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TITLE

Biotribological Testing and Analysis of Articular Cartilage Sliding against Metal for Implants

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KEYWORDS

cartilage, metal implants, tribology, wear, gene expression, metabolic activity

SUMMARY

This protocol describes the preparation, biotribological testing, and analysis of osteochondral cylinders sliding against metal implant material. Outcome measures included in this protocol are metabolic activity, gene expression and histology.

ABSTRACT

Osteochondral defects in middle-aged patients might be treated with focal metallic implants. First developed for defects in the knee joint, implants are now available for the shoulder, hip, ankle and the first metatarsalphalangeal joint. While providing pain reduction and clinical improvement, progressive degenerative changes of the opposing cartilage are observed in many patients. The mechanisms leading to this damage are not fully understood. This protocol describes tribological experiment to simulate a metal-on-cartilage pairing and comprehensive analysis of the articular cartilage. Metal implant material is tested against bovine osteochondral cylinders as a model for human articular cartilage. By applying different loads and sliding speeds, physiological loading conditions can be imitated. To provide a comprehensive analysis of the effects on the articular cartilage, histol ogy, metabolic activity and gene expression analysis are described in this protocol. The main advantage of tribological testing is that loading parameters can be adjusted freely to simulate in vivo conditions. Furthermore, different testing solutions

might be used to investigate the influence of lubrication or pro-inflammatory agents. By using gene expression analysis for cartilage-specific genes and catabolic genes, early changes in the metabolism of articular chondrocytes in response to mechanical loading might be detected.

INTRODUCTION

The treatment of osteochondral defects is demanding and requires surgery in many cases. For focal osteochondral lesions in middle-aged patients, focal metallic implants are a viable option, especially after the failure of primary treatment, like bone marrow stimulation (BMS) or autologous chondrocyte implantation (ACI)[1]. Partial surface replacements can be considered salvage procedures that can reduce pain and improve the range of motion[2]. These implants are typically composed of a CoCrMo alloy and are available in different sizes and offset configurations to match the normal anatomy[3]. While initially developed for defects on the medial femoral condyle in the knee, such implants are now available and in use for the hip, ankle, shoulder, and elbow[4–6]. For a satisfactory outcome, it is crucial to assess the mechanical joint alignment and condition of the opposing cartilage. Furthermore, correct implantation without protrusion of the implant has been shown to be fundamental[7].

Clinical studies demonstrated excellent short-term results in terms of pain reduction and improvement of function in middle-aged patients for various locations[5,6,8]. Compared with allograft implantation, focal metal implants allow early weight bearing. However, the opposing articular cartilage showed accelerated wear after in a considerable number of patients[9,10]. Hence, even with proper placement, in many cases degeneration of the native cartilage seems inevitable, while the underlying mechanisms remain unclear. Similar degenerative changes have been observed after bipolar hemiarthroplasty of the hip[11] and are increased with activity and loading[12].

Tribological experiments provide the possibility to study such pairings in vitro and simulate different loading situations occurring under physiological conditions[13]. The use of osteochondral pins offers a simple geometry model to investigate the tribology of articular cartilage sliding against native cartilage or any implant material[14] and might further be used in whole joint simulation models[15]. Metal-on-cartilage pairing show accelerated cartilage wear, extracellular matrix disruption, and decreased cell viability in the superficial zone compared with a cartilage-on-cartilage pairing[16]. Damage to the cartilage occurred mainly in the form of delamination between the superficial and middle zones[17]. However, the mechanisms leading to cartilage degeneration are not fully understood. This protocol provides a comprehensive analysis of the biosynthetic activity of articular cartilage. By the determination of metabolic activity and gene expression levels of catabolic genes, early indications for cartilage breakdown might be identified. The advantage of in vitro tribological experiments is that loading parameters can be adjusted to imitate various loading conditions.

Hence, the following protocol is suitable to simulate a metal-on-cartilage pairing, representing an experimental hemiarthroplasty model.

PROTOCOL

1. Preparation of metal cylinders

1.1. Analyze cylindrical cobalt-chromium-molybdenum (CoCrMo) rods fulfilling the standard specifications for surgical implants for their chemical composition using scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy per manufacturer's protocol to confirm provided values.

NOTE: The elemental composition of the CoCrMo alloy used for this experiment is 65% Co, 28% Cr, 5% Mo and 2% others.

1.2. Wet grind the samples with silicon carbide grinding paper starting with a grain size of 500. Use grinding paper in increasing order up to a grain size of 4000.

1.3. Polish the cylinder with 3 µm and 1 µm paste to achieve a surface roughness that is within the tolerance level of surface finish requirements for metallic surgical implants (ISO 5832-12:2019) and total and partial joint replacement implants (ISO 21534:2007).

NOTE: The average surface roughness is determined using a confocal microscope.

1.4. Cut CoCrMo rods (Ø of 6 mm) to cylinders with a length of 10 mm.

2. Harvesting of osteochondral cylinders

2.1. Use bovine stifle joints from skeletally mature animals (aged 18-24 months at the time of sacrifice) and keep them contained and cooled until dissection within 24 h after sacrifice.

NOTE: Joints are purchased from the local butcher. The joint remains closed until dissection.

2.2. To harvest cylindrical osteochondral plugs under aseptic conditions, disinfect the knee and perform an arthrotomy and expose the medial femoral condyle.

NOTE: The dissection has to be performed with caution not to damage the articular surface.

2.3. Inspect the articular surface for macroscopic damages.

NOTE: Discard the sample if the cartilage lacks its whitish, smooth and glossy appearance or if there is blistering, fissures or larger defects.

2.4. Align the cutting tube perpendicular to the articular surface of the weight-bearing area and drive the device into the cartilage and subchondral bone by firm strokes with a hammer. At 15 mm penetration depth, twist the device clockwise with a sudden motion.

2.5. Remove the device, insert the white knob and screw it in until the bottom end of the osteochondral plug is visible.

2.6. Mark the anteroposterior orientation of the samples with a sterile marker in order to arrange the osteochondral cylinder accordingly during testing.

NOTE: The three-dimensional collagen network and its complex architecture facilitate the unique mechanical properties of articular cartilage and should be considered in the orientation of the samples.

2.7. Rinse the sample with phosphate-buffered saline (PBS) to wash off blood and fat tissue.

2.8. Repeat the steps mentioned above to harvest the desired number of osteochondral plugs (8 mm diameter, 15 mm length).

NOTE: Typically, 9 to 12 osteochondral cylinders can be harvested from the weight bearing area on the medial femoral condyle.

2.9. Place the samples in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, supplemented with antibiotics (penicillin 200 U/mL; streptomycin 0.2 mg/mL) and amphotericin B 2.5 µg/mL and store them at 4 °C until testing to maintain viability.

2.10. Analyze control osteochondral plugs immediately after harvesting to establish baseline values (see analysis section).

3. Tribological testing

3.1. Perform the experiments using a commercially available reciprocating tribometer with a cylinder-on-plate configuration. Requirements for the device are vertical loading and adjustable load and sliding speed. Furthermore, a liquid cell enables to perform the tests in a lubricating solution.

