Comparison of four methods for generating decellularized equine synovial extracellular matrix

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OBJECTIVE
To evaluate 4 methods for generating decellularized equine synovial extracellular matrix.

SAMPLE
Villous synovium harvested from the femoropatellar and medial femorotibial joints of 4 healthy adult horses < 7 years of age. Synovial samples were frozen (~80°C) until used.

PROCEDURES
Synovial samples were thawed and left untreated (control) or decellularized with 1 of 4 methods (15 samples/horse/method): incubation in 0.1% peracetic acid (PAA), incubation in 0.1% PAA twice, incubation in 1% Triton X-100 followed by incubation in DNase, and incubation in 2M NaCl followed by incubation in DNase. Control and decellularized samples were examined for residual cells, villous integrity, and collagen structure and integrity by means of histologic examination and scanning electron microscopy; cell viability was evaluated by means of culture and exclusion staining. Decellularization efficiency was assessed by testing for DNA content and DNA fragment size.

RESULTS
Incubation in PAA once preserved the synovial villous architecture, but resulted in high DNA content and retention of large (> 25,000 base pair) DNA fragments. Incubation in Triton and incubation in NaCl resulted in low DNA content and short (< 200 base pair) DNA fragments, but destroyed the synovial villous architecture. Incubation in PAA twice resulted in low DNA content and short DNA fragments while retaining the synovial villous architecture.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that of the methods evaluated, incubation in 0.1% PAA twice was the best method for generating decellularized equine synovial extracellular matrix. (Am J Vet Res 2016;77:1332–1339)

Abbreviations

| bp | Base pair |
| ECM | Extracellular matrix |
| PAA | Peracetic acid |

In recent years, there has been intense interest in the use of decellularized ECM for tissue and organ replacement and in reconstructive surgical procedures, with research focusing on both specific tissues (eg, skin, esophageal mucosa, small intestinal submucosa, and umbilical vein) and entire organs (liver, heart, and lung). The ECM is composed of molecules secreted by resident cells and provides a 3-D matrix that supports those cells while also functioning as a medium for signal transfer to and between cells, influencing their proliferation and migration. The ECM is regarded as being in a state of dynamic equilibrium that is central to normal tissue and organ development. Thus, following transplantation, decellularized ECM is readily integrated through the natural tissue turnover process.

Generating decellularized ECM requires a process that removes cells and antigenic components yet retains the 3-D structure and composition, without residual toxicity, so that the decellularized ECM can promote residence and growth of homologous cells following transplantation without causing adverse reactions. Studies have shown that the method of decellularization affects the properties of decellularized ECM. Because most ECM components are relatively conserved across mammalian species, allogenic or xenogenic transplantation of decellularized ECM is associated with a low risk of host immune responses. However, insufficient removal of cell components can result in inflammation and poor results after implantation. Therefore, authors have suggested that to minimize or avoid adverse reactions, decellularized ECMs used for transplantation should lack any visible nuclear material on histologic and scanning electron microscopic examination and should contain < 50 ng of double-stranded DNA/mg of ECM (on a dry weight basis), with all DNA fragments < 200 bp in length.

Decellularization methods that have been described range from mechanical (ie, agitation and pressure) to chemical, with chemical methods involving...
the use of various acids, bases, hypotonic solutions, hypertonic solutions, and detergents, alone or in combination.\textsuperscript{1,16} Biological agents such as enzymes have been found to incompletely remove cell fragments but are a good adjunct to other methods. There is a concern that enzyme residues may cause adverse reactions in vivo, but this is a common concern for most decellularization agents.

To date, cartilage ECM has not been found to be a satisfactory structural scaffold for repair of cartilage defects.\textsuperscript{17} Thus, interest has focused on synovial ECM as an alternative to cartilage ECM. The synovium has 2 distinct layers—an intima and subintima, with the intima consisting of a continuous layer of lining cells and the subintima consisting of blood vessels, fat cells, and fibroblasts, along with a few lymphocytes or macrophages.\textsuperscript{18} The synovial ECM consists of a fine fibrillar matrix with a few type I collagen fibers overlying a deeper layer relatively rich in type I collagen.\textsuperscript{18} Synovium plays an important role in the regeneration and degeneration of cartilage\textsuperscript{19,20} and contains relatively hardy, highly metabolic cells that readily proliferate and secrete a variety of substances such as hyaluronan, growth factors, and interleukin-1 receptor antagonist.

