard et al.²². qPCR was performed in a 10 μl reaction, consisting of 3 μl of 50 times diluted cDNA, 1 μM of forward and reverse primers, and 5 μl of SYBR Green (Bio-Rad, Veenendaal, The Netherlands). Gene expression was normalized to PPIA, identified as being most stable in porcine under mechanical loading.²⁰ Differential gene expression between loading and static control was evaluated by normalizing the relative gene expression (ΔCT) of the loaded sample to ΔCT of the time-matched control sample.

[Table 1 around here]

2.3.4. Proteoglycan dynamics

Fixed charged density A noninvasive imaging technique based on equilibrium partitioning of an ionic contrast (EPIC-μCT) was used to monitor the cartilage matrix fixed charge density (FCD) during the culture. The method relies on the electrochemical interaction between the FCD present in the cartilage matrix and an ionic contrast agent resulting in a non-uniform distribution of the ionic contrast agent that is inversely related to the density of the negatively charged PGs. At least 16 h prior to scanning, explants were incubated with 2.5 ml of 1:1 Hexabrix 320 and chondrogenic medium, exceeding the time required for full equilibration²³. All scanning was performed in air using a μCT 40 (Scanco, Brüttsellen, Switzerland) at 45 kVp, 177 μA, 200 ms integration time, and a voxel size of 18 μm (figure 2b). Each explant was scanned in 25 min. After segmenting cartilage
from the environment by simple thresholding (Scanco Medical software), the average x-ray attenuation was calculated for the cartilage volume to obtain a measure of the overall PG content (Matlab R2012-R2014; The Mathworks®, Natick, MA). To visualize the spatial variation in PG content across the full cartilage thickness, the superficial surface of the 3D segmentation was rotated into the XY-plane (figure 2c) and attenuation values were averaged per XY-slice (figure 2d).

[Figure 2 around here]

**PG loss** Every medium change, medium was collected and stored at -80°C until analysis. Prior to assaying the medium for PG content, samples (200 µl) were digested overnight (~16 h) in 200 µl digestion buffer (5 mM L-cystein, 5 mM EDTA, 100 mM phosphate buffer (pH 6.5), 140 µg Papain). PG content in the medium was determined with a DMMB assay. The digested samples were centrifuged for 10 minutes at 12.000 rpm and 5 times diluted in 1:1 digestion buffer (without Papain) and chondrogenic medium to obtain proper dilutions. After the diluted samples were centrifuged for 5 minutes at 12,000 rpm, 40 µl of the supernatant and 150 µl of DMMB solution (46 µM 1,9 dimethylmethylene blue, 40.5 mM Glycin, 40.5 mM NaCl, pH 3.0) was pipetted into a 96-well plate, along with a series of standards with known PG (chondroitin sulfate from shark cartilage, Sigma, C4384) concentration (0-25 µg/ml). To obtain a measure of the concentration of PG, the absorbance values at 540 nm and 595 nm (Synergy HT plate reader (BioTek)) were subtracted from each other.

**PG distribution** After thawing, cryosections (7 µm) were fixed in 3.7% 0.1 M phosphate buffered formaldehyde (5 min) and washed with water (2 min). Proteoglycans were stained for 8 min with 0.1% safranin O and imaged (Zeiss Axio Observer Z1, 1 pixel=1.079 µm with a 10×/0.45 NA EC Plan-Apochomat lens).
2.4 Statistical Analyses

Statistics were performed with IBM SPSS Statistics 22. Data was tested for normality (Shapiro-Wilk test), equal variances (Levene's test), and sphericity (Mauchly’s test, only for the repeated factor ‘time’). Gene expressions were tested for significant differences with a one-sample t-test, or a nonparametric Wilcoxon signed-rank test (H0: μ=1) depending on the normality of the groups. Differences in relative gene expression (ΔΔCT) of > 2× or < 0.5× were considered biologically relevant. Total (relative) PG content (based on μCT data) and rate of PG loss (based on DMMB assay) were tested for significant differences using two-way ANOVA with repeated measures (repeated factor ‘time’, random factor ‘loading condition’) and subsequent post hoc tests using within-subject contrasts for adjacent time points and independent t-tests for loading condition. Statistical significance was assumed for p<0.05.

