1-1-2016

Investigating the Antigen Removal Process of Porcine Cartilage in Preparation of Creating an Osteochondral Xenograft

Bradley Jeffery Kindred

Investigating the antigen removal process of porcine cartilage in preparation of creating an osteochondral xenograft

By

Bradley Jeffery Kindred

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biomedical Engineering
in the James Worth Bagley College of Engineering

Mississippi State, Mississippi

December 2016

Investigating the antigen removal process of porcine cartilage in preparation of creating an osteochondral xenograft

By

Bradley Jeffery Kindred

Approved:

_____
Steven H. Elder
(Major Professor/Graduate Coordinator)

_____
Chartrisa LaShan Simpson
(Committee Member)

_____
Jun Liao
(Committee Member)

_____
James Ryan Butler
(Committee Member)

_____
Jason Keith
Dean
Bagley College of Engineering

Name: Bradley Jeffery Kindred

Date of Degree: December 9, 2016

Institution: Mississippi State University

Major Field: Biomedical Engineering

Major Professor: Dr. Steven Elder

Title of Study: Investigating the antigen removal process of porcine cartilage in preparation of creating an osteochondral xenograft

Pages in Study 46

Candidate for Degree of Master of Science

With Athletes and individuals developing osteoarthritis and chondral defects at younger ages, long term treatments are in high demand. Total knee replacements only last for 10-15 years, so younger individuals would need to have multiple knee replacements within their lifetime. Allograft transplantation has shown to last long term and have high success rates, but the lack of donors and the possibility of damaging other areas of the knee to obtain tissue grafts has become a large concern. Xenografts derived from porcine cartilage is cost effective and the supply is abundant. Two antigen removal processes were examined: a short term antigen removal process to maintain the mechanical stability of the tissue, and a long antigen removal process to minimize the risk of triggering an immune response. The antigen removal processes were compared, and the future precautions were determined to enhance the probability of creating a viable osteochondral xenograft preparation technique.

DEDICATION

This thesis is dedicated to my grandfather, Keith Millikan. He has always wanted me to succeed in academics, and I know this is an accomplishment that he would be proud of. He passed away before I earned my first degree, so I would like to dedicate this accomplishment to him.

ACKNOWLEDGEMENTS

I would like to say thank you and acknowledge the funding provided by NIH NIAMS under the award number R15AR066926.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

BACKGROUND.

Osteoarthritis and osteochondral allograft transplantation

Osteoarthritis is a degenerative disease the effects 15% of the current population over 60 years of age (1). It is characterized by damage to the articular cartilage due to trauma, or gradual degeneration over time (2). The main symptoms are pain and discomfort and can cause dramatic lifestyle changes that cause people to lose their active lifestyles. Full and partial knee replacements are one major treatment that a lot of individuals, especially over the age of 60, but due to its risk of early failure, researchers have been looking for long term solutions to treating osteoarthritis (3). Osteochondral allograft transplantation has been a fairly new treatment for osteoarthritis and other cartilage and osteochondral defects (4). The allograft transplantation procedure entails taking an osteochondral plug from another donor or from a non-weight bearing portion of the patient's knee and replacing the patients damaged section of cartilage (5). Early studies have shown positive results in younger patients. One in particular showed positive results in children (ranging from 13-20 years of age) with osteochondral defects. After surgery, all of the children were able to the activities of daily living at 6 months and were able to fully return to sports after 12 months post-surgery (6). With the increasing numbers of knee pain due to damaged cartilage in younger athletes, the demand for a long-term treatment for younger individuals is also increasing. However, the lack of

donors and the fact that cartilage is taken out of a different portion of the knee raises concerns (7). Damaging one portion of the knee to repair another seems counterproductive, and finding a new donor source, such as porcine cartilage, can greatly increase the supply of viable osteochondral tissue grafts and helps eliminate the damage done to portions of the knee when samples are taken from the patient.

Preparation and uses of cartilage xenografts

Xenografts are created using tissue derived from animals other than human donors (8). Because these tissues are from another species, certain steps have to be taken in order to ensure that the tissue will survive within the host. If the Xenografts aren't prepared properly, they have the chances of triggering immune responses upon implantation or can fail mechanically over time (9). In order to prevent graft rejection, the xenograft must undergo some form of an antigen removal process. Antigens are certain molecules within a tissue that invoke an immune rejection following implantation into the patient (10). The antigens are recognized as foreign and the immune symptom labels the graft as foreign and tries to eliminate it from the body. The goal of the antigen removal process of a xenograft is to remove as much of the immunogenic material from the tissue while still keeping the extracellular matrix (ECM) fully intact (11). Keeping the ECM intact not only allows the tissue to function mechanically upon implantation, but a functional ECM also allows for efficient cell seeding, cell migration, and helps the scaffold attach and grow with the body soon after implantation. Soft tissues are relatively easy to decellularize due to their low density and the ability of the detergents to flow easily in and out of the tissue (12). However, cartilage is very dense and has low porosity which prevents the flow of the detergent to reach deep within the tissue (13). In order to

allow the detergent to reach deep within the tissue, the cartilage tissue has to be exposed to the detergent for extended amounts of time (14). Although this will better decellularize the tissue, it can have detrimental effects on the ECM of the tissue (15). Currently, there are no other materials that can match the mechanical properties of native cartilage. Because it is a weight bearing tissue, the mechanical properties of the implanted scaffold are vital in the long term success of the xenograft. That is why it is important to find an efficient antigen removal process that not only removes all of the immunogenic components of the tissue so that the tissue isn't quickly rejected from the body, but also ensure long term survival by making sure the tissue is mechanically stable (16). Most decellularization techniques require a detergent to lyse the cells and wash out the cellular components of the tissue (17). Detergents such as sodium dodecyl sulfate (SDS) and Triton X-100 to disturb the cell membrane and cause cell lysis. However, these detergents have shown to negatively affect the biomechanics of the xenografts. Controlling the concentrations of the detergents and limiting the time of exposure to the detergents will help preserve the ECM (13). However, while doing so, the xenograft could be insufficient at removing all of the antigens and cause the xenograft to be rejected after implantation.

Preparation and uses of cartilage xenografts

There are a few different ways that researchers have tried to help mask and/or remove the antigens from the xenograft tissue. For the antigen removal process of cartilage, the primary detergent used is sodium dodecyl sulfate (SDS). SDS is used in low concentrations and is found to be very effective at removing most of the antigens from various different tissues, especially cartilage (11). One of the problems with using

detergents like SDS, is that although they are very good at removing the cellular components and proteins from the tissues, they do have a negative effect on the extracellular matrix of the tissue (13). SDS is known to also break down cartilage and GAG when the tissue is exposed to the detergent for a long period of time and/or when the SDS solution is in higher concentrations(>2%) (13). So, although the antigens are removed and the tissue is fully decellularized, the xenograft would fail mechanically if it were to be implanted into the host. That's one reason why researchers are also exploring other options.

One alternative used to reduce the chances of triggering an immune response is using cross-linking to make the antigens and proteins less detectable within the xenograft (18). Instead of removing the proteins from the tissue, cross-linking masks them to try and hide them from the immune system from the host. If the cross-linking is sufficient, then the body will not be able to recognize the antigens and the chances of triggering an immune response lowers (19). One advantage to cross-linking is that it does not use harsh solutions that deteriorate the extracellular matrix of the tissue scaffold. Studies have showed that after cross-linking tissue samples, the biomechanical properties were not significantly different than that of the control samples (11). However, studies also suggest that it does fully protect against immune responses after an extended period of time (20). The glutaraldehyde cross-linked samples were able to overcome hyperacute and acute rejection responses, but were still being recognized by the immune system and ultimately ended in graft failure (13).

Novel techniques and procedures need to be introduced in order to create a standard for cartilage and bone antigen removal procedures, as the current techniques

used either fully remove all of the antigens from the tissue or keep the biomechanical properties intact, but there needs to be a way to do both. If the tissue is not 100% decellularized, there will always be a chance that the body will recognize it as foreign and react accordingly. However, without a xenograft that acts and performs exactly like native tissue, the graft will fail mechanically over time. Researchers are focusing on finding ways to remove most if not all the antigens as well as keep all of the biomechanical properties intact.

Antigen removal processes for xenografts

The body has different ways of rejecting scaffolds after implantation based on different aspects of the biochemistry within the scaffold. Depending on how well the antigens are masked or removed from the tissue, the specific antigens remaining within the tissue after implantation, the cellular components that are present within the scaffold, and the quality of the immune system of the host will determine the type of rejection that occurs (21). The types of rejections are determined by the time the scaffold takes to cause a rejection response, and the rejection responses have specific characteristics that determine which response will occur.

One type of rejection is Hyperacute rejection (HAR). HAR is a rejection that happens very quickly and soon after tissue implantation within the host (22). This is particularly caused by antigen recognition within the tissue. The α-gal antigen is one of the primary antigens that triggers an immune reaction when a foreign tissue, in particular bone and cartilage, is implanted within another host (23). When the antigen removal process doesn't rid the tissue of all of the antigens, there will always be a chance to

trigger hyperacute rejection. Some studies have shown that the more extensive the antigen removal (AR) process, the less chance of triggering HAR.

Another type of rejection is acute immune rejection of the xenograft. These types of rejections typically take longer to occur (days to weeks to occur). If the HAR does not occur because the cross-linking and antigen removal processes were successful in removing the α-gal, but there are still surface proteins or cellular components that are still present within the tissue, then an acute immune rejection is likely to occur (24). Acute immune rejection is a sign that all or most of the α-gal is eliminated from the tissue, but there are still some cellular components left within the tissue, and the tissue will still fail due to immune rejection (24).