To our knowledge, there have been no published reports evaluating methods of generating decellularized synovial ECM. The purpose of the study reported here, therefore, was to evaluate 4 methods of generating decellularized equine synovial ECM. The 4 methods evaluated consisted of incubation in 0.1% PAA (1XPAA method), incubation in 0.1% PAA twice (2XPAA method), incubation in 1% Triton X-100 followed by incubation in DNase (Triton method), and incubation in 2M NaCl followed by incubation in DNase (NaCl method). Control and decellularized samples were examined for residual cells, villous integrity, and collagen structure and integrity by means of histologic examination and scanning electron microscopy; cell viability was evaluated by means of culture and trypan blue exclusion staining. Decellularization efficiency was assessed by testing for DNA content and DNA fragment size. We hypothesized that all 4 methods would decellularize the synovium but that the PAA methods would be superior in retaining the synovial structure.

Materials and Methods

Synovial samples

Villous synovium was aseptically harvested from the femoropatellar and medial femorotibial joints of 4 healthy adult (< 7 years old) horses that were euthanized for reasons unrelated to orthopedic problems and did not have any history or any currently apparent signs of lameness. Synovium was dissected from the underlying fat and fibrous layer of the joint capsule and placed in Dulbecco modified Eagle medium\textsuperscript{4} for transport. The joints were macroscopically inspected for any abnormalities, including but not restricted to abnormal synovial fluid, thickened or inflamed synovium, hemorrhage in or around the joint, and visible cartilage damage. Any joints with abnormalities were excluded as sources of synovium. For joints without abnormalities, all villous synovium that could be obtained was harvested.

Synovium was pooled by horse and divided under a dissecting microscope into 1 X 1-cm sheets. Sheets were then cut into standard discs with an 8-mm biopsy punch. Thickness of the individual discs ranged from 5 to 7 mm.

All synovial samples from each horse were randomly assigned to a control group (no processing) or to 1 of the 4 decellularization methods such that at least 15 samples from each horse were allotted to each of the 5 treatment groups (minimum of 75 synovial samples/horse). Synovial samples were frozen at -80°C in an ethanol container with no medium and used within 1 month. Extra samples were maintained at -80°C and, if needed as a replacement, were used within 2 months.

Decellularization of synovial samples

Synovial samples were decellularized with 1 of 4 methods. The 1XPAA method consisted of incubation at 37°C in 0.1% PAA and 4% ethanol for 6 hours with mechanical agitation, as described.\textsuperscript{22} Samples were then washed twice (15 minutes each time) with PBS (0.9% NaCl) solution and twice (15 minutes each time) with deionized water. For the 2XPAA method, samples were incubated and washed as described for the 1XPAA, and the incubation and washing steps were then repeated. For the Triton method,\textsuperscript{23} samples were incubated at 37°C in 1% Triton X-100 for 24 hours with mechanical agitation. They were then washed as described for the 1XPAA method, incubated overnight at 37°C in PBS solution containing 70 U of DNase/mL, and washed a final time. They were then sterilized by incubating them at 37°C in 0.2% PAA for 2 hours. For the NaCl method, samples were treated as described for the Triton method, except that 2M NaCl was substituted for Triton X-100.

Following decellularization, synovial samples were cut with an 8-mm biopsy punch, frozen at -80°C in an ethanol container with no medium and examined within 1 month.

