Osteochondral Allograft Preservation in a Serum-free Chemically-defined Media

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By

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

Osteochondral Allograft Preservation in a Serum-free Chemically-defined Media

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a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

My family has played a huge part in my life; without their support I would not be here today. There are two people that I most dearly wish could see where I am today. Papa Tom and Nana Dorothy Garrity passed away during my college years, and I would give anything to have just five more minutes with them. As a young child, my interest in biology and anatomy was first ignited by Papa Tom in the fish cleaning house after a long day on Lake George in Minnesota. My interest in orthopaedics was further solidified by observing the improvement in function and quality of life after my grandfather Bart Schiermeyer's total knee replacement.

My parents love and support have been constant throughout every step of my life. Even when I have been away at school, they have never been more than a phone call away. Without their support it is hard to say where I would be today. To my sister's, Ellen and Katie: thank you for sitting through those all-day wrestling tournaments and supporting me through the years. I have a great deal of pride in being a good example for you two. My girlfriend Katie Milles has been a constant fixture in my life since 2006. Many late nights have been spent after our respective sports' practices were over, and this pattern continues today as I continue in medical school and she in dental school. I could not ask for a better girlfriend.

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OSTEOCHONDRAL ALLOGRAFT PRESERVATION IN A SERUM-FREE CHEMICALLY-DEFINED MEDIA

Joseph T. Garrity

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ABSTRACT

Articular cartilage has a very limited capacity as compared to other biological tissues to repair and regenerate because of its avascular nature. Chronic degradation of articular cartilage may result in osteoarthritis, the most common debilitating disease worldwide. In order to alleviate severe pain symptoms and dysfunction associated with osteoarthritis, treatment strategies to replace rather than repair the tissue have been developed. Osteochondral allografts (OCAs) allow the transplantation of whole cartilage tissue into a defect with viable cells, or chondrocytes that will maintain the cartilage matrix. Fresh OCAs have demonstrated greater than 75% clinical success in the treatment of articular cartilage lesions. Currently allografts are stored at 4°C and used within 28 days, however, FDA-mandated disease testing requires 14 days of screening which decreases the effective window for implantation to 14 days. The purpose of this study was to evaluate OCAs stored up to 56 days in a Control (DMEM supplemented with ITS, Sodium Pyruvate, L-ascorbic acid, and MEM Non-essential amino acids) and Test (DMEM

supplemented ITS+, Sodium Pyruvate, L-ascorbic acid, MEM Non-Essential amino acids, dexamethasone, boron, and TGF-β3) media preparation at 4°C and 37°C. Media was changed and collected every 7 days. At Days 0, 28, and 56, full thickness cartilage was evaluated for cell viability, GAG and HP content, and histologic evaluation. Media was collected at Days 7, 14, 21, 28, 35, 42, 49, and 56 for GAG content of the media, a measure of GAG release from tissue. Media collected on Days 7, 28, and 56 was also evaluated for NO, PGE2, MMP, and cytokine content. Cell viability as well as tissue GAG and HP content were maintained at Day 0 levels up to Day 56 in the 37°C Control media. GAG release of tissues at 37°C indicated active metabolism in the tissue. The profile of cytokines released during storage by OCAs stored in the 37°C Control media may be a preliminary step towards a screening protocol for testing OCA viability during storage. Our work showed that storage in a serum-free chemically-defined media at 37°C can maintain OCAs \ better than the current tissue banking protocol for storage.

CHAPTER 1: LITERATURE REVIEW

Articular cartilage pathology is a common finding in the orthopedic world and these lesions have a very limited capacity to heal. Curl et al. looked at records from 31,516 knee arthroscopies and found chondral lesions in 63% of the arthroscopies performed.[1] Another study, a prospective cohort examining 1,000 patients that underwent knee arthroscopy, found chondral or osteochondral lesions in 61% of these patients.[2] In a retrospective study from Poland, 25,124 arthroscopies of the knee were evaluated and cartilage lesions were seen in 60% of these operations.[3] In 993 patients with a median age of 35 years, articular cartilage pathology was found in 66% of their knees during arthroscopy. The most commonly reported activity among these patients was sports participation.[4] A viable treatment option is needed for these patients, many of whom are young and active individuals, who are at a higher risk for developing OA later in life because of these chondral lesions. Osteoarthritis (OA) is a leading cause of disability in the United States with an estimated 27 million people affected by this disease.[5] It also represents a huge burden in cost with associated treatment costs exceeding $60 billion annually.[5]

Articular cartilage injuries are difficult to treat because they have limited healing ability.[6] Articular cartilage injuries can be classified three different ways; cartilage matrix and cell injuries, chondral defects, and injuries that extend into the subchondral bone.[7] Chondrocytes are usually able to restore cell matrix proteoglycan in injuries that only

disrupt a portion of the cartilage matrix.[8] The natural response to chondral injuries is limited by the lack of blood vessels and reparative cells in articular cartilage but can sometimes be repaired in a biologically appropriate manner. Lastly, injuries that extend into the subchondral bone cause hemorrhage and the formation of a fibrin clot. The fibrin acts as a scaffold for repair.[9] Inconsistency is a major problem in the biological repair of articular cartilage and many times the repair tissue that fills this defect does not maintain the mechanical properties of native hyaline cartilage.[10] This tissue is commonly classified as hyaline-like or fibrocartilage with inferior mechanical properties to the native cartilage.[7] The aim of this thesis is to investigate a new culture media for the storage of osteochondral allografts.

Other treatment options for articular cartilage defects

The primary treatment for chondral lesions is typically arthroscopic lavage and debridement.[11] Symptomatic improvement of joint pain and function was noted in most patients after undergoing these procedures.[12,13] These two procedures remove loose cartilage and debris yet do not repair the damaged articular cartilage or prevent the advancement of disease. Therefore they are unsatisfactory for a long term solution. Unfortunately, it has also been shown that arthroscopic lavage and debridement do not improve pain or function compared to placebo arthroscopy.[14]

The microfracture technique was first introduced in 1997 and showed good functional improvement in an eleven year follow-up.[15] Unfortunately, the defect is not replaced with hyaline cartilage after the procedure but rather filled with fibrocartilage.[16] Also,

significant deterioration in clinical outcome has been seen after eighteen months.[17] Results from this procedure are not satisfactory to be a long term solution. Osteochondral autografting has shown some promise in the treatment of articular cartilage defects, especially small defects. However, differences in thickness, donor-site morbidity, and donor-recipient structural differences are complications with using this procedure that decrease the effectiveness of this procedure.[18] In a recent study using sheep this procedure resulted in the rapid degeneration of the transplanted cartilage as well as chondrocytes near the repair site.[19] There are currently not enough long term studies evaluating the efficacy of this procedure.

Many of the potential patients for OCA have few options besides total joint replacement. This type of procedure improves pain and mobility but is not appropriate for a young and active individual. There is also the concern of implant wear, osteolysis, and peri-prosthetic failure.[20,21]

Osteochondral allografts (OCAs) have gradually increased in popularity among orthopedic surgeons because they are the only option for treatment of articular cartilage lesions that replaces damaged tissue in a biologically appropriate manner. The defect is filled with hyaline cartilage instead of hyaline-like or fibrocartilage. It has also been demonstrated that chondrocytes in the OCA are viable after implantation in the patient.[22-25]

History

OCAs have been used for treatment of articular cartilage pathology for more than a century. Lexer was the first to use OCA in 1908 and had a reported a success rate of

approximately 50%.[26] However, difficulties associated with technical aspects of the surgery, among other issues, contributed to the discontinuation of the procedure. Tumor surgeons then began using the technique again in the 1970s. Despite early successes during the resurrection of this procedure, it was not used widely because of the limiting factor of tissue availability.[27] Until 1998 when OCA became commercially available from several tissue banks, the use of fresh OCA in North America was restricted to two institutions which maintained their own systems for retrieving, processing, and storing tissues for their own clinical use.[28-30] This review will focus on small fragment OCA for articular cartilage pathology in which minimal bone is left on the allograft speed integration and avoid other issues with transplanting bone. Larger OCAs with ancillary bone used to replace hemijoints or entire condyles in oncologic or massive trauma cases are not the focus of this review.

Indications

Injuries that involve both cartilage matrix and chondrocytes have an inferior repair response that does not regenerate normal hyaline cartilage.[31] OCA is a technique that is indicated for treating articular cartilage defects of full-thickness and greater than 1 cm^2.[32,33] Conditions that can cause lesions or defects of this nature include trauma, ostechondritis dissecans (OCD), avascular necrosis, and focal degenerative disease. OCA is most commonly used in the knee, but indications for use in the ankle, hip, elbow, and shoulder are being investigated. There has also been success using OCA to salvage previously failed procedures such as microfracture, autologous chondrocyte implantation, and osteochondral autograft transfer.[34]

Contraindications

Conversely, there are some important considerations before implementing OCA. Advanced multicompartmental arthrosis at any age or activity level is currently a relative contraindication for OCA. Uncorrected meniscal or ligament pathology and axial malalignment of the lower limb also appear to be contraindications for OCA of the knee.[34] Inflammatory diseases such as rheumatoid arthritis and corticosteroid induced osteonecrosis are also contraindications for performing OCA.[35] However there has been recent success in repairing OC defects due to steroid induced osteonecrosis.[36]

Success with OCA

The use of OCAs to treat articular cartilage defects is an alluring approach due to structural integrity, native tissue composition and architecture, and capabilities for integration. The bone portion of the graft will incorporate with host bone via creeping substitution and the articular cartilage can maintain a functional level of viability by receiving adequate nutrition via diffusion of synovial fluid. Importantly, the avascular nature of articular cartilage in conjunction with its dense, specialized extracellular matrix surrounding the chondrocytes allow this tissue to be transplanted without immunosuppression of the patient. Based on best current knowledge, functional survival of OCAs after transplantation primarily depends on chondrocyte viability and mechanical properties of the graft at the time of implantation into the patient. Incorporation of the graft bone into host bone and preservation of cartilage viability must then be maintained for long term success.

Incorporation of the osseous portion of the OCA functions to anchor the graft in place. The osseous portion of small grafts in replaced by host bone in a process called creeping substitution.[37] Larger grafts, such as replacement of an entire condyle, are reinforced but not replaced by host bone.[38] Osteocytes typically do not survive storage without surgical revascularization, which decreases the immunogenicity of the OCA.[39] In an animal model study, Glenn et al. reported that 89% of canine OCAs had full incorporation of bony trabeculae into surrounding native bone at three and six months post-implantation.[40] It has also been shown that the bone-to-cartilage ratio does not influence the quality of the graft.[41]

OCAs have been reported to survive at least twenty five years with a stable, integrated osseous base, viable donor chondrocytes, and preservation of functional extracellular matrix. [25] Jamali et al. published a case report in which donor cells from a fresh OCA survived for twenty-nine years after OCA procedure.[24] Also, biopsies from fresh OCAs up to 6 years post-transplantation have shown RNA synthesis and proteoglycan (PG) production.[22]

One concern among surgeons and researchers is the effect storage has on the OCA *in vivo.* This was especially true during the advent of FDA-required mandatory disease testing when tissue banks were forced to hold OCA for an average of 14 days.[42] Previous outcome studies with positive results had all been done involving fresh OCA transplanted within a week of donor death and historically within days of excision.[22] Subsequent studies have shown positive results with prolonged fresh stored OCA for 20 days.[43] Historically, fresh OCAs were typically harvested, stored at 4°C in Ringer's

lactate, and implanted within 24 hours of donor death. Now, OCAs are still considered fresh after 28 days of storage at 4°C while awaiting final disease testing.