3.2. Determine the contact pressure in the CoCrMo-on-cartilage system using a pressure measurement film. Place the pressure measurement film at the interface and apply static load for 30 s to determine initial contact pressure, contact size and shape. Owing to the convexity of the metal cylinder and the articular cartilage, the initial contact area has an elliptical shape in this configuration.

NOTE: The pressure measurement film reacts to the applied pressure showing red discoloration of zones where the threshold pressure is reached or exceeded. For 1 N of load, the contact pressure was determined around 2 MPa by visual comparison with defined contact pressures.

3.3. Fix the osteochondral cylinders on the bottom sample holder with the marking aligned with the sliding direction, and mount the CoCrMo cylinders onto the upper load cell.

3.4. Add the testing solution (PBS with 3 g/L hyaluronic acid) into the liquid cell that results in

submerging the osteochondral cylinder and covering the metal-cartilage sliding interface.

3.5. Set the testing parameters (prescribed normal force, stroke and sliding speed), which are then applied and maintained throughout the test.

NOTE: The stroke length of the reciprocating motion must be set according to the contact area to create a migrating contact area (MCA). For plugs that are 8 mm in diameter, a 2 mm stroke allows adequate rehydration of the cartilage.

3.6. Start reciprocal sliding of the CoCrMo cylinder against the articular cartilage immersed in the lubricating solution with the set loading parameters.

3.7. Monitor the coefficient of friction (COF) during the experiments.

NOTE: The COF is assessed automatically but can be calculated using the equation $\mu=F/W$ (μ - coefficient of friction; F - frictional force; W - normal load applied by the system).

3.8. Terminate the experiment after the desired testing period.

3.9. Remove the osteochondral plug from the sample holder, rinse it with PBS and store it in medium until further biological analysis (see below).

3.10. Submerge control samples in the testing solution at room temperature for the duration of the test and analyze together with samples that have been exposed to mechanical loading.

4. Analysis

NOTE: Osteochondral cylinder are analyzed for metabolic activity and gene expression to investigate biological activity; histology is performed to study cartilage surface integrity and the underlying matrix.

4.1. Histology

4.1.1. For histological analysis, immerse the osteochondral plugs in 4% buffered formaldehyde solution at room temperature until further processing.

4.1.2. Rinse the samples with PBS and place them into a plastic vessel.

4.1.3. Add an excess of the ready-to-use decalcifier-solution so that all samples are covered.

4.1.4. Apply constant agitation for 4 weeks for complete decalcification.

4.1.5. After decalcification, embed the samples in water soluble glycols and resins and store them at −80 °C.

4.1.6. Obtain 6 µm sections by cryosectioning transversal to the contact area.

4.1.7. Subsequently, prepare the samples for Safranin O staining and Fastgreen counterstaining using a manufacturer's protocol.

4.1.8. Capture histological images using a microscope and process using imaging processing software.

4.2. Metabolic activity

NOTE: The metabolic activity of chondrocytes in the articular cartilage are investigated with an XTT-based ex vivo toxicology assay.

4.2.1. Rinse the osteochondral plug using PBS and place the sample in a Petri dish.

4.2.2. Place a 24-well plate on a scale and zero the scale.

4.2.3. Cut off the cartilage from the osteochondral graft with a scalpel in one piece.

4.2.4. Bisect the cartilage in two equal pieces so that the contact area is equally distributed onto both cartilage pieces and mince one half to 1 mm^3 pieces. The second half is used for gene expression analysis.

4.2.5. Transfer the minced cartilage into one well of the prepared 24-well plate and determine the tissue weight.

4.2.6. Repeat the steps mentioned above for each sample and add 1 mL of growth medium to each well of the plate.

4.2.7. Add the XTT solution (490 µL of XTT labelling reagent and 10 µL of activation reagent) according to the manufacturer's instruction and mix.

4.2.8. Incubate the plate at 37 °C and 5% CO_2 for 4 h.

4.2.9. After incubation, remove the supernatant and transfer it to a 5 mL tube.

4.2.10. Extract the tetrazolium product by adding 0.5 mL of dimethyl sulfoxide (DMSO) to the cartilage tissue in the 24-well plate and apply continuous agitation for 1 h at room temperature.

4.2.11. Remove the DMSO solution and pool it with the previously collected XTT solution.

4.2.12. Transfer 100 µL of the sample in triplicates in a 96-well plate on a plate reader and measure the absorbance at a wavelength of 492 nm and a reference wavelength at 690 nm.

4.2.13. Normalize the resulting absorbance values to the wet weight of each sample and perform analysis using software.

4.3. Gene expression analysis

4.3.1. RNA isolation

NOTE: RNA isolation is carried out using a commercial kit (**Table of Materials**) according to the instructions provided by the manufacturer with small amendments.

4.3.1.1. Mince the second half of the cartilage tissue obtained from the osteochondral plug into small pieces.

4.3.1.2. Transfer them to a tube containing ceramic beads and 300 µL of Lysis Buffer (containing 1% β-mercaptoethanol).

NOTE: The samples can be frozen in liquid nitrogen until further processing.

4.3.1.3. Thaw the samples for 2 min and use the commercial lyser for homogenization of the tissue. Apply 6500 rpm for 20 s (homogenization step) four times with a 2 min cooling phase after each run (at 4 °C using the commercial lyser cooling device) to fully disrupt the tissue.

4.3.1.4. Add 20 µL of proteinase K and 580 µL of RNase-free water to each tube and incubate them at 55 °C for 30 min.

4.3.1.5. Centrifuge the samples for 3 min at 10,000 x *g* and transfer the supernatant to a 1.5 mL tube.

4.3.1.6. Add 0.5 volumes of 90% ethanol to each tube and mix.

4.3.1.7. Transfer 700 µL of the sample to an RNA binding column placed in a 2 mL collection tube and centrifuge at 8,000 x *g* for 15 s.

4.3.1.8. Discard the flow-through and repeat the centrifugation step for the complete lysate.

4.3.1.9. Add 350 µL of Buffer RW1 to the column, centrifuge at 8,000 x *g* for 15 s, and discard the flow-through.

4.3.1.10. Mix 10 µL of DNase stock solution and 70 µL of Buffer RDD. Add the solution to the RNA purification membrane and incubate it at room temperature for 15 min.

4.3.1.11. Add 350 µL of Buffer RW1 to the column and centrifuge at 8,000 x *g* for 15 s. Discard the flow-through.

4.3.1.12. Add 500 µL of Buffer RPE and centrifuge at 8,000 x *g* for 15 s. Discard the flow-through.

4.3.1.13. Add 500 µL of Buffer RPE to the RNA purification column and centrifuge at 8,000 x *g* for 2 min.

4.3.1.14. Place the column in a 1.5 mL collection tube and add 30 µL of RNase-free water. Centrifuge at 8,000 x *g* for 1 min.

