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Fabrication of decellularized cartilage-derived matrix scaffolds

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RE: Submission of manuscript

Dear Editors,

With this letter we would kindly respond to your invitation to submit a methods paper regarding the fabrication of decellularized cartilage-derived matrix scaffolds. We have previously described the chondrogenic potential of these scaffolds in our publication in “Multipotent Stromal Cells Outperform Chondrocytes on Cartilage-Derived Matrix Scaffolds” *Cartilage* 2014.

In this paper we describe the fabrication of this scaffold into detail and would like to further elaborate on some steps in the JoVE video.

The manuscript has not been previously published and is not under consideration elsewhere. All authors have read and approved all versions of the manuscript, its content, and its submission to the *JoVE*.

Thank you for your consideration. We look forward to your comments.

Sincerely yours,

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TITLE:

Fabrication of Decellularized Cartilage-derived Matrix Scaffolds

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KEYWORDS:

Decellularization, cartilage, scaffolds, osteochondral, tissue engineering, extracellular matrix

SUMMARY:

Decellularized cartilage-derived scaffolds can be used as a scaffold to guide cartilage repair and as a means to regenerate osteochondral tissue. This paper describes the decellularization process in detail and provides suggestions to use these scaffolds in *in vitro* settings.

ABSTRACT:

Osteochondral defects lack sufficient intrinsic repair capacity to regenerate functionally sound bone and cartilage tissue. To this extent, cartilage research has focused on the development of regenerative scaffolds. This article describes the development of scaffolds that are completely derived from natural cartilage extracellular matrix, coming from an equine donor. Potential applications of the scaffolds include producing allografts for cartilage repair, serving as a scaffold for osteochondral tissue engineering, and providing *in vitro* models to study tissue formation. By decellularizing the tissue, the donor cells are removed, but many of the natural bioactive cues are thought to be retained. The main advantage of using such a natural scaffold in comparison to a synthetically produced scaffold is that no further functionalization of polymers is required to drive osteochondral tissue regeneration. The cartilage-derived matrix scaffolds can be used for bone and cartilage tissue regeneration in both *in vivo* and *in vitro* settings.

INTRODUCTION:

Articular cartilage defects in the knee caused by traumatic events can lead to discomfort, and above all can have a large impact on the lives of the young and active population[1-3]. Moreover, cartilage damage at a young age may lead to a more rapid onset of osteoarthritis later in life[4]. Currently, the only salvage therapy for generalized osteoarthritis of the knee is joint replacement

surgery. As cartilage is a hypocellular, aneural, and avascular tissue, its regenerative capacity is severely limited. Therefore, regenerative medicine approaches are sought after to aid and stimulate the regenerative capacity of the native tissue. For this purpose, scaffolds are designed and used as either a cell-carrier or as an inductive material that incites differentiation and regeneration of tissue by the body's native cells[5].

Decellularized scaffolds have been widely studied within regenerative medicine[6]. It has had some success, for example, in aiding the regeneration of skin[7], abdominal structures[8], and tendons[9]. The advantage of using decellularized scaffolds is their natural origin and their capacity to retain bioactive cues that both attract and induce cell differentiation into the appropriate lineage required for tissue repair[6,10]. Moreover, since extracellular matrix (ECM) is a natural biomaterial, and decellularization prevents a potential immune response by removing cellular or genetic content, issues regarding biocompatibility and biodegradability are overcome.

Cartilage-derived matrix (CDM) scaffolds have shown great chondrogenic potential in *in vitro* experiments when seeded with mesenchymal stromal cells[11]. In addition, these scaffolds have shown the potential to form bone tissue through endochondral ossification on ectopic locations in *in vivo* settings[12]. As CDM scaffolds guide the formation of both bone and cartilage tissue, these scaffolds may hold potential for osteochondral defect repair in addition to cartilage repair.

This article describes a protocol adapted from Yang *et al.* (2010)[13] for the production of decellularized CDM scaffolds from equine stifle cartilage. These scaffolds are rich in collagen type II and devoid of cells, and do not contain any glycosaminoglycans (GAGs) after decellularization. Both *in vitro* and *in vivo* experiments on (osteo)chondral defect repair can be conducted using these scaffolds.

PROTOCOL:

For this protocol, equine stifle cartilage was obtained from horses that had died from other causes than osteoarthritis. Tissue was obtained with permission of the owners, in line with the institutional ethical regulations.

Note: This protocol describes the fabrication of scaffolds from decellularized equine cartilage, which can be used for applications such as *in vitro* tissue culture platforms or for *in vivo* implantation in regenerative medicine strategies. The enzymatic treatment steps must be performed in the described chronological order.

1. Harvesting of Articular Cartilage from Donor (Cadaveric) Joints

1.1. Ahead of the harvesting step, prepare 1 L of cartilage washing solution, consisting of sterile phosphate-buffered saline (PBS), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% (v/v) Amphotericin B.

1.2. Wear gloves and a lab coat during the entire harvesting procedure, since the donor can carry pathogens.

1.3. Obtain an intact (cadaveric) joint for cartilage isolation.

Note: At this point, the skin around the joint is still intact. Equine stifle cartilage obtained from horse was used in this protocol, but other joints from other species can also be used.

1.4. Remove the skin that is located around the joint area of interest by using a scalpel and a surgical tweezer. In addition, remove excessive fat and muscle tissue if necessary, without damaging the underlying joint. Optionally, transfer the prepared cadaver to a biological safety cabinet.

1.5. To perform an arthrotomy, *i.e.*, a separation of the joint, first define the location where the joint articulates by performing extension and flexion of the joint.

1.6. Carefully remove more layers of fat, muscles, and tendons with a scalpel and surgical tweezer to open up the joint area.

1.7. Make an incision to reach the joint cavity.

Note: The joint cavity is filled with synovial fluid, which can drip from the cavity when performing a correct incision.

1.8. Continue to open up the joint completely by cutting through the tendons that keep the joint together.

1.9. Inspect the cartilage for any macroscopic damage.

Note: If the articular cartilage does not have a glossy and smooth appearance or if evident blistering, clefts, or defects are present, discard the cartilage.

1.10. Use a sterile scalpel to remove the cartilage from the subchondral bone. Cut all the way down to the subchondral bone to also remove the deep zone of the cartilage (**Figure 1**). To prevent the cartilage from drying out, regularly drip cartilage washing solution on the cartilage.

Note: At this time, the size of the cartilage slices does not matter.

1.11. Collect the cartilage slices in 50 mL tubes containing previously prepared cartilage washing solution (**Figure 2A**).

1.12. After collecting the cartilage from all the areas of interest, snap freeze the cartilage slices in liquid nitrogen for 5 min.

1.13. Transfer the cartilage slices into 50 mL tubes and immediately lyophilize the cartilage slices for 24 h in a freeze dryer. Set the freeze dryer at approximately 0.090 mbar while the ice condenser is -51 °C (**Figure 2B**).

1.14. Store the cartilage slices in a dry place at room temperature until further use.

Note: Up to the point of scaffold formation, the solutions and cartilage do not have to be prepared and treated in a sterile way.

2. Creating Decellularized Cartilage Particles

2.1. Submerse the lyophilized cartilage slices in liquid nitrogen.

2.2. Directly grind the samples either manually or by a milling machine. When grinding the cartilage slices by hand, use a mortar and pestle and grind the slices for approximately 45 min until they are pulverized (**Figures 2C** and **2D**). When grinding automatically, use a milling machine at its pre-set speed for a few seconds up to a minute, until the snap-frozen cartilage slices are pulverized.

Note: Pre-cool the mortar or the grinding compartment of the milling machine by adding liquid nitrogen. When using a milling machine, make sure that all particles are ground, and that no particles stay in the bottom of the grinding compartment.

2.3. Sieve the cartilage particles to get rid of larger parts by using a sieve with a 0.71 mm mesh size.

2.4. Store the particles in a dry place at room temperature.

3. Enzymatic Decellularization with Trypsin 0.25%-EDTA

3.1. Prepare the digestion solution, consisting of 0.25% trypsin with EDTA supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% (v/v) Amphotericin B. Store the solution at 4 °C.

Note: Trypin is a protease that degrades proteins residing in the cartilage tissue. This enzymatic step will open up the dense cartilage structure.