Chronic rejection of the xenograft happens more than a few weeks after implantation (25). Chronic rejection is typically characterized by activation of the T and B cells by existing major histocompatibility complex (MHC) molecules still being present within the tissue (26). MHC molecules are typically present on the surface of donor cells. MHC molecules don't trigger by being recognized by antibodies; instead, the MHC molecules are recognized by the helper T cells and B cells, and they trigger the chronic immune response that eventually ends in tissue rejection and failure (26).

A tissue that is immunologically privileged typically has little to no vascularity, nerves, and lymphatic supplies (27). When the tissue is immunologically privileged, it means that the body's immune system has a hard time immunologically regulating the specific tissue. For example, the cornea is an immunologically privileged area of the eye that contains molecular components that also help create an immune-privileged environment (28). For Cartilage, some studies have suggested that because it has low

vascularity, nerves, and lymphatic supplies that it should be immune-privileged (27). Being immune-privileged, the tissue should have a lower chance of rejection and also take longer for the body to start the rejection process. Also, it has been shown that cartilage has much lower α-gal presence than other tissues, which lowers its chances of causing HAR after implantation (29). HAR are typically caused by the presence of α-gal within the tissue and quick recognition by the host's immune system (30). So, in order to try and prevent HAR, researchers have been targeting the elimination of α-gal from bone and tissue samples.

As stated before, cartilage already has low α-gal but because of its density, it is harder to remove. With bone, there is a much higher presence of α-gal, but it is very porous which would typically mean that it would be a lot easier to remove it from the tissue (31). Also, another way that researchers are trying to avoid HAR is by using tissue derived from pigs that have been bred specifically to eliminate or drastically lower their α-gal antigens (32).

To try and avoid the acute immune rejection, a heavy decellularization process is typically used (33). Because the acute immune rejection is associated with the presence of cellular surface proteins from the donor, emphasis should be made on developing a protocol that tries to not only eliminate the antigens within the tissue but all of the cells and cellular components as well. For Cartilage, using an SDS solution to decellularize the tissue has shown promising results in eliminating most if not all of the cellular components (13).

Chronic immune rejection is most prevalent with the implantation of the cartilage xenograft (34). Cartilage has lower α-gal content and is immune-privileged, but due to

the lack or porosity makes it harder to remove all of the antigens from the tissue during decellularization. This is why an antigen removal process needs to be designed specifically for cartilage; other decellularization processes simply will not work for this type of tissue. Typically, the procedure to prevent chronic immune rejection is to fully decellularize the tissue and rid it of all of the proteins, molecules, and cellular components from the donor (35). Also, immunosuppressants have been shown to help reduce the ability of the T and B cells to recognize the tissue as foreign and start the rejection process (36). Because there are many different factors that set off different rejection pathways, there needs to be specific steps in the protocol to address each type of pathway to help ensure that the tissue will not be rejected immediately as well as months after implantation.

Recently, the studies of using a cartilage xenograft for orthopedic and osteoarthritic conditions has unlocked a lot of knowledge that had previously been unknown. There are many different ways researchers are investigating the antigen removal process, such as using detergents such as SDS, snap-freezing, and also using cross-linkers to try and hide antigens and proteins from the host's immune system. Because researchers have tried different ways of preparing xenografts, results have shown that there are various different ways the body recognizes the tissue and attempts to reject it from the body (37). If the α-gal is not sufficiently masked or eliminated from the tissue, the body will have a hyperacute response and reject the scaffold almost immediately. If the α-gal is sufficiently eliminated, but other proteins and cellular components from the host are still present, then the host's immune system will typically elicit an acute immune rejection response. For chronic immune rejection to occur, the

host's immune system, specifically the T and B cells, will recognize certain MHC molecules still being left in the tissue (26). Another factor in the antigen removal process is not only to eliminate all of the cellular components but to leave enough of the ECM intact that the xenograft won't fail mechanically once it is implanted. Some studies show that using SDS is great for decellularizing the cartilage, but it also has highly negative effects on the extracellular matrix (13). Creating a viable protocol would need to focus on eliminating the α-gal to prevent HAR, eliminating surface proteins and cellular components to prevent acute immune rejection, eliminating other MHC components that trigger chronic immune rejection, all while making sure that it will not damage the extracellular matrix of the cartilage xenograft. There is a high demand for bone and cartilage xenografts to help treat trauma and diseases such as osteoarthritis (38). There has been promising strides in identifying an antigen removal process for cartilage and bone, but there will need to be some new approaches and ideas to create an efficient antigen removal process that will work a majority of the time.

Specific aims

The specific aims of this study are to gather valuable information about the effects of the different antigen removal processes on the immunogenicity and biomechanics of the tissue. Research has shown that most antigen removal processes are efficient at fully decellularizing the cartilage or efficient at maintaining the ECM of the cartilage, but not efficient at both. This experiment is designed to compare an antigen removal process that is designed for full decellularization and compare that to an antigen removal process that is designed to maintain a mechanically stable xenograft. The project is broken down into 3 specific aims:

- Examine a short term antigen removal process that is designed to remove the antigens and cellular components from the tissue while still keeping the cartilage biomechanically stable.
- Examine a long term antigen removal process that is designed to maximize the decellularization of the cartilage without concern over the biomechanical stability of the xenograft.
- Comparing the two antigen removal processes, analyzing what would need to be done to move forward with each process to create an implantable xenograft, and concluding which antigen removal process would be best to move forward with.

CHAPTER II

METHODS OF HARVESTING SAMPLES, ANTIGEN REMOVAL, AND TESTING

Harvesting the osteochondral plugs

The osteochondral plugs were harvested from the stifle joints of porcine animals that had been obtained from a local meat processor. The plugs were 5 mm in diameter, and the height varied greatly due to the amount of bone that had been harvested with the plug. The aims of this study are directed towards the cartilage and its weight and cross-sectional area, so the varieties in length did not negatively affect this study.

Antigen removal processes

All of the samples in the controls and the experimental groups underwent a wash to clean the plugs osteochondral plugs. Directly after harvesting, the samples were placed in PBS at room temperature and agitated for one hour (twice). The samples were then placed into 10% hydrogen peroxide at 37 °C overnight. The next day, the samples were washed 3 times in dH_2O and ECA for 20 minutes each wash. The samples were then separated into three groups: the control group, the long term exposure group (LE), and the short term exposure group (SE). The controls were stored at 4 °C while the two experimental groups went through their antigen removal processes. The Long term exposure group had an antigen removal processes specifically designed for maximum antigen removal regardless of the effects towards the ECM. The Short term exposure

group, however, had an antigen removal process that was designed specifically towards antigen removal but minimizing the effects caused to the ECM.

Immediately following the last PBS wash, the SE group was placed in a 25 mL low SDS concentration (10 mM Tris-HCl (pH 7.6), 1 mM PMSF, 0.05% SDS 5 mM MgCl2, 0.5 mM $CaCl_2$, 12.5 mg DNase I, 1.25 mg/ml RNase, 1% antibiotic-antimycotic mixture solution) for 6 hours at 37 °C with agitation. After the 6 hours had passed, the samples were washed twice for 30 min each in dH_2O. The samples were sterilized in a peracetic acid solution (10 mL 2% peracetic acid, 5 mL 100% ethanol, 5 mL dH_2O) for 4 hours. The samples were then equilibrated in PBS until the peracetic acid levels in the PBS fell below 1 mg/L, which was measured using peracetic acid test strips. After equilibration, the samples were stored at 4 °C.

The LE group, directly after the PBS wash, was placed in a 0.2% hyaluronidase solution (10 mM Tris-HCl (pH 6), 0.2% hyaluronidase, 1 mM PMSF, 1% antibiotic-antimycotic mixture) for 48 hours at 37 °C with agitation, changing solution after the first 24 hours. After the 48 hours in the hyaluronidase solution, the samples were moved to a 1% SDS solution (10 mM Tris-HCl (pH 7.6), 1 mM PMSF, 1% SDS, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$) for another 48 hours at 37 °C with agitation, changing solutions after the first 24 hours. The samples were then moved to a DNAse/RNAse solution (10 mM Tris-HCl (pH 7.6),1 mM PMSF, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mg/ml DNase I, 0.05 mg/ml RNase, 1% antibiotic-antimycotic mixture) for 48 hours at 37 °C with, changing solution after the first 24 hours. The samples are washed twice for 30 minutes in dH_2O. The samples are then sterilized in a 2% peracetic acid solution for 4 hours at room

temperature. After sterilization, the samples are washed in PBS until the concentration of peracetic acid concentration falls below 1mg/L. The samples are then stored at 4°C.

Methods of biochemistry testing

To test the differences in the biochemistry between the two antigen removal processes, three different assays were used to quantitate specific molecular components of each antigen removal process as well as controls. The cartilage of each osteochondral plug was cut off the bone using a razor blade. To obtain a baseline to compare them, the samples from each group were freeze-dried and weighed by group. Weighing the samples before testing allowed us to compare the amount of DNA, collagen, and Glycosaminoglycan (GAG) per milligram (mg) of body weight.

To determine the amount of DNA content was left in each experimental group, the samples were first digested in a papain digestion buffer (10 mL PBE, 15.8 mg L-cysteine hydrochloride, 50 µL papain) overnight at 60 °C. The samples were then spun at 10,000 g for 3 min in the centrifuge. To Quantify the DNA in each sample, 100 µL of the papain digested sample was added to a clean cuvette with 2 mL of 1x TNE buffer with .2 ug/ml Hoechst dye and the fluorescence was read using the GloMax®-Multi Jr Single Tube Multimode Reader by Promega. The sample readings were then compared to those of the standards that were created using known concentrations of calf thymus DNA.