Characterization of decellularized synovial samples

Histologic examination—Control and decellularized synovial samples (3 samples/horse/treatment group) were thawed and then fixed in neutral-buffered 10% formalin and sectioned at a thickness of 8 μm. Sections were stained with H&E or picrosirius red and examined in a blinded fashion by two of the authors (NAR and ALB) working together to assign scores for histologic appearance. For sections stained with H&E, scores ranging from 0 to 4 were assigned for villous integrity (0 = villi not clearly identifiable on the synovial surface; 1 = villi clearly identifiable...
along 25% to 50% of the synovial surface; 2 = villi clearly identifiable along 50% to 75% of the synovial surface; 3 = villi clearly identifiable along > 75% of the synovial surface; and 4 = villi clearly identifiable along the entire extent of the synovial surface) and cell loss (0 = cell nuclei found in > 75% of the outer layer of the synovial surface; 1 = cell nuclei found in 50% to 75% of the outer layer; 2 = cell nuclei found in 25% to 50% of the outer layer; 3 = cell nuclei found in < 25% of the outer layer; and 4 = cell nuclei not found in the outer layer). Sections stained with picrosirius red were examined under polarized light and scored on a scale from 0 to 4 for collagen structure and integrity (0 = < 25% of collagen [pink fibrils] clearly intact and aligned; 1 = 25% to 50% of collagen clearly intact and aligned; 2 = 50% to 75% of collagen clearly intact and aligned; 3 = > 75% of collagen clearly intact and aligned; and 4 = all collagen clearly intact and aligned).

Scanning electron microscopy—Control and decellularized synovial samples (3 samples/horse/treatment group) were thawed and fixed for 24 hours at 4°C in 2.5% glutaraldehyde in 0.1M PBS solution with 0.1M sucrose. Samples were then postfixed for 1 hour at 22°C with 100% osmium tetroxide, washed twice in PBS solution, and dehydrated with graded ethanol solutions (50%, 70%, 80%, 95%, and 100%). Follow-
ing the dehydration process, samples were dried with hexamethyldisilazane and sputter-coated with a gold-palladium microlayer. Samples were then examined by two of the authors (NAR and ALB) working together using a scanning electron microscope equipped with a field-emission gun electron source. Surface topography was evaluated subjectively, and villi were reported to be present or absent.

**Evaluation of cell viability**—Control and decellularized synovial samples (3 samples/horse/treatment group) were thawed and placed in 6-mm inserts designed to fit into standard 12-well culture plates and were incubated for 3 days at 37°C in minimum essential medium α containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin (250 ng/mL). On day 3, wells were examined with a microscope by one of the authors (NAR), and cells adhered to the plate or growing from synovial samples were counted, with results verified by another author (ALB) working independently who was blinded to treatment group.

Synovial samples were then digested at 37°C for 5 hours in 0.02% collagenase in Hank balanced salt solution. Cells were filtered through a 70-µm cell strainer, stained with trypan blue, and counted by one of the authors (NAR). Cell viability was calculated as the percentage of cells that remained unstained.

**Quantification of DNA content**—A commercial kit was used to extract DNA from 5-mg (wet weight) portions of control and decellularized synovial samples (3 samples/horse/treatment group). Samples were first fully pulverized in lysis buffer and proteinase K; all subsequent steps were performed according to the manufacturer’s instructions. A spectrophotometer was then used to determine DNA content of each of the samples.

**Determination of DNA fragment size**—For determination of DNA fragment size, DNA was extracted from control and decellularized synovial samples (3 samples/horse/treatment group) as described for quantification of DNA content. Purified DNA (100 ng/10 µL) from each sample was then separated by means of gel electrophoresis on a 3% agarose gel with 0.5% ethidium bromide. Gels were examined by means of UV transillumination, and fragment size was compared with a reference (200 to 25,000 bp) ladder.

**Figure 2**—Photomicrographs of equine synovial samples that were untreated (control; first row) or decellularized with the 1XPAA (second row), 2XPAA (third row), Triton (fourth row), or NaCl (fifth row) method and stained with H&E (left column) or picrosirius red (right column) stain. Notice that with the 1XPAA method, the villous architecture was retained, but cellular debris (nuclear fragments, pyknotic cells, and pyknotic nuclei; arrow) was present on the synovial surface, whereas with the 2XPAA method, the villous architecture was retained and no cellular debris was seen (arrow). With the Triton and NaCl methods, no synovial villi were distinguishable, and only collagen bundles were seen. H&E and picrosirius red stains; bars = 20 µm.