4.3.1.15. Store the isolated RNA at -80 °C until cDNA synthesis.

4.3.2. cDNA synthesis

NOTE: To synthesize complementary DNA (cDNA) from messenger RNA (mRNA) a commercial kit (**Table of Materials**) was used. RNA from bacteriophage MS2 was added to stabilize isolated RNA during cDNA synthesis.

4.3.2.1. Thaw and mix the reagents. The composition for a single reaction is shown in **Table 1**.

4.3.2.2. Add 16 µL of RNA sample to the volume for a single reaction (14 µL).

4.3.2.3. Perform cDNA synthesis in a thermal cycler using the following parameters: 10 min at 25 °C (primer annealing), 60 min at 50 °C (DNA synthesis), 5 min at 85 °C (denaturation) and 5 min at 20 °C (cooling phase).

4.3.2.4. Store cDNA at -20 °C until real-time quantitative polymerase chain reaction (RT-qPCR).

4.3.3. RT-qPCR

NOTE: For RT-qPCR of bovine samples, primers and probes were designed by using commercial Real-Time qPCR software (e.g., IDT) for the genes GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), COL2A1 (Collagen type 2), ACAN (Aggrecan), COL1A1 (Collagen type 1), MMP-1 (Matrix Metalloproteinase-1), and MMP-13 (Matrix Metalloproteinase-13). Bovine primers and double quenched probes were provided by IDT. The reagents used for a single reaction to evaluate the efficiency and gene expression are displayed in **Table 2**.

4.3.3.1. Dispense the master mix of a single reaction (9 µL) to each well of a 96-well PCR plate and add 1 µL of cDNA to each reaction. Perform tests for each sample in triplicates.

4.3.3.2. Close the PCR plate using sealing oil and centrifuge at 877 x *g* for 10 min at 4 °C.

4.3.3.3. Perform RT-qPCR using a precision thermal cycler with the following protocol: 95 °C for 10 min, 45 cycles of amplification (95 °C for 10 s, annealing for 30 s, cDNA synthesis), and 37 °C for 30 s.

NOTE: Specific annealing temperatures are required for each primer.

4.3.3.4. Use GAPDH along with the target genes to confirm efficiency.

4.3.3.5. Use the provided software to calculate the efficiency of each gene.

4.3.3.6. Normalize the cycle threshold (CT) values to the expression of the reference gene GAPDH and use the ΔΔCT method for quantification.

REPRESENTATIVE RESULTS

The contact area and contact pressure must be confirmed using a pressure measurement film (**Figure 1**). Physiological loading condition can be confirmed by comparing with reference imprints for defined contact pressures. During testing, the coefficient of friction is monitored constantly. With a migrating contact area, a low friction coefficient can be maintained for at least 1 h (**Figure 2**). Using Safranin O staining the extracellular matrix composition and structure can be determined (**Figure 3**). The intensity of Safranin O staining is proportional to the proteoglycan content. Fast Green counterstains the non-collagen sites and provides a clear contrast to the Safranin O staining. The proteoglycan content varies over the articular surface but should be uniform throughout the tissue section in baseline samples (**Figure 3A**). Control samples submerged in the testing solution show extraction of GAGs, which can be counteracted by mechanical loading (**Figure 3B, 3C**). Metabolic activity of the bovine articular chondrocytes is independent of the harvesting site, but shows an increase with mechanical loading compared with unloaded controls (**Figure 4**). The gene expression levels of cartilage-specific genes (COL2A1, ACAN) increase with physiological loading conditions, whereas catabolic genes (COL1A1 and MMP13) are upregulated with stationary contact area (**Figure 5**).

FIGURE AND TABLE LEGENDS

Table 1: Reagents for a single reaction for cDNA synthesis.

Table 2: Reagents for the Master Mix for a single PCR.

Figure 1: Pressure measurement of the initial contact area at the metal-cartilage interface before testing. Due to the convexity of the metal cylinder and the articular surface and its elastic properties, the initial contact area is elliptical. During sliding, this initial contact area moves with a stroke of 2 mm, resulting in a larger area that is exposed to mechanical loading; scale bar = 2 mm.

Figure 2: Time-depended coefficient of friction (duration 1 hour) for seven samples tested at 8 mm/s sliding speed and 1 N load (2 MPa contact pressure). Each colored line represents the COF of one osteochondral cylinder. The observed variability is within the limits for biological samples.

Figure 3: Histological cross-sections of bovine osteochondral samples stained with Safranin-O and Fast Green. (**A**) Baseline samples show high GAG content throughout the articular cartilage.

(**B**) Control sample submerged in testing solution without mechanical loading show less Safranin-O staining in the middle zone, indication an extraction of proteoglycans. (**C**) Tested samples show higher GAG content compared with controls, indicating mechanical stimulation; scale bar = 250 µm

Figure 4: Metabolic activity of bovine articular chondrocytes after tribological testing with different loading variations and controls. The horizontal dotted line represents baseline levels. The nonparametric Kruskal–Wallis test was performed for comparison between testing groups followed by Dunn's post hoc test in case of significance. *$p < 0.05$. This figure has been modified from Stotter et al.[18].

Figure 5: Gene expression of cartilage-specific genes after tribological testing with different loading conditions and controls. COL2A1=collagen type 2; ACAN=aggrecan; COL1A1= collagen type 1; MMP13= matrix metalloproteinase 13. The expression levels were normalized to the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The horizontal dotted lines represent baseline expression levels. The nonparametric Kruskal–Wallis test was performed for comparison between testing groups followed by Dunn's post hoc test in case of significance. *$p < 0.05$. This figure has been modified from Stotter et al.[18].

DISCUSSION

Focal metallic implants represent a salvage procedure for osteochondral defects, especially in middle-aged patients and after failed primary treatment. Although clinical studies demonstrated promising short-term results, one observed complication is damage to the opposing, native cartilage[10]. Cadaver and biomechanical studies show clear evidence that proper implantation with flat or slightly recessed positioning maintains natural contact pressures[19]. Tribological experiments provide a possibility to test various cartilage pairings in vitro. In such, loading conditions, lubrication, material pairings and duration might be adjusted as desired.

Bovine cartilage is available in high quantity at the local abattoir. The cellularity and the zonal structure are very similar to the human femoral condyles[20]. However, proteoglycan content is site-specific, whereas gene expression levels have been shown to be uniform over the articular surface. In this protocol, osteochondral plugs were harvested from the weight bearing area. Cartilage thickness, collagen architecture and resulting tribological properties show regional differences over the articular surface[16]. The limitation of using osteochondral plugs in an unconfined loading setup with a disrupted collagen network and changed fluid pressurization compared with whole joint models need to be considered.