To determine the amount of collagen present in the samples, the samples were first digested in the papain digestion buffer overnight at 60 °C. The samples were then spun at 10,000 g for 3 min in the centrifuge. Then, 45 µL of the digestate of each sample was put in a screw-cap vial and 50 µL of 2 N NaOH was added. The standards were then

prepared by adding known amounts of hydroxyproline to screw-cap vials and 50 µL of 2 N NaOH was added to each tube. The samples and standards were then hydrolyzed using the autoclave at 120 °C for 20 minutes. After the samples had cooled to room temperature, 450 µL of .056 M Chloramine T reagent was added to each sample and allowed to oxidize for 25 minutes at room temperature after mixing gently. 500 µL of 1 M Ehrlich's reagent (3g p-dimethylaminobenzaldehyde, 13 mL, brought up to 20 mL with 1-proponol/perchloric acid (2:1 v/v) solution) was added to each sample, mixed, and incubated at 65 °C for 20 min. Then, 200 µL was taken from each sample and placed in a 96 well plate. The absorbance was then read at 550 nm using the µQuant™ Microplate Spectrophotometer by Bio-Tek Instruments, Inc. The collagen content was then calculated by assuming that 12.5% of the collagen in hydroxyproline.

To determine the amount of GAG present in the samples, the samples were first digested in the papain digestion buffer overnight at 60 °C. The samples were then spun at 10,000 g for 3 minutes. The supernatant was then collected. 50 µL of each sample was put in their own microcentrifuge tube and brought up to 100 µL with papain. 1 mL of the Blyscan dye reagent (provided in the Blyscan sulfated glycosaminoglycan assay kit) was then added to each sample. The samples were then put on a shaker to agitate for 30 minutes. The samples were then spun on the microcentrifuge at 12,000 rpm for 10 minutes. This forms a pellet at the bottom of each microcentrifuge tube. The supernatant was then discarded, leaving only the pellet. 500 µL of the Blyscan dissociation reagent was added to each sample, and the samples were vortexed to release the dye that was captured within the pellet. The samples are then centrifuged at 12,000 rpm for 5 minutes. 200 µL of each sample was added to a 96 micro well plate. The plate was then placed in

the μQuant™ Microplate Spectrophotometer by Bio-Tek Instruments, Inc. The plate absorbance level was read at 656 nm. The Absorbance levels of the samples were compared to that of the standards to calculate the amount of GAG (μg/mg) in each sample.

To show the quality of the collagen and proteoglycans within the tissue after each antigen removal process within the tissue, the samples were stained using two different types of staining processes. The fast green-safranin O staining was done to show the quality of the proteoglycans, and the picro-sirius red staining was done to show the quality of the collagen. This staining was done to see if the antigen removal processes alter the quality of the cartilage and proteoglycans.

The samples were first sent to the Veterinary School of Medicine at Mississippi State University to mount the tissue on histology slides. Once received back from the Veterinary School, the slides began prep for staining. The slides were deparaffinized and hydrated by being placed in xylene for 3 minutes (2 cycles), 1:1 xylene:ethanol solution for 3 minutes, 100% ethanol for 3 minutes (2 cycles), 95% ethanol for 3 minutes, 70% ethanol for 3 minutes, and then 50% ethanol for 3 minutes. The slides were first stained with Weigert's iron hematoxylin working solution for 10 minutes. Then, the slides were washed in running tap water for 10 minutes. For the slides being prepped for picro-sirius red staining, the slides were covered in picro-sirius red stain for an hour, and then rinsed in acetic acid. For the slides being prepped for fast green-safranin O staining, the slides were covered in Fast green stain for 5 minutes, washed in 1% acetic acid for 20 seconds, and then stained in 0.1% safranin O solution for 5 minutes. The excess stain was removed gently from the slides using a chem-wipe. The slides were dehydrated and

cleaned with 95% ethanol, 100% ethanol, and xylene for 2 changes each and 2 minutes in each. Coverslips were then mounted using mounting medium and sealed with fingernail polish. Images were captured on the Leica DM2500 microscope located in Dr. Elder's Office.

Methods of identifying protein content

To analyze the immunohistochemistry of the antigen removal processes, an electrophoresis/western blot was conducted to look for the amount of vimentin left after AR, and samples were also immunostained to highlight the α-gal content within the tissue after the antigen removal processes. Vimentin content was targeted because it is an antigen that is plentiful within various tissues, especially cartilage. Knowing the amount of vimentin that each antigen removal process was able to remove the cellular components within the tissue that may cause an immune reaction if implanted within another host. A-Gal content was investigated because studies have shown that xenografts that contain small amounts of the α-gal have a high chance of causing a hyperacute response causing the graft to fail. The elimination of these two antigens are key to preventing hyperacute and acute immune reactions.

To stain samples to show the levels of α-gal within the tissue, the samples were first sent to the Veterinary School of Medicine at Mississippi State University to mount the tissue on histology slides. Once received back from the Veterinary School, the slides began prep for staining. First the samples were deparifinated and rehydrated using Xylene and ethanol and washed for 15 minutes in cold water. Then, the slides were covered in a 0.1% pronase solution (0.1% pronase in PBS) for 10 minutes. The slides were rinsed twice in PBS for 3 minutes each cycle before they were blocked with 10%

horse serum in PBS for 10 minutes. The slides were rinsed two more times for 3 minutes each wash. The primary antibody was diluted 1:5 dilution of α-gal in 1% BSA in PBS and was added to cover the sections (one slide was not covered as a control). The slides were then incubated overnight at 4 °C. After incubation, the slides were washed 5 times in PBS for 5 minutes each wash. The secondary antibody was prepared at 1:100 dilution of IgM in PBS to cover the sections of the slides and incubated in a dark cabinet for 2 hours. After incubation, the slides were washed for 30 minutes, changing the PBS every 5 minutes. The propidium iodide counterstain was prepared at 1:1000 dilution in PBS and then applied to the slides for 10 minutes in a dark cabinet. Following incubation, the slides were washed in PBS for 30 minutes, changing the PBS every 5 minutes. The slides were then dipped in 95% ethanol 10 times, 100% ethanol 10 times, 100% xylene 10 times, and then dipped again in a separation 100% xylene solution 10 times to prepare the slides for mounting. Cover slips were mounted with a mounting medium and then sealed using nail polish. Images of the staining were captured using the Leica DM2500 microscope located in Dr. Elder's office.

For the Electrophoresis / Western blot, samples were first freeze dried overnight and the dry weight was recorded of each sample. Each group was divided in two subgroups in order to have enough protein to analyze within each group (Control 1, Control 2, SE 1, SE 2, LE 1, LE 2). The samples were then homogenized in a tris-triton lysis buffer. The homogenate was then spun in the centrifuge at 10,000 g for 3 minutes, and the supernate was collected for testing. The amount of sample that was used in the electrophoresis was normalized by its weight. This was done in order to eliminate the chances that the differences in the weight of the samples would be a cause to the

differences in the vimentin shown between the sample groups. The electrophoresis was run using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell and the Mini-PROTEAN® TGX gels (both from BIO-RAD). The running buffer used was a 1X solution of the Tris/Glycine/SDS running buffer (BIO-RAD Cat. No. 1610732). The samples were diluted 3 parts sample with 1 part 4X Laemmli Buffer solution (900 µL Bio-Rad 4x Laemmli Sample Buffer Catalog #161-0747, 100 µL 2-mercaptoethanol solution). The samples were then placed in boiling water for 5 minutes to denature the protein. After letting the samples cool to room temperature, they were added to their specific wells within the gel. The gels were run at 100 V for the first 15 minutes, and the run for 120 V until the samples had run all the way down the gel. After the run has completed, the gel is removed from its cast and equilibrated in the prechilled towbin transfer buffer (3.03 g of 25 mM Tris, 14.4 g of 192 M Glycine, 200 mL 20% methanol, adjust volume up to 1 liter with dH_2O). The Immun-Blot® PVDF Membrane (Bio-Rad Cat. #1620174) was first wet in 100% methanol for 5 minutes to ensure wetness before being equilibrated in the transfer buffer for 20 minutes. Thick filter paper was also put in a container with transfer buffer to wet the filter paper. The transfer sandwich was made first setting one thick filter paper on the Semi-dry Transfer cell. Then the membrane was placed on top of the filter paper and smoothed down using a cell culture scraper. The gel was then placed on top of the membrane, and the final thick filter paper was added on top of the gel and smoothed down. The semi-dry transfer was run at 20 V with a .25 limit for an hour. After the transfer was completed, the membrane was placed in 5% dried milk blocking solution at 37° C for two hours. The membrane was then washed twice in PBS-Tween for 5 minutes each wash. The primary antibody (AMF-17b, Developmental

Studies Hybridoma Bank, The University of Iowa) was diluted 1:100 in 1% BSA in PBST. The membrane was then incubated in the diluted primary antibody overnight at 4° C. The membrane was then washed twice in PBST for 5 minutes each wash. The secondary antibody (goat anti-mouse HRP conjugate from Bio-Rad) was diluted 1:6000 in 1% BSA in PBST. The membrane was then incubated in the secondary antibody for 2 hours. After two more washes in PBST, the membrane was incubated in the chemiluminescent substrate (Thermo Scientific™ Cat. #32109) for 5 minutes, and then the samples were revealed using exposure to x-ray film.

Methods of biomechanical testing

Biomechanics testing was done to identify what the impact of the antigen removal processes had on the mechanics on the tissue. The instantaneous stiffness and equilibrium stiffness was calculated of each sample using the Mach-1.

