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Objective: To study mechanical overload of mature meniscal tissue under normal and pro-inflammatory conditions in vitro.

Method: Three days after a single unconstrained compression (strain: 25–75%, strain rate 1/s) of meniscal explants from 16 to 24 months-old cattle combined with interleukin-1 treatment (IL-1, 10 ng/ml) release of glycosaminoglycans (GAGs; dimethylmethylene blue (DMMB) assay), lactate dehydrogenase (LDH; cytotoxicity detection kit), and nitric oxide (NO; Griess assay), as well as gene transcription (quantitative reverse transcription polymerase chain reaction (RT-PCR)) and numbers of cells with condensed nuclei (CN; histomorphometry) were determined.

Results: Mean peak stresses during compression were about five (25%), 11 (50%), and 30 MPa (75%), respectively. GAG and LDH release and numbers of CN increased whereas NO production and mRNA levels of matrix metalloproteinase (MMP)-2, -3 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 decreased strain-dependently after compression. IL-1 induced an increase in GAG and NO release as well as MMP-2, -3 and ADAMTS-4 levels, but had no impact on the LDH release and slightly increased numbers of CN. However, in combination with compression the tissue responses were reduced and LDH and CN levels were increased compared to IL-1 alone.

Conclusion: Our data suggest that a single impact compression induces cell damage and release of GAG and reduces the NO production and transcription of certain matrix-degrading enzymes. It also reduces the capacity of meniscal tissue to respond to IL-1, which might be related to the cell damage and suggests that the compression-related GAG release might rather be the result of immediate extracellular matrix-damage than a cell-mediated event. This, however, needs to be confirmed in future studies.

Introduction

Introduction

Knee menisci play a crucial role in the joint function, given that meniscal damage is associated with the development and exacerbation of osteoarthritis (OA). In a healthy knee joint, the menisci transmit 45–75% of the axial load, and even after partial meniscectomy the local peak stress on the tibial plateau is severely increased. It is therefore essential to understand the pathomechanisms involved in meniscal destruction, in order to prevent degeneration and conserve the meniscal function.

Mechanical overload seems to play a major role in meniscal degeneration. Several in vitro studies have shown that mechanical stimulation on a physiological level can promote extracellular matrix production, whereas mechanical injury might induce degradation of proteoglycans, cell damage, and changes of gene transcription in meniscal tissue. Dynamic compression of meniscal tissue from 18 weeks-old pigs (0–0.1 MPa stress or 0–20% strain applied with 1 Hz for 2 h) altered for example the release of glycosaminoglycan (GAG) and nitric oxide (NO) or the transcription of several genes involved in extracellular matrix metabolism and degradation strain-dependently within 24 h. Single load compression of immature bovine meniscal tissue showed that a single impact (50% strain, 1/s strain rate, unconfined conditions) introduces immediate cell death and down-regulates the transcription of several matrix-damaging enzymes within 4 h, and a single impact (40% strain, strain rates 0.5–50%/s; confined
mediated mechanism. NO synthesis was also associated with incubation with IL-1 contradicts that response using an NO-anabolic pathways in a porcine meniscal model and that co-
showed that physiological levels of dynamic compression induced transcription in meniscal tissue. We therefore decided to
also inhibited the intrinsic meniscal repair response, and trig-
eter studies and compressed them by a single load using a loading
device that had been described previously for the compression of that
tissue using varying strains, even though the meniscal response to
articular cartilage explants.