3.2. Fill the 50 mL tubes with the pulverized cartilage particles up to a volume of approximately 7.5 mL per tube.

3.3. Incubate the cartilage particles with the prepared digestion solution at 37 °C for 24 h in total, while refreshing the digestion solution every 4 h.

3.3.1. First, add 30 mL of the digestion solution to each 50 mL tube that contains cartilage particles.

3.3.2. Then, resuspend the particles by using a vortex or pipet so that all particles are exposed to the enzymatic solution.

3.3.3. Incubate the samples for 4 h at 37 °C on a roller.

3.3.4. To refresh the digestion solution, centrifuge the tubes for 20 min at 3113 x g to cause sedimentation of the cartilage particles. Discard the supernatant.

Note: The supernatant will become clearer with every trypsin incubation period.

3.3.5. Repeat step 3.3.1-3.3.4 until the particles have been incubated with the digestion solution for 6 cycles of 4 h.

3.4. After the final cycle, remove the supernatant after centrifuging and wash the particles in 30 mL of cartilage washing solution twice. Centrifuge the particles for 20 min at 3113 x g between each of the washes.

4. Enzymatic Decellularization with Nuclease Treatment

4.1. Prepare a 10 mM Tris-HCl solution at pH 7.5 in deionized water.

4.2. Add 50 U/mL deoxyribonuclease and 1 U/mL ribonuclease A to the Tris-HCl buffer, to obtain the nuclease solution.

Note: This step is performed to specifically degrade deoxyribonucleases and ribonucleases.

4.3. To remove the cartilage washing solution from the cartilage particles (step 3.4), centrifuge for 20 min at 3113 x g, and remove the supernatant.

4.4. Add 30 mL of the nuclease solution to the cartilage particles and stir the particles through the solution, making sure that all particles are exposed to the nuclease solution.

4.5. Incubate the samples on a roller for 4 h at 37 °C.

4.6. Remove the nuclease solution by centrifuging the cartilage particles for 20 min at 3113 x g and discard the supernatant.

4.7. Wash the samples by adding 30 mL of 10 mM Tris-HCl solution without the deoxyribonuclease and ribonuclease A to the cartilage particles. Resuspend the particles and leave the samples on a roller for 20 h at room temperature.

5. Detergent Decellularization

5.1. Make the detergent solution by dissolving 1% (v/v) octoxynol-1 in PBS.

5.2. Remove the Tris-HCl solution from the cartilage particles by centrifuging for 20 min at 3113 x g and discarding the supernatant.

5.3. Add 30 mL of the prepared 1% detergent solution to the cartilage particles. Resuspend the cartilage particles gently to avoid foaming of the solution.

Note: This step breaks down cellular membranes.

5.4. Incubate the samples on a roller for 24 h at room temperature on a roller.

5.5. Remove the detergent solution by centrifugation for 20 min at 3113 x g and discard the supernatant.

5.6. To remove all of the remnants of the decellularization solutions, wash the cartilage particles in 6 cycles of 8 h in 30 mL of cartilage washing solution. Perform washing on a roller at room temperature.

5.6.1. To change to the next wash, centrifuge the suspension for 20 min at 3113 x g, discard the supernatant and add fresh cartilage washing solution.

5.7. Leave the last wash in the tubes and store the decellularized cartilage particles at -80 °C.

6. Creating Scaffolds from the Decellularized Particles

6.1. If the particles have been stored at -80 °C, thaw the closed tubes that contain the cartilage particles in warm water before creating the scaffolds.

6.2. Transfer the cartilage particles with a small ladle to a cylindrical mold, for example, a plastic vial with a diameter of 8 mm and a height of 2 cm.

6.3. When placing the cartilage particles into the plastic mold, press all the air-bubbles out to avoid cavities in the scaffold, and fill the mold until the edge.

Note: It will be more difficult to take the scaffolds out of a metal mold after scaffold formation, which can lead to cracks in the scaffold.

6.4. Freeze the molds with cartilage particles for 10 min at -20 °C.

6.5. Lyophilize the cartilage scaffolds within their molds for 24 h in a freeze dryer.

6.6. After lyophilization, take the scaffold out of the mold (**Figure 3**) and cross-link them with ultraviolet (UV) light at 30 cm distance and 365 nm overnight.

6.7. In order to use the scaffolds for *in vitro* cell culture or *in vivo* implantation, sterilize the scaffolds with, for example, ethylene oxide (EtO) gas.

Note: EtO sterilization is performed by an external party.

7. Characterization of the Decellularized Scaffolds with Histological Stainings

Note: To ensure complete decellularization and to visualize the remaining natural character of the cartilage, perform several histological stainings before using the scaffolds in any experiment, including hematoxylin and eosin (H&E) staining to ensure decellularization, Safranin-O staining to visualize residual GAG presence, collagen type I immunohistochemistry to differentiate between collagen content, and collagen type II immunohistochemistry to differentiate between collagen content.

7.1. Cut the scaffolds in thin slices of approximately 3 mm with a scalpel.

Note: If the scaffolds are cut in larger or smaller sizes, the durations of the dehydration cycles need to be adapted.

7.2. Embed the scaffolds in a drop of 4% (w/v) alginate and induce cross-linking by adding a similar volume of 3.7% non-buffered formalin that contains 20 mM $CaCl_2$.

Note: Alginate embedding makes the scaffold slices more rigid compared to the washing steps prior to paraffin embedding. In case the scaffolds have been cell-seeded or *in vivo* implanted, alginate embedding is not necessary, as the composition of the scaffolds will be resistant enough due to ECM incorporation by the cells.

7.3. Dehydrate the samples by placing them in a graded ethanol series, starting with one-hour cycles of 70%, 96%, 96%, 100%, and 100% ethanol, followed by 2 one-hour cycles of xylene, and ending with 2 one-hour cycles of paraffin at 60 °C.

7.4. After dehydration, paraffin-embed the samples in a mold and cool the samples down.

7.5. Cut the samples with a microtome in slices of 5 μm thick.

7.6. Before staining, rehydrate the samples by placing them in a returned graded ethanol series. Start with two washes of xylene for 5 min, followed by 2 washes of 100%, 95%, and 70% ethanol for 3 min per wash. Finally, wash the samples 3 times in water for 2 min.

Note: In case of using alginate to process samples for paraffin embedding, make sure to wash off the alginate with 10 mM citric acid prior to rehydration of the paraffin sections.

7.7. Stain the samples with hematoxylin and eosin, Safranin-O, collagen I, and collagen II as previous described[11].

8. Characterization of the Decellularized Scaffolds with Quantitative Analyses

8.1. Obtain papain digests of the scaffolds as described previously[11].

8.2. Perform an assay with a fluorescence-based DNA quantification kit to measure the double-strand DNA content of the scaffolds to ensure complete decellularization. Follow the protocol provided by the manufacturer. Express the amount of DNA per dry weight of the scaffold.

8.3. Perform a dimethylmethylene blue assay to quantify the remainder of the GAGs within the scaffold, as described previously[11]. Express the amount in GAG per DNA.

9. Seeding of the Decellularized Scaffolds

9.1. Cut sterilized scaffolds from step 6.7 into 3 mm thick slices.

9.2. Transfer the scaffolds in separate wells of a 6-well plate.

9.3. Rehydrate scaffolds with cell culture medium by pipetting 1 mL of medium on top of the scaffold and let it soak for 30 min. Use either chondrocyte or mesenchymal stem cell (MSC) expansion medium.

Note: Use the same medium for scaffold rehydration as for cell culture of the cells that will be seeded. Chondrocyte expansion medium consists of Dulbecco's modified media (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL fibroblast growth factor-2 (FGF-2). MSC expansion medium consists of minimum essential medium alpha (α-MEM) with 10% heat-inactivated FBS, 0.2 mM L-ascorbic acid 2-phosphate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL FGF-2.

9.4. Prepare 3×10^6 cells in 100 μL of medium, which is the required volume for each scaffold with a diameter of 8 mm and a height of 3 mm.

Note: Multiple cell types from different species can be used for seeding. When using chondrocytes or mesenchymal stromal cells, isolate the cells as previously described[11]. When using chondrocytes, make sure that they have not been expanded past the P1 passage in order to minimize the number of dedifferentiated chondrocytes. When using mesenchymal stromal cells, they must be tested on their ability for multi-lineage differentiation, as previously described[14].

9.5. Pipet 50 μL of prepared cell suspension on top of the pre-soaked scaffold and incubate the scaffold for 1 h at 37 °C.