The Mach-1 was calibrated before use. Before each sample was run, it was thawed in PBS at room temperature for 20 minutes. The cartilage was then cut away from the bone at the cartilage/bone interface using a razor, and loaded into the chamber with PBS to be tested. The thickness was first measured electronically via the Mach-1. The thickness of each sample was then used to calculate the rmap amplitude at 5% of the thickness of the cartilage per step for 5 total steps per sample. The data was recorded, saved, and later analyzed in Microsoft Excel.

CHAPTER III

RESULTS

Biochemistry testing

The DNA content of each sample was calculated and then normalized by the weight of the sample. This eliminated the weight of the samples as a possible independent variable during this test. Comparisons of the mean DNA content can be seen in Figure 3.1.

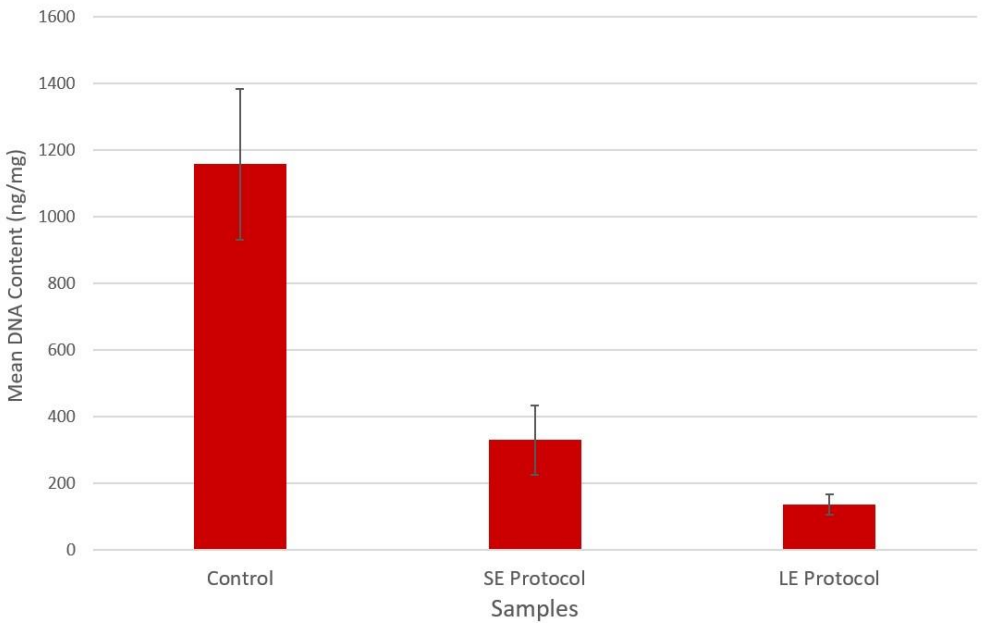

Figure 3.1 The effects of antigen removal on DNA content

There were a total number of 18 control samples, 14 SE samples, and 22 LE samples used in the DNA analysis. The mean DNA content of was 1157.848 ng/mg for the controls, 349.968 ng/mg for the SE samples, and 135.7828 ng/mg for the LE samples. The Standard Deviations were 226, 103.7698, and 31.139 ng/mg respectively. T-tests were run to compare the data between each sample group. The t-test revealed a p-value of less than .001 in each comparison, which indicated a significant difference in mean DNA content between all three sample groups. This data analysis of the number of samples, mean DNA content, and standard deviation is represented in Table 3.1.

Table 3.1 DNA assay analysis

	Control	SE protocol	LE protocol
Number of Samples	18	14	22
Mean DNA content (ng/mg)	1157.848	329.968	135.7828
Standard Deviation	226	103.7698	31.13936

T-tests: Controls vs SE $P<.001$, Controls vs LE $P<.001$, SE vs LE $P<.001$.

The collagen content of each sample was calculated during the chloramine-T assay and then normalized to its weight to eliminate weight as an independent variable during testing. Comparisons of the mean collagen content after antigen removal can be seen in figure 3.2.

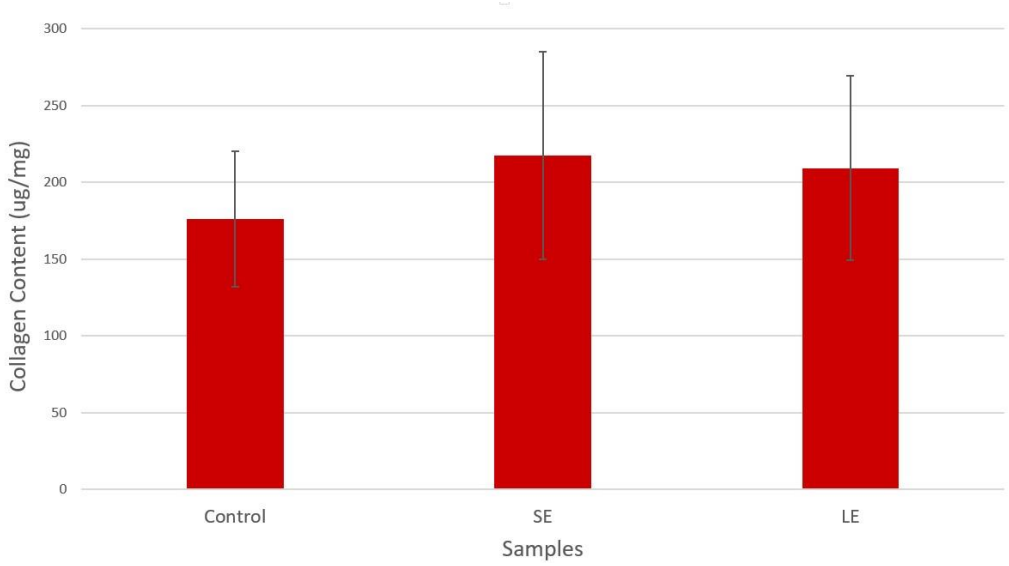

Figure 3.2 The effects of antigen removal on collagen content

There were a total number of 17 control samples, 14 SE samples, and 16 LE samples used during the chloramine-T assay. The mean collagen content was 176.067 ug/mg for the controls, 217.423 for the SE group, and 209.219 for the LE group. The standard deviations were 44.359 ug/mg for the controls, 67.588 for the SE group, and 60.163 for the LE group. An ANOVA analysis was run to compare the three sample groups, and the p-value was .069. The P-value was greater than 0.05 which indicates that the three groups showed no significant difference between one another. These values can be seen in Table 3.2.

Table 3.2 Chloramine-T assay analysis

	Control	SE Protocol	LE Protocol
Number of Samples	17	14	16
Mean Collagen Content (ug/mg)	176.067	217.423	209.219
Standard Deviation	44.359	67.588	60.163

ANOVA: P-value=0.069.

The GAG content was calculated after running the Blyscan glycosaminoglycan assay and was normalized to the weight of each sample to eliminate the weight as an independent variable. The mean GAG content of each sample group can be seen in Figure 3.3.

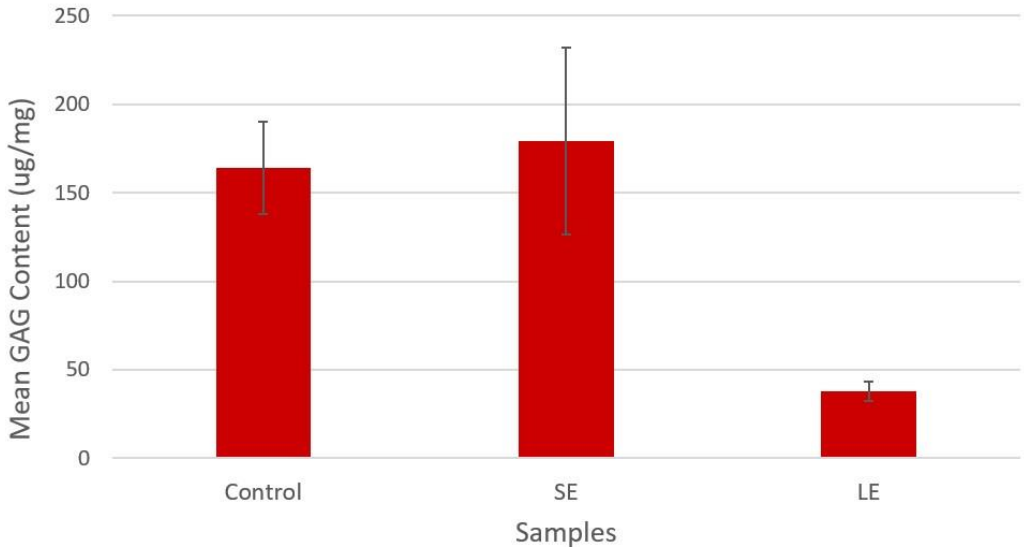

Figure 3.3 The effects of antigen removal on GAG content.

For the Blyscan GAG assay, 17 control samples, 14 SE samples, and 16 LE samples were used. The mean GAG content was 164.0844 ug/mg for the controls, 179.333 ug/mg for the SE group, and 30.529 ug/mg for the LE group. The standard deviations for each sample group were 26.00 ug/mg, 52.60 ug/mg, and 8.392 ug/mg respectively. T-tests were run to compare the data between each experimental group. When comparing the controls vs. Se samples, $p=0.369$. When comparing the controls vs LE group and the SE group vs the LE group, both p-values were less than .001. This indicated that there was a significant difference between the GAG content of the Controls

and LE samples as well as a significant difference between the SE samples and the LE samples. However, there was no significant difference between the controls and the SE samples. This data can be seen in Table 3.3.

