Submission ID #: 61304
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18697768

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

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps.

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **138km**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Christoph Stotter:** Our protocol makes it possible to test the sliding of metal for orthopedic implants against articular cartilage, thereby investigating the effects of focal implants or hemiarthroplasty on the biosynthetic activity of articular chondrocytes.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Christoph Stotter:** The main advantage of this technique is that it provides a comprehensive insight into both the tribological properties and biological effects of mechanical loading in a metal-on-cartilage pairing.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Christoph Stotter:** This technique might complement the clinical findings after surgical procedures like implantation of focal metallic implants to treat an osteochondral defect of the knee or hemiarthroplasty of the hip.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Videographer: This interview statement is optional. If you don't have time, don't film it.*

Introduction of Demonstrator on Camera

1.4. **Christoph Stotter:** Demonstrating the procedure will be Bojana Simlinger, a scientist from our partner institution, the Austrian Excellence Center for Tribology, and Christoph Bauer, a post-doc from my laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Tribological Testing

2.1. Use a commercially available reciprocating tribometer with a cylinder-on-plate configuration, vertical loading capabilities, and adjustable load and sliding speed **[1]**. Additionally, a liquid cell is required to perform the tests in a lubricating solution **[2]**. Fix the osteochondral cylinders on the bottom sample holder with the marking aligned with the sliding direction **[1]**. NOTE: Text in red moved from 2.3, author says it belongs here

2.1.1. Tribometer.

2.1.2. Liquid cell.

2.3.1. Move here

2.2. Begin by determining the contact pressure in the cobalt-chromium-molybdenum-on-cartilage system using a pressure measurement film **[1]**. Place the pressure measurement film at the interface and apply a static load for 30 seconds to determine initial contact pressure, contact size, and shape **[2]**. *Videographer: This step is important!*

2.2.1. WIDE: Establishing shot of talent at the lab bench preparing to perform the contact pressure test.

2.2.2. Talent placing the pressure film at the interface and applying a static load.

2.3. ~~**[1]**.~~ Mount the cobalt-chromium-molybdenum cylinders onto the upper load cell **[2]**.

2.3.1. Talent fixing the osteochondral cylinders on the sample holder. NOTE: Move this before 2.2.1.

2.3.2. Talent mounting the CoCrMo cylinders onto the upper load cell.

2.4. Add the testing solution into the liquid cell to submerge the osteochondral cylinder and cover the metal-cartilage sliding interface **[1-TXT]**. Set testing parameters such as prescribed normal force, stroke, and sliding speed, which will be maintained throughout the test **[2]**.

2.4.1. Talent adding testing solution into the liquid cell. **TEXT: PBS with 3 g/L hyaluronic acid**

2.4.2. Talent setting the testing parameters.

2.5. Start reciprocal sliding of the metal cylinder against the articular cartilage immersed in the lubricating solution **[1]**, monitoring the coefficient of friction throughout the experiment **[2]**. *Videographer: This step is difficult and important!*

2.5.1. Talent starting the sliding.

2.5.2. COF readout during the experiment.

2.6. Terminate the experiment after the desired testing period **[1]**. Remove the osteochondral plug from the sample holder **[2]**, rinse it with PBS **[3]**, and store it in medium until further biological analysis **[4]**.

2.6.1. Talent ending the experiment.

2.6.2. Talent removing the osteochondral plug from the sample holder.

2.6.3. Talent rinsing the plug with PBS.

2.6.4. Talent storing the plug.

2.7. Keep control samples in the testing solution at room temperature for the duration of the test and analyze them together with the samples that have been exposed to mechanical loading **[1]**.

2.7.1. Talent putting a control sample in the testing solution.

3. Metabolic Activity Analysis

3.1. Place a 24-well plate on a scale and zero it **[1]**. Rinse the osteochondral plug with PBS and place it in a Petri dish **[2]**, then use a scalpel to cut the cartilage from the graft in one piece **[3]**. *Videographer: This step is difficult and important!*

3.1.1. Talent placing the plate on the scale and zeroing the scale.

3.1.2. Talent rinsing the osteochondral plug with PBS, then placing it in a Petri dish.

3.1.3. Talent cutting the cartilage from the osteochondral graft.

3.2. Bisect the cartilage in two equal pieces so that the contact area is equally distributed onto both cartilage pieces **[1]** and mince one half into approximately 1-millimeter cubed pieces. Use the second half for gene expression analysis **[2]**.