9.6. Carefully turn the scaffold upside down, pipet the remaining 50 µL of cell suspension on this side of the scaffold and incubate for 1 h at 37 °C.

9.7. After incubation, add 3 mL of medium to the wells, and culture the cell-seeded scaffolds at 37 °C. Handle the culture plate gently to avoid detachment of the cells.

9.8. Culture the scaffolds for the period of time that is required for the experiment and change the medium 2-3 times a week. Pipet slowly and as far away from the scaffold as possible.

9.9. After culturing of the cell-seeded scaffold, cut the scaffolds in half and process them for both histology and biochemical analyses.

REPRESENTATIVE RESULTS:

Decellularization of CDM scaffolds must always be confirmed using histological stainings as well as using DNA quantification to measure the amount of DNA remnants. Insufficient decellularization might lead to undesired immunological responses that influence the results in *in vivo* settings[15-17]. For this specific decellularization method, DNA was below the detection range, which started at 57.8 ± 10 ng/mg DNA/dry weight (n = 3). Full decellularization using this protocol will lead to the production of a scaffold that is rich in collagen type II (**Figure 4C**) and has no cells (**Figure 4A**) or GAGs (**Figure 4B**). Healthy equine cartilage is displayed as comparison, stained for Safranin-O (**Figure 5A**) and collagen II (**Figure 5B**).

The scaffold must display a macroscopically homogeneous porosity. Air bubbles will lead to easily detectable large holes in the scaffold and are, therefore, carefully removed. These large holes in the scaffold may have a detrimental impact on the mechanical properties and lead to inhomogeneous cell attachment upon seeding. Successful production of the scaffold also involves a freeze-drying step lasting for at least 24 h; this will lead to a scaffold that has a white appearance (**Figure 3**). In case of insufficient lyophilization, the scaffolds will have a yellowish color and no clear pores can be observed.

In order for the scaffold to be used for *in vivo* applications and *in vitro* cell-seeding, cell integration with the scaffold, as well as cellular functionality, must be shown. Here, scaffolds were seeded with MSCs that produced ECM after 4 (**Figures 6A-D**) and 6 (**Figure 6E-H**) weeks of *in vitro* culturing. Formation of GAG, collagen II, and a peripheral collagen I, as well as the presence of cells, was shown. Additionally, the specificity of collagen II is displayed in **Figure 5B**, where cartilage but not bone is stained positive for collagen II in an osteochondral plug.

FIGURE AND TABLE LEGENDS:

Figure 1: Equine knee after removing full-thickness cartilage. The cartilage is removed from the condyles using a scalpel until the calcified cartilage layer that cannot be cut using a scalpel is reached.

Figure 2: Sequential steps in creating decellularized cartilage-derived matrix particles. (**A**) Cartilage slices that have been removed from the condyles are washed in an antibiotic-infused solution. (**B**) Cartilage slices are lyophilized, and now have a white and paper-like appearance. (**C**) Snap-freezing of the lyophilized cartilage is performed right before (**D**) pulverizing cartilage particles by hand-milling using a mortar and pestle. Step D can also be done with automatic milling. This figure has been modified from Benders *et al.*[11].

Figure 3: The final product, a decellularized cartilage-derived matrix scaffold. This cylindrical scaffold is 2 cm high and has a diameter of 8 mm. The scaffold has a clear porous structure. The left and right picture display a scaffold from two different angles. Note that no large holes are present at the surface of the scaffold as all of the air bubbles were removed prior to lyophilization. This figure has been modified from Benders *et al.*[11].

Figure 4: Histological characterization of the scaffold. (**A**) H&E staining shows ECM particles of different sizes and the absence of cells. (**B**) Safranin-O staining shows that no GAGs have been retained in the decellularization process. (**C**) Collagen type II immunolocalization reveals that the decellularized particles are rich in collagen type II. All scale bars represent 500 μm. This figure has been modified from Benders *et al.*[11].

Figure 5: Histological representation of healthy equine cartilage. (**A**) An osteochondral plug stained with Safranin-O and Fast Green shows bone without GAGs (green), cartilage with GAGs (red), and the calcified cartilage layer in between. (**B**) A collagen II-stained osteochondral plug stains cartilage but not bone. Scale bars represent 500 μm.

Figure 6: Neo-matrix formation on the scaffold after 4 and 6 weeks of culture using mesenchymal stromal cells. After 4 (**A-D**) and 6 (**E-H**) weeks of culture, newly formed matrix is rich in cells (A+E), collagen II (B+F), and GAGs (C+G), as can be observed with H & E, collagen II, and Safranin-O stainings, respectively. IN addition, collagen I is present after both 4 (D) and 6 (H) weeks in the periphery of the scaffold. Cell density as well as the amount of matrix deposition is higher at the periphery. All scale bars represent 500 μm. This figure has been modified from Benders *et al.*[11].

DISCUSSION:

The ECM of articular cartilage is very dense and quite resilient to different enzymatic treatments. The multi-step decellularization protocol described in this article addresses such resistance and successfully generates decellularized matrices. To achieve that, the process spans over several days. Many decellularization processes have been proposed for different types of tissues[18], and this article describes a protocol suitable for the decellularization of cartilage. In this protocol, it is, however, necessary to follow the enzymatic treatment with the detergent steps in order to remove all cells. The amount of DNA is diminished remarkably in the first few steps involving the treatment with trypsin, and leaving out these steps will not result in proper decellularization[11].

Note that this protocol is based on the decellularization of equine cartilage tissue. The activity of enzyme solutions used was found sufficient for the adequate removal of the equine

chondrocytes. However, despite the conservation of the matrix composition across species, the protocol may have to be adjusted for decellularization of cartilage from other animals due to the differences in the amount of naturally residing chondrocytes[19]. For example, cartilage of smaller animals is known to have a higher cell content, and may, therefore, require a more aggressive decellularization process. A particular reason for choosing equine cartilage to create decellularized scaffolds is that equine and human cartilage show clear resemblance in thickness, cell density, and biochemical make-up[20].

To ensure a reproducible product, several assessment criteria may be important to determine whether complete decellularization has been achieved. In this protocol, both an H & E staining and biochemical quantification were used to evaluate the residual amount of DNA in the end product. Other researchers have also proposed to determine the size of the remaining DNA, with a maximum of 200 bp in length for quality control[21], or a residual DNA amount of less than 50 ng/mg dry tissue weight[18,22]. Regardless, alterations to the protocol must always be followed up with histological evaluation and quantitative assays to determine the effect of decellularization, as well as the remaining ECM products.

The main limitation of this protocol is that, the thorough decellularization procedure involving the exposure to trypsin leads to extensive loss of GAGs. Even though trypsin does not cleave GAGs, the reason for the loss of GAGs can be the opening up of the cartilage tissue by trypsin cleaving proteins that anchor or encapsulate GAGs. ECM components like GAGs are important for retaining water in articular cartilage, and therefore play a significant role in the biomechanical resilience of the tissue[4]. Protocols that aim to reduce the loss of GAGs throughout the decellularization process will affect the thoroughness of the decellularization process.

After decellularization, cell integration and function have been shown in cell-seeded scaffolds. Previous research has shown that matrix production by chondrocytes on this scaffold is unsatisfactory, especially when compared to the abundant matrix deposition by mesenchymal stromal cells[11]. As cartilage-like tissue is deposited on the scaffold, this new matrix is generally first deposited in the periphery of the scaffold before invading the rest of the scaffolds. This can be clearly observed on the histological slices where a cell-rich periphery, rather than a cell-rich center, is often seen (**Figure 6**). However, this effect may be reduced when using perfusion bioreactors for cell seeding and to enhance nutrient exchange. As matrix deposition occurs, the scaffolds will also assume a glossier appearance, becoming more mechanically consistent and less brittle. As such, they can be easily cut using a scalpel without falling apart. The properties of the newly formed matrix can be evaluated using both histological stainings and quantitative assays. As no GAGs are left after the decellularization process, all of the GAGs that can be quantitatively measured will be a product of neosynthesis.