Immune response

Currently, OCA are not HLA defined or blood-type matched before transplantation from donor to recipient.[34] Isolated chondrocytes will elicit an immune response, however, the extracellular matrix of intact hyaline cartilage effectively " hides" chondrocytes from the host immune system.[44] Therefore, intact hyaline cartilage is a relatively immunologically privileged tissue and the host does not become sensitized to the allograft.[45] Fresh OCAs takes advantage of this property of the tissue by transplanting viable chondrocytes inside an intact extracellular matrix. However, bone and marrow components are not immunologically privileged. Prior to transplantation steps are taken to minimize bone volume, and grafts are lavaged to remove any leftover marrow components.[46,47] The response is also limited because very few osteocytes are able to survive without vascularization, effectively reducing the immune response.[39] Further, osteocytes have been shown to be less resistant to preservation at 37°C.[48] The immune system accommodates OCAs because of the lack of viable osteocytes after storage and an intact extracellular matrix. Graft size plays a role in immune response, as larger grafts tend to have a stronger immune response .[49] Small fragment OCAs with minimal bone, therefore, minimize the immunogenic load. Among storage methods, frozen OCAs elicit less immune response than fresh OCA yet this response is not necessarily rejection, but rather an accommodation of the tissue.[47]

Retrieval studies in humans have consistently shown that patients tolerate the OCA immunologically, despite no HLA or blood type matching, and show no histologic evidence of rejection.[37,50] However, a study using MRI found that those patients with anti-HLA antibodies had a poorer graft-host interface than those people without anti-HLA antibodies[45] Despite the evidence of immune response to OCAs, the clinical relevance is unknown. [47,51] When taking into account the relative success OCA procedures have had in the past, it seems that the immune responds with accommodation of the tissue. Importantly, when examining failed OCAs there was no evidence of an immune response to the allograft tissue.[37,50,52] One would assume that if the immune system is rejecting these tissues, a response would be especially evident in failures. Modulation of the variable immune response seen in OCA may prove to be efficacious in the future, however, there needs to be more knowledge and research to determine the effects of the immune system on this allograft tissue. OCAs generally always tolerate transplantation immunologically, and the use of small-fragment OCA minimizes the host response to donor tissue.

Current tissue bank storage

The American Association of Tissue Banks establishes guidelines for procurement and processing of allograft tissue. All tissue bank facilities are required by law to register with the FDA which allows for federal oversight and inspection.[53] Also, accredited tissue banks should be following Current Good Tissue Practice set up by the FDA. Recently tissue banks began withholding allograft tissues from surgeons for 14 days to allow for complete disease testing before releasing tissue for

transplantation.[34,43,54] OCAs are harvested within 24 hours of donor death and aseptic techniques are used during the entire process from donor to patient. Acceptable donors are between the ages of 15 to 40 years old with articular surfaces that pass a visual inspection for quality.[42]

Detailed medical, social, and sexual histories of potential donors are acquired prior to transplantation.[55] As of August 2007, donors are screened for HIV type 1 and 2 antibody, hepatitis B surface antigen, total antibody to hepatitis B core antigen (IgG and IgM), HTLV-I/HTLV-II antibody, hepatitis C antibody, and a syphilis assay. Also, nucleic acid-amplification tests (NATs) are required for HCV and HIV-1. The window period, (i.e. time between infection and detection of virus by the screening tests), is seven days for the NATs, therefore seven days is the minimum time for withholding the allograft. Bacterial cultures are also obtained, and tissue is discarded if there is a positive culture for *Clostridium* or *Streptococcus Group.* [53] Before the newest testing protocol, OCA were sometimes held as long as 40 days after harvest.

After the Ball et al. landmark article in 2004 demonstrating the superiority of tissue culture media over lactated Ringer's, tissue banks began storing OCAs in tissue culture medium.[52,56] Storage in Lactated Ringer's solution at 4°C was standard practice prior to this article. The critical time point for graft implantation is 28 days, after this time point cell viability in the prolonged fresh stored OCA begins to fall below 70%.[57] Unfortunately because of the current time constraints for transplantation of tissue and stringent testing guidelines, large amounts of donor tissue are discarded.[58]

Storage

Preservation Method	Tissue Viability	Immune Response
Frozen	None	↓↓
Cryopreserved	~21%, limited to superficial layer	↓
Lactated Ringer's	60-90% after 7 days	↑
Tissue Culture Media 4°C	27-83% after 28 days	↑
Tissue Culture Media 37°C	~60% after 28 days	Unknown

Table 1: Comparison of OCA storage methods

The goal of a storage media for OCA is to maximize chondrocyte viability and maintain tissue properties until the tissue can be transplanted. The maintenance of chondrocyte viability is important for successful clinical outcome. Malinin and colleagues demonstrated that OCAs transplanted after 21 days of storage had more degenerative changes after 6 weeks *in vivo* than the OCAs transplanted before 21 days of storage.[59]

Freezing is commonly used as a preservation method for bulk allograft procedures such as oncologic or major trauma cases where it is necessary to have tissues available for emergencies.[7] The method of storing OCAs by freezing the grafts at -80°C kills nearly all viable osteocytes and chondrocytes during the freezing process.[39,60-64] This method of storage is not promising for small fragment OCAs that rely on high chondrocyte viability for successful outcomes. In the past, freezing OCAs seemed like an attractive approach because it could extend the storage period and potentially increase availability. In practice, however, freezing OCAs is complicated by the formation of ice

that destroys chondrocytes.[65] The high water content of articular cartilage negatively affects chondrocyte survival during the freezing process because of this ice formation.

It is not surprising the other properties of the tissue would be affected by the lack of viable chondrocytes after freezing. Chondrocytes are responsible for the maintenance of the extracellular matrix in articular cartilage and the cartilage matrix begins to breakdown in the absence of viable chondrocytes. The biomechanical properties of deep frozen OCA are inferior to fresh OCA.[66] Acosta et al. found increased levels of matrix metalloproteinases (MMPs) in frozen allografts indicating active degradation of the extracellular matrix.[67] One beneficial effect of storage at -80°C is the decreased immunogenicity of the graft.[39] Frozen allografts are still attractive outside of oncology because they allow for more flexibility in scheduling and grants more time for thorough disease testing.

The addition of cryopreservative agents, such as ethylene glycol, glycerol, or dimethyl sulfoxide (DMSO), allows the grafts to maintain higher chrondrocyte viability than freezing alone. Hypothetically, these supplements should help prevent ice formation during the freezing process, therefore, increase viability. It has been shown that frozen OCAs where no ice is formed will have good cell recovery after re-warming.[68] The addition of glycerol and DMSO increased chondrocyte viability when compared to freezing without cryopreservative agents but the surviving chondrocytes are typically isolated in the superficial layer. [62,64] Ohlendorf et al. demonstrated that even with survival of chondrocytes in the superficial layer there is no survival in the middle or deep layers of articular cartilage.[65] This is likely due to the inability of the cryopreservative agents to penetrate articular cartilage. Full penetration of DMSO and glycerol into articular

cartilage requires longer than one hour but letting tissue absorb the cryopreservative agents for longer periods of time increases the risk of toxicity.[69,70] Research continues in this area because of the ability to increase schedule flexibility and allow for thorough disease testing.

Malinin et al. cryopreserved allografts with glycerol and then froze them for at least 24 hours. The six cryopreserved OCAs were then transplanted into canine knees and examined at a later date. The cartilage appeared normal macroscopically but there was a loss of chondrocyte nuclei microscopically 4 months after transplantation. Encouraging data from this study was the complete healing of bone after 4 months and the absence of a chronic inflammatory reaction.[71] Csonge and colleaugues were able to maintain chondrocyte viability of 21.6% after cryopreservation of articular cartilage.[72]

By the end of the 20th century, fresh cold-stored OCA became available commercially, greatly increasing the availability of tissue.[34] Prior to this, the use of fresh OCA was limited to two institutions in North America.[30] Fresh OCAs have shown the best outcomes because of high chondrocyte viability and superior material properties maintained during storage. Traditionally, fresh OCAs were transplanted very soon after donor death. Until the FDA started requiring disease testing, it was standard practice to store at 4°C in Lactated Ringer's solution and transplant within forty-eight hours.[73] Sammarco et al. demonstrated that OCA stored with this method had close to 100% chondrocyte viability at the time of transplantation.[74] However, tissue banks started holding OCAs for a minimum of 14 days to allow for adequate viral and bacterial disease testing as mandated by the FDA. Subsequently, surgical transplantation was delayed by 3 to 6 weeks after initial tissue recovery.[42] This prolonged fresh storage led to a subsequent

decrease in cell viability among other tissue properties. There was concern for some time because no long term studies had been done to show the effect prolonged storage had on the OCA *in vivo*. There have since been prospective studies looking at prolonged fresh stored allografts that showed success.[43,52,54,58,75] Unfortunately, laboratory studies have shown that allograft quality decreases with increased storage time.[56,76,77] Improvements must be made in tissue culture technique and media constituents to maintain OCAs as similar to *in vivo* tissue after storage as possible. Currently, maintenance of chondrocyte viability near 70% until transplantation is the major goal of OCA storage. Studies have shown the ability of storage media to maintain this amount of viability for 28 days.[30,56,77] Therefore, it is the current goal to utilize fresh stored OCAs within 30 days.[43,75]

Fetal bovine serum (FBS), a common supplement to tissue culture media included proteins with growth factors, hormones, amino acids, sugars and lipids. The addition of FBS to storage media at 4°C has been shown to significantly improve chondrocyte viability in certain preparations. Allografts stored in media with FBS maintained a chondrocyte viability of 67% while the serum-free media maintained only 27.3% of chondrocytes after 28 days of storage.[78] Another study involving the storage of sheep knees at 4°C in tissue culture media with FBS evaluated cell viability over the course of 60 days. Chondrocyte viability was 98% at Day 8, 81% at Day 29, and 52% at Day 60.[30] Storage of canine OCAs in culture media with FBS held at 4°C was shown to maintain cell viability ~95% of fresh levels up to 14 days. However, cell viability was also shown to decrease thereafter to 65-90% at Day 28.[57] Similarly Malinin et al. demonstrated a chondrocyte viability of nearly 100% at 7 days, ~70% at 14 days, and less than 40% after 21 days in the storage of human OCA.[59] Pearsall et al. examined chondrocyte viability in

refrigerated OCAs stored in culture media and supplemented with FBS at the time of transplantation. The average length of storage was 30 days and average chondrocyte viability at the time of transplantation was 67%.[76]

FBS has shown positive results in maintaining tissue properties and chondrocyte viability of OCA during storage. Proteoglycan synthesis, measured by $^{35}SO_4$ uptake, of human OCA stored in media supplemented with FBS at 4°C was maintained at levels of fresh control after 28 days of storage. OCAs stored in serum-free media had a significant decrease in proteoglycan synthesis.[78] Storage at 4°C in DMEM with supplemental FBS was also shown to maintain histologic characteristics and a biomechanical properties of fresh control canine OCA for 28 days.[79] Wayne et al. found that GAG content, proteoglycan content and histologic appearance did not deteriorate over a period of 60 days when stored at 4°C in tissue culture media supplemented with FBS.[80] Amiel et al. determined that type II collagen and glycosaminoglycan (GAG) were synthesized and maintained at Day 0 levels in canine OCA for 28 days after storage at 4°C.[81] However, it has been shown that s-GAG content per dry weight decreased from 34% on Day 1 to 21% on Day 60 in OCAs stored in tissue culture media supplemented with FBS at 4°C.[30]

Other studies involving the storage of OCA in media supplemented with FBS have shown mixed results. Storage of canine OCA showed a significant decrease in proteoglycan synthesis after 28 days of storage even with FBS supplementation.[57] In another study examining storage at 4°C, the addition of FBS to tissue culture medium did not seem to have much effect on the GAG content of OCAs stored for 28 days.[78] An early study evaluating articular cartilage after storage at 4°C in media supplemented with FBS and antibiotics showed a significant decline in GAG content from fresh controls after 5

days of storage.[82] These studies highlight some of the benefits of supplementation with FBS to storage media.

In addition, it is important to evaluate the effect that storage has on OCAs *in vivo.* Oates et al. showed that canine OCA stored for 14 days in DMEM supplemented with FBS at 4°C maintained their biochemical and biomechanical composition *in vivo* just as well as the grafts that were implanted fresh.[83]

There has been a trend to move away from the use of FBS because of variability in its contents, possibility of disease transmission, and possible immunogenic reaction. Iatrogenic transmission of Creutzfeldt-Jakob disease has occurred through other procedures. Though there are no reports to date of Creutzfeldt-Jakob disease transmission through infected bovine serum, the risk remains.[84,85] Despite the positive effects of FBS media supplementation, the risks outweigh the benefits of using this storage supplement and there has been a subsequent movement away from this supplement, especially in humans. Therefore, there is a need to find a serum-free chemically-defined media that will maintain OCA in a manner similar to FBS.