Pro-inflammatory cytokines, such as interleukin-1 (IL-1), are
another key factor in the development of degenerative joint
diseases. IL-1 has been found in elevated levels in the synovial fluid of OA and rheumatoid arthritis joints. In articular cartilage
a combination of a single load compression and IL-6 or IL-1-treat-
ment, resulted in synergistic catabolic effects. Shin et al. showed that physiological levels of dynamic compression induced
anabolic pathways in a porcine meniscal model and that co-
incubation with IL-1 contradicts that response using an NO-
mediated mechanism. NO synthesis was also associated with
apoptosis in meniscal cells following partial meniscectomy. IL-1
also inhibited the intrinsic meniscal repair response, and trig-
eter proteoglycan degradation, NO production and catabolic gene
transcription in meniscal tissue. We therefore decided to
include the combination of a single load compression and IL-1-
treatment in the present study, in order to see what the effects of
such a combination are in a mature bovine meniscal tissue model.
GAG and LDH release, NO production, gene transcription of certain
genes and the amount of cells with condensed nuclei (CN) (a non-
specific morphological feature of cell death) were then
measured after an incubation time of 3 days.

To our knowledge this is the first study showing (1) strain-
dependent effects of a single compression on mature meniscal
tissue in vitro and (2) that a single load injury impairs the IL-1-
related response of meniscal tissue.

Method

Isolation of meniscal explants

Menisci of 16–24 months-old cattle procured by a local abattoir
were isolated as described previously (see Lemke et al. for detailed
graphical explanation). Four full thickness tissue cylinders
(10 mm in diameter) were punched perpendicular to the bottom
surface of each meniscus (leaving out the vascularized meniscal
base). Tissue disks 1 mm in thickness including the original
meniscal bottom surface were sliced off the cylinders using a sterile
scalpel blade, and four to five smaller explants (3 mm in diameter)
were obtained from this disk using a biopsy punch (HEBUnedical,
Tuttlingen, Germany). Weight and thickness of explants were
measured and for every single experiment the total of up to 60
explants (from one animal, two knee joints including medial/lateral
menisci) were randomized among the different experimental
groups (for further details see statistical analysis).

Single impact compression

Explants were compressed individually in an unconfined
culture medium-containing polysulfate chamber installed in
a computer-controlled loading device as described previously.
The platen had a larger diameter than the explants. A single
displacement ramp (strain rate 1 mm/s with different strains: 25–
75% of sample thickness) was applied, and the maximum strain
was maintained for 10 s. Afterward the platen returned to the
starting position. This protocol was selected based on (1) reports
that 25% strain exceeds the normal strain experienced by the
meniscus during physiological loading and (2) previous injury
studies on articular cartilage explants and meniscal explants.
Stresses were recorded during compression by the computer software as described elsewhere. After compression three explants/well of a 24-well plate were placed in 1 ml fresh
culture medium (except for the histology study where one explant
was cultured per 96-well plate in 250 μl) and incubated for 3 days at
37°C in an atmosphere of 5% CO2 with or without 10 ng/ml IL-1α
(R&D systems). The medium consisted of Dulbecco’s Modified Eagle’s Medium supplemented with 10 mM of hydroxyethyl
piperazineethanesulfonic acid (HEPES) buffer, 1 mM of sodium
pyruvate, 0.4 mM of proline, 50 μg/ml of ascorbic acid, 100 U/ml of
penicillin G, 100 μg/ml of streptomycin sulfate and 250 μg/ml of
ampicillin B.

Measurements of GAG release and NO synthesis

Cumulative GAG release into the culture supernatant was
determined photometrically using the dimethylmethylen blue (DDMB) dye assay at a wavelength of 525 nm (Photometer Ultra-
spec II, Biochrom, Cambridge, UK) using shark chondroitin-sulfate
as standard. Values were presented as μg GAG/mg wet weight of
the explants.

Release of NO into the culture supernatant was determined by
measuring nitrite accumulation using the Griess reagent (1%
sulfanilamide and 0.1% N-(1-naphthyl)-ethylen diamine-
dihydrochloride in 5% H3PO4, Sigma–Aldrich, St. Louis, MO, USA).
Hundred-microliter of each sample and 100 μl Griess reagent were
mixed and incubated for 15 min, and the absorption was deter-
mimed in an automated plate reader (Slt Reader 340 ATTC, SLT-
Labinstruments, Achterwehr, Germany) at 550 nm. Sodium nitrite
(NaNO2, Merck, Darmstadt, Germany) was used to generate a
standard curve for quantification. Values were presented as μmol
NO/mg wet weight of the explants.