The scaffolds produced using this decellularization protocol provide an off-the-shelf solution and can be implanted without the necessity of cell-seeding prior to implantation. However, when applied as a treatment for (osteo)chondral defects, the biomechanical properties will have to be enhanced to diminish the chance of indentation of the construct in the early phases of articular loading. Co-implantation with protective layers on top, or other reinforcement strategies may

need to be implemented. In the future, further refinement of the protocol may enhance the regenerative potential of the scaffold. For instance**,** the retention of GAGs, the preservation of collagen fiber orientation, or combination with other biomaterials to reinforce these scaffolds may be beneficial to allow for an improved and smoothly regenerated articular surface. Consequently, these scaffolds may play a role as components of the next generation of regenerative grafts for the treatment of (osteo)chondral defects**.**

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

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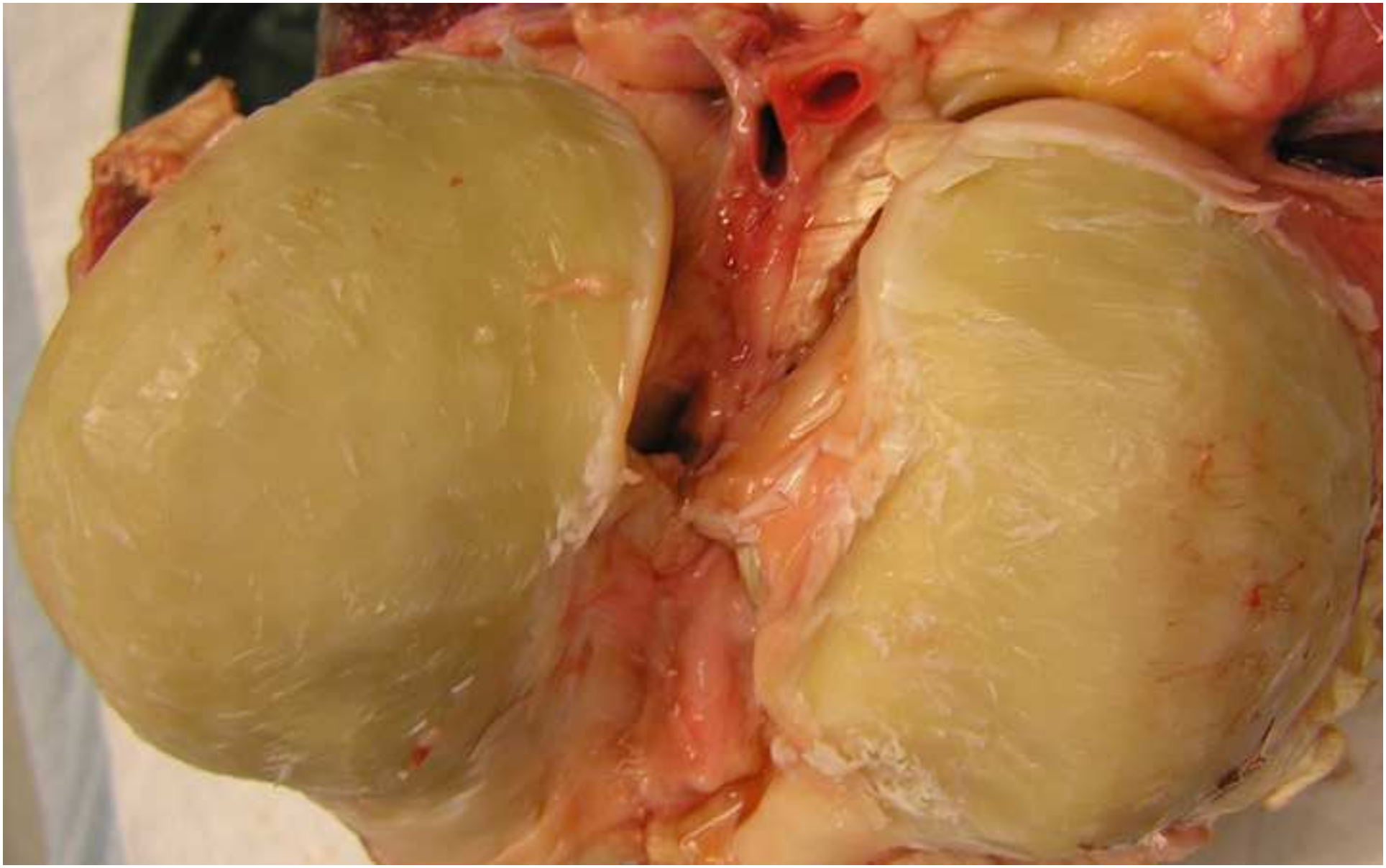

Figure 2

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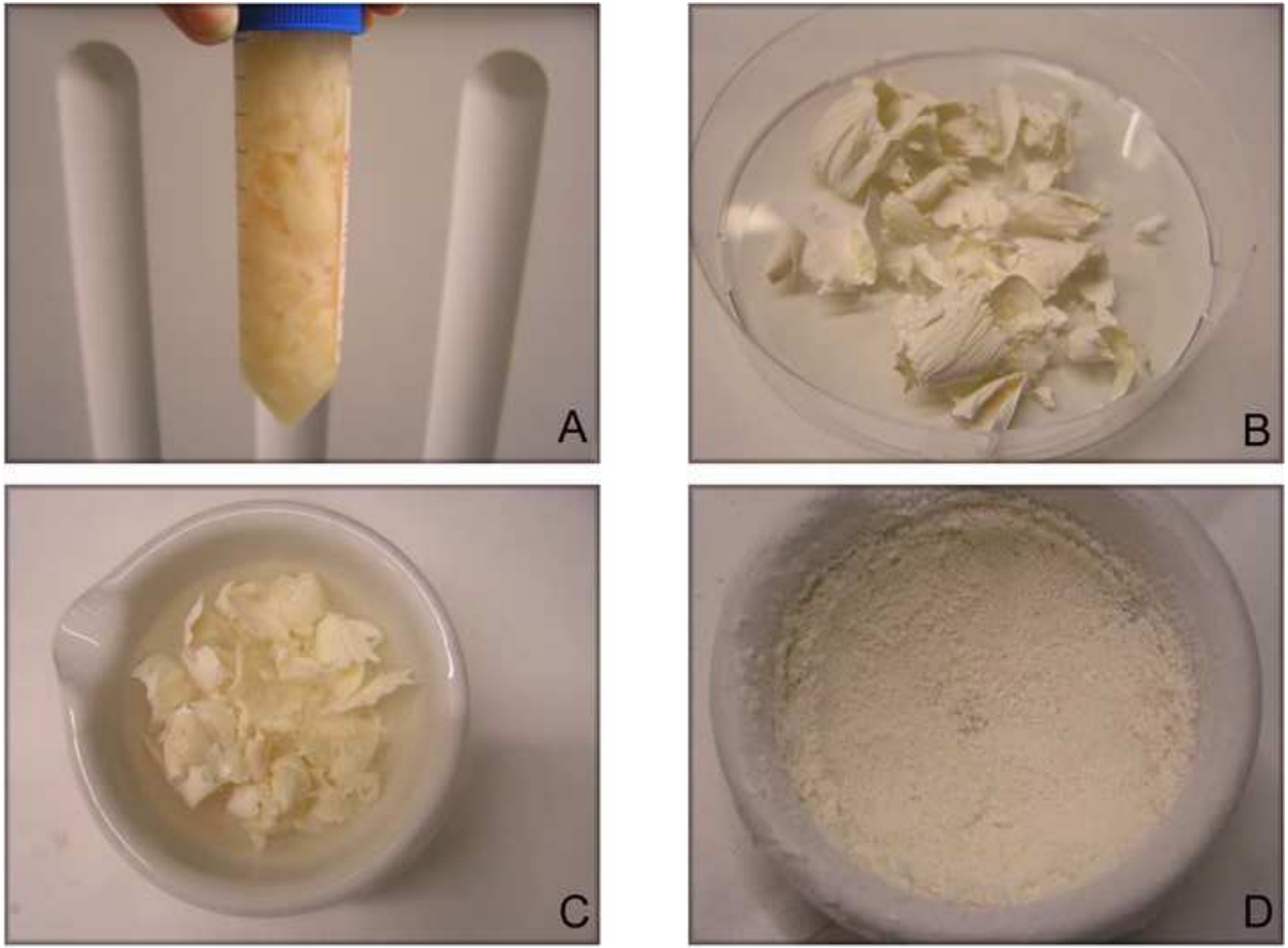