Serum-free preparations have shown promise in OCA storage especially with a chemically defined supplementation to improve tissue viability and material properties. Tissue banks have since changed their storage protocol from Lactated Ringer's solution to tissue culture medium, following the work done by Ball and coworkers.[56] OCAs stored in tissue culture media maintained a higher percent of viable chondrocytes when compared to those stored in Lactated Ringer's solution. They demonstrated that human OCA plugs stored for 28 days at 4°C in a serum-free culture media maintained chondrocyte viability at nearly 85% whereas Lactated Ringer's solution struggled to

maintain 30% chondrocyte viability over the same time period.[56] Teng et al. confirmed these findings by showing that juvenile bovine OCA stored in culture media only containing DMEM has significantly higher cell viability after 2 weeks than Lactated Ringer's solution.[86] Human femoral condyle OCAs were stored in serum-free culture medium at 4°C. Chondrocyte viability was 98% at Day 14 and maintained at 70% on Day 28.[77] Allen, et al. examined human OCAs at the time of surgical implantation after an average storage time of 20 days and found 80% viability after storage in serum free culture media at 4°C.[87]

Tissue culture media maintains the biochemical and biomechanical properties of OCAs better than those stored in Lactated Ringer's solution. In the past serum free media did not seem very promising, a study evaluating porcine OCA found significant deterioration of histologic characteristics and proteoglycan synthesis after 7 days.[88] Since this article there have been other publications supporting the maintenance of tissue properties in serum-free culture media with the addition of supplements to the media. Proteoglycan synthesis was significantly better in OCAs stored in tissue culture media supplemented with ascorbic acid and glutamine when compared to those stored in Lactated Ringer's.[56] Cartilage matrix properties, GAG content and biomechanical parameters, were maintained at levels of fresh controls in human OCAs stored for 28 days in serum-free culture media at 4°C.[77] Allen et al. further confirmed the maintenance of extracellular matrix and other tissue properties during storage at 4°C in tissue culture media for an average of 20 days when they showed that GAG content and indentation stiffness was maintained at fresh levels.[87]

Author	Preservation Method	% Viability (Day 7)	% Viability (Day 14)	% Viability (Day 21)	% Viability (Day 28)
Allen*	DMEM 4°C	-	-	82%	-
Ball	Lactated Ringer's 4°C	91	80	-	29
	DMEM 4°C	-	91	-	83
Linn	DMEM+FBS 4°C	-	-	-	61
	DMEM+FBS+ etanercept 4°C	-	-	-	68
Pallante	DMEM+FBS 4°C	-	85	-	40
	DMEM 37°C	-	-	-	60
Pennock	DMEM+FBS 4°C				68
	DMEM 4°C				27
Teng	Lactated Ringer's 4°C	58	20	-	-
	DMEM 4°C	67	55	31	15
	DMEM+FBS 4°C	94	86	84	45
	DMEM+IGF-1	70	59	56	5.9
	DMEM+ZVA D-fmk	73	63	52	16.1
Williams, J	DMEM+FBS 4°C	-	94-98	75-98	65-90
Williams, R**	DMEM+FBS 4°C	98	80		81
Williams, S.	DMEM 4°C	98	97	-	70

Table 2: Comparison of storage media preparations in literature

*Day 20

**Days 8,15,29

Continuous modifications of the storage medium used for preserving OCA have occurred. Robertson et al. found that pro-apoptotic genes were upregulated during storage at 4°C that coincided with decreases in chondrocyte viability and proteoglycan synthesis.[89] Since this article, supplementation with apoptosis inhibitors has been an active area of research in the storage of OCA. Teng et al. used the apoptosis inhibitor

ZVAD-fmk as a supplement to DMEM. The supplemented media had significantly higher cell viability than DMEM alone at week 3; however, it was unable to maintain a significantly higher cell viability than DMEM alone at week 4.[86] Etanercept, a cytokine inhibitor, increased chondrocyte viability in the superficial layer and decreased the expression of TNF-α when used as a supplement to tissue culture media.[90] Bae et al. examined the effects that the addition of epigallocatechin-3-O-Gallate (EGCG) had on rabbit OCA stored at 4°C. Those grafts stored in media supplemented with EGCG maintained cell viability and GAG content better than the media without EGCG.[91] Nitric oxide synthesis was elevated during storage and had a detrimental effect on proteoglycan synthesis.[92] Inhibition of nitric oxide synthesis using a supplementation to tissue culture media is another area to be explored.

Recently, research has explored the possibility of storing OCAs at 37°C. Similar to the resurrection of OCAs in the 1970's, tissue culture techniques are being experimented with again. Pallante et al. found higher cell viability after 28 days in the articular cartilage from OCA stored at 37°C compared to 4°C. There was only a modest decrease in cell viability between the fresh control and grafts stored at 37°C. Storage at 37°C maintained cartilage thickness, GAG content, and collagen content compared to fresh controls after 28 days of storage.[93] Bian et al. found that culturing bovine OCA at 37°C in a serum-free medium could actually increase stiffness, as measured by the equilibrium modulus, and GAG content. This media was also supplemented with dexamethasone.[94] Another study demonstrated that OCA could maintain chondrocyte viability at Day 0 levels for up to 28 days when stored at 37°C with DMEM+, FBS, and cycloheximide. Unfortunately, sulfated GAG content decreased significantly from Day 0

levels.[95] Bastian et al. compared chondrocyte and osteocyte viability in tissue stored at 37°C. They found few live cells in bone, a beneficial effect that would decrease the immune response initiated during transplantation, and viable chondrocytes in the cartilage layer.[48]

Retrieval studies

Author	Storage	Diagnosis	No. of Knees	Avg. time to failure (years)
Gross	4°C	Trauma	6	<1
			11	2.9
			24	12
Kandel		OA,ON, OCD	44	1-7
Oakeshott	4°C	OA, ON	18	2.9
Williams	4°C	Multiple	14	3.5

Table 3: Selected retrieval studies from literature

Gross et al. inspected 41fresh OCA used in the repair of posttraumatic defects. These OCAs were transplanted to the patient within 72 hours post-harvest. In the meantime they were stored in Ringer's lactate at 4°C. Failures were classified into three groups: early, mid-term, and long-term retrievals. Early failures were due to decreased chondrocyte viability and nonfunctional ECM whereas later failures were due to fracture or incomplete remodeling. Long term survival of OCAs was associated with viable chondrocytes, ECM preservation, and graft bone replaced by host bone.[25] Williams et al. examined articular cartilage from failed OCA. These retrieved grafts had been stored at

4°C in Lactated Ringer's solution and transplanted within 5 days of donor death. Chondrocyte viability was maintained at above 80% in the transplanted tissue after an average 3.5 years in the patient. Metabolic activity and cartilage integrity was also sustained from the time of transplantation to retrieval.[52] The orthopedic transplant program began in 1972 at Mount Sinai Hospital in Toronto. One hundred eight patients had fresh small-fragment OCAs transplanted in their knees. Twenty-two of these failed; however, data is only available for 18 of these patients. Available failures were examined histologically, and viable donor chondrocytes and cartilage matrix were seen up to nine years post-transplantation.[37] Kandel et al. examined 22 patients whose OCA had failed. A total of 44 OCAs were evaluated at the time of failure. These OCAs were harvested from the donor and transplanted to the patient within 24 hours of donor death. Procedures were as follows: seven tibial plateau grafts, three femoral condyle grafts, 13 bipolar grafts, and two bipolar bicompartmental grafts. Viable chondrocytes were seen up to 7 years post-transplantation. Also, viable donor chondrocytes were seen in the retrieved OCAs 7 years post-transplantation.[50]

Importantly there was no evidence of an immune reaction to the OCAs evaluated in these retrieval studies, further indicating that this is an immunologically privileged tissue.

Clinical Outcomes

Author	Storage Medium	Time from harvest to transplantation (days)	Number of Cases	Follow-Up (years)	Outcomes	5 year survival
Aubin	4°C in Lactated Ringers	2	60	10	85% survival	95%
Chu	4°C in Lactated Ringers	2-6	43	6.2	93% survival	
Davidson	4°C in tissue culture media	36	10	3.3	100% survival	
Garrett	4°C in tissue culture media	4	17	3.5	94% survival	
Ghazavi	4°C in Lactated Ringers		126	7.5	86% survival	95%
Gortz	4°C in tissue culture media	5-21	28	5.6	89% survival	
Gross	4°C in Lactated Ringer's	3	60	10	80% survival	95%
LaPrade	4°C in tissue culture medium	20.3	23	3	100% survival	
McCulloch	4°C in Lactated Ringer's	24	25	~3	96%	
McDermott	4°C in Lactated Ringer's	1	48	6	75% success	
Meyers			59	2-10	77% success	
Williams	4°C in tissue culture medium	30	19	4	100% survival	

Table 4: Outcome studies after implantation of OCAs

There was early success using OCA, even with sub-optimal storage methods. McDermott et al. prospectively evaluated the first one hundred OCA procedures performed from 1972-1983. These OCAs were stored in Lactated Ringer's at 4°C up to 24 hours. Overall, the procedure was mildly successful. The success rate in the trauma group was most promising at 75% success.[35] Myers and colleagues found a success rate of 77% after 2-10 years post-transplantation of osteochondral allografts in 58 patients.[96] Recently, McCulloch et al. found success in using OCAs stored in Lactated Ringer's for an average of 24 days. In this prospective study, 25 patients received prolonged fresh stored OCAs and showed significant improvement in function after an average follow up of 35 months. Only one allograft was reported as a failure and 88% of the OCAs showed incorporation of host bone at the time of follow up.[75]

OCAs were used to correct defects in patients due to osteochondritis dissecans (OCD) in 17 patients. Grafts were stored in tissue culture medium at 4°C and transplanted to patient within four days of donor death. There was a survival rate of 94% after an average three and a half years.[97]

Fresh stored OCAs have proven to be a viable treatment option in many outcome studies. Fresh small-fragment OCAs were used to correct posttraumatic defects in 126 knees. These grafts were harvested within 24 hours of donor death and stored at 4°C in Ringer's lactate. After an average follow-up of 7.5 years, the OCAs had a success rate of 86%. Failures were associated with worker's compensation cases and bipolar grafts.[98] Chu et al. evaluated 43 unipolar OCAs that were stored in Ringers lactate at 4°C and transplanted within 2-6 days of donor death. After an average of 6 years, 84% of unipolar OCAs were rated good or excellent. Among the 43 OCAs transplanted only one was

rated a failure.[99] Sixty patients were involved in this study evaluating the use of OCAs stored in Ringers lactate at 4°C for large posttraumatic defects (>3cm^2). In these patients, 84% had excellent or good scores after an average follow up of 10 years.[73] In another example of OCAs having great success in the repair of post-traumatic defects, distal femoral osteochondral allografts were stored similarly to the previous two studies above. In 60 patients receiving femoral condyle grafts with an average follow up of 10 years, 80% of these survived.[100]

In what used to be a contraindication to this procedure, fresh OCAs were used to repair 28 knees due to steroid-associated osteonecrosis. The average time to follow up was 5.6 years and at this time there was an 89% survival rate. Donor tissue was procured within 24 hours of death, stored in tissue culture media at 4°C, and transplanted within 5-21 days.[36]

The following studies were important because they confirmed that OCAs were still successful *in vivo* after prolonged fresh storage at 4°C. Nineteen patients whose articular cartilage defects were corrected with OCA stored at 4°C in tissue culture medium for an average of 30 days were evaluated. The average follow-up period was 4 years and 100% of the grafts survived. These patients also had increased functional scores based on the Activities of Daily Living Scale of the Knee Outcome Survey.[54] Davidson and colleagues evaluated eight patients, two of which had bilateral OCA, who received femoral condyle OCAs stored for an average of 36 days in serum-free culture media at 4°C. A second-look arthroscopy was performed 40 months after transplantation and a biopsy was taken from the donor and native cartilage. Cell viability between native and donor cartilage was not statistically different. Also on clinical assessment, OCAs

improved function.[58] LaPrade et al. examined patients whose femoral condylar articular cartilage defects were treated with OCAs stored at 4°C for an average of 20 days in serum-free culture medium. Significant clinical and functional improvements were noted after an average follow up of 3 years. The procedure was a success in all of these patients.[43] These studies provided the much needed confirmation that prolonged fresh stored OCAs were still a viable treatment option.

Articular cartilage pathology is a common finding in the orthopedic world and current treatments are not sufficient to repair cartilage defects in an appropriate manner. OCAs are a promising treatment but improvements need to be made to prolong the fresh storage of tissue. Viability decreases rapidly after 28 days and disease testing takes 14 days on average, this leaves roughly a 14 day window for transplantation of tissue that severely hampers scheduling and tissue availability to patients.[42,57] This thesis will examine the effect of OCA storage at 37°C with different media supplementations. Tissue will be evaluated at 28 and 56 days for viability, biochemical contents, and biomechanical properties. Media will also be evaluated to determine differences in the protein release from tissue in different media preparations.