Cell viability measurements

The cell viability was assessed using (1) a biochemical and (2)
a histomorphometric assay. (1) The release of lactate dehydro-
genase (LDH) was measured in the culture supernatants by
measuring the LDH activity with the Cytotoxicity Detection Kit
(Roche). Hundred-microliter of media was added to the same
amount of kit reagent in 96-well plates; the optical density was
measured at 500 nm using the same plate reader as for the GAG
measurements. The OD readouts were normalized to the tissue
weight after subtraction of media background values. Mean
control values were set to 100%, and all values were calculated as
% of control. (2) Explants were fixed overnight in 4% para-
formaldehyde, embedded in paraplast, and serial histological
sections (7 μm thick) were prepared and stained with Mayer’s
hematoxylin for the quantification of cell death, as previously
described for cartilage explants. In brief, three sections from
each explant disk were evaluated. Using a Zeiss Axioshot
microscope (Zeiss, Wetzlar, Germany) with a 40× objective,
normal and CN of cells were counted in three optical fields in
each section (one was located in the center of the explant
sections and two were located on both sides of the central field
without overlapping). Values from each field were recorded and
used for the calculation of the relative number of condensed cells (of total). Encoded labels were used on all samples to ensure blind scoring.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene to determine gene expression levels, as described previously. Meniscal explants from each group were pooled and frozen immediately in liquid nitrogen. Total RNA was extracted after pulverization of the tissue using the TRIzol reagent (1 ml/100 mg wet weight tissue; Invitrogen, Carlsbad, CA, USA) followed by extraction with chloroform and isopropanol precipitation. Extracted RNA was quantified spectro-photometrically at OD260/OD280 nm. Before real-time RT-PCR was performed using the Qiagen QuantiTect SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions the extracted RNA was digested with DNase (65°C for 10 min; Promega, Madison, WI, USA) to remove any traces of DNA. Bovine primers (Table I) were used at a concentration of 0.5 μM.

Conditions for real-time RT-PCR were as specified by manufacturer’s description: reverse transcription 30 min at 50°C; PCR initial activation step 15 min at 95°C; denaturation 15 s at 94°C; annealing 30 s at 60°C; extension 30 s at 72°C; optional: data acquisition 30 s at melting temperature 70–78°C.

Differences of mRNA levels between control and stimulated samples were calculated using the ΔΔCT-method. ΔCT represents the difference between the CT (cycle of threshold) of a target gene and the reference gene (GAPDH). The ΔΔCT value is calculated as the difference between ΔCT from the stimulated samples and the control.

Statistical analysis

A total of 438 explants from eight animals were used for the study: 180 explants in the dose-response experiments (strain 25–75%) in three independent experiments (60 explants/experiment). In each experiment the 60 explants were from two knee joints (including medial and lateral menisci) randomly distributed among the four experimental groups (15 explants/group, compressed individually, but cultured subsequently in groups of four independent experiments (3 × 60, 1 × 48 explants/ experiment, also randomly distributed and cultured as described above). Therefore, four or five measurements were made per experimental group and experiment (n = 19 for all four experiments together). Explants were pooled per group and experiment and used for mRNA isolation (n = 3/type of experiment). Thirty explants were fixed for histological evaluation (n = 5/group).

Results

Compression of meniscal explants

Explants of approximately 1 mm thickness (min. 0.9 mm, max. 1.2 mm; wet weight min. 8 mg, max. 11 mg) were compressed individually and stress (MPa) was recorded ([Fig. 1(A)]) shows examples of stress vs time curves for 25%, 50% and 75% strain, respectively). At all strain levels the stress rapidly increased and peaked as a result of the tissue compression. After reaching the final strain the stress decreased during the following 10 s of static compression, indicating equilibration of the tissue. Mean values (±S.E.M.) for the peak stresses were 25% strain: 4.9 ± 0.35 MPa, 50% strain: 11.2 ± 0.57 MPa, and 75% strain: 30.5 ± 1.12 MPa [Fig. 1(B)].