Table 3.3 Blyscan GAG assay analysis

	Control	SE protocol	LE protocol
Number of samples	17	14	16
Mean GAG content (ug/mg)	164.084	179.333	30.529
Standard Deviation	26.0	52.60	8.392

T-Tests: Control vs SE P=.369, Control vs LE P<.001, SE vs LE P<.001.

The picoserius red staining of the controls and antigen removal processed samples revealed no visible difference in the collagen content or structure between controls or the antigen removal processed samples. This can be seen in Figure 3.4.

Figure 3.4 Picoserius red staining for collagen in the cartilage samples

The safranin-O fast green stain shows visual differences in proteoglycan content between the samples. The SE antigen removal process looks to keep most of the proteoglycans intact, whereas the LE antigen removal stained samples seem to have very low levels of proteoglycans. This can be seen in figure 3.5.

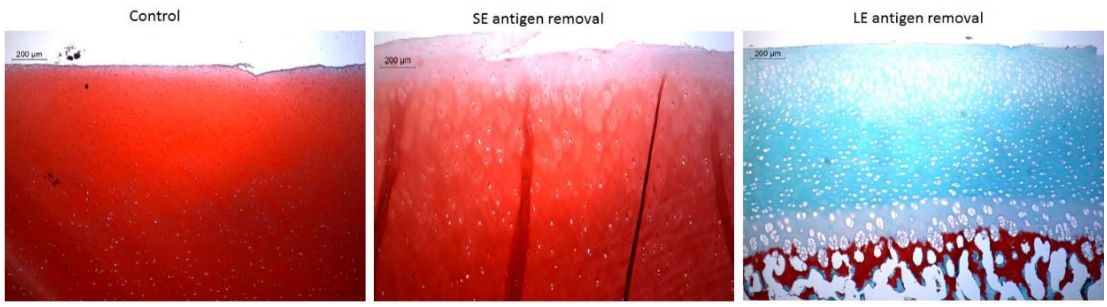

Figure 3.5 Safranin-O and fast green staining of cartilage samples

Identifying protein content

The immunofluorescent staining was done to show expression of the nuclei of the cells (red) and the α-gal (green) within the tissue. The fresh bone sample shows a significant amount of both nucei and α-gal content, whereas the fresh cartilage sample shows no signs of α-gal being present within the tissue, but the nuclei are still shown to be present. This can be seen in figure 3.6.

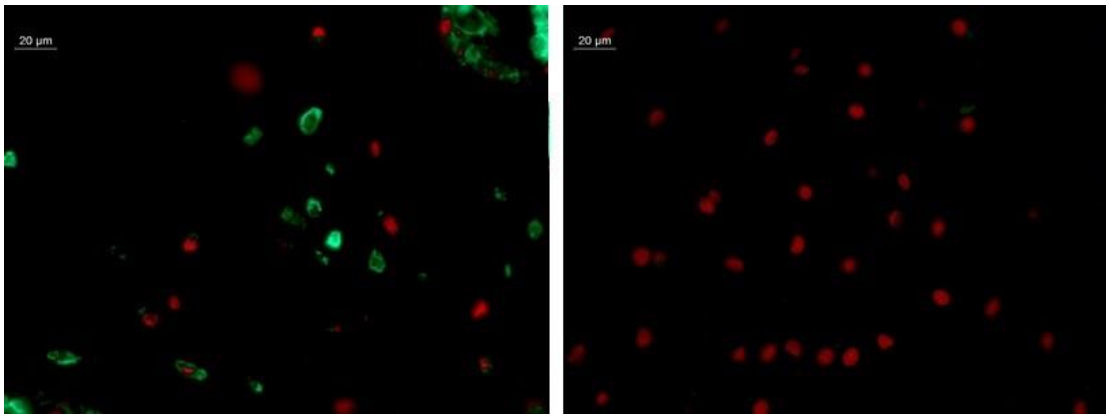

Figure 3.6 Immunofluorescent staining of fresh bone (left) and fresh cartilage (right).

The vimentin content of each experimental group was qualitatively measured during the analysis of the western blot. The two controls showed very high signaling in the area where the vimentin is anticipated on being based on molecular weight. Lane 1 for the SE sample group shows very low signaling within the same area, but there was no visible signal in the second SE lane. There was no visible signal in the appropriate area for vimentin the either of the LE sample lanes. The blot can be seen in figure 3.7.

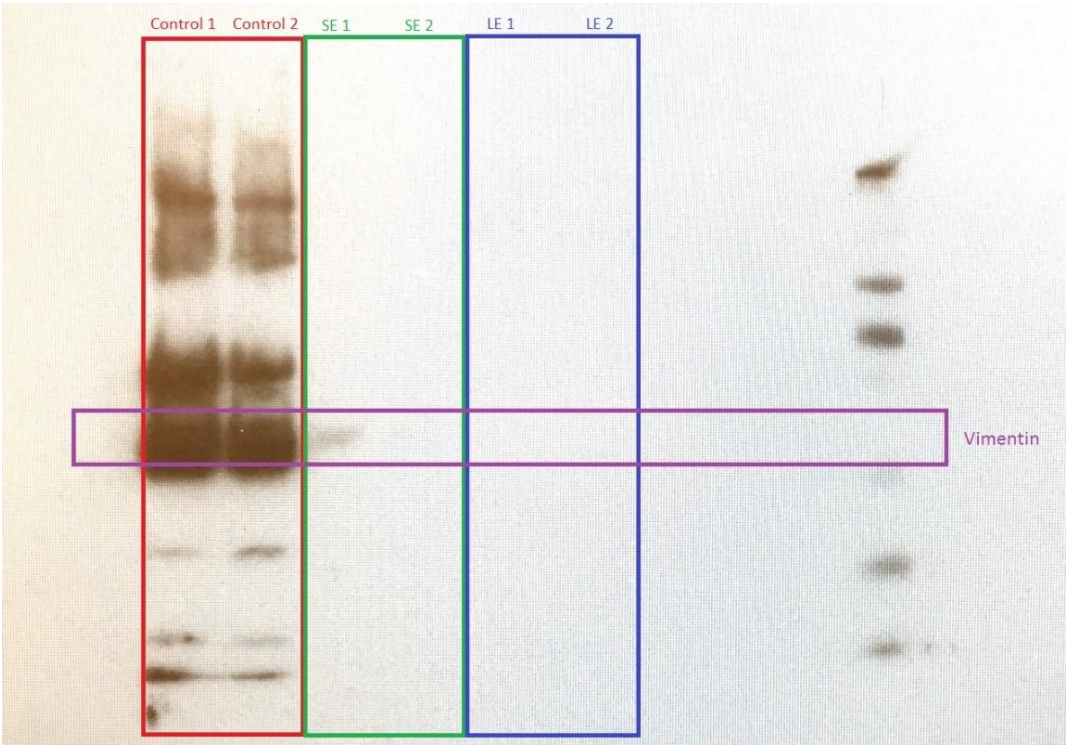

Figure 3.7 Western analysis of vimentin

Biomechanical testing

The biomechanical testing was able to measure the instantaneous stiffness, relaxation modulus, and the relaxation time of the samples. When reviewing the

instantaneous stiffness moduli of the sample groups, the modulus of the SE antigen removal protocol was not significantly different than the controls, but the instantaneous modulus of LE antigen removal protocol was significantly lower than the moduli of the controls and SE antigen removal than the controls, but the LE antigen removal samples showed significantly lower instantaneous stiffness. The SE antigen removal samples were significantly stiffer than the LE samples. This figure can be seen in Table 3.4.

Table 3.4 Biomedical Analysis

	Control	SE antigen removal	LE antigen Removal
Mean Instantaneous Modulus (MPa)	0.6061	0.37567	0.11955
St. Dev.	0.23577	0.13002	0.11928
Mean Relaxation Modulus (MPa)	0.07172	0.00369	0.00117
St. Dev.	0.06749	0.00128	0.00117
Mean Relaxation Time (Seconds)	266.2425	2335.2925	104.568
St. Dev.	155.565	133.515	30.6437

Instantaneous Moduli T-Tests: Control vs SE P=0.51, Control vs LE P<0.001, SE vs LE P=0.12. Relaxation Moduli ANOVA test P=0.75. Relaxation Time T-Tests: Control vs SE P=0.6758, Control vs LE P=0.0204, SE vs LE P=0.2711.

CHAPTER IV

ANALYSIS AND DISCUSSION

Biochemistry

The DNA analysis revealed that the mean DNA content between the controls, the SE samples, and the LE samples were all significantly different from one another. The Control had the most DNA content per mg of sample, and the other two groups were significantly less. The SE group also had significantly higher amounts of DNA per mg of sample when compared to the LE group. The LE protocol was significantly better at eliminating the DNA from the samples than the SE group. This result is what was expected due to the higher concentration of SDS and the longer antigen removal process.

The Chloramine-T assay showed that the collagen content between the controls and either antigen removal group were not significantly different than one another. Both antigen removal processes were able to maintain the collagen within the tissue. This is one crucial step in maintaining the extracellular matrix in order for the tissue to hold up mechanically if it were to be used as a cartilage xenograft.