CHAPTER 2

STUDY 1: OSTEOCHONDRAL ALLOGRAFT PRESERVATION IN A SERUM-FREE CHEMICALLY DEFINED MEDIA

1. Experimental Purpose and Hypothesis

Despite the prevalence of articular cartilage pathology, science has struggled with finding a treatment to halt the progression to OA. To date, reports exist on the cell viability, biochemical, biomechanical, molecular, and histologic properties during the storage of OCA, however, there is not one that characterizes all of these properties. It is the purpose of this study, therefore, to take a comprehensive look at the effects of a Control and Test media at 4°C or 37°C after 28 and 56 days of storage. This study will also evaluate the effects of different storage media or temperatures on OCA metabolism based on cytokines, chemokines, and inflammatory mediators. We hypothesized that the OCA stored at 37°C in the Test media would have better maintenance of cell viability and tissue properties than the other storage medias or temperatures.

2. Materials and Methods

a. Tissue Harvest and Culture

Osteochondral allografts (OCAs) were aseptically harvested within four hours of death from medial and lateral femoral condyles and tibial plateaus of 10 adult canine cadavers euthanized for reasons unrelated to this study. Allografts were stored overnight in

Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 1X insulin-transferrin-selenium (ITS, BD Biosciences, San Jose, CA), 1X penicillin-streptomycin-Fungizone™ (PSF), L-Glutamine, 0.9mM Sodium Pyruvate, 50µg/mL L-ascorbic acid, and MEM Non-Essential amino acids (Invitrogen, Carlsbad, CA) at 37°C, 95% humidity, and 5% CO2. Day 0 Control OCA were aseptically harvested from one femoral condyle and tibial plateau of 5 adult canine cadavers euthanized for reasons unrelated to this study. These OCAs were stored overnight in the media described previously and evaluated the following day.

The volume of each OCA (n=40) was measured and multiplied by 25-30 times that amount to determine the volume of media for each allograft. The OCA were cultured in a Control media (DMEM supplemented with ITS 0.9mM Sodium Pyruvate, 50µg/mL L-ascorbic acid, and MEM Non-essential amino acids) or in the Test media (DMEM supplemented with 1X insulin-transferrin-selenium-bovine serum albumin-linoleic acid [ITS+, BD Biosciences, San Jose, CA], 0.9mM Sodium Pyruvate, 50µg/mL L-ascorbic acid, MEM Non-Essential amino acids, 10.0µM dexamethasone sodium phosphate [APP Pharmaceuticals, Schaumburg, IL], 250 µg/mL sodium tetraborate [Sigma Aldrich, St. Louis, MO], and 2.5 ng/mL rhTGF-β3 [R&D Systems, Minneapolis, MN]).

Once the OCAs were aseptically processed they were preserved in either Control media or Test media at 4°C or 37°C for 28 or 56 days. Each stored specimen had its own contralateral control on the opposite leg. The media were changed every 7 days and media samples were saved for later analysis. At each time point full-thickness cartilage was evaluated for chondrocyte viability, biochemical analysis, and biomechanical analysis.

b. Chondrocyte Viability

Full thickness cartilage from each storage group was used to determine chondrocyte viability. Quantititative analysis of chondrocyte viability was determined by manually counting live and dead cells from images taken at 10X magnification using an Olympus F view II camera and Micro Suite Basic Edition software. CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, Invitrogen, Carlsbad, CA) was used to visualize live cells and ethidium homodimer-1 (EthD-1, Invitrogen, Carlsbad, CA) to visualize dead cells. Percent live cells were determined by taking the total number of live cells divided by the total amount of cells in the sample.

c. Glycosaminoglycan (GAG) Assay

After PBS wash, cartilage plugs were blotted with a paper wipe and weighed on balance to obtain the wet weight. Dry weight was determined after lyophilization for 24 hours. Cartilage tissue was the then digested in 50µL papain solution for 3 hours at 60°C. Sulfated-GAG (s-GAG) concentration within the cartilage matrix was determined using aliquots of digest solution using the 1,9 dimethylmethylene blue (DMMB) dye-binding assay. S-GAG content was determined from the ratio of s-GAG to total tissue dry weight[101] and reported at µg GAG/mg dry weight.

The DMMB assay was used to determine the amount of s-GAG release into media after each week of storage using the samples obtained during weekly media changes. Media GAG was reported as µg GAG normalized by multiplying by media volume used for storage.

d. Hydroxyproline (HP) Assay

The hydroxyproline (HP) content was determined using a colorimetric assay modified to a 96-well format. HP content was used as a measure of total collagen content.[102] A 50-µL aliquot of the digest solution was mixed with 50 µL 4N NaOH and the mixture was autoclaved for 20 minutes at 121° C to hydrolyze the sample. The sample was then mixed with the chloramine T reagent and incubated at 25° C for 25 minutes, followed by mixing with Ehrlich aldehyde reagent. The chlorophore was developed at 65° C for 20 minutes. The absorbance was then read at 550 nm using a Synergy HT (Bio-TEK, Highland Park, VT) and the samples were compared with an HP standard to determine the HP concentration of the sample. Results were standardized to tissue dry weight and reported as µg HP/mg dry weight.

e. Nitric Oxide (NO) assay

Nitrite concentrations were measured spectrophotometrically using the Griess reaction (Promega Corp., Madison, WI) as described by the manufacturer. The concentrations were used to determine nitric oxide synthesis. A 25µL aliquot of medium diluted with 25µL of phosphate buffered saline was incubated with 50µL of 0.1% sulfanilamide in 5% phosphoric acid, then with 50µL of 0.1% N-1-naphthylethylenediamine dihydrochloride for 5 minutes at room temperature. Absorbance was then read at 520nm. Values were reported as µg NO/mL and then normalized for each sample by multiplying the concentration by amount of media used for storage.

f. PGE2 assay

Concentrations of PGE_2 in the media samples were quantified by a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The samples and standards were incubated with 50µL of goat polyclonal anti-mouse IgG antibody and 50µL of

acetylcholinesterase-linked tracer. The plate was covered with a plastic film and incubated for 18 hours at 4°C. Before developing the plate, Ellman's Reagent was reconstituted with UltraPure water. The plate was rinsed with Wash Buffer and Ellman's Reagent was added to the wells. The plate was covered and allowed to develop in darkness while on an orbital shaker for 60-90 minutes. The absorbance was measured at a wavelength of 410nm. Sample concentrations were determined from the standard curve and reported as picogram (pg) per mL as noted in the manufacturer's directions . PGE2 was then normalized to media volume by multiplying pg PGE2/mL by the volume of media the OCA used for storage.

g. Cytokine assays

An aliquot of femoral condyle and tibial plateau storage media from every group at Days 7, 28, 56 was thawed. Each aliquot was analyzed using a multiplex canine cytokine and chemokine immunoassay (Millipore Corp., Billerica, MA) based on the xMAP platform (Qiagen Inc, Valencia, CA) for GM-CSF, IFN-γ, IL-7, IL-15, IL-2, IP-10, IL-4, KC, IL-6, IL-8, IL-10, IL-18, MCP-1, and TNF-α according to the manufacturers' directions. Media samples were mixed with anti-chemokine and cytokine monoclonal antibody-charged, small, polystyrene microspheres in a 96 well plate. Following an overnight incubation at 4° C, a polyclonal secondary antibody was added and also streptavidin-phycoerythrin. The median fluorescence intensity (MFI) was determined for each sample and concentration was reported as pg/mL. The values were then normalized by multiplying the concentration by the amount of media used to store the OCA. Another aliquot of femoral condyle storage media from every group at Days 7, 28, and 56 was analyzed using a multiplex human matrix metalloproteinase (MMP) immunoassay

based on the xMAP platform (Qiagen Inc, Valencia, CA) for 5 MMPs: MMP1, MMP2, MMP3, MMP9, MMP13. This assay has been previously shown within our laboratory to cross-react with samples of canine origin [103]. Values were reported as pg cytokine or MMP and normalized by multiplying the concentration by the amount of media used to store the OCA.

h. Biomechanical Testing

At each end point, 4mm plugs were removed from the articular cartilage and immediately put in a -80°C freezer until biomechanical testing could be done.[56] The dynamic modulus of cartilage specimens were determined by unconfined compression with loading to 10% strain at a rate of 0.05% per second, after an initial 0.02-N tare load (elastic modulus, or Eγ). Dynamic modulus (G*) was measured by superimposing 2% peak-to-peak sinusoidal strain at 0.1 Hz. Values were reported as milli-Pascals (MPa).

i. Histologic Assessment

For histologic evaluation, 2 mm-thick sagittal sections of the OCA were cut and fixed in 10% formalin, and then placed in a decalcifier in Surgipath Decalcifier II. After decalcification and subsequent routine histologic processing, each specimen was embedded in paraffin, and sectioned through the sagittal plane with 5µm thickness. These samples were then stained with hematoxylin and eosin (H&E), toluidine blue (T blue), and Masson's trichrome. Each section was subjectively assessed by one investigator. Each section was qualitatively assessed for cartilage architecture, GAG content, and collagen.

j. Immunohistochemistry

As described before, 2 mm sagittal sections were cut and fixed in 10% formalin. After fixation was complete, samples were decalcified in 10% disodium ethylenediaminetetraacetic (EDTA) acid. After decalcification and subsequent routine histologic processing, each specimen was embedded in paraffin, and sectioned 5 μm through the sagittal plane. For immunohistochemical analysis, unstained sections were deparaffinized in xylene and rehydrated in graded ethanol solutions. The samples were permeabilized with a 0.1% trypsin solution at 36° C for 60 minutes and then blocked with a 10% bovine serum albumin at 40° C. Slides were incubated overnight at 4° C in predetermined dilutions of the primary antibodies: collagen type II (rabbit polyclonal antibody, Abcam, Cambridge, UK) and proteoglycan (mouse anti-human antibody, Millipore Corp., Billerica, MA).

The next day slides were rinsed in Tris-buffered saline before being incubated with the secondary antibody. Collagen type II was labeled with goat anti-rabbit fluorescein isothiocyanate (FITC, Millipore Corp., Billerica, MA) and proteoglycan was labeled with goat anti-mouse rhodamine (Millipore Corp., Billerica, MA). Samples were coverslipped and reviewed using fluorescent light microscopy. Negative controls were used as comparison in which the primary (but not secondary antibody) was omitted from the slides to see if any stain was due to fluorescence aside from the target region. Immunohistochemical images were subjectively assessed.

k. Statistical Analysis

Statistical analyses were done using the SigmaStat® computer software program (San Rafael, CA). Data were pooled for each endpoint, Day 28 and Day 56, and comparisons were made between the four storage media and Day 0 controls. A one way ANOVA

using Tukey post-hoc comparisons was used for statistical analysis and significance set at $p<0.05$.

A two-tailed, equal variance T-test was used to determine statistical significance in the biomechanical data. Significance was set at $p<0.05$.

3. Results

a. Chondrocyte Viability

i. Femoral condyle OCAs stored in the 37° C Control media had significantly higher cell viability at Day 28 than 4°C Control and 4°C Test (p=0.016, p=0.01). At Day 56, the 37° C Control media had significantly higher cell viability than 4°C Control (p=0.008), 4°C Test(p=0.015), and 37°C Test (p=0.023).

At Day 28, the Day 0 viability was significantly higher than 4°C Control (p=0.007) and 4°C Test (p=0.01) media. At Day 56, the Day 0 viability was significantly higher than 4°C Control (p=0.032), 4°C Test (p<0.001), and 37°C Test (p=0.002).

ii. Tibial plateau OCAs stored in the 37° C Control media had significantly higher cell viability at Day 28 than both 4°C Control (p=0.039) and 4°C Test (p<0.001) media. Also, those stored in the 37°C Test media had significantly higher viability than OCA stored in the 4°C Test (p=0.019) media. At Day 56, the 37°C Control media had significantly higher cell viability than 4°C Control (p=0.034), 4°C Test (p<0.001), 37°C Test (p=0.015) media.

At Day 28, the Day 0 viability was significantly higher than 4°C Control (p=0.008), 4°C Test (p=0.008), and 37°C Test (p=0.001) media. At Day 56, the Day 0 viability was significantly higher than 4°C Control (p=0.008), 37°C Control (p=0.043), 4°C Test (p=0.008), and 37°C Test (p=0.008) media.

iii. See Tables on p.52. See Figures on pp. 62-67.

b. Tissue GAG content

i. Femoral condyle OCAs had no significant differences in tissue GAG content at Day 28. At Day 56, The OCAs stored in 37°C Test media had significantly less tissue GAG content than 4°C Control (p=0.027) and 37°C Control (p=0.033) media.