**Figure 3**—Mean ± SEM histologic scores for cell loss (black bars), villous integrity (gray bars), and collagen structure and integrity (striped bars) for synovial samples from 4 horses that were untreated (control) or were decellularized with 1 of 4 decellularization methods (3 samples/horse/treatment group).
Statistical analysis

Numerical data (ie, histologic scores for villous integrity, percentage cell viability, and DNA content) were analyzed by means of the Shapiro-Wilk method to determine whether they were normally distributed. Two-factor ANOVA was performed to determine whether values varied with horse or treatment group. However, because horse was found to not be a significant factor, data were reanalyzed by means of 1-way ANOVA followed by the Tukey test. All analyses were performed with standard software; values of \( P < 0.05 \) were considered significant. Data are expressed as mean ± SEM.

Results

Cell viability

Examination of frozen and then thawed control synovial samples following incubation for 3 days revealed cells adhered to the plate but there was no cell growth, and all cells that were present stained with trypan blue, indicating that they were not viable. For all 4 decellularization methods, no residual intact cells or cell growth was seen after incubation for 3 days.

Histologic examination

Histologic examination of control synovial samples revealed a lining layer of synoviocytes that was 1 to 2 cell layers thick, a central arteriole and venule in loose connective tissue consisting of fat cells, and a fine fibrillar matrix in the interstitium (Figure 1). Synovial samples decellularized with the 1XPAA and 2XPAA methods generally showed retention of villous integrity (Figure 2), whereas there was a loss of villous integrity in samples decellularized with the Triton and NaCl methods. In addition, cellular debris was seen on the surface of synovial samples decellularized with the 1XPAA method.

Villous integrity score was significantly \( (P < 0.01) \) higher for the control, 1XPAA, and 2XPAA groups than for the Triton and NaCl groups (Figure 3). Similarly, collagen structure and integrity scores for the control, 1XPAA, and 2XPAA groups were significantly \( (P < 0.001) \) higher than scores for the Triton and NaCl groups. On the other hand, all 4 decellularization methods resulted in substantial cell loss, with cell loss scores for all 4 decellularization groups significantly \( (P < 0.001) \) higher than cell loss scores for the control group.

Scanning electron microscopy

Scanning electron microscopy confirmed that the villous structure was maintained in synovial samples decellularized with the 1XPAA and 2XPAA methods, but was lost in synovial samples decellularized with the Triton and NaCl methods (Figure 4).

DNA content

Mean DNA content was significantly \( (P < 0.001) \) higher for the control and 1XPAA groups than for the 2XPAA, Triton, and NaCl groups (Figure 5).
DNA fragment size

Large (> 25,000 bp) DNA fragments were recovered from control samples and samples decellularized with the 1XPAA method (Figure 6). In contrast, only small (< 200 bp) DNA fragments were recovered from samples decellularized with the 2XPAA, Triton, and NaCl methods.

Discussion

Results of the present study suggested that, of the 4 decellularization methods evaluated, the 2XPAA method was the best method for generating decellularized equine synovial ECM, in that it resulted in low residual cellularity and low DNA content but preserved the synovial villous architecture. In contrast, the 1XPAA method preserved the synovial villous architecture but resulted in high DNA content, and the Triton and NaCl methods resulted in low DNA content but also resulted in a loss of villous integrity.

The 1XPAA, Triton, and NaCl decellularization methods were chosen for inclusion in the present study because they have previously been used to obtain ECM from tissues that are structurally similar to synovium, with the 1XPAA method used to generate small intestinal submucosa ECM, the Triton method used to generate urinary bladder ECM, and the NaCl method used to generate ECM from the umbilical vein of human. These tissues, like synovium, have an architecture that is relatively loose and only 2 to 3 cell layers thick. In the present study, however, none of these 3 methods resulted in low residual cellularity and low DNA content while preserving the villous structure required to generate an ECM scaffold capable of supporting living cells. Our findings are in line with results of a previous study showing the importance of tailoring the decellularization method to the specific tissue of interest.