3. Results

Immediately after loading, a strong staining of denaturated collagen was observed in the loaded region, which persisted throughout the culture period (figure 3, black arrow). The non-loaded constructs did not show any signs of collagen damage in the middle and superficial zones during the culture period. No dead cells were found prior to loading and in the non-loaded controls (figure 3). At 24 h after loading, dead cells were found ~2.5 mm around the indented zone, which is slightly larger than the ø2 mm indenter. This area of dead cells persisted throughout the culture period but did not increase over time.

Overall, expressions of all examined genes were near the detection limit. Loading was determined to have a significant effect on several genes 6 h and 24 h after loading (figure 4). At 6 h, COL2 was downregulated (p=0.007, fc=0.31), and MMP13 was upregulated (p=0.028, fc=6.28) in loaded constructs.
groups compared to controls. After 24 h, both the downregulated COL2 and upregulated MMP13 returned to their normal values (i.e. fc ~1). ACAN was significantly upregulated at 24 h (p=0.028, fc=19.25), but not at 6 h.

[Figure 4 around here]

The safranin O staining showed a uniform distribution of proteoglycans prior to loading and 48 h after loading, and in the non-loaded controls at all time points (figure 3). After 9 days, staining suggested PG loss in the loaded group (figure 3). The quantified PG distributions from the μCT data were similar in all samples: after a decline beginning at the surface, PG content gradually increased towards the deeper zones at ~25% of the cartilage thickness (figure 5). Prior to loading, statistical differences in PG content were found between the control and loaded group in the superficial zone (p=0.04), but not in the middle and the deep zone (figure 6a, c-e). After 2 days (p=0.004) and 9 days (p=0.002), total PG content significantly dropped in the loaded group compared to the control group (figure 6a). The same effect was observed in the middle zone (figure 6d), along with a temporal increase in PG content in the control group (p=0.014). The effect of loading became most apparent after 9 days in the superficial zone (figure 6c, p<0.001). At each time point, PG content in the loaded group was lower than the control group (figure 6a, c-e), indicating an inherent difference between the superficial and middle zones in the patellofemoral groove (controls) vs. femoral condyles (loaded) (figure 6c-e).

[Figure 5 around here]

Overall, all individual samples showed a similar pattern in rate of PG loss into the culture medium (independent of loading), with a steadily decreasing rate after 4 days (figure 6b). After 2 and 4 days of culture, the rate of PG loss in the loaded group appeared to be elevated compared to the non-
loaded controls, but these differences were not statistically significant (p=0.157 at day 2). The reduction in PG loss from day 4 to day 7 was independent of loading (p=0.011), but a lasting decrease in PG loss from day 7 to day 9 was only observed in the control group (p=0.009) (figure 6b). Cumulatively, PG losses of ~1.2 mg were found independent of loading at the end of the culture period.

[Figure 6 around here]

4. Discussion
The objective of this study was to evaluate the early response of articular cartilage to mechanically induced damage. The applied loading protocol evoked an early cell response that expressed itself in cell death in and around the indented area, and in changes in matrix-related gene expression profiles, showing an initial catabolic effect within 24 hours, changing into a repair response with elevated ACN expression after 48 hours. In the matrix, the loading induced loss of superficial and mid-zone PG matrix and denaturation of the collagen matrix in the indented area.

The in vitro system that was used to apply repetitive indentation loading of cartilage and to monitor early events after overloading was newly designed. Longer culture periods are required in future studies to also capture secondary regenerative responses. The system is suited to keep osteochondral constructs in culture while they are scanned using μCT or mechanically perturbed on a tensile tester. This allows for repeated measures with higher statistical power. Osteochondral plugs from the patellofemoral groove and femoral condyles are used as a model of healthy cartilage. Osteochondral constructs are preferred over chondral explants, because the attachment of cartilage to bone provides a more physiological mechanical condition during mechanical loading.