At Day 28, there were no significant differences between Day 0 controls. However, at Day 56 the Day 0 OCAs had significantly more tissue GAG content than those stored in 37°C Test (p=0.003) media.

ii. At Day 28, tibial plateau OCAs stored in the 4°C Control media had significantly higher tissue GAG content than 37°C Control (p=0.008) and 37°C Test (p=0.021) media. At Day 56, OCAs stored in the 4°C Control media had significantly higher tissue GAG content than 4°C Test (p=0.017) and 37°C Test (p=0.003) media. Also at Day 56, the 37°C Control media had significantly more tissue GAG content than 37°C Test (p=0.016) media.

There were no significant differences from Day 0 controls at Day 28. At Day 56, Day 0 OCAs had significantly higher tissue GAG content than 4°C Test (p=0.02) and 37°C Test (p=0.008) media.

iii. See Tables on p.53. See Figures on p. 68

c. Media GAG Content

i. Femoral condyle OCAs stored in 37°C Test media had significantly more GAG release into media than those stored in 37°C Control media at Days 21(p=0.003) and 28 (0.001). Those stored in the 37°C Test media had significantly more GAG release than 4°C Control at Days 7 (p<0.001), 14 (p<0.001), 21 (p<0.001), 28 (p<0.001), 35 (p=0.016), 42 (p=0.016), 49 (p=0.016), 56 (p=0.016). OCAs stored in 37°C Test media had significantly more GAG release than those stored in the 4°C Test media at Days 7 (p=0.004), 14 (p=0.001), 21(p<0.001), and 28 (p=0.001). Those stored at 37°C Control media had significantly more GAG release than those stored in the 4°C Test media at Days 7 (p=0.011), 14 (p=0.025), 28 (p=0.026), 49 (p=0.008), and 56 (p=0.032). OCAs stored in 37°C Control media had significantly more GAG release than those stored in 4°C Control media at Days 7 (p<0.001), 14 (p<0.001), 21 (p<0.001), 28 (p<0.001), 35 (p=0.008), 42 (p<0.001), 49 (p<0.001), 56 (p=.008). Lastly, OCAs stored in 4°C Test media had significantly more GAG release than 4°C Control media on Days 7 (p=0.005), 14 (p<0.001), 21 (p<0.001), 28 (p=0.001), 35 (p=0.008), 42 (p=0.023), 49 (p=0.026), and 56 (p=0.041).

ii. Tibial plateau OCAs stored in 37°C Test media had significantly more GAG release into media than those stored in 37°C Control media at Days 21 (p=0.001), 28 (p=0.003), and 35 (p=0.045). Those stored in the 37°C Test media had significantly more GAG release than 4°C Control at Days 7 (p=0.002), 14

(p<0.001), 21 (p<0.001), 28 (p<0.001), 35 (p=0.016), 42 (p=0.008), 49 (p=0.016), and 56 (p=0.008). OCAs stored in 37°C Test media had significantly more GAG release than those stored in the 4°C Test media at Days 7 (p=0.032), 14 (p<0.001), 21 (p<0.001), 28 (p=0.001), 35 (p=0.016), 49 (p=0.011), and 56 (p=0.018). Those stored at 37°C Control media had significantly more GAG release than those stored in the 4°C Test media at Days 7 (p=0.01), 14 (p=0.004), 21 (p=0.017), 28 (p=0.003), 49 (p=0.015), and 56 (p=0.024). OCAs stored in 37°C Control media had significantly more GAG release than those stored in 4°C Control media at Days 7 (p<0.001), 14 (p<0.001), 21(p<0.001), 28 (p<0.001), 35 (p=0.013), 42 (p=0.008), 49 (p=0.008), and 56 (p=0.002). Lastly, OCAs stored in 4°C Test media had significantly more GAG release than 4°C Control media on Days 7 (p=0.01), 14 (p=0.01), 21 (p=0.001), 28 (p=0.001)

iii. See Tables on p. 54. See Figures on p. 69

d. Hydroxyproline (HP) content

i. There were no significant differences between femoral condyle OCA storage group's HP content Day 28 or 56.

Also, there were no significant differences at any time point from Day 0.

ii. At Day 28, tibial plateau OCA stored in 37°C Test media had significantly higher HP content than 37°C Control (p=0.016). There were no significant differences between HP content of tibial plateaus at Day 56.

At Day 28, OCAs stored in 37°C Test media had significantly higher HP content than Day 0 ($p=0.044$). At Day 56, OCAs stored in 4°C Test media had significantly higher HP content than Day 0 ($p=0.046$).

iii. See Tables on p.55. See Figures on p.70

e. Media NO content

i. Femoral condyle OCAs stored in 37°C Control media had significantly more NO release at Day 7 than 4°C Control ($p=0.002$) and 4°C Test ($p=0.013$) media. There were no significant differences at Day 28 or 56.

ii. Tibial plateau OCAs stored in 37°C Control media had significantly more NO release at Day 7 than 4°C Control ($p=0.001$) and 4°C Test ($p=0.024$) media. Also at Day 7, NO release was significantly higher in the OCAs stored in 37°C Test media than those stored in 4°C Control media ($p=0.01$). At Day 28, OCAs stored in 37°C Test media continued to have significantly more NO release than those stored in 4°C Control media ($p=0.004$). There were no significant differences at Day 56.

iii. See Tables on p.56. See Figures on p.71

f. Media PGE2 content

i. There were no significant differences between femoral condyles at Day 7. At Day 28, OCAs stored in the 37°C Control media had significantly more PGE2 release than 4°C Control ($p=0.02$) and 4°C Test ($p<0.001$) media. Also at Day 28, 4°C Control media had significantly more PGE2 release than 4°C Test

(p=0.005) media. Finally, the OCAs stored in 37°C Test media had significantly more PGE2 release than 4°C Test (p=0.007) media.

At Day 56, OCAs stored in 37°C Control had significantly more PGE2 release than those stored in 4°C Control (p=0.041) and 4°C Test (p=0.023) media.

ii. At Day 7, there was no significant differences between tibial plateau storage groups.

At Day 28, the OCAs stored in 37°C Test media had significantly more PGE2 release than those stored in 4°C Control (p<0.001), 37°C Control (p=0.003), and 4°C Test (p<0.001) media. Also at Day 28, those stored in 37°C Control media had significantly more PGE2 release than 4°C Control (p=0.021) and 4°C Test (p<0.001) media. Finally, the OCAs stored in 4°C Control media had significantly more PGE2 release than 4°C Test (p<0.001) media.

At Day 56, the OCAs stored in 37°C Control media had significantly more PGE2 release than those stored in 4°C Control (p<0.001) and 4°C Test (p<0.001) media.

iii. See Tables on p.56. See Figures on p.72

g. Media MMP content

i. MMP-2 release was significantly higher from OCAs stored in the 37°C Test media than 4°C Control (p=0.008) and 4°C Test (p=0.008) media on Day 7. Also at Day 7, the 37°C Control had significantly higher levels of MMP-2 release than 4°C Control (p=0.008) and 4°C Test (p=0.008) media.

At Day 28, OCAs stored in 37°C Control media had significantly higher levels of MMP-2 release than 4°C Control ($p=0.008$) and 4°C Test ($p<0.001$) media.

At Day 56, OCAs stored in 37°C Control media had significantly higher levels of MMP-2 release than 4°C Control ($p=0.008$) and 4°C Test ($p=0.008$) media.

ii. MMP-3 release into media was significantly higher from OCAs stored in 37°C Control media than those stored at 4°C Control media at Days 7 ($p=0.031$), 28 ($p=0.048$), and 56 ($p=0.008$).

The MMP-3 release into media was significantly higher from OCAs stored in 37°C Control media than those stored in 4°C Test media at Days 28 ($p=0.032$) and 56 ($p=0.008$).

iii. MMP-13 release into media was significantly higher from OCAs stored in 37°C Control media than 4°C Control at Days 7 ($p=0.008$), 28 ($p=0.008$), and 56 (0.008).

MMP-13 release into media was significantly higher from OCAs stored in 37°C Control media than 4°C Test at Days 7 ($p=0.016$), 28 ($p=0.008$), and 56 ($p=0.008$).

Also, on Day 28 the release of MMP-13 into media was significantly higher from OCAs stored in 37°C Control media than 37°C Test media ($p=0.016$).

iv. See Tables on p. 57. See Figures on p. 73

h. Media cytokine release

i. Femoral condyles

1. OCAs stored in 37°C Control media had significantly higher release of KC than those stored in the 4°C Control media at Days 7 (p=0.011), 28 (p=0.008), and 56 (p=0.008). Also, OCAs stored in 37°C Control media had significantly higher KC release than those stored in 4°C Test media at Days 28 (p=0.008) and 56 (p=0.008).
2. IL-6 release from media was significantly higher from OCAs stored in 37°C Control media than those stored in 4°C Control media at Day 7 (p=0.032). No other significant differences were found.
3. At Day 7, IL-8 levels were significantly higher in 37°C Control media than 4°C Control (p=0.01), 4°C Test (p=0.019), and 37°C Test (p=0.036) media. Also, the 37°C Test media had significantly higher levels of IL-8 than the 4°C Control (p=0.025) media.

   At Day 28, IL-8 release from OCAs into media was significantly higher in those stored in the 4°C Test media than 4°C Control (p=0.032) media.

   At Day 56, 37°C Control media had significantly higher release of IL-8 than 4°C Control (p=0.008) and 4°C Test (p=0.008) media.
4. At Day 7, MCP-1 release from OCA stored in 37°C Control media was significantly higher than 4°C Control (p=0.008), 4°C Test (p=0.008), and 37°C Test (p=0.008) media. Also on Day 7, release of MCP-1 from OCAs stored in 37°C Test media at than those stored in 4°C Control (p=0.015) media.

At Day 28, MCP-1 release was significantly higher from OCAs stored in 37°C Control media than those stored in the 37°C Test media. Also at Day 28, release of MCP-1 from OCAs stored in 4°C Test media was higher than release from those stored in 37°C Test media.

At Day 56, MCP-1 release from OCA stored in 37°C Control media was significantly higher than 4°C Control (p=0.008), 4°C Test (p=0.008), and 37°C Control (p=0.008) media.

5. See Tables on p. 58. See Figures on p. 74

ii. Tibial plateaus

1. At Day 7, release of KC was significantly higher in 37°C Control than 4°C Control (p=0.001) and 4°C Test (p=0.004) media. Also, 37°C Test media had significantly higher release of KC than 4°C Control (p=0.007) and 4°C Test (p=0.024) media.

   At Day 28, release of KC was significantly higher in 37°C Control than 4°C Control (p=0.008) and 4°C Test (p=0.008) media. Also, 37°C Test media had significantly higher release of KC than 4°C Control (p=0.008) and 4°C Test (p=0.008) media.

   At Day 56, release of KC was significantly higher in 37°C Control than 4°C Control (p=0.008) and 4°C Test (p=0.008) media. The 37°C Test media had significantly higher release of KC than 4°C Control (p=0.032).

2. There were no significant differences in IL-6 release at any time point.

3. At Day 7, 37°C Control media stored at had significantly higher levels of IL-8 release than 4°C Control (p=0.002), 4°C Test (p=0.002), and 37°C Test (p=0.016) media. Also at Day 7, the 37°C Test media had significantly higher levels than 4°C Test (p=0.032) media.

   At Day 28, the 37°C Test media had significantly higher levels of IL-8 release than 4°C Control (p=0.008), 4°C Test (p=0.008), and 37°C Control (p=0.032) media. Also at Day 28, the 37°C Control media had significantly higher levels of IL-8 than 4°C Control (p=0.008) and 4°C Test (p=0.008) media.

   At Day 56, the 37°C Control media had significantly higher levels of IL-8 release than the 4°C Control (p=0.002) media.

4. At Day 7, the 37°C Control media had significantly more MCP-1 release than 4°C Control (p=0.008), 4°C Test (p=0.008), and 37°C Test (p=0.013) media. Also at Day 7, the 37°C Test media had significantly more MCP-1 release than 4°C Control (p=0.018) and 4°C Test (p=0.027) media.

   At Day 28, the 37°C Control media had significantly more MCP-1 release than 4°C Control (p=0.008), 4°C Test (p=0.008), and 37°C Test (p<0.001) media. Also at Day 28, the 37°C Test media had significantly more MCP-1 release than 4°C Control (p=0.008) and 4°C Test (p=0.008) media.

At Day 56, the 37°C Control media had significantly more MCP-1 release than 4°C Control ($p=0.008$), 4°C Test ($p=0.008$), and 37°C Test ($p<0.001$) media. Also at Day 56, the 37°C Test media had significantly more MCP-1 release than 4°C Control (0.008) media.