It is widely accepted that the goal when preparing ECM scaffolds is to maintain the structural matrix while retaining as many of the biological factors (eg, proteins, growth mediators, and anti-inflammatory cytokines) that promote cell adhesion and differentiation as possible, yet remove underlying molecules that may promote host reactivity. Because the 1XPAA method retained the villous synovial architecture but had residual DNA content, we elected to also test the 2XPAA method. Ongoing work in our laboratory has shown that ECM generated with the 2XPAA method and seeded with synoviocytes produced substantial proliferation and translocation of cells. Thus, retention of the synovial villous architecture appeared to be a good marker for structural integrity of decellularized synovial ECM.

Destruction of the synovial villous architecture by the Triton and NaCl methods in the present study was unanticipated because these methods have been used previously to generate ECM from tissues with architectures similar to that of synovium. Triton X-100 is one of the most widely used nonionic surfactants that permeabilize living cell membranes and that, by lysing cells, allow for the extraction of proteins and other cellular organelles. In the present study, the loss of the synovial villous architecture with the Triton method suggested that the synovial villous architecture could be dependent on lipid interactions between cells and the ECM. A similar argument could be made to explain the loss of synovial villous architecture with the NaCl method, in that 2M NaCl is known to disrupt membranes through high osmotic pressure and to cause denaturation of proteins. In contrast, both PAA methods resulted in retention of the synovial villous architecture. Peracetic acid is thought to disrupt the osmotic function of the lipoprotein cytoplasmic membrane, rupture cell
walls,27,28 and oxidize sensitive sulphydryl bonds as well as double bonds in enzymes, proteins, and other biological compounds. Presumably, these effects of PAA were sufficient to remove the necessary cell components but retain the structure of the ECM.

With the Triton and NaCl methods in the present study, both the specific chemical agent used and the treatment time may have contributed to the loss of the synovial villous architecture. Both methods involved a 36-hour processing time, whereas the 2XPAA method could be completed in 12 hours.

Importantly, the present study was not designed to evaluate the effects of various individual factors on the overall outcomes of the 4 decellularization methods. For example, it is possible that the additional wash step used in the 2XPAA method may not have been necessary, and simply using an incubation time of 12 hours might have been sufficient to obtain the same results. Further work is needed to identify the optimal method for generating decellularized synovial ECM. Nevertheless, the 2XPAA method used in the present study was easy to perform and resulted in decellularized ECM that has shown promising results in other studies performed in our laboratory.

Peracetic acid concentrations of 0.1% to 0.2% have been used in many decellularization protocols as a final wash to sterilize the ECM. In those studies, PAA sterilized the ECM but left the important structural and biological components intact, allowing for successful ingrowth of cells.29-32 Thus, an additional advantage of the 2XPAA method used in the present study is the elimination of the need for an additional sterilization step. The loss of biologically active proteins after various decellularization protocols has been studied for several tissue types,33,34 and the effect of sterilization with PAA on the biologically active protein content has been studied in some tissues.35 To our knowledge, however, the effects of PAA on biologically active protein content when used as a single agent for decellularization of synovium have not been determined.

Finally, although decellularization methods used in the present study have been shown to result in low amounts of residual chemicals, the presence of residual chemicals and their potential toxic effects were not studied. The chemicals used were chosen because of their abilities to damage cells; hence, any residue may cause cell damage or death, decreasing the ability of cells to migrate into and thrive in ECM generated with these methods.24,36 Future studies should include assays to quantify the presence of residual chemicals in decellularized samples.

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Footnotes

b. Sigma-Aldrich Corp, Steinheim, Germany.
c. Collagenase type II, Gibco, Gaithersburg, Md.
d. Nova NanoSEM 400, FEI, Hillsboro, Ore.
e. Corning Costar Transwell, Sigma-Aldrich Corp, Steinheim, Germany.
f. QIAamp DNA mini extraction kit, Qiagen, Hilden, Germany.
g. NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, Del.
h. GelDoc, Kodak Inc, Rochester, NY.
i. SPSS, version 22.1, IBM Analytics, Fullerton, Calif.

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