Release of GAGs

In the untreated control group a mean GAG release of 7.9 μg/mg wet weight was found, which increased with increasing strain of compression ([Fig. 2(A)]; Pearson’s correlation coefficient r = 0.667; P < 0.05). Compression with a 25% strain failed to increase the GAG release; however, a single compression with 50% and 75% strain led to an increase by 12% and 32%, respectively, compared to the untreated control group.

In a separate set of experiments the meniscal explants were treated with or without a combination of a single compression (50% strain; strain rate 1 mm/s) and IL-1 (10 ng/ml). The incubation with IL-1 served as an internal control, because our group had shown an increase in GAG release from meniscal explants by IL-1-treatment previously. Additionally, these experiments should show the impact of combined compression and IL-1-treatment. Stimulation with IL-1 resulted in a significant 188% increase in GAG release [Fig. 2(B)], whereas compression of the explants induced a 31% higher GAG concentration in the supernatants compared to the control. The combination of compression and IL-1-treatment led to a significantly lower release of GAG compared to the IL-1 stimulation alone. However, GAG release was still significantly higher (by 134%) than the untreated control group.

NO synthesis

The untreated control group displayed a mean release of 0.032 μmol NO/mg wet weight. In contrast to the GAG release,
A single compression had an adverse effect on NO levels (Pearson’s correlation coefficient $r = -0.352$): 25% strain led to a slight decrease by 10%, but compressions with 50% and 75% strain lowered the NO synthesis significantly by 24% and 30%, respectively [Fig. 2(C)].

In the experiments with combined compression/IL-1-treatment, stimulation with IL-1 increased NO levels significantly by 149% [Fig. 2(D)], while compressions with 50% strain decreased the NO release by 10% in comparison to the untreated control (which was less decrease than that found in the pure mechanical overload experiment, see above). A combination of IL-1 and compression resulted in a significant decrease of NO levels compared to the stimulation with IL-1 alone; however, these NO levels were still significantly higher than those of control cultures (by 84%).

**Transcription of matrix-degrading enzymes and matrix molecules**

Compression led to a strain-dependent decrease in the mRNA levels of matrix metalloproteinase (MMP)-2, -3, and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 [Fig. 3(A)]. MMP-2 dropped to 0.86- (25% compression), 0.31- (50%) and 0.22-fold (75%) levels compared to control tissue (Pearson’s correlation coefficient $r = -0.788$), MMP-3 to 0.37, 0.17, and 0.06 (Pearson’s correlation coefficient $r = -0.7$), and ADAMTS-4 to 0.3, 0.14 and 0.15 (Pearson’s correlation coefficient $r = -0.332$), respectively. The incubation with IL-1 (10 ng/ml) increased transcription levels of MMP-2, -3, and ADAMTS-4 5.3-fold, 12.4-fold, and 6.3-fold, respectively [Fig. 3(B)]. Compression (strain 50%) again reduced the mRNA levels of MMP-3 and ADAMTS-4 like in the first set of experiments, but did not show a consistent effect on the
MMP-2 transcription. The combination of compression and IL-1-treatment, however, showed the same trend as discovered before in the GAG and NO measurements, which was a slight decrease of mRNA levels compared to those induced by IL-1 alone (MMP-2 3.3-fold, MMP-3 5.5-fold, and ADAMTS-4 4.5-fold higher than control).

The transcription levels of the matrix molecules aggrecan and type II collagen had been measured in the combined experiments [Fig. 3(B)]. Compared to controls (set to 1) IL-1 reduced the mRNA levels of these molecules to 0.24 and 0.17, respectively. Compression (strain 50%) slightly decreased the transcription levels of these molecules (aggrecan 0.89 and type II collagen 0.54 compared to controls = 1). The combined treatment of the explants with IL-1 and compression resulted in mRNA levels similar to those of IL-1-treatment alone (aggrecan: 0.25; type II collagen: 0.23 compared to controls).