5. See Tables on p.59. See Figures on p.75.

i. Biomechanical Analysis

i. At Day 28, femoral condyle Day 0 Control's elastic modulus was significantly higher than 4°C Control ($p=0.003$), 37°C Control ($p=0.004$), and 4°C Test (0.002) media. Dynamic modulus of Day 0 Controls was significantly higher at Day 28 than full thickness cartilage stored in 37°C Control media. However, at Day 56 there were no significant differences between femoral condyle storage groups in elastic or dynamic modulus. See Tables on p. 60 and Figure on p.76.

ii. At Day 28, full thickness cartilage from tibial plateaus elastic modulus of Day 0 Controls was just barely statistically significantly higher than those stored in 37°C Control ($p=0.049$) media. Also, the dynamic modulus from Day 0 controls was significantly higher than those stored in 37°C Control ($p=0.0017$) media at Day 28. At Day 56, the dynamic modulus was significantly less in OCAs stored in 37°C Control media than 4°C Test ($p=0.0278$) and 37°C Test ($p=0.0363$) media. See Tables on p.606161 Figure on p.77

j. Histology

i. Qualitative light microscopy analysis of femoral condyle OCA histology sections. Slight degenerative changes were seen in the architecture in OCAs

H&E staining on Day 28. The loss of normal architecture was pronounced as well as a loss of cells in the superficial layer on Day 56 of OCAs in the 37°C Test media. There was a loss of T-blue staining in the superficial layer of the OCAs stored in 37°C Control media at Days 28 and 56 compared to Day 0. Also at Day 56, the OCAs in 4°C Test media had less stain in the superficial layer. OCAs stored in 37°C Test media had a pronounced loss of T-blue stain at Day 28 and 56. Trichrome stain was maintained throughout storage. See Figures pp.78,80-85

ii. Qualitative light microscopy analysis of tibial plateau OCA histology sections. Slight degenerative changes were seen in OCAs H&E staining on Day 28 and 56. There was a slight loss of T-blue staining in OCAs stored in 37°C Control, 4°C Control, and 37°C Test media at Day 28. At Day 56 there was a pronounced loss of T-blue staining in OCAs stored in 4°C Test and 37°C Test media compared to Day 0 staining. Trichrome stain was maintained throughout storage. See Figures on pp.79, 86-91.

k. Immunohistochemistry

i. Qualitative light microscopy analysis of femoral condyle and tibial plateau immunohistochemistry samples. The samples showed Collagen II throughout all layers and proteoglycan was also evident upon examination of all samples. See Figures on pp.92-96.

4. Discussion

In this study, the 37°C Control media preserved the tissue better than all other storage groups. Similar to the work by Teng and colleagues, the basic media provided the best environment for OCA preservation.[86] This work also confirmed that of Pallante et al. that demonstrated a high cell viability by storing OCAs at 37°C.[93] However, this study used a serum-free chemically-defined media to achieve this high cell viability.

	4°C Control	37°C Control	4°C Test	37°C Test
Cell Viability	Negative	**POSITIVE +++**	Negative	Positive
ECM	Positive	Positive	Positive	Negative
Biomechanics	**Positive ++**	Positive	**Positive ++**	**Positive ++**
Biomarkers				
Inflammatory	Negative	Positive	Negative	Negative
Cytokines	Negative	**Positive ++**	Negative	Negative
MMP's	Negative	**Positive ++**	Negative	Negative
Histology	Positive	Positive	Positive	Positive

Table 5: Summary of results from storage of femoral condyle OCAs.

	4°C Control	37°C Control	4°C Test	37°C Test
Cell Viability	Negative	**Positive ++**	Negative	Positive
ECM	**Positive ++**	Positive	Positive	Negative
Biomechanics	**Positive ++**	Positive	**Positive ++**	**Positive ++**
Biomarkers				
Inflammatory	Negative	**Positive ++**	Negative	Positive
Cytokines	Negative	**Positive ++**	Negative	Positive
Histology	Positive	Positive	Positive	Positive

Table 6: Summary of results from storage of tibial plateau OCAs.

Currently, OCAs stored in serum-free media at 4°C maintain GAG content and ~70% cell viability up to 28 days. However the cell viability in OCAs stored after 28 days at 4°C is significantly less than Day 0 levels, which is consistent with the OCAs stored at 4°C in our study.[56,77] The chondrocyte viability of femoral condyle OCAs were maintained at Day 0 levels in the 37°C Control media up to 56 days and viability was significantly higher in this media than all other groups. The 37°C Control media was able to maintain Day 0 levels of viability only to 28 days in tibial plateaus, however, it still maintained significantly higher viability than any other storage group. It was previously shown that storage of OCAs in media at 37°C could maintain high cell viability up to 28 days.[93] We confirmed these findings and improved upon them by maintaining cell viability at Day 0 levels up 56 days. OCA storage at 37°C has previously demonstrated the ability to maintain GAG and HP content up to 28 days of storage.[93,94] In this study, tissue glycosaminoglycan (GAG)

and hydroxyproline (HP) content of femoral condyles, both measures of extracellular matrix quality, were maintained at Day 0 levels in 37°C Control media after 56 days of storage. GAG and HP content of tibial plateaus was also maintained at Day 0 levels in 37°C Control media up to 56 days. The GAG and HP content of OCAs stored at 4°C was maintained despite a decrease in cell viability. This would indicate a decrease in metabolism. Otherwise, they would have a decrease in GAG or HP similar to OCAs that had a decrease in GAG content when stored in 37°C Test media.

GAG content of media is a measure of GAG release from tissue. The lower tissue GAG content of OCAs stored in 37°C Test media corresponded with significantly higher levels of media GAG release than the 4°C groups and the 37°C Control, we hypothesize that this is due to stress or lack of extracellular matrix maintenance by chondrocytes from OCAs stored in the 37°C Test media. The 37°C Control media maintained tissue GAG but had significantly more media GAG release than OCAs stored at 4°C, this is likely similar to normal metabolism. The low levels of media GAG release in OCAs stored at 4°C could be because the cells have entered a quiescent state. These general trends were seen in both femoral condyle and tibial plateau OCAs. The release of GAG into media could be from the production of smaller GAG molecules or catabolic release from tissues. The low amounts of GAG release into media from OCAs stored at 4°C support the earlier hypothesis of decreased metabolism in these tissues.

Previous work has shown that NO release into media has a detrimental effect on proteoglycan synthesis of OCAs.[92] The NO release was elevated at Day 7 from

femoral condyle OCAs stored in 37°C Control compared to those stored at 4°C. A similar trend was seen in tibial plateau OCA. The upregulation in media NO release compared to 4°C groups was likely due to surgical insult during the removal of these OCAs rather than storage dynamics. On the other hand, PGE2 release showed a time dependent increase in release from femoral condyles and tibial plateaus OCAs. At Day 7 there were no significant differences between storage groups of femoral condyles and tibial plateaus. However, at Day 28 and 56 the femoral condyle OCAs stored in 37°C Control media had significantly more PGE2 release into media than both 4°C Control and 4°C Test media. Among tibial plateau OCAs at Day 28, those stored in 37°C Test media had significantly higher PGE2 release than any other group and the 37°C Control media had significantly higher PGE2 release than tibial plateau OCAs stored in 4°C Control and 4°C Test media. Surprisingly, at Day 56 the only significant differences between tibial plateau OCA storage groups was a significantly higher release from those stored in 37°C Control than 4°C Control or 4°C Test media. The correlation between tissue viability and increased PGE2 release from OCAs stored in 37°C Control media indicate that this could be a marker for tissue viability. Also, it is logical that there would be less PGE2 release from OCAs stored in the Test media because it is supplemented with dexamethasone, a potent corticosteroid.

High levels of MMP-2, 3, and 13 continued to be released from femoral condyle OCAs stored in 37°C Control media throughout the course of this study. This media also maintained chondrocyte viability at Day 0 levels, therefore, these MMPs could be markers for tissue viability during preservation.

The cytokines KC, IL-8, and MCP-1 remained elevated throughout this study in 37°C Control media, indicating that they could be markers of tissue viability because of the high chondrocyte viability maintained in this media. IL-6 was only elevated on Day 7 and this elevation was likely due to inflammation during surgical removal. Similar trends in cytokine elevation were seen during both femoral condyle and tibial plateau OCA storage. Biomarker research is being continued in our lab, the measurement of proteins released in to media could allow tissue banks to assess OCA quality without insulting the tissue. This is important because currently there is no way to individually assess the quality of OCAs during storage, with biomarkers it may be possible to determine the quality of each individual OCA.

The lesser amount of NO, PGE2, MMP's, and cytokines from OCAs stored in media at 4°C further support the hypothesis that OCAs have a decreased metabolism at 4°C. Whether or not this is similar to *in vivo* metabolism is yet to be determined.

At Day 28, the elastic and dynamic moduli of full thickness cartilage from femoral condyle and tibial plateau OCAs stored in 37°C Control media was unable to maintain the stiffness of Day 0. However, at Day 56 these values were not statistically significant. These results are confounding and could be due to group differences or errors in testing. However, there has been a correlation seen with maintenance of biomechanical properties using dexamethasone as a supplement to media. This is similar to reported data in the literature.[94] This would explain why the Test groups maintained their biomechanical qualities through Day 56 to that of Day 0 controls.

Correlations were seen in the histological staining and biochemical results of OCA samples. Among femoral condyle OCAs sections, the loss of cells in the

superficial layer of OCAs stored in 37°C Test media at Day 56 corresponded with a decrease in cell viability using CellTracker™ Green CMFDA and ethidium homodimer-1. Also, the significant drop in GAG content at Day 56 of OCAs stored in 37°C Test media from Day 0 levels as measured with the DMMB assay corresponded well with the decreased T-blue staining of those samples at Day 56. Trichrome staining was maintained through storage and this correlated with the biochemical measurements. In tibial plateau histologic staining there were some similar correlations to biochemical measurements. There were some minor degenerative changes noted with H&E staining at Day 56. The most pronounced correlation was again in the T-blue staining, there was profoundly less staining in OCAs stored in 4°C Test and 37°C Test media which correlated to the significantly less than Day 0 levels of GAG found using the DMMB assay. As found with femoral condyle OCA section Trichrome staining, the staining was maintained throughout storage which correlated with the biochemical data.

Immunohistochemistry helped confirm the type of collagen measured biochemically and the production of proteoglycan through the different layers of cartilage. The samples indicate that collagen II was not replaced with collagen I during storage because of the strong green fluorescence of Collagen II throughout storage. Proteoglycan was also evident upon examination of all samples.

CHAPTER 3: FUTURE DIRECTIONS

The findings in the current study can be translated to human OCA research as animal models are commonly used in orthopedic research. Research is ongoing in our laboratory with different storage temperatures and supplements to storage media. Despite the 37°C Control media with basic supplementation proved to be superior in this particular study further research needs to be done to assess the best media preparation for tissue preservation. Unpublished data from our laboratory suggests that one of the supplements of the Test media actively kills chondrocytes. This is logical considering the time-dependent decrease in cell viability of OCAs stored in the 37°C Test media. Studies have shown that apoptotic pathways are upregulated during storage, therefore, many media supplements to inhibit apoptosis to prolong storage and tissue quality have been investigated.[89] These studies have shown mixed results so far.[86,90]

Currently our lab is attempting to identify a panel of cytokines that are released from OCAs into the storage media that would allow tissue bankers to assess the viability of the tissue prior to transplantation. Further study will be needed to determine which cytokines are upregulated by healthy tissue during storage. Research is also ongoing using alamarBlue to assess tissue viability without disturbing it during storage.

Most importantly, we have begun *in vivo* canine studies transplanting OCAs at Day 28 and 56 to assess their efficacy and the effect of storage on the allografts. Time will tell which storage media composition yields the best clinical results *in vivo*.

TABLES

Table 7: Average percent chondrocyte viability of femoral condyle and tibial plateau OCA from Day 0 Controls ± SEM

	Day 0
Femoral condyle	77.23482±3.907
Tibial plateau	85.83275±2.107

Table 8: Average percent chondrocyte viability of femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media ± SEM.

	Day 28	Day 56
4C Control	39.73122±9.507	27.38466±11.882
37C Control	76.39993±2.675	69.55185±3.253
4C Test	33.32883±12.435	23.27365±7.376
37C Test	56.51616±9.739	27.80691±10.192

Table 9: Average percent chondrocyte viability of tibial plateau OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media ± SEM.