The Blyscan Glycosaminoglycan assay revealed that the GAG content of the LE group was significantly lower than the controls and SE samples. The SE group showed a significantly higher amount of GAG than the LE group but the levels were not significantly different than that of the controls. This assay has shown that the LE protocol has a negative effect on the GAG content of the cartilage, whereas the SE

samples does not have a negative effect. Because there is significantly lower GAG in the samples that went through the long exposure antigen removal process, these samples should have a poor ability to retain water. The loss of GAG greatly affects the porosity of the cartilage. Because fluid will be able to flow easier in and out of the cartilage after the LE antigen removal process, the cartilage will typically be less stiff. Because the samples that underwent the SE antigen removal process showed no significantly lower GAG content when compared to the GAG, they should be able to retain water, absorb forces, and function mechanically the same as the control samples.

As seen in Figure 3.4, the collagen content of the three groups does not look to be any different from one another. This, along with the Chloramine-T assay, provides evidence that these two antigen removal processes do not have a negative effect on the collagen of the cartilage. Maintaining the collagen will not only help the cartilage perform mechanically, but also help with cell seeding and migration as high collagen content provides a matrix that allows cells to adhere to the tissue properly.

Identifying protein content

In the western blot used to identify the vimentin within the samples, you can easily see the thick protein bands within both control lanes. Due to its intensity, we can assume that the controls had very high levels of vimentin within the samples. When looking at the SE lanes, you can barely see the vimentin within the first lane of the SE group, but the band does not seem to be present within the second lane. This shows that the SE group has gotten rid of a lot of the vimentin within the samples if you compare the intensity of the bands. The small amount of vimentin within the first lane does show that the elimination of vimentin may not be 100%. If vimentin is still present within the

cartilage xenograft after implantation, it could cause an immune rejection of the scaffold. The LE group shows no sign of vimentin within its two lanes. The LE antigen removal process should be sufficient enough in removing the vimentin, and the cartilage that underwent the LE antigen removal process should have a very low chance causing an immune rejection response after implantation.

In the immunofluorescent staining of the fresh cartilage and fresh bone samples, it is easy to see the differences between the presences of the α-gal in the two different samples. The fresh bone showed a high amount of α-gal being present within the tissue. However, the fresh cartilage sample showed little-to-no signs of having α-gal within the tissue. Because the α-gal content within the cartilage is minute, the risk of the cartilage being the cause of a hyperacute rejection reaction after implantation being very low as well. However, because the cartilage will be implanted as an osteochondral plug, the decellularization process must get rid of a sufficient amount of α-gal from the bone so it also has a very low chance of causing an immune rejection reaction. More test will have to be run on the bone to be able to tell if the decellularization protocols tested in this study have a significant impact on the α-gal content of the bone section of the osteochondral xenograft.

Biomechanics

The biomechanics tests showed some similarities and some differences between the controls, the SE antigen removal group, and the LE antigen removal group. These similarities and differences between the three groups. The instantaneous stiffness of cartilage is greatly due to its biphasic properties. Because of the density of the cartilage and its water retention capabilities, its instantaneous strength comes from the ability of

water to flow freely out of the tissue when it takes an instantaneous load. The hydrostatic pressure builds and supports most of the load. If the extracellular matrix becomes degraded, the water will be able to flow more freely in and out of the tissue without building up the hydrostatic pressure. Without this buildup of pressure, the instantaneous stiffness will be decreased. The relaxation moduli relates to the stiffness of the cartilage after all of the water has been forced out of the tissue. When all of the water is forced out of the tissue, the extracellular matrix has to now hold up that load. If the relaxation stiffness moduli is lowered, that indicates that the decellularization techniques has a negative impact on the extracellular matrix's biomechanical abilities. Furthermore, because the collagen is the primary support system of the cartilage, a lowered relaxation moduli indicates that the collagen was negatively impacted during the decellularization process. The mean relaxation time shows how quickly the water is forced out in each step and how quickly the load becomes supported mainly by the extracellular matrix of the cartilage. The mean relaxation time gives information about the tissue's ability to hold water and how well the tissue builds up the hydrostatic pressure. If the water flows out freely, the hydrostatic pressure will be decreased, also decreasing the biomechanical stiffness of the cartilage under instantaneous loads. The more quickly the tissue relaxes indicates that the water flows more quickly out of the tissue. The longer the tissue takes to relax, the more hydrostatic pressure has built up within the tissue, and the more stiff the cartilage acts under instantaneous loads. This evidence should relate closely with the instantaneous modulus of the cartilage as well.

When looking at the instantaneous moduli, the control samples and the SE group were not significantly different than one another. However, the LE group showed a

significantly lower instantaneous modulus than that of the controls and SE group. The lower instantaneous modulus indicates that the LE samples weren't as able to build up the hydrostatic pressure as much as the other sample groups. As indicated in the biochemistry testing, the GAG content was also significantly lower within this sample group as well. The less GAG content would make the cartilage samples less dense, and because GAG gives the cartilage the ability to attract and hold onto water, the decrease in GAG would also reduce the cartilage samples' ability to attract and retain water. Because the SE group was not significantly different than the controls when comparing GAG and collagen content, the densities of the two should be relatively the same. Therefore, it makes sense that the instantaneous moduli of the two groups would be very similar.

The mean relaxation moduli of the three groups were not significantly different than one another. This indicates that the strength and integrity of the extracellular matrices of the samples tested were not mechanically different than one another. When looking back at the collagen content assay in table 3.2, the collagen content was not significantly different between the three groups. Because the collagen content was not significantly different between the groups, it makes sense that the mean relaxation moduli of the three groups would also be statistically the same as one another.

When comparing the mean relaxation times of the three sample groups, the LE samples took significantly less time to relax than the other two groups. The SE group relaxation time was not significantly different than the controls. The lowered relaxation time of the LE group indicates a significantly lower density of the extraxellular matrix of the LE group than that of the SE and controls. The LE did show significantly lower GAG

than the control and SE groups, which would allow water to flow quicker out of the tissue.

Short term exposure review

The short exposure antigen removal process eliminated a substantial amount of DNA from cartilage without eliminating significant amounts of cartilage and GAG. The DNA content of the samples after the short term exposure were significantly lower than the controls, but there was still enough residual DNA left over that there is still a possibility of causing an immune rejection response due to if the samples were implanted into a host. However, studies have shown that the main reasons for immune response are foreign antigen recognition, and xenografts have survived after implantation without 100% DNA removal. The GAG content was not significantly different than that of the controls, which shows that the short exposure antigen removal process does not have a negative effect on the GAG within the tissue. Biomechanically, this is a good thing because the GAG contributes to greater stiffness. However, when it comes time to seed the xenograft with chondrocytes or mesenchymal stem cells, the lack of porosity will make it very hard for the cells to seed deep within the tissue. If the GAG content were lower, the ECM would be more porous, and therefore be a better scaffold for cell seeding and cell migration. The low porosity does help in providing an immunopriveledged tissue that will help reduces the chances of eliciting an immune response rejection, however it hinders the seeding and migration ability of the cells within the tissue.

When analyzing the immunohistochemistry of the short exposure antigen removal process, there seemed to be some signs of vimentin within the cartilage after decellularization. The levels of vimentin seemed to be very low when compared to the

controls, and one lane of the western didn't show any signs of having vimentin. This shows that the SE antigen removal process is efficient at eliminating immunogenic proteins within the cells of the tissue. There was a slight sign in the first lane of the SE group which does make an immune response possible after implantation, but the levels look to be a lot lower than before, and the density of the cartilage should help reduce the chances of triggering an immune response. Based on previous studies the levels of α-gal within porcine cartilage is already quite low, and that is confirmed in the image of the control sample in figure 3.6. The elimination of the majority of the vimentin shows that the SE antigen removal process is efficient in eliminating extracellular immunogenic proteins from the cartilage, but there is still a chance of causing an acute immune reaction. The lack of α-gal and significantly lower vimentin levels within the short exposure samples will greatly lower the chances of the cartilage triggering a hyperacute immune reaction and acute immune response, which is the two quickest form of immune rejection response and is typically triggered by recognition of foreign antigens. However, other studies show that porcine bone has a much higher α-gal content than that of the cartilage, and the effectiveness of the removal of the α-gal protein from the bone would give a better idea if the osteochondral plug would cause a hyperacute or acute reactions after the short exposure antigen removal process.

The biomechanical testing showed that the instantaneous stiffness and relaxation stiffness was not significantly lower than that of the controls, which provides evidence that the short antigen removal process does not have a negative effect on the biomechanics of the cartilage. This is very important because the articular cartilage is a

high weight bearing tissue, and hindered ECMs can cause weight bearing tissues to fail quickly.

Long term exposure review

The long term exposure was efficient at removing the DNA and GAG from the cartilage samples. The significant reduction of the residual DNA is a good identification of an efficient decellularization technique, and also is a good indication that the chances of triggering an immune response due to DNA content will be lowered. The GAG content was significantly lower than the controls. The mean GAG content was less than 20% than that of the controls. Eliminating this much GAG greatly increases the porosity of the tissue. This increase in porosity will allow for cell seeding and migration deep into the tissue. However, the loss of GAG could greatly reduce the biomechanical ability of the tissue and could lead to mechanical failure after implantation. The collagen seemed to be unaffected by the long exposure antigen removal process which will help cells adhere to the tissue, but the strength of the ECM will still be greatly reduced due to the loss of the GAG.

After analyzing the western blot membrane, there seemed to be no vimentin signal in the lanes containing the samples that underwent the long exposure antigen removal procedure. Because there was such strong signal in the controls, this western blot provides strong evidence that the long exposure antigen removal process is efficient at removing cytoplasmic proteins from the cartilage. Fully eliminating vimentin and other cytoplasmic proteins is crucial in avoiding acute rejection responses after implantation, and based on the western blot analysis, the LE antigen removal process should yield a xenograft that will have a low chance of causing an acute rejection

response. The lack of α-gal within the cartilage also provides evidence that the chances of triggering a hyperacute immune rejection response is quite low. Because α-gal is the main reason for hyperacute rejection and the main cause of porcine-derived graft rejection, the chances of the cartilage triggering a hyperacute rejection after undergoing the LE antigen removal process should be low.