3.2.1. Talent cutting the cartilage into two pieces.

3.2.2. Talent mincing one of the cartilage pieces.

3.3. Transfer the minced cartilage into one well of the prepared 24-well plate and determine the tissue weight **[1]**. Repeat this process for each sample and add 1 milliliter of growth medium to each well of the plate **[2]**.

3.3.1. Talent placing the minced cartilage into a well in the plate.

3.3.2. Talent adding medium to the already filled wells in the plate.

3.4. Add 500 microliters of XTT solution to each well and mix **[1-TXT]**. Then, incubate the plate at 37 degrees Celsius and 5% carbon dioxide for 4 hours **[2]**.

3.4.1. Talent adding XTT solution to a few wells. **TEXT: 490 µL of XTT labelling reagent + 10 µL of activation reagent**

3.4.2. Talent putting the plate in the incubator and closing the door.

3.5. After incubation, remove the supernatant and transfer it to a 5-milliliter tube **[1]**. Extract the tetrazolium product by adding 500 microliters of DMSO to the cartilage tissue in each well **[2]** and apply continuous agitation for 1 hour at room temperature **[3]**.

3.5.1. Talent removing the supernatant from the well and placing it in the tube.

3.5.2. Talent adding DMSO to a few wells.

3.5.3. Plate agitating at room temperature.

3.6. Remove the DMSO solution and pool it with the previously collected XTT solution **[1]**. Transfer 100 microliters of each sample in triplicates to a 96-well plate **[2]**. Use a plate reader to measure the absorbance at a wavelength of 492 nanometers and a reference wavelength of 690 nanometers **[3]**. *Videographer: This step is important!*

3.6.1. Talent adding the DMSO from a well to the 5 mL tube with the XTT solution.

3.6.2. Talent adding sample to wells in a 96-well plate

3.6.3. Talent putting the plate in a plate reader and measuring absorbance.

4. Gene Expression Analysis

4.1. To isolate the RNA, mince the second half of the cartilage tissue obtained from the osteochondral plug into small pieces **[1]**. Transfer the tissue to a tube containing ceramic beads and 300 microliters of Lysis Buffer with 1% beta-mercaptoethanol **[2]**.

4.1.1. Talent mincing the second half of the cartilage.

4.1.2. Talent putting the tissue in the tube with the beads and lysis buffer.

4.2. Use a commercial lyser to homogenize the tissue, applying 6500 rpm for 20 seconds four times, with a 2-minute cooling phase after each run **[1]**. Add 20 microliters of proteinase K and 580 microliters of RNase-free water to each sample **[2]** and incubate them at 55 degrees Celsius for 30 minutes **[3]**. *Videographer: This step is difficult and important!*

4.2.1. Talent homogenizing the tissue with the lyser. NOTE: Cooling phase was added after homogenization

4.2.2. Talent adding proteinase K and water to the sample, with the proteinase K container in the shot.

4.2.3. Talent putting the samples to incubate.

4.3. Centrifuge the samples for 3 minutes at 10,000 x *g* **[1]** and transfer the supernatant to 1.5-milliliter tubes **[2]**. Add 0.5 volumes of 90% ethanol to each tube and mix **[3]**, then transfer 700 microliters of the sample to an RNA binding column in a 2-milliliter collection tube **[4]** and centrifuge it at 8,000 x *g* for 15 seconds **[5]**.

4.3.1. Talent putting the samples in the centrifuge and closing the lid.

4.3.2. Talent transferring the supernatant to a new tube.

4.3.3. Talent adding ethanol to the sample and mixing it.

4.3.4. Talent transferring some of the sample to an RNA binding column.

4.3.5. Talent placing the tube with the column in the centrifuge. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 4.5.4 and 4.6.4.*

4.4. Discard the flow-through and repeat the centrifugation step for the rest of the lysate **[1]**. Add 350 microliters of Buffer RW1 to the column **[2]**, centrifuge it at 8,000 x *g* for 15 seconds, and discard the flow-through **[3]**.

4.4.1. Talent discarding the flow through from the tube. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 4.5.4.*

4.4.2. Talent adding Buffer RW1 to the column, with the buffer container in the shot. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 4.5.3.*

4.4.3. Talent taking the column out of the centrifuge and discarding the flow through. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 4.6.2.*

4.5. Mix 10 microliters of DNase stock solution and 70 microliters of Buffer RDD **[1]**. Add the solution to the RNA purification membrane and incubate it at room temperature for 15 minutes **[2]**. Then, add 350 microliters of Buffer RW1 to the column, centrifuge it at 8,000 x *g* for 15 seconds **[3]**, and discard the flow-through **[4]**.