Reported ranges of static peak normal stresses (0.5 to 5 MPa) are relatively similar across different species. Therefore, the present findings are considered indicative for damage development in human cartilage. Osteochondral constructs were compressed to induce mechanical damage (25 N, 5
cycles at 120 mm/min). These loads correspond to average peak stresses of 8 MPa and loading rates around 14 MPa/s. In a joint, physiological loading rates are in the range of 6 to 25 MPa/s for walking, reaching peak normal stresses of 3 to 11 MPa\textsuperscript{33}. Thus, the average applied pressures resembles the high end of physiological loading. However, because loading was applied with a small diameter indenter (\(\varnothing 2\) mm), the local strains under the indenter likely exceed the strains that occur in vivo, where loading conditions are more confined and distributed over larger areas.

Dynamic mechanical loading is considered vital for healthy cartilage to maintain its load-bearing function. It may be speculated that immobility following injury could reduce the regenerative response. Extending the culture protocol with dynamic loading for longer culture periods could potentially reveal beneficial effect of dynamic loading on tissue regeneration, and bridge the gap between short-term in vitro and long-term in vivo studies. However, the effects will likely depend on the nature of the dynamic loading protocol, similar to the effects observed in tissue engineering studies.

The present study applies immobile conditions after injury. In most cases, the damage protocol was indeed found to result in microscopic collagen damage starting at or slightly below the surface (figure 3), consistent with previous studies\textsuperscript{3}. Under indentation with a small diameter indenter, the superficial layer experiences tension, loading the superficial collagen fibres. Indeed, some samples showed collagen damage at the surface, which is in line with previous studies\textsuperscript{25,26}. Differences between samples might be due to differences in cartilage thickness, as strains in thinner cartilage are higher, and therefore thinner cartilage is more susceptible to collagen damage as a direct consequence of overloading\textsuperscript{17}. Previous studies have shown that positive staining of the Col2-3/4m antibody, but not of the Col2-3/4C\textsubscript{short}, occurs superficially after overloading\textsuperscript{17}, and these patterns resemble the damage patterns found in this study. Therefore, the collagen damage that is observed is thought to be the immediate result of excessive loading. To study enzymatic denaturation of collagen, which might occur over time, Col2-3/4C\textsubscript{short} staining and measurements of the levels of collagenases are advised in future mid-long term studies.
The attained load levels were high with mean peak stresses of 8 MPa and stress rates around 14 MPa/s. Such a cyclic loading regime resulted in a loss of cell viability around the indented area (figure 3), indicating that the threshold load to retain cell viability was exceeded. This agrees with Chen et al.\textsuperscript{27} who found 48 h after loading (5 MPa at 60 MPa/s for 2 min) a significant increase in apoptotic cells.

The paired observations of the release of PGs in the culture medium revealed a common pattern in both control and loaded samples. The rate of PG loss after day 0 peaked at 4 days after loading, and then steadily decreased (figure 6b). This pattern was also observed by Ewers et al.\textsuperscript{28} within a 4 day period after impacting $\phi 6.35$ mm chondral plugs at 40 MPa/s up to $\sim$40 MPa. Under loading, they found PG release to the medium up to $85 \pm 7$ $\mu$g/ml per day, comparable to the $47 \pm 12$ $\mu$g/ml per day in the present study. Similar to the finding of Ewers et al.\textsuperscript{28}, the difference in PG release between the control and loaded group was most evident 2 days post-trauma, and it coincides with previous findings where loss of PGs was found $\sim$48 h after excessive indentation loading\textsuperscript{4}.

The mechanism behind the release of PGs is not fully understood, but excessive loss is considered the result of an imbalance in metabolism, degradation of aggrecan, and increased porosity of the cartilage matrix as a result of collagen network damage. Based on histology, PG loss was found to increase from the articular surface into the deeper zones with time and loading (figure 3). This coincides with EPIC-µCT data, showing changes in the superficial and middle zones but not in the deep zone (figure 6c-e). The typical depth-wise profile of the contrast agent concentration (figure 5) is in line with other studies\textsuperscript{29,30} and with the optical density profiles based on safranin O stained cartilage\textsuperscript{31}.