Cell viability

The release of LDH activity increased significantly with increasing strain of compression of the meniscal explants, indicating damage to the cellular membranes [Fig. 4(A)]. There was a significant positive correlation between the release of LDH and the increase in strain of compression (Pearson’s correlation coefficient \( r = 0.728 \)). While a 25% compression did not alter the release
of LDH, a 50% and 75% compression increased the release significantly 1.69-fold and 2.19-fold, respectively compared to the control. IL-1, on the other hand, did not alter the LDH release significantly [Fig. 4(B)]. However, in combination (IL-1 + 50% compression) the LDH levels were comparable to the levels found in cultures of explants that had been compressed only.

Cells with CN were counted as an indicator of cell damage and Fig. 4(C) shows examples of normal and CN. About 9% of the cells had CN in control cultures [Fig. 4(D)], which increased dose-dependently with increasing strain of compression (Pearson’s correlation coefficient r = 0.905). While the increase in the 25% strain group was not significant, the amount of CN in the 50% and 75% groups increased significantly (3.8- and 5.4-fold, respectively) compared to the control. IL-1 increased the amount of CN (2.2-fold, but not significantly), and in combination with 50% strain compression CN levels were comparable to that of compression alone (3.9-fold higher than control).

Discussion

We have studied the influence of a single load compression on meniscal tissue explants from mature cattle in vitro under serum-free conditions, and found a strain-dependent release of GAG in the subsequent 3 days of culture, which suggests a strain-dependent damage introduced to the tissue. In previous single load studies GAG release had either not been measured, or there was no significant increase in GAG release, which could be due to the lower strain that had been used (40%), the confined conditions of the loading device, or the fact that the authors used serum in the media and tissue from immature animals. Others, however, found a strain-dependent increase in GAG release within 24 h after 2 h/1 Hz dynamic compression in an immature pig model with even lower strains (20%), which suggests that dynamic loading might trigger GAG release differently or that the immature pig model is more sensitive to injurious loading. We additionally incubated the tissue with IL-1 and confirmed previous work where GAG release was significantly increased by the cytokine. However, the combination of single compression and IL-1 failed to show levels of GAG release which could represent the sum of GAG release induced by IL-1 and compression alone; GAG release was rather decreased compared to the IL-1-treatment alone, which suggests that compression might interfere with the IL-1-related pathways or even damages the tissue so that it is not able to respond properly to the cytokine any more. These findings are different to studies with articular cartilage where a combination of IL-1 and single load compression showed synergistic effects on the GAG release. This suggests that meniscal tissue and articular cartilage respond differently to combinations of compression and cytokine-treatment. Killian et al. showed that the increased transcription of matrix-degrading enzymes as a response to dynamic compression of meniscal tissue is mediated by autogenous IL-1 expression. The fact that co-treatment of single injury and IL-1 did not lead to higher responses in our study suggests again, that the dynamic compression model and the single load model trigger some different events.