4.5.1. Talent mixing the DNase stock with Buffer RDD.

4.5.2. Talent adding the mix to the RNA purification column.

4.5.3. *Use 4.4.2.*

4.5.4. *Use 4.4.1.*

4.6. Add 500 microliters of Buffer RPE to the RNA purification column **[1]** and centrifuge it at 8,000 x *g* for 15 seconds. Discard the flow-through **[2]**, then add another 500 microliters of Buffer RPE to the RNA purification column **[3]** and centrifuge it at 8,000 x *g* for 2 minutes **[4]**.

4.6.1. Talent adding buffer RPE to the column, with the buffer container in the shot. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 4.6.3.*

4.6.2. *Use 4.4.3.*

4.6.3. *Use 4.6.1.*

4.6.4. *Use 4.3.5.*

4.7. Place the column in a new 1.5-milliliter collection tube **[1]** and add 30 microliters of RNase-free water **[2]**. Centrifuge the tube at 8,000 x *g* for 1 minute to elute the RNA **[3]**.

4.7.1. Talent placing the column in a new tube.

4.7.2. Talent adding water to the column.

4.7.3. Talent putting the tube in the centrifuge and closing the lid.

4.8. Synthesize cDNA using a commercial kit. Thaw and mix all reagents, add the RNA sample **[1]**, and perform the reaction in a thermal cycler as described in the text manuscript **[2]**.

4.8.1. Talent mixing the reagents from the cDNA synthesis kit.

4.8.2. Talent putting reaction tubes in a thermal cycler and closing the lid.

4.9. Add 9 microliters of RT-qPCR master mix and 1 microliter of cDNA to each well of a 96-well plate, with each sample in triplicates **[1]**. Close the PCR plate with sealing oil and centrifuge it at 877 x *g* for 10 minutes at 4 degrees Celsius **[2]**. Perform RT-qPCR in a precision thermal cycler according to manuscript directions **[3]**. *Videographer: This step is important!*

4.9.1. Talent adding master mix and cDNA to a few wells in the plate.

4.9.2. Talent putting the sealed plate in the centrifuge and closing the lid.

4.9.3. Talent programming the thermal cycler. NOTE: Shot of PCR software screen was taken here

Results

5. Results: Tribological Testing and Analysis of Bovine Osteochondral Samples

5.1. Prior to testing, the contact area and contact pressure at the metal-cartilage interface must be confirmed using a pressure measurement film. Physiological loading condition can then be confirmed by comparing the obtained imprint with reference imprints for defined contact pressures **[1]**.

5.1.1. LAB MEDIA: Figure 1.

5.2. A low friction coefficient can be maintained for at least 1 hour with a migrating contact area **[1]**.

5.2.1. LAB MEDIA: Figure 2.

5.3. The extracellular matrix composition and structure can be determined with Safranin O staining **[1]**. The intensity of Safranin O staining is proportional to the proteoglycan content. The proteoglycan content varies over the articular surface but should be uniform throughout the tissue section in baseline samples **[2]**.

5.3.1. LAB MEDIA: Figure 3.

5.3.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize A.*

5.4. Control samples submerged in the testing solution show extraction of glycosaminoglycans, which can be counteracted by mechanical loading **[1]**.

5.4.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize B and C.*

5.5. Metabolic activity of the bovine articular chondrocytes is independent of the harvesting site but shows an increase with mechanical loading **[1]**.

5.5.1. LAB MEDIA: Figure 4.

5.6. The gene expression levels of cartilage-specific genes increase with physiological loading conditions **[1]**, whereas catabolic genes are upregulated with stationary contact area **[2]**.

5.6.1. LAB MEDIA: Figure 5. *Video Editor: Emphasize the graphs for COL2A1 and ACAN.*

5.6.2. LAB MEDIA: Figure 5. *Video Editor: Emphasize the graphs for COL1A1 and MMP13.*

Conclusion

6. Conclusion Interview Statements

6.1. **Christoph Stotter:** Following this procedure additional analyses can be performed, including determination of cartilage wear products, and analysis of the articular surface with scanning electron microscopy.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

6.2. **Christoph Stotter:** In a follow-up project, our study group is investigating new implant materials and designs, including 3D-printed metallic implants. Furthermore, we try to optimize lubrication of articular cartilage in various tribological pairings.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Videographer: This interview statement is optional. If you don't have time, don't film it.*