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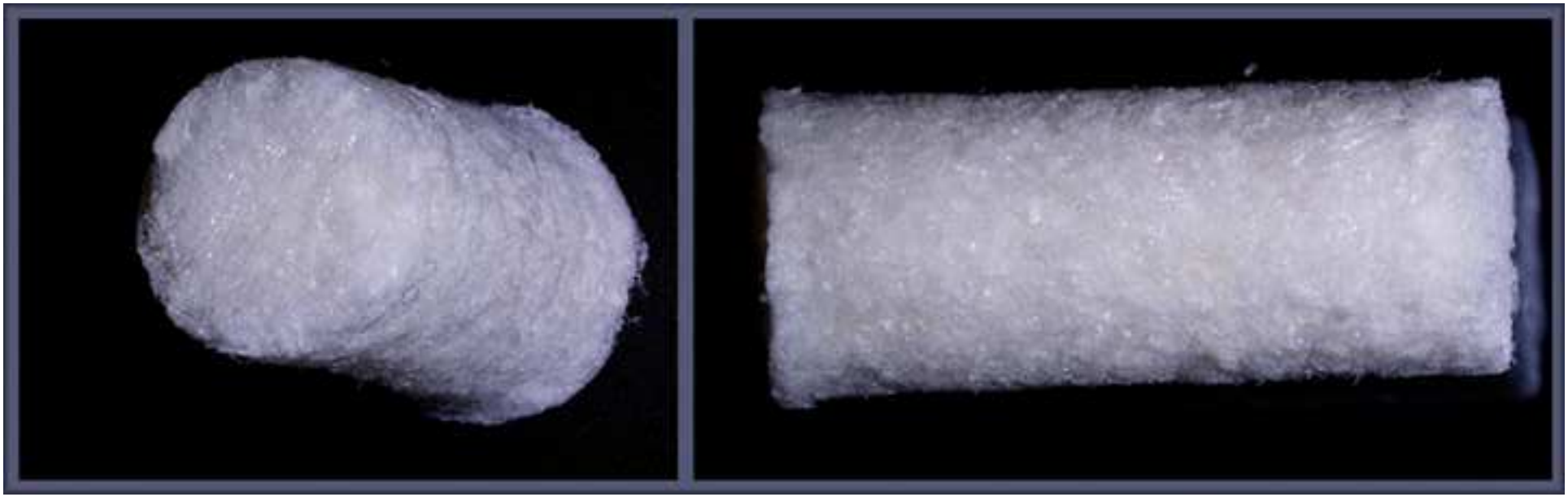

Figure 4

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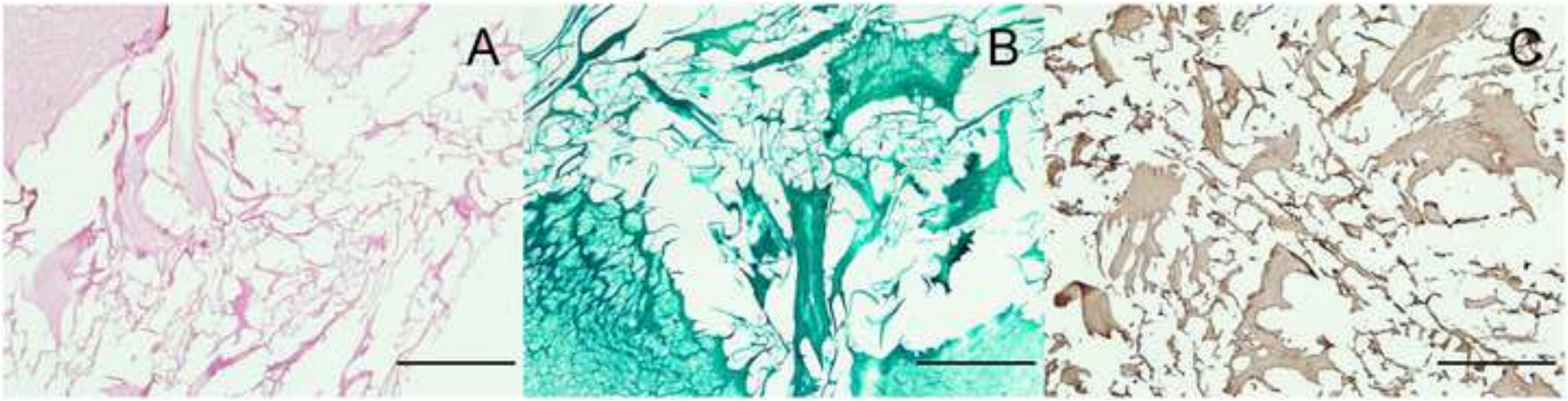

Figure 5

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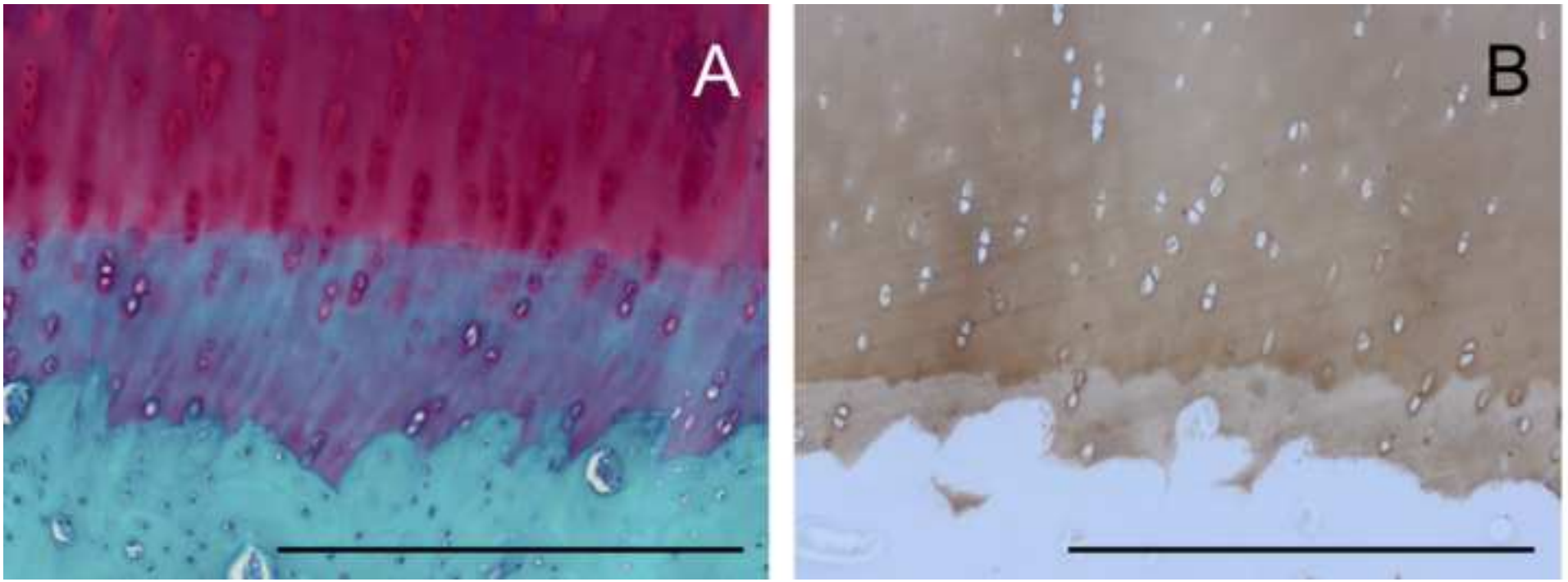