This is, to our knowledge, the first study that uses EPIC-µCT to monitor PG content over time in living osteochondral explants in an in vitro test set-up. Therefore, validation of the approach in a pilot study was required, which showed that Hexabrix at the concentration used here, did not affect cell viability (data not shown). However, a few samples used for µCT scanning showed loss of superficial PGs, suggesting a possible interaction of the contrast agent with the matrix.
Interestingly, this pattern was only observed in the loaded constructs but not in the static controls. It might be that the structural integrity of the superficial zone is decreased due to loading, but that the PGs are still entrapped in the network of collagen fibres. Adding a relatively small ionic contrast agent that interacts with the negative charges of the PGs might result in expel of PGs from the matrix that would otherwise not occur. This hypothesis requires further study.

The use of more moderate loading protocols might retain cell viability, but it is questionable whether subtler effects of loading on PG dynamics and gene expressions can be detected. Harvesting procedures, donor and donor-site variations may mask an effect of the loading regime.

For example, cartilage from the patellofemoral groove (controls, 1.74±0.47 mm thick) was found to contain higher fixed charge density than cartilage from the femoral condyles (loaded, 1.66±0.31 mm thick), resulting in a biased control group. One might consider harvesting non-loaded controls from the same region, but this decreases the number of samples that can be harvested from one knee, thereby increasing donor variations. Previous studies found elevated levels of PG release immediately after dissection, which ameliorated after ~3 days\textsuperscript{12}. Indeed, the current study found higher rates of PG loss in the 4-days period prior to loading (data not shown). To minimize effects of harvesting, mechanical damage was induced 4 days after harvesting and data on PG loss that were obtained during the period before mechanical perturbation were excluded from analysis.

A custom-made puncher (⌀3 mm) mountable to the indenter tip allowed isolation of exactly the loaded tissue for qPCR analysis of full-thickness cartilage. However, it is likely that injured cells were mainly located in the superficial zones of the loaded area (figure 3). Therefore, the current gene expressions are considered an underestimation of the changes in gene expression in the superficial zone of the loaded samples, and the effect of the perturbation would be more pronounced if the superficial zone was studied separately. However, isolating intact RNA from cartilage is difficult. The low cellularity requires a lot of tissue, and the abundant matrix clogs the filter. Yet, the amount of RNA that was isolated in the present study from one half of the isolated tissue (~8.5 mg) may be sufficient to show effects in two or three cartilage layers in the future.
Gene expression in loaded samples was significantly different from that in the non-loaded controls during the first 24 h (figure 4). MMP3 and MMP13 were slightly upregulated while COL2 was slightly downregulated after 6 h. At 24 h, COL2 and MMP levels returned back to equilibrium, and ACAN gene expression increased, indicating a regenerative response that starts with a 24 h delay. Increased ACAN expression at 24 h following impact (9 MPa at >1000 MPa/s) has been observed before in the tissue adjacent to the impact site, but not at the impact site itself\textsuperscript{32}. The difference with the present study might indicate the existence of a threshold loading rate. Apparently, under high impact loading, an irreversible degenerative process is initiated, which does not occur at the present stress rates (~14 MPa/s) at similar peak stresses (~8 MPa).

The current study enhances our understanding of tissue failing and repair mechanisms in mechanically overloaded cartilage. After an initial degenerative response, the chondrocytes in the loaded area initiate a healing process after 24 h at the gene expression level. However, maintenance and repair at the matrix level was not seen within a 9 day period after loading, probably because of reduced cell viability. The developed procedures open new possibilities for future research, for instance to determine thresholds of damage that may or may not regenerate, or to explore effects of repeated and dynamic loading regimes over longer culture times to bridge the gap between short-term in vitro and long-term in vivo studies. Ultimately, such insight may result in improved treatment strategies for cartilage damage.

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**Role of the funding source**

The study was performed using internal funding sources. None of the authors have professional or financial affiliations that may bias this study.
Conflict of interest

The authors have no conflict of interest with publication of the data in the present manuscript.

References

3. Donkelaar CC van, Parraga Quiroga JM, Henao Murillo L, et al. 2014. The relationship between loading rate and damage. 7th World Congress of Biomechanics, Boston (MA), USA.


Figure Legends

Figure 1: Schematic diagram showing the culture time of the osteochondral plugs with the time points for the analysis of PG dynamics (EPIC-µCT, PG loss, and PG distribution), viability, collagen damage, and gene expressions.