In the majority of tribological studies, PBS alone is used as testing solution to generate more robust data. PBS is a buffer solution with isotonic osmolarity and helps to maintain a constant pH during biological experiments. Using PBS with hyaluronic acid provides boundary lubrication and reduced friction[21]. Accordingly, synovial fluid reduces the friction coefficient and improves fluid pressurization compared with saline[22]. The friction coefficient depends on various system properties, demonstrated by the classic Stribeck Curve. The Stribeck Curve relates the friction coefficient and viscosity, speed and load and presents the basic lubrication regimes: boundary,

mixed, and hydrodynamic lubrication. Boundary lubrication can be obtained with PBS alone as lubricating fluid, but loading parameters would need to be adjusted accordingly. The COF delivered from the tests are average values over the stroke. Thus, it can be assumed that different lubrication conditions occur during the cycle. During standstill at reversal position, boundary conditions might be prevailing, while mixed lubrication might be predominant during sliding. Based on absolute duration during the sliding cycle, the latter would have had more influence on the mean COF value.

To investigate physiological conditions occurring in joints during daily activities, loading conditions can be adjusted accordingly in the tribometer software. Pressure sensitive measurements should be used to confirm the desired contact pressures. Reported femorotibial contact pressures range between 1 MPa during standing and up to 10 MPa during downhill running[23]. With a focal resurfacing, implant pressures are just slightly elevated compared with healthy joints[24]. Reported relative sliding velocities during the gait cycle are reported up to 100 mm/s with high variations during the different phases. This means that relative joint movements exceed the velocities that can be applied in this tribological setup. To mimic natural kinematic conditions and contact pressures in healthy knee joints, loading conditions range from 1 to 10 MPa contact pressure and 5 to 100 mm/s sliding speed. However, while high loads can be applied in this experimental setup, the range of sliding velocities is limited. Pathological loading conditions, both overload and inadequate loads, might also be simulated. Low sliding velocities or static loading equate immobilization, while higher loads represent nonphysiological mechanical stimulation.

As enzymatic digestion can affect the expression of cartilage-specific genes, a nonenzymatic tissue homogenization is described in this protocol. During cDNA synthesis, in addition to the instructions, RNA from bacteriophage MS2 is added for stabilization purposes. Gene expression levels, but not proteins, were analyzed to detect early changes in the biosynthetic activity of articular chondrocytes. In addition to histological sections and metabolic activity, these assays provide comprehensive information on the effects of mechanical loading on articular cartilage.

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DISCLOSURES

The authors declare that they have no competing interests.