	Day 28	Day 56
4C Control	50.34013±7.944	44.90302±8.84
37C Control	78.05448±7.934	71.59964±5.549
4C Test	35.2419±9.982	27.68835±5.526
37 Test	66.21004±3.334	40.24481±8.551

Table 10: Average total tissue GAG content of femoral condyle and tibial plateau OCA from Day 0 Controls. Values reported at µg GAG/mg dry weight ± SEM.

	Day 0
Femoral condyle	107.7861±7.304
Tibial plateau	137.7271±9.7

Table 11: Average total tissue GAG content of femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported at µg GAG/mg dry weight ± SEM.

	Day 28	Day 56
4C Control	122.8825±19.743	133.1977±25.756
37C Control	111.6715±14.021	143.1201±30.69
4C Test	110.1583±24.073	62.03961±26.277
37 Test	83.16426±19.015	45.47233±13.401

Table 12: Average total tissue GAG content of tibial plateau OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported at µg GAG/mg dry weight ± SEM.

	Day 28	Day 56
4C Control	171.2841±10.095	152.3022±16.362
37C Control	114.1398±12.558	128.2485±15.465
4C Test	143.9543±14.799	83.25958±16.189
37 Test	107.9645±19.546	70.95953±10.651

Table 13: Average total media GAG content of femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as µg GAG normalized to media volume ± SEM.

	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
4C	854.9848 ±172.532	477.7734 ±78.762	579.1158 ±114.09	332.0003 ±75.346	278.5124 ±60.473	346.1762 ±110.025	317.036± 112.893	385.8885 ±112.52
37C	5753.316 ±948.84	3833.465 ±652.417	3029.647 ±420.621	2501.676 ±360.597	3269.766 ±715.03	2349.219 ±297.125	2159.224 ±277.83	2960.621 ±580.85
4T	2708.496 ±500.802	2065.177 ±317.792	2567.112 ±414.895	1463.629 ±227.772	1808.188 ±432.89	1413.792 ±364.126	956.9396 ±206.414	1229.131 ±326.86
37T	6287.376 ±942.359	6127.096 ±921.607	6987.114 ±1025.93	5197.367 ±759.837	5674.968 ±2120.3	4102.255 ±1687.68	2831.696 ±1174.82	2360.426 ±758.61

Table 14: Average total media GAG content of tibial plateau OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as µg GAG normalized to media volume ± SEM.

	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
4C	1033.86 ±275.9	540.4845 ±103.51	573.419 ±105.46	418.7695 ±103.25	529.1133 ±207.04	425.5679 ±121.84	344.6821 ±72.096	352.0595 ±156.41
37C	6894.78 ±1253.1	3882.545 ±897.335	3080.33 ±378.57	2507.286 ±344.72	2283.831 ±508.77	2292.311 ±464.11	1993.245 ±387.54	2526.65 ±472.29
4T	2410.77 ±350.61	1470.071 ±250.46	1813.59 ±295.81	1203.139 ±175.88	1324.185 ±296.00	1078.968 ±296.34	661.8652 ±184.33	1014.907 ±269.38
37T	4926.32 ±1021.9	5657.802 ±754.86	7002.99 ±943.73	5050.089 ±663.89	6211.998 ±1571.7	5148.572 ±1306.3	3653.359 ±889.9	4917.372 ±1289.6

Table 15: Average total tissue HP content of femoral condyle and tibial plateau OCA from Day 0 Controls. Values reported at µg HP/mg dry weight ± SEM.

	Day 0
Femoral condyle	11.91843±1.227
Tibial plateau	40.6185±2.877

Table 16: Average total tissue HP content of femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported at µg HP/mg dry weight ± SEM.

	Day 28	Day 56
4C Control	4.966775±3.385	8.60919±3.12
37C Control	4.604439±2.615	7.295832±0.87
4C Test	4.418919±2.351	8.66096±1.219
37 Test	4.29748±2.08	7.799685±13.4

Table 17: Average total tissue HP content of tibial plateau OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported at µg HP/mg dry weight ± SEM.

	Day 28	Day 56
4C Control	3.601321±4.991	7.227135±3.991
37C Control	4.472227±0.637	5.962451±1.281
4C Test	4.455807±1.466	7.810117±1.967
37 Test	5.746179±4.966	9.727519±3.903

Table 18:Average total media NO release from femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values are reported as µg NO normalized to media volume ± SEM.

	Week 1	Week 4	Week 8
4C Control	736.6113±68.073	686.2217±61.996	598.1128±104.521
37C Control	1318.999±207.125	784.2455±80.748	634.9471±127.484
4C Test	874.7158±73.666	766.5193±62.189	699.6534±113.744
37C Test	1039.877±136.857	849.5451±106.753	611.9189±159.639

Table 19: Average total media NO release from tibial plateau OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values are reported as µg NO normalized to media volume ± SEM.

	Week 1	Week 4	Week 8
4C Control	809.2675±56.491	744.926±58.811	689.932±101.887
37C Control	1281.37±110.078	883.7199±71.759	750.1224±120.607
4C Test	926.1565±93.647	873.754±96.101	763.3694±130.691
37C Test	1223.406±133.405	1089.2±87.283	856.1071±132.931

Table 20: Average total media PGE2 release from femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values are reported at pg PGE2 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	54675.95±7464.59	21185.26±3764.99	7759.531±1944.25
37C Control	212267.5±142181	37794.3±5278.64	25875.64±7204.38
4C Test	82508.65±23370.4	6880.983±999.13	5455.854±1175.76
37C Test	67104.18±8594.49	91327.57±37739.3	20761.6±7284.24

Table 21: Average total media PGE2 release from tibial plateau OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values are reported at pg PGE2 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	75787.15±11438.8	22226.35±3997.93	8560.867±1649.8
37C Control	153910.7±76036.2	37918.52±4935.16	29954.85±3470.33
4C Test	65416.93±8060.61	5705.997±1003.75	5811.483±1290.86
37C Test	75811.44±6889.43	74898.33±9393.39	35580.02±9315.97

Table 22: Average total MMP-2 release into media from femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported at pg MMP-2 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	0	0	0
37C Control	153958.5±37736.5	292994.9±53631.3	263089.4±45634.4
4C Test	0	13990.1±12921.9	0
37C Test	59948.38±16676.8	130577.4±59874.8	123596.8±51162.3

Table 23: Average total MMP-3 release into media from femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg MMP-3 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	94571.89±16082.5	54393.95±12073	19232.52±3071.65
37C Control	177446.9±27195.5	142543±35775.5	100779.7±26088.7
4C Test	117673±13986.2	51958.3±6008.09	12129±2633.83
37C Test	135019.8±26479.8	77571.01±33822.1	73335.35±30777.1

Table 24: Average total MMP-13 release into media from femoral condyle OCA from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg MMP-13 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	385.2±385.2	489.06±489.06	0
37C Control	105142.9±62010	143367.2±81277.5	74079.6±58175.2
4C Test	6991.14±5080.11	546.48±546.48	0
37C Test	16273.65±13130.9	10764.64±5874.13	6064.263±3593.61

Table 25: Average total KC release into media from femoral condyle OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg KC normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	35839.64±12510.4	782.3425±293.94	0
37C Control	161730.4±80531.9	54114.84±33164.7	43393.61±38258.1
4C Test	71423.98±17239.5	1401.487±483.369	196.5833±80.828
37C Test	93415.49±24005.4	10779.41±6945.65	542.2523±230.394

Table 26: Average total IL-6 release into media from femoral condyle OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg IL-6 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	0	134.005±134.005	0
37C Control	18058.86±16332	260.4001±222.548	203.9278±203.928
4C Test	178.2294±178.229	0	0
37C Test	322.7981±322.798	16.36226±16.362	0

Table 27: Average total IL-8 release into media from femoral condyle OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg IL-8 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	84703.56±27.558.1	8851.474±1781.41	3908.329±690.816
37C Control	616286.9±155837	62776.87±32056.7	248675±230742
4C Test	147783.3±35381.9	52677.65±19246.2	4739.466±589.113
37C Test	213769.4±38113.8	76329.05±30401.3	9537.231±3795.2

Table 28: Average total MCP-1 release into media from femoral condyle OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg MCP-1 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	17117.94±6808.09	223.0721±137.737	0
37C Control	337971.8±201621	112706.3±35524.7	779136.5±702580
4C Test	36880.68±11813.7	105476±32909.4	0
37C Test	55999.71±10675.1	12365.87±5452.89	2294.431±1243.35

Table 29: Average total KC release into media from tibial plateau OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg KC normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	55743.58±14448.02	435.7188±120.984	0
37C Control	206594±27486.8	24963.64±7651.155	8897.978±4097.876
4C Test	70108.81±19238.95	1173.049±456.11	161.3898±99.792
37C Test	151775.3±22108.21	26087.22±3784.482	3307.926±1479.349

Table 30: Average total IL-6 release into media from tibial plateau OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg IL-6 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	170.5721±105.471	0	0
37C Control	3226.535±1761.21	0	0
4C Test	103.7029±67.112	0	0
37C Test	1198.069±1088.43	0	0

Table 31: Average total IL-8 release into media from tibial plateau OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg IL-8 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	122942.1±31795.2	6332.919±865.342	4799.72±721.303
37C Control	856149.7±155412	83794.43±9031.85	36872.8±7051.5
4C Test	144207±30915.6	9492.261±2378.37	13339.88±7626.24
37C Test	320935.5±80297.6	194190.8±59767.1	26141.69±8641.95

Table 32: Average total MCP-1 release into media from tibial plateau OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as pg MCP-1 normalized to media volume ± SEM.

	Day 7	Day 28	Day 56
4C Control	17845.25±6534.05	0	0
37C Control	196833.7±37990.6	148236.6±15031.4	118378.3±13034.1
4C Test	21078.91±7574.95	0	24384.28±23459.1
37C Test	66618.41±15004	29127.07±9645.46	8687.532±2765.91

Table 33: Average elastic and dynamic modulus of femoral condyle OCA full thickness cartilage Day 0 Controls. Values reported as MPa ± SEM.

	Day 0
Elastic Modulus	7.0003±0.9511
Dynamic Modulus	6.8636±1.3677

Table 34: Average elastic and dynamic modulus of tibial plateau OCA full thickness cartilage Day 0 Controls. Values reported as MPa ± SEM.

	Day 0
Elastic Modulus	5.0272±0.8811
Dynamic Modulus	5.25612±0.6445

Table 35: Average elastic modulus of full thickness cartilage from femoral condyle OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as MPa ± SEM.

	Day 28	Day 56
4C Control	3.6438±0.4647	4.7890±0.6145
37C Control	2.8953±0.7688	5.0866±1.0182
4C Test	3.4082±0.3617	5.9492±0.4939
37 Test	4.3353±1.0453	5.9106±1.0773

Table 36: Average dynamic modulus of full thickness cartilage from femoral condyle OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as MPa ± SEM.

	Day 28	Day 56
4C Control	3.5980±0.6123	6.4207±0.8562
37C Control	3.7219±0.5577	7.9304±1.3175
4C Test	4.0177±0.8681	7.3900±0.5646
37 Test	4.9933±1.2246	6.5325±1.5861

Table 37: Average elastic modulus of full thickness cartilage from tibial plateau OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as MPa ± SEM.

	Day 28	Day 56
4C Control	2.9583±0.5495	3.9255±1.1505
37C Control	2.4948±0.4214	6.9864±1.6632
4C Test	4.2700±0.6932	7.6691±1.3883
37 Test	3.3419±1.0252	10.0256±2.6771

Table 38: Average dynamic modulus of full thickness cartilage from tibial plateau OCAs from 4°C Control, 37°C Control, 4°C Test, and 37°C Test storage media. Values reported as MPa ± SEM.