Figure 6

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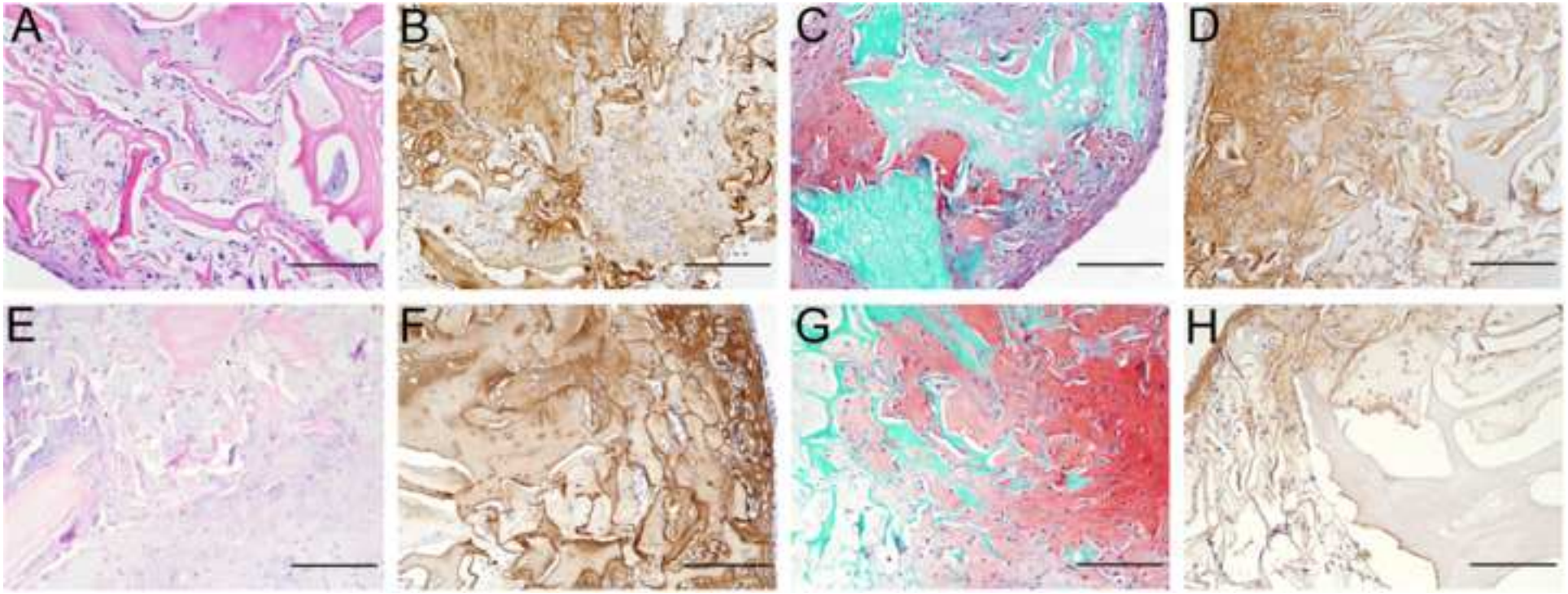

Name of Material/ Equipment	Company	Catalog Number
Cadaveric joint		
Sterile phosphate-buffered saline (PBS)		
Penicillin-Streptomycin	Gibco	15140
Amphotericin B	Thermo Fischer Scientific	15290026
Liquid nitrogen		
Trypsin-EDTA (0.25%), phenol red	Thermo Fischer Scientific	25200072
Tris-HCl pH 7.5		
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich	DN25
Ribonuclease A from bovine pancreas	Sigma-Aldrich	R6513
Triton X-100 (octoxynol-1)	Sigma-Aldrich	X100
Papain	Sigma-Aldrich	P3125
Trisodium citrate dihydrate	Sigma-Aldrich	S4641
Alginate	Sigma-Aldrich	180947
Formalin		
$CaCl_2$		
Ethanol		
Xylene		
Paraffin		
Ethylene oxide sterilization	Synergy Health, Venlo,	
Multipotent Stromal cells/chondrocytes from equine donors		
MEM alpha	Thermo Fischer Scientific	22561
L-ascorbic acid 2-phosphate	Sigma-Aldrich	A8960
DMEM	Thermo Fischer Scientific	41965
Heat inactivated bovine serum albumin	Sigma-Aldrich	10735086001
Fibroblast growth factor-2 (FGF-2)	R & D Systems	233-FB
DNA quantification kit (Quant-iT PicoGreen	Thermo Fischer Scientific	P7581

1,9-Dimethyl-Methylene Blue zinc chloride d	Sigma-Aldrich	341088
Freeze-dryer	SALMENKIPP	ALPHA 1-2 LD
Analytical mill	IKA	A 11 basic
mortar/pestle	Haldenwanger 55/0A	
Roller plate	CAT	RM5
Centrifuge (for 50 mL tubes)	Eppendorf	5810R
Capsule (cylindric mold)	TAAB	8 mm flat
Superlight S UV	Lumatec	2001AV
Incubator		
Microtome		
Sieve (mesh size 0.71 mm)	VWR	34111229
Scalpel		
Scalpel holder		
Small laddle		

	Comments/Description
	This can be obtained as rest material from the local butcher or veterinary center.
	MSCs and chondrocytes can be isolated from donor joints that are rest material, coming from the local butcher or veterinary center.

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Author(s): KEM Benders, ML Terpstra, R Levato, J Malda

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Department:	Orthopaedics
Institution:	University Medical Center Utrecht
Article Title:	Fabrication of decellularized cartilage-derived matrix scaffolds
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Utrecht, august 22 2018

Rebuttal document for revision of manuscript JoVE58656

Dear editor and reviewers,

We would like to thank you for thoroughly peer-reviewing and providing feedback on the manuscript. The comments are very useful and have improved the quality of the manuscript. We appreciate the time, work and precision spend on peer-reviewing the manuscript. The title is 'Fabrication of decellularized cartilage-derived matrix scaffolds' and the work is produced by K.E.M. Benders, M.L. Terpstra, R. Levato, J. Malda. Below, you will find a discussion and a response for each of the comments of the editor and reviewers in **bold**. Also, the adapted sentences according to the comments are presented in *italic with inserted words in italic and underlined.*

Yours sincerely,
Prof. dr. J. Malda

Author's response to peer-review comments

Editorial comments

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The manuscript has now been thoroughly read to avoid the presence of spelling and grammar mistakes.

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Thank you for your comment. We will ask for specific permission for each used and already published figure. Since one additional figure was added, we are currently asking for new permission. We will provide you with specific permission for all figures before the publication process.

3. Figure 3: Please define the left and right panels in the figure legend.
The left and right picture has been specified as follows:
The left and right picture display a scaffold from two different angles.

4. Please provide an email address for each author.
Each author is now provided with an e-mail address in section 'AUTHORS & AFFILIATIONS'.

5. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

The centrifuge speed was changed from 4000 rpm to 3113 g for all of the five cases.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Picogreen, Triton, etc.

- **Triton is now changed to octoxynol-1.**
- **Picogreen is now refered to as:**
    - *8.2) Perform an assay with a fluorescence-based DNA quantification kit to measure the double-strand DNA content of the scaffolds to ensure complete decellularization. Follow the protocol provided by the manufacturer. Express the amount of DNA per dry weight of the scaffold.*
- **In the tabe of materials two trademark symbols have been removed.**

7. Please revise the protocol (lines 62-68, 1.2.1, 1.7, 3.4.1, etc.) to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

- **The imperative tense has been applied for the steps**
    - **1.1-1.14, 2.1-2.4, 3.2, 3.4, 5.7, 6.1-6.7, 7.1, 7.3 (some step-numbers have been changed due to adaptations to the manuscript)**
    - **In addition, extra information has been added below the steps as 'Note: …', which were added below the steps 1.3, 1.7, 1.9, 1.10, 1.14, 2.2, 3.1, 3.3.4, 4.2, 5.3, 6.3, 6.7, 7.2, 7.3, 7.7, 9.1, 9.3, 9.4.**
- **Safety procedures**
    - *1.2) Wear gloves and a lab coat during the entire harvesting procedure, since the donor can carry pathogens.*

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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.

More information was added for the following steps

- **1.2-1.8, 1.13, 2.2, 3.2, 9.3**

A reference was added to provide information for the steps

- **7.8, 8.1, 8.3, 9.4**

9. 1.1: What volume of washing solution is needed?

The following volume has been added:

1.1) Ahead of the harvesting step, prepare 1 L of cartilage washing solution…

10. 1.2: Please specify the source of knee joint and describe how to perform an arthrotomy.

Extra information is now provided regarding performing the knee section, 1.4-1.8. Also, the joint source has been clarified.

1.3) … *Note: for this protocol equine stifle cartilage was obtained from horses that had died from other causes than osteoarthritis, with permission from the owner. Beside horse stifle joints, also other joints from other species can be used in this protocol.*

11. 1.4: What container is used?
For this step, 50 ml tubes were used.
1.11) Collect the cartilage slices in 50 ml tubes containing previously prepared cartilage washing solution (Figure 2A).

12. 2.2: What can be used to sieve the cartilage particles?
The maze size has been added (0,71 mm), as well as the product information in the materials list.