The instantaneous stiffness of the cartilage after the LE antigen removal process was significantly lower than that of the controls. This was to be expected due to the significantly less amounts of GAG within the tissue. The GAG is essential to the stiffness of the cartilage due to its ability to attract and retain water. This, and the increased porosity due to less GAG allows the water to flow more easily in and out of the tissue, significantly decreasing the amount of hydrostatic pressure, which in turn decreases the stiffness of the tissue. The lowered stiffness is something that will need to be addressed before the tissue can be suitable for osteochondral implantation.

SE antigen removal vs. LE antigen removal

The LE antigen removal process was significantly better than the SE antigen removal process at removing the DNA from the cartilage. The LE antigen removal process was also significantly better at eliminating the vimentin from the tissues when compared to the SE antigen removal process. Because the LE antigen removal process was significantly better at eliminating the DNA and cytoplasmic proteins and was efficient at eliminating α-gal, the LE antigen removal process should give the cartilage a better chance of avoiding an immune rejection response after implantation into the host. The SE antigen removal process could be an efficient process that won't cause an immune response, but the LE antigen removal process was more efficient at eliminating

cellular proteins and components from the tissue giving it a better chance of avoiding rejection.

The collagen content of the cartilage samples after the antigen removal processes were not any different from one another or the controls which shows that the antigen removal processes do not have a negative effect on the collagen content within the cartilage. The GAG content was significantly different between the two antigen removal processes. The LE antigen removal process eliminated significantly more GAG than the SE antigen removal process. The SE antigen removal processes was not significantly different than the controls, which shows that the SE antigen removal process was able to maintain the GAG content whereas the LE antigen removal process was efficient at eliminating the GAG from the cartilage samples. The SE antigen removal group has better biomechanical potential due to the maintained GAG, whereas the LE antigen removal group has greater porosity and cell seeding and migration ability due to the significant decrease in GAG content. Although the SE antigen removal group should perform better mechanically, evidence shows that the low porosity and high GAG content can cause insufficient cell migration deep within the cartilage tissue. However, the LE antigen removal group should allow cells to migrate deep within the graft, but the decrease in GAG greatly decreases the biomechanical stiffness and stability of the cartilage, which was shown in the biomechanical testing of the samples.

Possibilities of use for osteochondral transplantation

The short term exposure antigen removal process removed a significant amount of DNA, and vimentin from the tissue and showed that it was biomechanically stable during biomechanical testing. However, the lack of porosity could hinder the tissues ability to

allow chondrocyte cells to migrate appropriately deep within the tissue which could lead to xenograft failure after implantation. The antigen removal process removed a significant amount of the immunogenic components of the tissue but there were still signs of vimentin, and DNA present within the tissue, which still will make the scaffold susceptible to triggering an immune response from the body. So, although the scaffolds would most likely be biomechanically stable after implantation, the xenograft still has potential to trigger an immune response and also may hinder cell migration, which are large concerns.

The long exposure antigen removal process also removed a significant amount of DNA, vimentin and α-gal from the sample tissue, but the samples displayed significantly stiffness during biomechanical testing. This antigen removal process was significantly better than the short term exposure group at removing the DNA, and the western blot showed no signs of vimentin within the samples after decellularization. This process would give the cartilage xenografts a greater chance of avoiding immune detection and triggering an immune response, but the biomechanical instability of the tissue would most likely cause the tissue to fail over time. The GAG content was significantly lowered, which hinders the biomechanics of the tissue but increases porosity to allow proper cell migration deep within the tissue. This process alone does not show any indication that it would contribute to long term success of a xenograft after implantation, but full antigen removal and proper cell seeing could become the first part of a multi-step preparation technique in creating an effective xenograft that will be long lasting within the host.

Conclusion

With athletes and individuals developing osteoarthritis and chondral defects at younger ages, long term treatments with minimal maintenance are growing in demand (39). Total and partial knee replacements only last for 10-15 years, so individuals needing treatments below the age of 40 would need to have multiple knee replacements within their lifetime (40). Allograft transplantation has shown to last long term and have high success rates, but the lack of donors and the possibility of damaging other areas of the knee to obtain tissue grafts has become a large concern (41). A successful osteochondral xenograft needs avoid triggering an immune response while also being able to withstand all of the biomechanical forces that the tissues undergoes on a day to day basis.

For a cartilage xenograft to be effective, it has to be able to withstand the day to day forces that the native cartilage goes though without triggering a rejection response after implantation. In order for it to achieve these two main goals, the integrity and strength of the extracellular matrix and the ability to keep the biphasic characteristics of native cartilage needs to be maintained. Also, the antigen removal process needs to rid the tissue of all cytoplasmic and extracellular antigens that may trigger an immune response. If any α-gal proteins are recognized by the body, it will cause a very quick rejection response that will start breaking down the xenograft, causing it to fail. The SE antigen removal process was very good at keeping the cartilage biomechanically stable, but some improvements could be made to enhance the ability of the process to remove or cover up more of the cytoplasmic proteins. The LE antigen removal process eliminated the cytoplasmic proteins efficiently, however, the biomechanical stability of the cartilage

was sacrificed. The LE antigen removal process would need improvements to be made to support the tissue mechanically, such as adding cross-linkers to the tissue. There has been some promising experiments where crosslinking can be used to make damaged cartilage more stable and better mimic the biomechanical properties of native, healthy cartilage.

The antigen removal processes also need to be evaluate for their effects on bone. The samples do come as osteochondral plugs to help ensure the scaffold can become easily anchored in after being implanted. This also means that the bone needs to be decellularized enough to not cause an immune response. As shown before, the α-gal content of natural porcine cartilage is very low. However, there was α-gal present within the natural bone. The bone sections underwent both the SE and LE antigen removal processes along with the cartilage, and sections of bone were immunostained to see if either antigen removal process rid the bone of the α-gal protein. When reviewing the image in 4.1, you can see that the bone section that underwent the LE antigen removal process has significantly less α-gal and nuclei present within the bone. The SE antigen removal process does not seem to remove the α-gal or nuclei very efficiently from the lacunae of the bone. This should affect the immunogenicity of the osteochondral plug, and more investigation needs to go into the antigen removal processes on the bone. Although the cartilage showed no signs of α-gal present, the bone still has a chance of causing a hyperacute reaction once implanted into the patient.

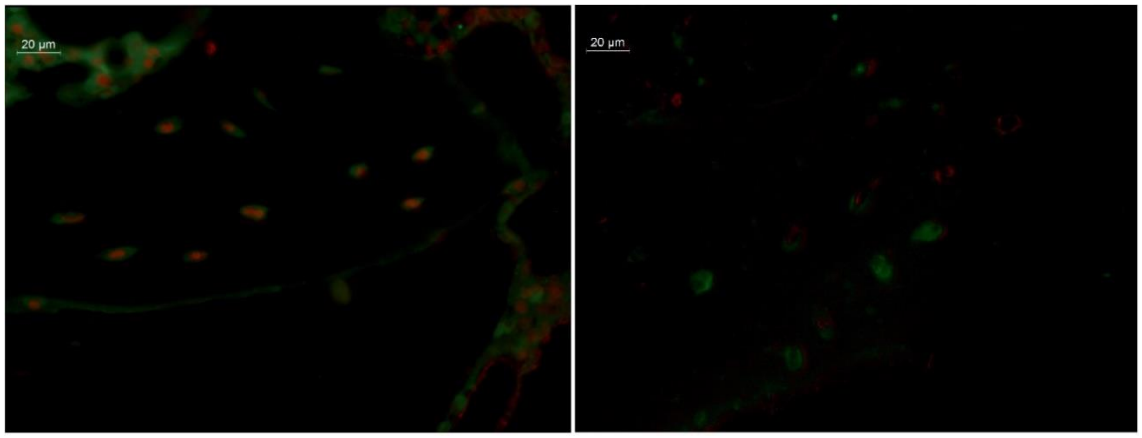

Figure 4.1 Immunostaining of bone samples after undergoing the short exposure (SE) antigen removal process (left) and the long exposure (LE) antigen removal process.

Future studies will need to be done to truly identify the probability of samples causing immune rejection after being processed with the short exposure and the long exposure antigen removal processes. Also, especially for the LE antigen removal process, additional processes need to be made to ensure the cartilage is biomechanically stable upon implantation. For the SE antigen removal group, future processes need to be made to enhance the tissues ability to seed cells properly and to make the scaffold more immunopriveledged so that the small amounts of DNA and cellular proteins can go unnoticed after implantation in to the patient. This study has shown that these two specific antigen removal processes alone are not sufficient enough in creating an osteochondral graft for the treatment of chondral lesions, but they have shown the ability to be the backbone and starting points for future processes.

REFERENCES

1. Dell'Isola, A., R. Allan, S. S. P. Marreiros, and M. Steultjens. "Identification of Clinical Phenotypes in Knee Osteoarthritis: A Systematic Review of the Literature." BMC Musculoskeletal Disorders 17.425 (2016): n. pag. BioMed Central. Web.

2. Han, W., D. Aitken, Z. Zhu, A. Halliday, X. Wang, B. Antony, F. Cicuttini, G. Jones, and C. Ding. "Hypointense Signals in the Infrapatellar Fat Pad Assessed by Magnetic Resonance Imaging Are Associated with Knee Symptoms and Structure in Older Adults: A Cohort Study." Arthritis Research & Therapy 18.234 (2016): n. pag. BioMed Central. Web.