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Figure 1

Click here to access/download;Figure;Fig1.pdf

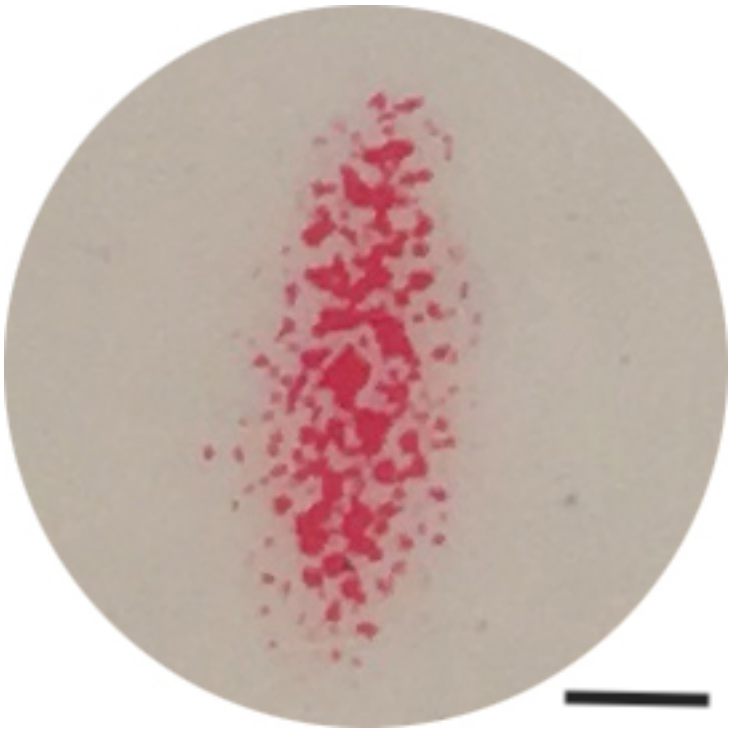


Figure 2

Click here to access/download;Figure;Fig2.pdf

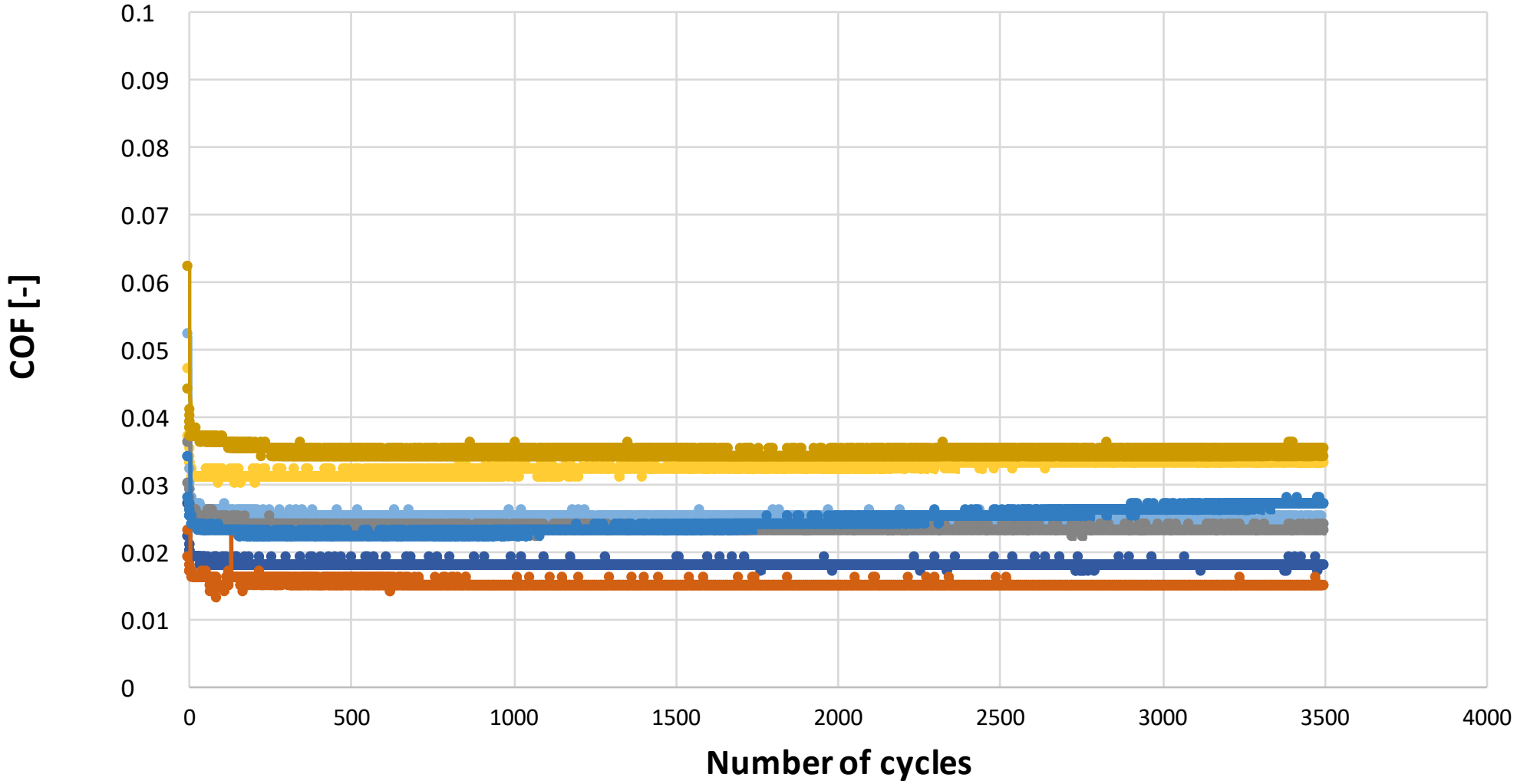


Figure 3

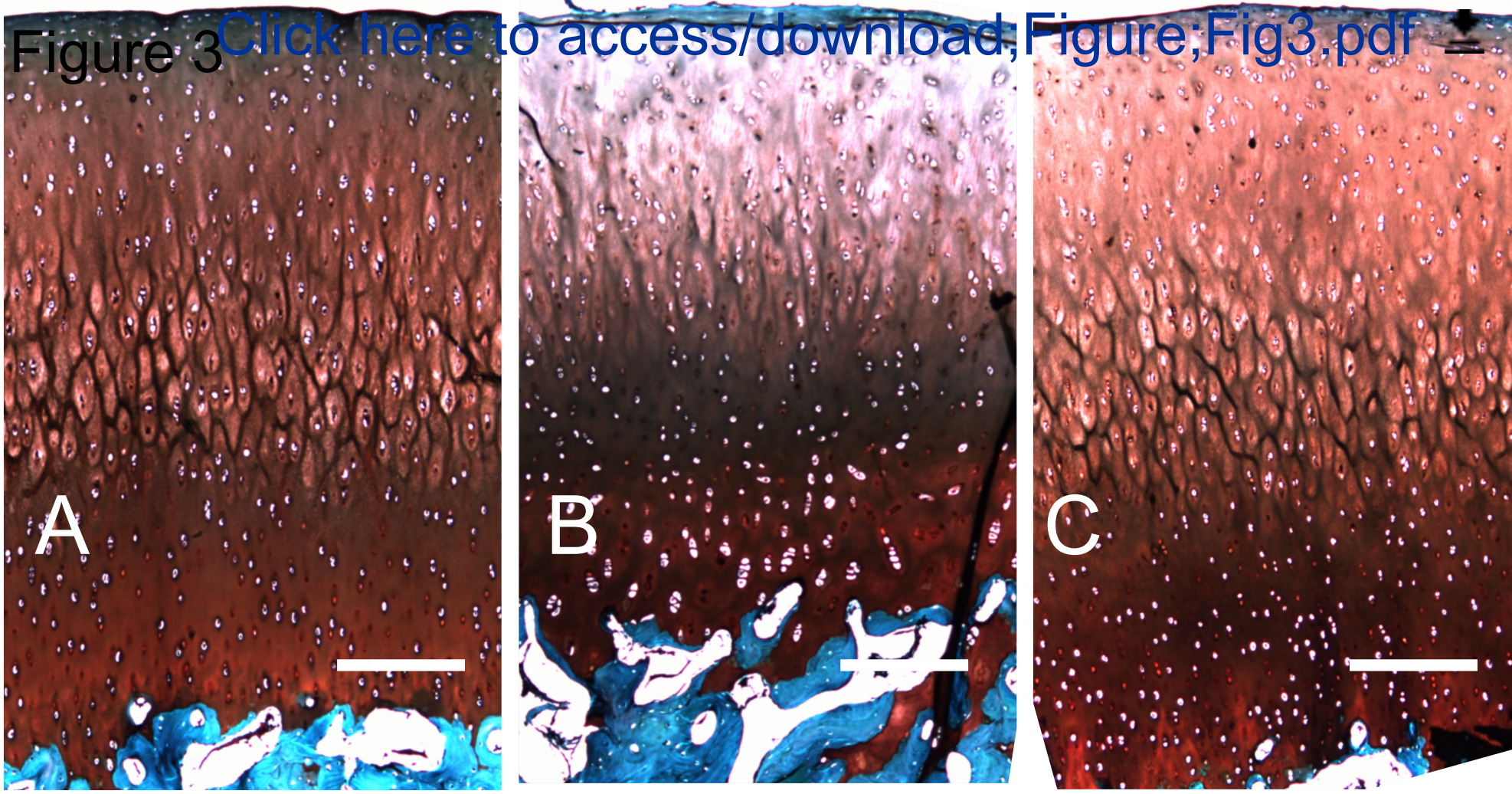


Figure 4

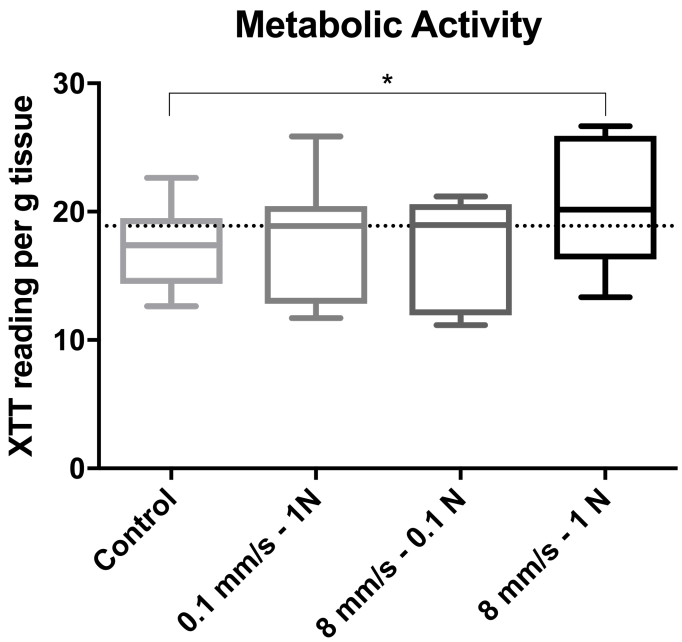


Figure 5

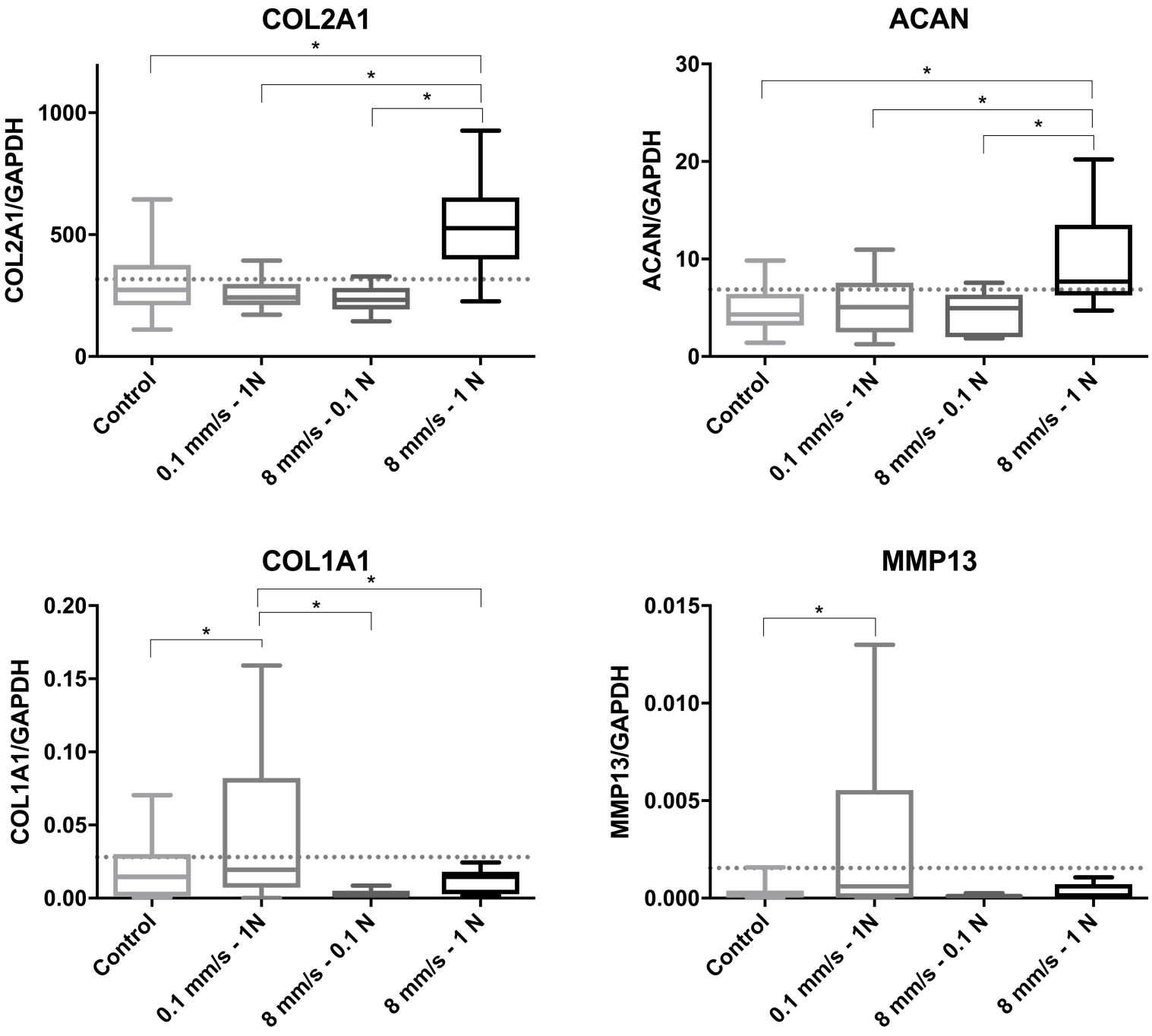


Table 1

	Volume (µl)
Transcriptor RT Reactions Buffer 5x conc.	6
Protector RNase Inhibitor 40U/µl	0.75
Deoxynucleotide Mix 10 mM each	3
Random Hexamer Primer 600 µM	3
Transcriptor Reverse Transcriptase 20 U/µl	0.75
MS2 RNA (0,8 µg/µl)	0.375
Nuclease free distilled water	0.125
Total volume	14

Table 2

	Volume (µl)
FastStart Probe Master 2X	5
Hydrolysis Probe 2,5 µM	1
Left PrimerGAPDH 5 µM	
Right Primer GAPDH 5 µM	
Nuclease free distilled water	3
Total Master Mix	9

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Amphotericin B	Sigma-Aldrich Chemie GmbH	A-2942-100ML	
buffered formaldehyde solution 4%	VWR	97131000	
Cell Proliferation Kit II (XTT)	Roche Diagnostics	11465015001	XTT-based ex vivo toxicology assay
CoCrMo raw material	Acnis International		CoCrMo rods 6mm in diameter
CryoStar NX70 Cryostat	Thermo Fischer Scientific		cryosectioning device
dimethyl sulfoxide (DMSO)	Sidma-Aldrich Chemie	D 2438-10ML	
Dulbecco's modified Eagle's medium	Sigma-Aldrich Chemie GmbH		medium
fetal bovine serum	Gibco		
Hyaluronic acid	Anika Therapeutics Inc.		component of lubricating solution
iCycler	BioRad		thermal cycler
Leica microscope DM-1000	Leica		microscope for histology
LightCycler 480 Sealing Foil	Roche Diagnostics		
LightCycler 96	Roche Diagnostics		thermal cycler for PCR
MagNA Lyser Green Beads	Roche Diagnostics	3358941001	
Osteochondral Autograft Transfer System (OATS	Arthrex Inc.		cutting tube for harvesting osteochor
osteosoft	Merck	1017279010	decalcifier-solution
Penicillin /Streptomycin	Sigma-Aldrich Chemie GmbH	P4333-100ML	
phosphate-buffered saline	Sigma-Aldrich Chemie GmbH		PBS