The idea that single load compression might damage the tissue so that it is not able to respond to the cytokine properly any more is also supported by our findings that both, NO production as well as transcription of matrix-degrading enzymes (and matrix molecules), were reduced after compression. The incubation with IL-1 served as an internal control and showed that the meniscal tissue is able to increase NO synthesis and mRNA levels of the enzymes under the given circumstances, but still these parameters were down-regulated by a single compression strain-dependently. This confirms data from Kisiday et al. who also found enzymes such as MMP-9 and -13 (but not MMP-3) or ADAMTS-4 and -5 to be reduced, but the authors used one strain only (50%) and immature tissue, which suggests that down-regulation of these enzymes by a single compression does not depend on the maturation of the meniscal tissue. The reduction of mRNA levels should lead to reduced enzyme activities in the longer term, which appears to be paradox, because these enzymes are usually thought to be part of degenerative pathways and actually prevent repair in meniscal tissue. It is therefore more likely that the reduction in mRNA levels is the result of an impaired cell function due to the injury. The increased levels of LDH and cells with CN in cultures of compressed explants support that hypothesis. Gupta et al. used dynamic compression and demonstrated a bias in the strain-dependent response for NO production and the same group found a strain-dependent transcription of several matrix-degrading enzymes. They concluded that dynamic compression with physiological levels of strain triggers anabolic events, whereas higher strains (such as 20%) turn into destructive pathways in the immature porcine model. We did not see such a bias in the response of the mature bovine tissue to different strains of single compression which suggests that there might be species- or maturation-dependent differences, or, which might be even more likely, that the single load model mimics a single traumatic event, whereas dynamic compression might simulate a range of joint conditions, starting at physiological mechanical stimulation and ending in different levels of meniscal tissue overuse, depending on the strain of compression.

Peak stresses increased strain-dependently in our study starting at 4.9 MPa (25% strain) up to 30.5 MPa (75% strain). With 50% strain the peak stress peaked up to 11.2 MPa (±0.57 S.E.M.), which is in the same range as described for immature bovine tissue using the same loading regime (15.6 MPa ± 0.4 S.E.M.), which suggests that maturation of meniscal tissue does not change the peak stress response of the tissue on a major scale. Nishimuta and Levenston used lower strain rates in their single impact model with immature tissue, and therefore found lower peak stresses (4.63 MPa, 40% strain, 0.5/s strain rate). The stress vs time curves in our study showed a typical shape that had also been found in other single load injury models using articular cartilage. There were no unexpected irregularities in the readout which would indicate injurious events during compression, such as fissuring, cracking or other sudden failure of the extracellular matrix, which corresponds well with the fact that the explants did not show any major structural changes macroscopically after compression (not shown). This had already been described in the other meniscal single load studies and suggests that meniscal tissue is very resistant to mechanical deformation. However, Nishimuta and Levenston clearly showed that in immature bovine meniscal tissue, despite the macroscopic integrity, the cells already get damaged, which would probably lead to subsequent degeneration of the tissue after a single impact trauma. Kisiday et al. found many dead cells after compression of immature tissue, but only looked at the surface of the explants. We also found a significant increase in cell damage depending on the strain of compression, which supports the conclusion of the previous studies and adds that in mature tissue a single load compression introduces down-regulation of several cell activities, including reduced NO production, lower levels of matrix-degrading enzymes or loss of the ability to respond to IL-1. Since (1) most of these down-regulated cellular activities are usually considered to promote degradation of proteoglycans, and (2) we found significant amounts of cell damage, we suggest that the increased release of GAG in the present study is the result of immediate matrix-damage rather than an activation of cells or enzymatic activities.
Taken together our study shows that (1) mature bovine meniscal tissue is affected by a single load compression strain-dependently by increasing release of GAG and cell damage, but reducing the NO production and transcription of certain matrix-degrading enzymes; and (2) single impact loads reduce the capacity of meniscal tissue to respond to IL-1, which — all together — suggests that the compression-related GAG release might rather be the result of immediate extracellular matrix-damage than a cell-mediated event triggered by mechanical stimulation of the cells. This, however, has to be investigated in further studies.

Author contributions

MH was involved in the study design, collecting, analyzing and interpretation of the data, drafting of the manuscript. JL collected data and helped with the corresponding analysis and interpretation of the data. MS and AJG were involved in the analysis and interpretation of the data and the critical revision of the manuscript. BK was involved in the study design, supervision of the study, analyzing and interpretation of the data and drafting of the manuscript. All authors have approved the final version of the manuscript for submission.

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Conflict of interest

All authors disclose any financial or personal relationship with other persons or organizations that could potentially or inappropriately influence (bias) their work and conclusions.

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