13. 2.2.1: Please specify the size of the cartilage particles used in the protocol. Please specify at which temperature the particles can be stored.
Regarding to the sieve that was used in this protocol, the particles size is not larger than 0,71 mm. In addition, the storage temperature has been added:
2.4) Store the particles in a dry place at room temperature.

14. 3.5, 5.6: What volume of PBS solution is used to wash?
The volume for washing has been added, which is 30 ml for both step 3.4 (former 3.5), and 5.6.

15. 6.5: What is the source of the UV-light and what is the wavelength range of the UV-light?
The UV light that was used is now specified in the materials list. In addition, the wavelength and distance are now added.
6.6) … *cross-link them with ultraviolet (UV) light at 30 cm distance and 365 nm overnight.*

16. 6.6: Please specify how to do ethylene oxide gas sterilization.
This was performed by an external company, from which the name is stated in the materials list.

17. 7.2, 9.1: What is used to cut?
The tool for cutting was added.
7.2) Cut the scaffolds in thin slices of approximately 3 mm with a scalpel.

18. 7.3: How to cross-link using formalin?
The method for cross-linking by using formalin has been specified below:
7.3) Embed the scaffolds in a drop of 4% (w/v) alginate and induce cross-linking by adding a similar volume of 3.7% non-buffered formalin that contains 20 mM $CaCl_2$.
Note: alginate embedding makes the scaffold slices more rigid regarding to the washing steps prior to paraffin embedding. In case the scaffolds have been cell seeded or in vivo implanted, alginate embedding is not necessary, as the composition of the scaffolds will be resistant enough due to ECM incorporation by the cells.

19. 7.4: Please specify the graded ethanol series used in this step.
The procedure has been added:
7.4) Dehydrate the samples by placing them in a graded ethanol series, starting with one-hour cycles of 70%, 96%, 96%, 100% and 100% ethanol, followed by 2 one-hour cycles of xylene, and ending with 2 one-hour cycles of paraffin at 60 ℃.

20. 7.5: More details are needed regarding how to perform different stainings.
For the staining protocols as well as the assays, references are added to former protocol. This is because the scope of this current protocol focuses on a new method for scaffold formation, rather than standardized laboratory protocols. However, references (11) are added to guide the reader to the corresponding protocols.

21. 8.2: Please add more details here. How to measure DNA content?
The DNA content was measured by a DNA quantification kit Picogreen that is specified in the materials list. Since no manufacturer's names are allowed in the manuscript, the DNA assay is referred to as follows:
8.2) Perform an assay with a fluorescence-based DNA quantification kit to measure the double-strand DNA content of the scaffolds to ensure complete decellularization. Follow the protocol provided by the manufacturer. Express the amount of DNA per dry weight of the scaffold.

22. 9.3.2: Please specify the medium used in the protocol.
The medium has now been specified for this protocol.
9.3) Rehydrate scaffolds with cell culture medium by pipetting 1 ml of medium on top of the scaffold and let it soak for 30 minutes. Use either chondrocyte or MSC expansion medium.
Note: use the same medium for scaffold rehydration as was used for cell culture of the cells that will be seeded. Chondrocyte expansion medium consists of DMEM with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml fibroblast growth factor-2 (FGF-2). MSC expansion medium consists of α-MEM with 10% heat-inactivated FBS, 0.2 mM l-ascorbic acid 2-phosphate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml FGF-2.

23. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.
The reference number are now all written as superscripts, and the order of the references are organized in the chronological order.

24. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
All the references have now been adapted to the above format.

25. References: Please do not abbreviate journal titles.
Adaptations have been made to the journal names, and they are now *italic*.

26. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
In the list of materials, two trademark/registered symbols have been removed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes a process to extract cartilage extracellular matrix and form it into scaffolds.

Major Concerns:
None

Minor Concerns:
Page 6 - last line. Safranin-O staining is said to confirm that there are no proteoglycans present in the extract, however this dye only stains the anionic glycosaminoglycans and provides no information of the presence of the proteoglycan protein cores. This sentence should be modified to indicate that no glycosaminoglycans are present. This error is also in the Figure 4 legend and should be corrected.

Thank you for pointing out this scientifically important error. We have changed proteoglycans to glycosaminoglycans (GAGs) in three locations:

- **First paragraph of the 'Representative results'**
- **Description of figure 4**
- **Description of figure 6 (former figure 5)**

Page 8 - last paragraph. Explain how trypsin treatment 'leads to extensive loss of GAGs' as trypsin does not cleave glycosaminoglycan chains.
Thank you for your suggestions. In the discussion section the following explanation was inserted: *'Even though trypsin does not cleave GAGs, the reason for the GAG-loss can be due to opening up the cartilage tissue by trypsin cleaving proteins that anchor or encapsulate GAGs.'*

Reviewer #2:

Manuscript Summary:
The authors present a detailed yet straight-forward method to both decellularise cartilage tissue and fabricate scaffolds from the resulting decell material. I feel that given the detail provided in this manuscript that the protocols detailed here could be readily reproduced by any tissue engineering lab as minimal specialist apparatus is required. Copy-editing of the manuscript is required as it contains a number of spelling and grammatical errors.

I have provided some comments below, these are in order to provide further clarification to the reader and aid them in performing the protocol.

Major Concerns:
None.

Minor Concerns:
Line 70 - Clearly state that this is equine tissue used in this protocol - perhaps move part of the section from the discussion on different species (line 376) into the introduction to clarify. Due to the amount of processing involved I would assume that the protocol will work across various species.
Thank you for your suggestion. We have inserted additional information about using equine tissue:

- **Last paragraph of the introduction: '***This paper describes a protocol adapted from Yang et al. (2010)[13] for the production of decellularized CDM scaffolds from equine stifle cartilage. These scaffolds are rich in collagen type II, devoid of cells and do not contain any glycosaminoglycans after decellularization. Both in vitro and in vivo experiments on (osteo)chondral defect repair can be conducted using these scaffolds.*
- **Beginning of the protocol: point 1.3.** *'Note: for this protocol equine stifle cartilage was harvested, however, also other joints from other species can be used.'*

Line 95 - Please provide more detail on the freeze drying parameters, temperature and vacuum set-points if applicable.

Thank you for your suggestion. The following information has been added:
'Set the freeze dryer at approximately 0,090 mbar while the ice condenser is -51 ℃…'

Line 97 - Are the slices stored at room temperate or at 4 degrees / -20 degrees?
The storage conditions have been added:
'1.14) Store the cartilage slices in a dry place at room temperature until further use.'

Line 107 - "grinding automatically" can the authors please clarify what device they use here. Is this a cryo-mill system?
More details have been provided now, and the automatic grinding machine is listed in the material's list:
2.2) Directly grind the samples either manually or by a milling machine. When grinding the cartilage slices by hand, use a mortar and pestle and grind the slices for approximately 45 minutes until they are pulverized (Figure 2C-D) When grinding automatically, use a milling machine at its pre-set speed for a few seconds up to a minute, until the snap-frozen cartilage slices are pulverized.
Note: pre-cool the mortar or the milling compartment of the milling machine by adding liquid nitrogen before the cartilage slices.
Note: when using a milling machine, make sure that all particles are grinded, and no particles stay in the bottom of the grinding compartment.

Line 114 - Are the particles stored at room temperate or at 4 degrees / -20 degrees?
The storing conditions have been added:
2.3) Store the particles in a dry place at room temperature.

Line 122 - Please specify a range of mass which can be used here (per tube). I assume that if doing a large prep you spread the cartilage particles out across a number of 50ml tubes?
The following information about sample size has been added:
3.2) Fill up 50 ml tubes with the pulverized cartilage particles up to a volume of approximately 7.5 ml per tube.

Line 129 - Can the authors provide more information as to what piece of equipment was used to vigorously agitate the sample, was it a vortex/shaker device?
Thank you for the comment. The equipment that was used is to find in the materials list. The follow specification was added in the manuscript:
3.3.3) Incubate the samples for 4 hours at 37 ℃ on a roller.

Line 151 - Is this water deionized? Perhaps re-phrase this sentence as demi-water would not be the most common term.
Demi-water has been changed into deionized water.

Line 195 - Are the cartilage particles lyophilised again or are they stored "wet"?
The sentence has been clarified:
5.7) Leave the last PBS wash in the tubes, and store the decellularized cartilage particles at -80 ℃.