Prescale Low Pressure	Fujifilm		pressure indicating film
RNeasy Fibrous Tissue Kit	QIAGEN	74404	
Synergy 2	BioTek Instruments		plate reader
Tetra-Falex MUST	Falex Tribology		Tribometer
Tissue- Tek O.C.T.	SAKURA	4583	embedding formulation
Transcriptor First Strand cDNA Synthesis Kit	Roche Diagnostics	40897030001	
β-mercaptoethanol	Sidma-Aldrich Chemie	M3148	

ıdral cylinders

Response to Editorial and Reviewers' comments

We would like to thank you and the reviewers for the valuable comments that we received in response to our submission (Manuscript Number JoVE61304). We have revised the paper and addressed the concerns according to the suggestions and believe that the manuscript has improved through the amendments and modifications. We hope that the new version of the article will be suitable for publication.

Editorial comments:
General:
Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread the manuscript and corrected spelling and grammar issues.

JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
We removed commercial language from the manuscript and the trademark symbols from the Table of Materials. In some cases, we found it vital to include the commercial names because of the amendments to the instructions provided by the manufacturer.

Protocol:
For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
We revised the manuscript accordingly and hope that description of each step is adequate and can be further used. If not, please let us know, as this is our first JoVE publication.

Specific Protocol steps:
Please provide more information (or a reference) about the x-ray spectroscopy (including the 'provided values'). Also, where do the CoCrMo cylinders come from?
More information on the spectroscopy has been included in the manuscript. The CoCrMo raw material was purchased by Acnis International and the provided composition values where then confirmed. The following table provides the detailed information (from Stojanovic et al, Acta Biomat). This information has also been included in the manuscript.