Figure 2: A, Osteochondral constructs were clamped in the inserts using silicon rings (red) and cultured in custom-made polycarbonate culture chambers. The closing cap allowed for sterile indentation via a silicon membrane (blue). B, The region of interest (green box, bar = 2.50 mm) was selected from the CT scan and C, the outer cartilage surface (yellow) of the thresholded volume was rotated into the XY plane. D, Averaging the x-ray attenuation for each XY-slice yields the proteoglycan content as a function of depth.

Figure 3: Representative images of Col2-3/4m (red = collagen damage, blue = cell nuclei), safranin O (orange = proteoglycans), and LDH (red = dead cells, green = viable cells) staining of full thickness articular cartilage in the static controls at the end of the culture period (top) and loaded constructs prior to loading (row 2), 2 days after loading (row 3) and at the end of the culture period (bottom). Bar = 250 μm.

Figure 4: Effect of loading on the mRNA expressions over a 24 h period represented in box plots containing 50% of the data (central mark = median). (H1: μ ≠ 1 with * p < 0.05, ** p < 0.01).

Figure 5: Typical spatial variation in proteoglycan content across full thickness cartilage using A, safranin O at day 9 (black curve represents averaged pixel intensity), and B, using µCT (0 mm = surface, averaged pixel intensity ± std) at day -3, 2, and 9, divided into a superficial (red), middle (green), and deep (blue) zone.

Figure 6: Dynamics in proteoglycan (PG) content (± std) in static controls (no pattern) and loaded constructs (pattern). A, Average PG content derived from µCT data in static controls and loaded constructs 3 days prior to loading, and 2 and 9 days after loading (figure 5). These results were divided into the superficial zone (20%, C), the middle zone (50%, D), and the deep zone (30%, E). B, Rate of PG loss into the culture medium in static controls and loaded constructs 2, 4, 7, and 9 days after loading. Asterisks indicate statistical differences (* p < 0.05, ** p < 0.01, *** p < 0.001).
Table 1: Gene names, their abbreviations, primer sequences, annealing temperatures, amplicon lengths, NCBI numbers, and intron spanning (I/S) conditions.20-22

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Abbreviation</th>
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<td><strong>Cartilage matrix</strong></td>
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<tr>
<td>Collagen, Type II, Alpha 1</td>
<td>COL2</td>
<td>F: AGAACCTGGAGACATCAAAGATATT</td>
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<td>R: TTCACCTTTTGTCAACCACGAT</td>
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<td>Aggrecan</td>
<td>ACAN</td>
<td>F: TCCCCAACAGAGGCTTCCAT</td>
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<td></td>
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<td>R: AGGTCGCTCCATTCAAGACA</td>
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<td><strong>Degradative enzymes &amp; inhibitors</strong></td>
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<tr>
<td>Matrix metalloprotease-3</td>
<td>MMP3</td>
<td>F: GATGTTGGTACTTCAGCAC</td>
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<td>R: ATCATTATGTACGGCCTCC</td>
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<td>Matrix metalloprotease-13</td>
<td>MMP13</td>
<td>F: CCAAAGGCTACAACCTTTTCTTG</td>
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<td>R: TGGGTCTTTGAGGTGGTCAA</td>
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<tr>
<td>ADAM metallopeptidase with</td>
<td>ADAMTS5</td>
<td>F: CGCTGCCACCACACTCAA</td>
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<tr>
<td>thrombospondin Type 1 motif 5</td>
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<td>R: CGTAGTGCTCCTCAGTGTCATCT</td>
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<tr>
<td><strong>Reference genes</strong></td>
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<tr>
<td>Peptidylprolyl isomerase A</td>
<td>PPIA</td>
<td>F: GCAGACAAAGTTCCAAGACAG</td>
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<tr>
<td></td>
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<td>R: AGATGCCAGGACCCGTATG</td>
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Amplicon length: 107-112; Tm (°C): 60; NCBI number: XM_001925959, NM_001164652, NM_001166308.1, XM_003129808.3, NM_007038.3, NM_214353.1; I/S: Yes.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6