Line 201 - Is a defined mass of cartilage or concentration added to each well? What gives the optimal scaffold?
The scaffolds are produced by adding as much cartilage in the mold as possible, while making sure to press all the air-bubbles out. No experiments have been done to investigate a higher particle density by applying more or less pressure. As for the protocol's decellularization steps, each tube can be filled up to approximately 7.5 ml.

3.2) Fill up 50 ml tubes with the pulverized cartilage particles up to a volume of approximately 7.5 ml per tube.

Line 214 - Please specify the UV light / lamp wavelength settings used to cross link the scaffolds. Are the scaffolds turned during the crosslinking procedure to expose all surfaces to the UV?
The UV light that was used is now specified in the materials list. In addition, the wavelength and distance are now added.
6.6) … *cross-link them with ultraviolet (UV) light at 30 cm distance and 365 nm overnight.*

Line 240 - Please state the % concentration of citric acid used here.
The citric acid concentration has been added: 10 mM: step 7.7).

Line 242 - Please state the section thickness used for slicing the samples.
The thickness of the histology slices has been added.
7.6) Cut the samples with a microtome in slices of 5 μm thick.

Line 261 - Did the authors use a DMMB kit for this assay? If so please include kit details in materials list.
In the current protocol no DMMB kit was used. Now a reference was added for performing the DMMB assay in step 8.3)
8.3) Perform a dimethylmethylene blue assay to quantify the remainder of the glycosaminoglycans (GAGs) within the scaffold, as was done before[11]. Express the amount in GAG per DNA.

Line 266 - Do the scaffolds necessary have to be cut to 3mm thick or can they be cut to whatever size is required?
Thank you for your comment. The scaffold size may have an effect on the dehydration protocol duration, as now clarified in a note. 7.2) … *Note: if the scaffolds will be cut in larger or smaller sizes, the durations of the dehydration cycles need to be adapted.*

Line 290 - Do the authors use a chondrogenic induction medium here (with TGFb3)?If so please state this.
The medium has now been specified for this protocol.
9.3) Rehydrate scaffolds with cell culture medium by pipetting 1 ml of medium on top of the scaffold and let it soak for 30 minutes. Use either chondrocyte or MSC expansion medium.
Note: use the same medium for scaffold rehydration as was used for cell culture of the cells that will be seeded. Chondrocyte expansion medium consists of DMEM with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml fibroblast growth factor-2 (FGF-2). MSC expansion medium consists of α-MEM with 10% heat-inactivated FBS, 0.2 mM l-ascorbic acid 2-phosphate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml FGF-2.

Figure 4 - Please include images of native equine cartilage with staining for H&E, Saf-o and IHC for type II collagen to further demonstrate the effect of the decell procedure in terms of removal of cells and GAG.
Thank you for your comment. Now, also a figure with healthy cartilage has been added, for the stains Safranin-O and collagen type II. The cells are also visible in the Safranin-O staining, for that the H&E staining was left out.

In addition, DNA removal is one of the key criteria that must be demonstrated in any decell protocol. The authors mention that the DNA levels are "diminished critically" but they do not state the DNA

level obtained.
Ref. 17 (Crapo et al.,) have a criteria of <50 ng dsDNA per mg ECM dry weight which is what multiple labs work towards when performing decell procedures.
Please include (either as a definitive number in the text or graphically represented in figure 4) the exact DNA per mg of DRY weight tissue after the decell procedure.
Thank you for this important comment. Regarding to this, two references have been added to state the criteria of decellularization, and DNA content is now included in the results section.
For this specific decellularization method, DNA was below the detection range, which started at 57,8 ± 10 ng/mg DNA/dry weight (n=3).

Reviewer #3:

Manuscript Summary:
The protocol is an interesting procedure for obtaining lab-made decellularized scaffold to be used in cell therapy or 3D culturing studies on cartilage cells. I think is interesting and suitable for publication.

Major Concerns:
The main concern is related with the steps used for the decellularization of the scaffolds. I think it would be interesting to state in the protocol which is the aim of every step. In addition, usually detergents are used for the breaking of cell membranes, so nuclease treatment could also be used after this, once the cell content have been released. It can be assumed that nucleases enters to the cell, but all of these should, at least be discussed in the discussion section.

The presence of collagen type II is a key feature for these scaffolds and their presence has to be carefully validated. Figure 4c should include, in my view, a negative control to demonstrate that the straining of the presented image is specific staining and not background staining.

Thank you for your comments on the manuscript. As for the explanation of protocol steps, details are now provided as stated at your related comment below. In addition, a Safranin-O and collagen II staining was now added for healthy cartilage. In the figure 5, an osteochondral plug was stained with collagen II, that shows a positive staining for cartilage, and a negative staining for bone. This confirms that the used collagen II staining does not show background, concluding that the decellularized scaffolds are not background staining.

Minor Concerns:
- A first part detailing the preparation of the solutions would help the reader
An overview of the used solutions can help the reader to understand the protocol, however, since the protocol runs over several days, it may as well confuse the reader. This is because many of the solutions need to be prepared fresh right before the consecutive steps. Therefore, we left the explanation for preparing the solutions in the original order to provide a protocol that can be followed in chronological order.

- step 2.1.2. This step could be a little more detailed. Any comments on the speed of the grilling machine?
Thank you for your comment. More details have been provided now, and the automatic grinding machine is listed in the material's list:
2.2) Directly grind the samples either manually or by a milling machine. When grinding the cartilage slices by hand, use a mortar and pestle and grind the slices for approximately 45 minutes until they

are pulverized (Figure 2C-D) When grinding automatically, use a milling machine at its pre-set speed for a few seconds up to a minute, until the snap-frozen cartilage slices are pulverized.
Note: pre-cool the mortar or the milling compartment of the milling machine by adding liquid nitrogen before the cartilage slices.
Note: when using a milling machine, make sure that all particles are grinded, and no particles stay in the bottom of the grinding compartment.

- Step 2.2 It is stated that the particles could be sieved, but nothing is stated about which particle sizes could be used for which application… It also could include some details about the procedures used for the sieving.
Now the sieve has been added to the material list, as well as the maze size (0,71 mm). Sieving was done for the purpose of removing large particles and clumps. The step has been changed like below.
2.3) Sieve the cartilage particles in order get rid of larger parts by using a sieve with a 0,71 mm mesh size.

- Related to the main concern mentioned above, it could be useful to include a brief explanation of the aim of every step (f.e. trypsin treatment is used for …)
Explanation of several steps have been added:
- **3.1) ...** *Note: trypin is a protease that degrades proteins residing in the cartilage tissue. This enzymatic step will open up the dense cartilage structure.*
- **4.2) …** *Note: this step is performed to specifically degrade deoxyribonucleases and ribonucleases.*
- **5.3) …** *Note: This step breaks down cells by breaking up membranes.*

- Step 6.1. What does it exactly mean? Suspend the particles in warm water?
This sentence has been clarified:
6.1) If the particles have been stored at -80 ℃, thaw the closed tubes that contain the cartilage particles in warm water before creating the scaffolds.

- Step 7.3 should be a little more explained (f.e. cross link with formalize containing 20 mM Ca Cl2... how exactly?)
The method for cross-linking by using formaling has been specified, as below:
7.3) Embed the scaffolds in a drop of 4% w/v alginate and induce cross-linking by adding a similar volume of 3,7% non-buffered formalin that contains 20 mM $CaCl_2$.
Note: alginate embedding makes the scaffold slices more rigid regarding to the washing steps prior to paraffin embedding. In case the scaffolds have been cell seeded or in vivo implanted, alginate embedding is not necessary, as the composition of the scaffolds will be resistant enough due to ECM incorporation by the cells.

- Step 9.2. It depends on your experiment. P1 chondrocytes would be good for some applications with these scaffolds.
P1 chondrocytes can indeed be useful in some cases, however, for this specific protocol we aimed for differentiated chondrocytes, since they were compared to MSCs in a former in vitro paper (11).

- Figure 5 can be completed with a figure showing scaffolds before and after the cell seeding and culture. It is important to show how the cells have produced the glycosaminoglycans. It could also include collagen type II immunostaining or some technique to show the expression of collagen type II.

An extra figure has been added. This presents 4 and 6-week cultured scaffolds with mesenchymal stromal cells, stained for H&E, Safranin-O, collagen I and II. (figure 6)