Elemental composition of the low-carbon CoCrMo alloy used in this study.

Element	Si	Mo	Cr	Mn	Co	C
Nominal, average wt.%	0.67	5.49	27.21	0.75	65.55	0.04
Measured, average wt.% ± SD	0.89 ± 0.17	4.90 ± 0.42	27.51 ± 0.30	0.84 ± 0.21	64.41 ± 0.61	*

* 1.45 ± 0.05 wt.% concentration of C due to contamination.

How do you determine if the roughness is within the tolerance level?
The surface roughness was determined using a confocal microscope. Tolerance levels are defined by the mentioned ISO documents for metallic implants. The described preparation resulted in a final Ra value of 15 ± 2 nm for the CoCrMo samples.

Figures:

Figure 2: Please use periods instead of commas for decimals (e.g., 0.01 instead of 0,01).
We made the according changes to the figure.

Figure 4, 5: What statistical test was used here?
The nonparametric Kruskal–Wallis test was performed for comparison between testing groups followed by Dunn's post hoc test in case of significance. The used test was included in the figure legend.

Table of Materials:
Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol. Please remove trademark (™) and registered (®) symbols from the Table of Materials.
We completed the information in the Table of Materials and removed trademark and registered symbols.

Reviewers' comments:
Reviewer #1:
Thank you for your valuable feedback on our manuscript. We addressed both major and minor concerns and hope that the revised version of the manuscript now provides the additional information.

Major Concerns:
The abstract refers to focal metallic implants, whereas the test protocol is more closely suited to a hemiarthroplasty (cartilage-on-metal) model. When a focal metallic implant is used, the interface between the metal implant and the cartilage should be considered as should the influence of the different modulus between the two materials and the effect this has on the opposing cartilage surface, simulating only the metal implant does not take this into consideration. The introduction should discuss the suitability of this protocol to simulate hemi-arthroplasty model.
We are aware of the limitations of this model for focal implants, especially at the implant-cartilage-interface. Hemiarthroplasty also represents a metal-on-cartilage situation ofter the implantation of a bipolar hip arthroplasty, for example. Accordingly, we included and discussed this in the introduction.

The work by Bowland et al should be considered and acknowledged in the introduction to this article. This work, carried out in both simple geometry (JOEIM 2018) and in a whole joint simulation model (J Biomaterials 2018) has investigated the influence of a stainless steel graft implanted either in a bovine plate or a porcine femur and has investigated how the introduction of this graft influences the wear and friction of the articulating surfaces.
Thank you very much for these references. Despite a comprehensive literature research in the course of our project, these study had not come to our attention. However, they provide in depth information on the topic and we included them in the introduction.

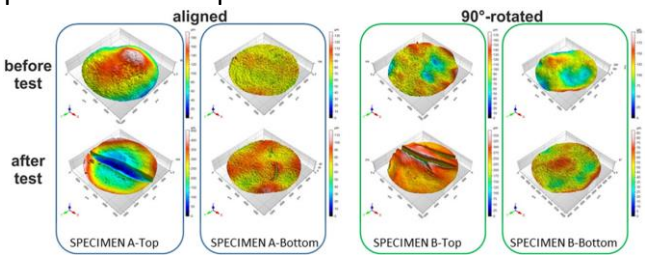
Minor Concerns:
The title largely reflects the protocol demonstrated, perhaps consider including 'bovine' in the title.

We discussed the title of the manuscript and decided not to include “bovine” to keep it short and comprehensive. Furthermore, the similarities in tribological properties of bovine and human cartilage are discussed in the manuscript.

The equipment list is very specific for some protocols for example, the microscopes used have been detailed however, for the tribological test, the details of the equipment are very vague, the level of detail given in the article should be consistent throughout.
We made the according changes in the manuscript. As suggested by the editor, commercial names were removed. Additionally, we included information on kinematics and applied loads for the tribological test.

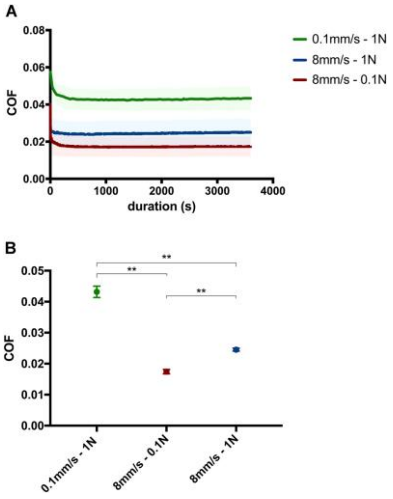
Consider whether a cartilage-on-cartilage model should be included as a control.
We have performed cartilage-on-cartilage experiments in previous studies. However, for specifically testing implant materials, in this case CoCrMo, and the effect of various loading conditions, we found that it would not give us additional information.

Could a method for assessing cartilage wear or to image the cartilage surfaces be included, this would be interesting to readers.
Cartilage wear can be assessed by volumetric wear or sGAG content. In a previous study investigating osteochondral cylinders and the effect of orientation during autologous osteochondral transplantation, we used Alicona 3D microscopy to image the surface (see below, Bauer et al, JOR). However, surface analysis beyond histological section was not planned for this protocol.



In Figure 1, indicate what is analysed from the fuji film measurements, is only the pink area analysed or is the contact area extrapolated from the pink region?
Figure 1 shows pressure measurement of the initial contact area at the metal-cartilage interface before sliding (static load). Due to the convexity of the metal cylinder and the articular surface and its elastic properties, the initial contact area is elliptical. During sliding, this initial contact area moves with a stroke of 2mm, resulting in a larger area that is exposed to mechanical loading. We changed the figure legend to be more clear. Additionally, we added a scale bar.

In Figure 2, include a legend detailing the lines, give details of the contact pressure applied and comment on whether the variability in this data is typical.
The figure legend was changed accordingly. Each coloured line represents the COF of one osteochondral cylinder (7 in total in this figure). The observed variability is within the limits for biological samples. Find below a figure for the time-dependent COF (mean±SD) with different loading conditions (from Stotter et al., JOR).



In Figure 3, please make labels B and C clearer.
The labels were moved for better visualization.

Update ISO 5832-12 to the 2019 reference
We updated to the 2019 ISO reference.

Please include a reference demonstrating the similarity between the human and bovine cartilage (lines 334-337).
A reference that demonstrates the similarity between human and bovine cartilage was introduced (Temple et al, BMC 2016).

Consider whether limitations of this method such as the use of bovine plugs rather than whole condyles which disrupts the collagen network and changes the fluid pressurisation within the cartilage should be considered.
We are aware that this protocol is not without limitations. One limitation is that this experimental set-up is not a whole joint model. During harvesting the collagen network might get damaged and the unconfined loading conditions might alter fluid pressurization. However, large sample numbers can be tested and reproducibility is high.
We included this limitation in the discussion section.

Reviewer #2:
Thank you for your detailed response. We addressed your concerns and believe that the manuscript quality improved through the changes.