3. Liao, K. M., and H. Y. Lu. "Complications after Total Knee Replacement in Patients with Chronic Obstructive Pulmonary Disease - a Nationwide Case Control Study." Medicine 95.37 (2016): n. pag. Wolters Kluwer. Web.

4. Dean, Chase S. et al. "Fresh Osteochondral Allograft Transplantation for Treatment of Articular Cartilage Defects of the Knee." Arthroscopy Techniques 5.1 (2016): e157–e161. PMC. Web.

5. Briggs, Dustin T. et al. "The Use of Osteochondral Allograft Transplantation for Primary Treatment of Cartilage Lesions in the Knee." Cartilage 6.4 (2015): 203–207. PMC. Web.

6. Lyon, Roger et al. "Can Fresh Osteochondral Allografts Restore Function in Juveniles With Osteochondritis Dissecans of the Knee?" Clinical Orthopaedics and Related Research 471.4 (2013): 1166–1173. PMC. Web.

7. Driban, Jeffrey B. et al. "Best Performing Definition of Accelerated Knee Osteoarthritis: Data from the Osteoarthritis Initiative." Therapeutic Advances in Musculoskeletal Disease 8.5 (2016): 165–171. PMC. Web.

8. Chu, Constance R., Michal Szczodry, and Stephen Bruno. "Animal Models for Cartilage Regeneration and Repair." Tissue Engineering. Part B, Reviews 16.1 (2010): 105–115. PMC. Web.

9. Crapo, Peter M., Thomas W. Gilbert, and Stephen F. Badylak. “An Overview of Tissue and Whole Organ Decellularization Processes.” Biomaterials 32.12 (2011): 3233–3243. PMC. Web.

10. Wong, Maelene L., and Leigh G. Griffiths. “Immunogenicity in Xenogeneic Scaffold Generation: Antigen Removal versus Decellularization.” Acta biomaterialia 10.5 (2014): 1806–1816. PMC. Web.

11. Cissell, Derek D. et al. “Antigen Removal for the Production of Biomechanically Functional, Xenogeneic Tissue Grafts.” Journal of biomechanics 47.9 (2014): 1987–1996. PMC. Web.

12. Fitzpatrick, John C., Peter M. Clark, and Franco M. Capaldi. “Effect of Decellularization Protocol on the Mechanical Behavior of Porcine Descending Aorta.” International Journal of Biomaterials 2010 (2010): 620503. PMC. Web.

13. Elder, Benjamin D., Sriram V. Eleswarapu, and Kyriacos A. Athanasiou. “Extraction Techniques for the Decellularization of Tissue Engineered Articular Cartilage Constructs.” Biomaterials 30.22 (2009): 3749–3756. PMC. Web.

14. Fermor, Hazel L. et al. “Development and Characterisation of a Decellularised Bovine Osteochondral Biomaterial for Cartilage Repair.” Journal of Materials Science. Materials in Medicine 26.5 (2015): 186. PMC. Web.

15. Cheng, Christina W., Loran D. Solorio, and Eben Alsberg. “Decellularized Tissue and Cell-Derived Extracellular Matrices as Scaffolds for Orthopaedic Tissue Engineering.” Biotechnology advances 32.2 (2014): 462–484. PMC. Web.

16. Sandor, M., H. Xu, J. Connor, J. Lombardi, J. R. Harper, R. P. Silverman, and D. J. McQuillan. "Host Response to Implanted Porcine-derived Biologic Materials in a Primate Model of Abdominal Wall Repair." Tissue Engineering Part A 14.12 (2008): 2021-031. PubMed. Web.

17. He, Ming, and Anthony Callanan. “Comparison of Methods for Whole-Organ Decellularization in Tissue Engineering of Bioartificial Organs.” Tissue Engineering. Part B, Reviews 19.3 (2013): 194–208. PMC. Web.

18. Xu, Xiu-Fang et al. “Effect of Carbodiimide Cross-Linking of Decellularized Porcine Pulmonary Artery Valvular Leaflets.” International Journal of Clinical and Experimental Medicine 7.3 (2014): 649–656. Print.

19. Piterina, Anna V. et al. “ECM-Based Materials in Cardiovascular Applications: Inherent Healing Potential and Augmentation of Native Regenerative Processes.” International Journal of Molecular Sciences 10.10 (2009): 4375–4417. PMC. Web.

20. Akens, Margarete K et al. “Long Term in-Vivo Studies of a Photo-Oxidized Bovine Osteochondral Transplant in Sheep.” BMC Musculoskeletal Disorders 2 (2001): 9. PMC. Web.

21. Revell, Christopher M., and Kyriacos A. Athanasiou. “Success Rates and Immunologic Responses of Autogenic, Allogenic, and Xenogenic Treatments to Repair Articular Cartilage Defects.” Tissue Engineering. Part B, Reviews 15.1 (2009): 1–15. PMC. Web.

22. Sprangers, B., M. Waer, and A. D. Billiau. "Xenotransplantation: Where Are We in 2008?" Kidney International 74.1 (2008): 14-21. ScienceDirect. Web.

23. Byrne, Guerard W et al. “Histopathologic Insights into the Mechanism of Anti-Non-Gal Antibody-Mediated Pig Cardiac Xenograft Rejection.” Xenotransplantation 20.5 (2013): 292–307. PMC. Web.

24. Zeyland, J., D. Lipiński, and R. Słomski. “The Current State of Xenotransplantation.” Journal of Applied Genetics 56.2 (2015): 211–218. PMC. Web.

25. Sandrin, M S et al. “Anti-Pig IgM Antibodies in Human Serum React Predominantly with Gal(alpha 1-3)Gal Epitopes.” Proceedings of the National Academy of Sciences of the United States of America 90.23 (1993): 11391–11395. Print.

26. Itakura, Go et al. “Controlling Immune Rejection Is a Fail-Safe System against Potential Tumorigenicity after Human iPSC-Derived Neural Stem Cell Transplantation.” Ed. Alysson Renato Muotri. PLoS ONE 10.2 (2015): e0116413. PMC. Web.

27. Galili, U., D. LaTemple, A. Walgenbach, and K. Stone. "Porcine and Bovine Cartilage Transplants in Cynomolgus Monkey: II. Changes in Anti-Gal Response during Chronic Rejection." Transplantation 63.5 (1997): 646-51. Wolters Kluwer. Web.

28. Kumar, A., and V. Kumar. "Immunological Aspects of Corneal Transplant." Immunological Investigations 43.8 (2014): 888-901. Taylor&Francis Online. Web.

29. Sutherland, Amanda J. et al. “The Bioactivity of Cartilage Extracellular Matrix in Articular Cartilage Regeneration.” Advanced healthcare materials 4.1 (2015): 29–39. PMC. Web.

30. Chen, S., B. Li, J. Li, X. Yin, H. Song, and L. Yuan. "Preparation of Xenogeneic Bone Graft and Its Osteoinductivity." Chinese Journal of Reparative and Reconstructive Surgery 23.3 (2009): 362-65. PubMed. Web.

31. Tasaki, M. et al. "High Incidence of Xenogenic Bone Marrow Engraftment in Pig-to-Baboon Intra-Bone Bone Marrow Transplantation." *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 15.4 (2015): 974–983. *PMC*. Web.

32. Liang, Fan et al. "Increased Levels of Anti-Non-Gal IgG Following Pig-to-Baboon Bone Marrow Transplantation Correlate with Failure of Engraftment." *Xenotransplantation* 20.6 (2013): 10.1111/xen.12065. *PMC*. Web.

33. Hill, Ryan C. et al. "Quantification of Extracellular Matrix Proteins from a Rat Lung Scaffold to Provide a Molecular Readout for Tissue Engineering ." *Molecular & Cellular Proteomics : MCP* 14.4 (2015): 961–973. *PMC*. Web.

34. Shimizu, Akira et al. "Pathologic Characteristics of Transplanted Kidney Xenografts." *Journal of the American Society of Nephrology : JASN* 23.2 (2012): 225–235. *PMC*. Web.

35. Byrne, Guerard W., and Christopher GA McGregor. "Cardiac Xenotransplantation: Progress and Challenges." *Current Opinion in Organ Transplantation* 17.2 (2012): 148–154. *PMC*. Web.

36. Thompson, P et al. "Alternative Immunomodulatory Strategies for Xenotransplantation: CD40/154 Pathway-Sparing Regimens Promote Xenograft Survival." *American Journal of Transplantation* 12.7 (2012): 1765–1775. *PMC*. Web.

37. Griesemer, Adam, Kazuhiko Yamada, and Megan Sykes. "Xenotransplantation: Immunological Hurdles and Progress toward Tolerance." *Immunological reviews* 258.1 (2014): 241–258. *PMC*. Web.

38. Seo, Seog-Jin et al. "Strategies for Osteochondral Repair: Focus on Scaffolds." *Journal of Tissue Engineering* 5 (2014): 2041731414541850. *PMC*. Web.

39. Antony, Benny et al. "Correlates of Knee Bone Marrow Lesions in Younger Adults." *Arthritis Research & Therapy* 18 (2016): 31. *PMC*. Web.

40. Losina, Elena et al. "Lifetime Medical Costs of Knee Osteoarthritis Management in the United States: Impact of Extending Indications for Total Knee Arthroplasty." *Arthritis care & research* 67.2 (2015): 203–215. *PMC*. Web.

41. Torrie, Arissa M. et al. “Osteochondral Allograft.” Current Reviews in Musculoskeletal Medicine 8.4 (2015): 413–422. PMC. Web.