Major Concerns:
Although the authors state that physiological loads and overload conditions can be simulated, very little guidance is given in the methods on how to determine the appropriate loads and speed to attain this. This is somewhat described later, but greater detail in needed in the methods as this is a very important aspect to the protocol.
The ultimate goal of this protocol is to simulate loading conditions occurring in vivo. Therefore, information on physiological and overload conditions are crucial for the reader. We provided more information in the discussion on the magnitudes of load and sliding velocity observed in healthy joints under physiological conditions (e.g. level walking) and overload situations. Ultimately, contact pressure is determined at the beginning of the experiments to confirm the desired values. For 1N of load, the contact pressure was

determined around 2MPa by visual comparison with defined contact pressures. This information was added to the protocol.

Very little is mentioned about the pros and cons of this protocol. Are there cases where this tribological experiment is not appropriate (for example, do the samples need to be a certain size)? Why were these assays picked to assess the cartilage and not others?
Thank you for this valuable feedback. We are aware that this protocol has certain pros and cons. The provided information on contact mechanics and loading conditions apply for samples with the specified dimensions. Naturally, the protocol might be adapted for larger samples with higher sliding speed and stroke length. The assays that were picked for this protocol have been established during the last years and previous studies. Over time, we have used various assays and methods. We found that gene expression is sensitive for detecting early responses in the biosynthetic activity of articular chondrocytes. We picked a selection of both anabolic and catabolic genes. For histology, we found that Safranin O staining with counterstaining provides the best information on proteoglycan content, collagen network and cellular distribution. Other stainings, like H&E or alcian staining, do not provide additional information and have certain drawbacks. With alcian staining, for example, distinguishing between different types of collagen is not possible.

Minor Concerns:
Line 56: You introduce the use of metal implants here. Can you briefly include a statement about why there is still an interest in metal implants when allograft implantation is an alternative treatment?
Certainly, allograft implantation is an alternative treatment. However, allografts are not easily available for use everywhere. Additionally, in certain countries the regulatory framework makes the use of osteochondral allografts difficult. Focal metallic implants provide an alternative treatment option that allows early weight bearing and provides fast recovery. We included a statement in the introduction section.

Line 82: You refer to provided values for the composition analysis. Can you provide these as a reference to readers?
The CoCrMo raw material was purchased by Acnis International and the provided composition values where then confirmed. The following table provides the detailed information (from Stojanovic et al, Acta Biomat). This information has also been included in the manuscript.

Elemental composition of the low-carbon CoCrMo alloy used in this study.

Element	Si	Mo	Cr	Mn	Co	C
Nominal, average wt.%	0.67	5.49	27.21	0.75	65.55	0.04
Measured, average wt.% ± SD	0.89 ± 0.17	4.90 ± 0.42	27.51 ± 0.30	0.84 ± 0.21	64.41 ± 0.61	*

* 1.45 ± 0.05 wt.% concentration of C due to contamination.

Line 88: How do you know that surface roughness is within tolerance requirements? Are you measuring this? If so, how?
The surface roughness is determined by analyzing the deviations of the real surface from its ideal form using confocal microscope. The described preparation resulted in a final Ra value of 15 ± 2 nm for the CoCrMo samples.

Line 95: You may want to add a note here to be careful not to damage the surface as this is very easy to do.
We included a note that highlights the importance of caution dissection.

Line 100: While you specify that plugs are obtained from the weight-bearing region of the condyle, a comment on regional differences in tribological properties would be appropriate.
We included a corresponding paragraph in the discussion that describes regional differences in cartilage thickness, collagen architecture and resulting tribological properties over the articular surface.

Line 113: Can you approximate how many plugs can be obtained from the condyle (or weight-bearing region)?
Typically, 9 to 12 osteochondral cylinder can be harvested from the weight bearing area on the medial femoral condyle.

Line 123: Is this tribometer purchased or made in-house? Although the video will show the tribometer, a schematic of the apparatus would be helpful here.
We used a commercially available tribometer for our experiments. As the video will show the experimental set-up and tribometer, we decided not to include a schematic of the apparatus in the manuscript.

Line 139: How does one determine stroke length? This is just another example of a parameter that can be adjusted, but it is unclear how it impacts the experiment.
The stroke length can be set like the other parameters. The stroke length of the reciprocating motion must be set according to the contact area to create a migrating contact area (MCA). For plugs with 8mm in diameter, a 2 mm stroke allows adequate rehydration of the cartilage. We included a note in the protocol.

Line 148: Are the plugs that are stored after tribology then used for the following assays? It's unclear and sometimes it appears that different plugs are used entirely for different parts of the protocol. Please specify what sample gets stored and used for which assay.
All the plugs that are tested in the tribometer are subsequently analysed. In parallel, free-swelling controls are submerged in testing solution for the duration of the tests and are then analysed together with the tested samples.
We included additional information for better comprehension.

Line 168: Can you provide hematoxylin & eosin and picrosirius red staining as well? These are also very important to assessing the state of the cartilage along with safranin-O.
We could provide H&E staining and Alcian staining. However, we have found that these staining do not provide much additional information to Safranin-O staining with counterstaining.

Line 236: Prior to storing, did you determine the purity and concentration of the RNA? This is a vital step that must be included in the protocol.
Determining purity and concentration of RNA before storing is a crucial step before cDNA synthesis. We determined purity and concentration in preliminary tests when establishing this protocol. Results showed purity without DNA (checked with PCR without RT). Concentrations are naturally low. However, during PCR amplification occurs early. The cycle threshold values are normalized to the housekeeping gene GAPDH. By strictly following the protocol, purity of the RNA can be assumed.

Line 283: The description of Figure 3A appears to be incorrect. Across the surface layer, GAG content looks content. In contrast, the GAG content changes as one looks deeper into the tissue.
Figure 3 shows histological sections of osteochondral samples. A) shows a baseline sample right after harvesting without mechanical loading. Compared with the second sample (B, free-swelling control), A) shows higher GAG content throughout the tissue with small variation between the different zones.

Line 288: Why did you choose to measure gene expression instead of the actual proteins?
Gene expression levels, but not proteins, were analysed to detect early changes in the biosynthetic activity of articular chondrocytes. In addition to histological sections and metabolic activity, these assays provide comprehensive information on the effects of mechanical loading on articular cartilage.

Line 340: Boundary lubrication can be obtained with a PBS-only lubricant, but the testing parameters would need to be adjusted. Since a Stribeck curve is not being shown here, it would be good to comment on why the loads/speeds used here are representative of boundary lubrication.
The different lubrication modes of articular cartilage with different loading conditions are now discussed in a dedicated paragraph in the discussion.

Figure 1: Please add a scale bar.
We added a scale bar (2mm) and changed the figure legend accordingly.

Figure 2: Please add a legend describing the differences in colour. Why were so many cycles performed?
The legend was changed accordingly. The cycles represent the testing duration of one hour.

Figure 3: Please define the arrow in panel C.
The arrow is not part of the figure. It seems that this arrow was created during the automated PDF creating during submission.