	Day 28	Day 56
4C Control	3.6327±0.5309	4.9548±0.6562
37C Control	2.2407±0.5029	5.3685±1.9378
4C Test	3.7415±0.7653	7.2236±1.5106
37 Test	3.6940±1.2916	10.0140±3.2835

FIGURES

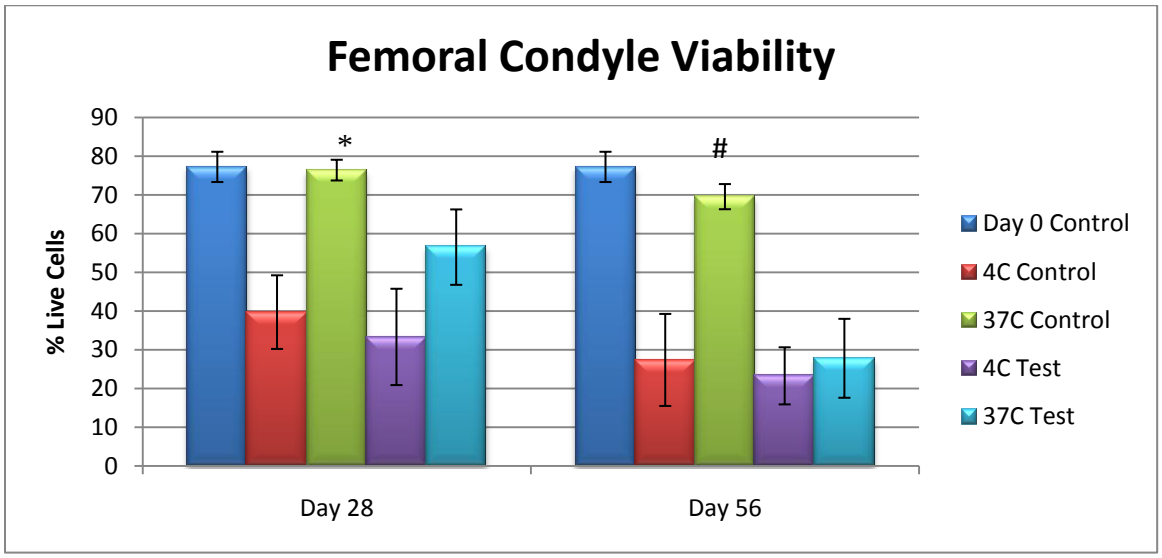


Figure 1: Chondrocyte viability (±SEM) of femoral condyles at Day 0, 28, and 56. At Day 28(*), OCAs from Day 0 and stored in 37°C Control media had significantly higher viability than 4°C Control and Test media. At Day 56(#), OCAs from Day 0 and stored in 37°C Control media had significantly higher viability than all other groups. ($p<0.05$)

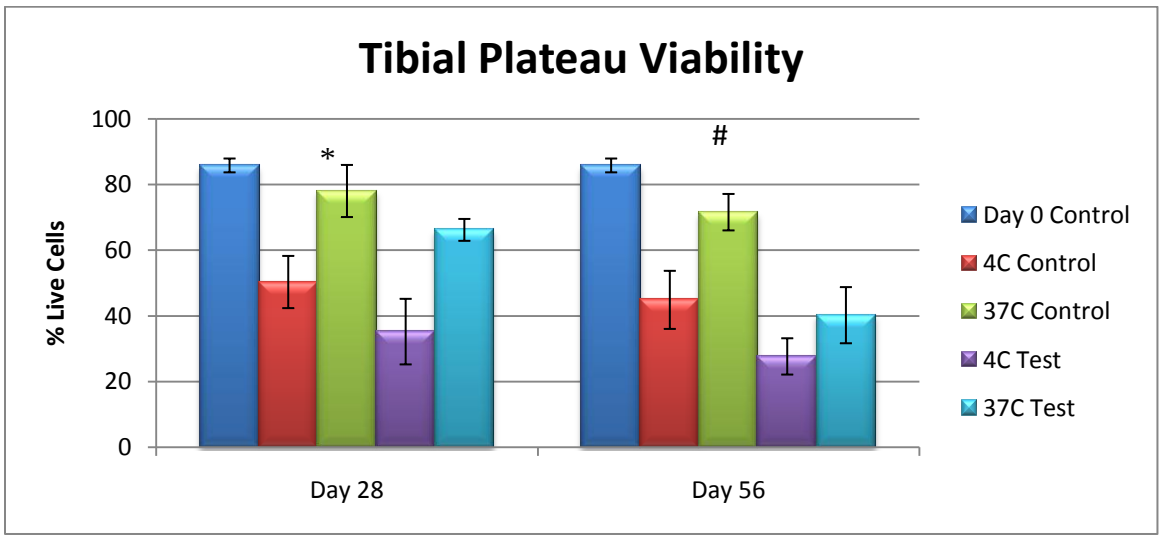
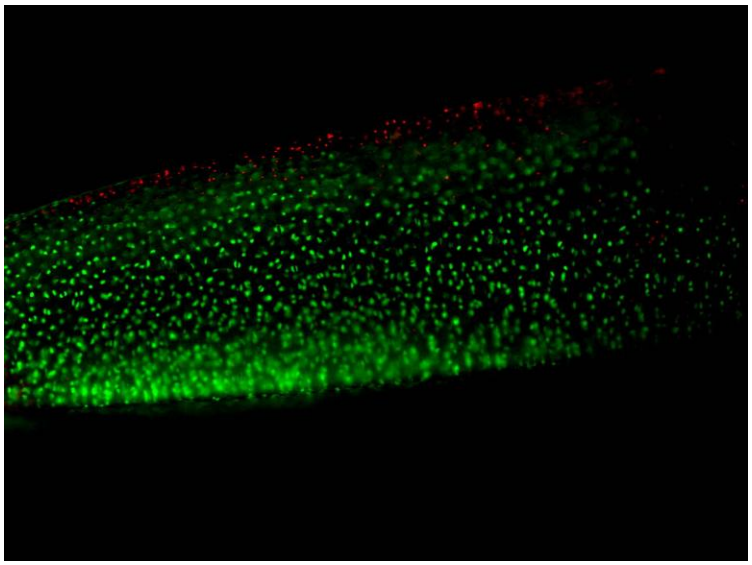
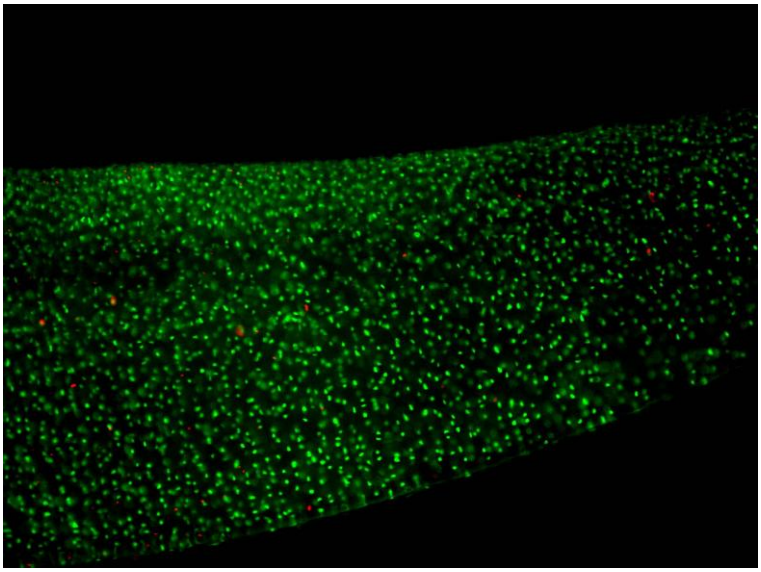


Figure 2: Chondrocyte viability (±SEM) of tibial plateaus at Day 0, 28, and 56. At Day 28(*), OCAs stored in 37°C Control media had significantly higher viability than both 4° storage groups, those in 37°C Test media had significantly higher viability than 4°C Test media, and Day 0 OCAs had higher viability than both 4° storage groups and 37°C Test media. At Day 56(#), OCAs stored in 37°C Control media maintained higher viability than all other storage groups yet no storage group was able to maintain Day 0 viability. ($p<0.05$)

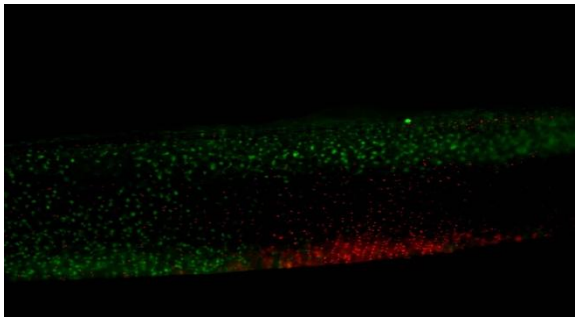


Femoral condyle

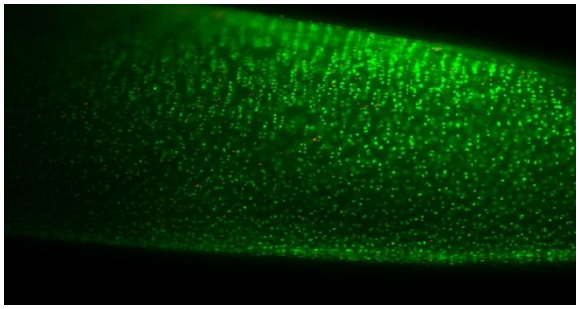


Tibial plateau

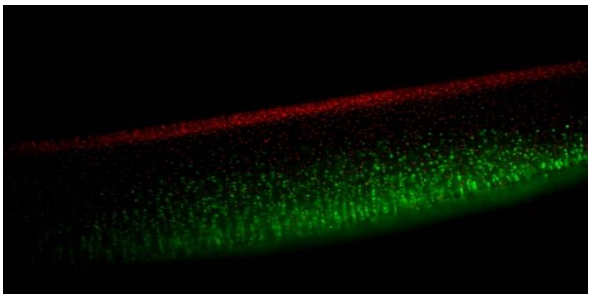
Figure 3: Representative images of chondrocyte viability from Day 0 Controls. (10X magnification)



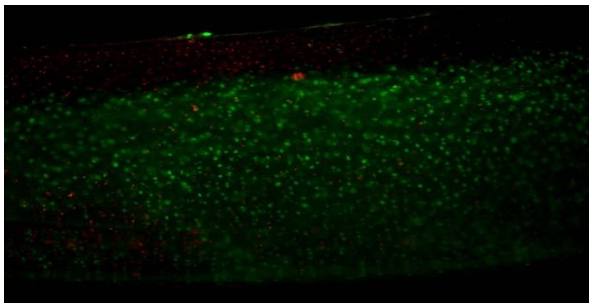
4°C Control



37°C Control

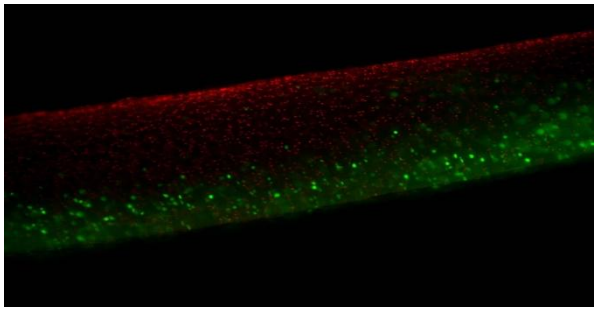


4°C Test

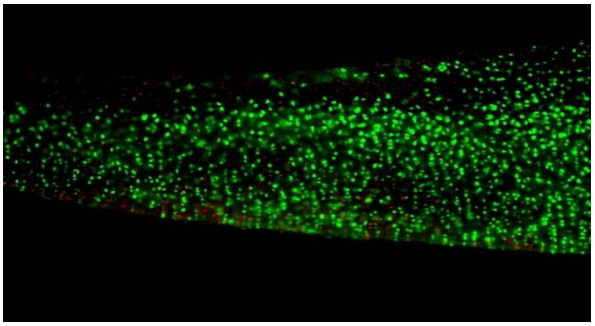


37°C Test

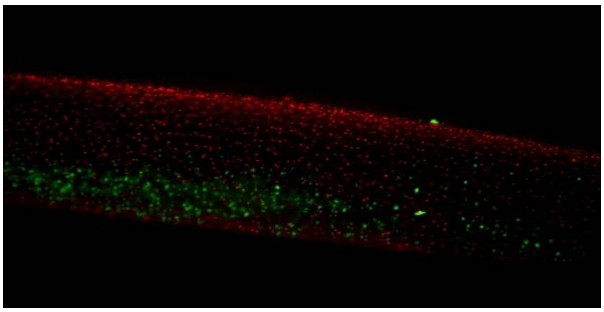
Figure 4: Representative images of femoral condyle chondrocyte viability from Day 28. (10X magnification)



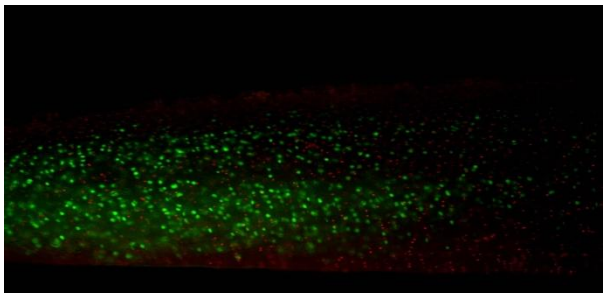
4°C Control



37°C Control

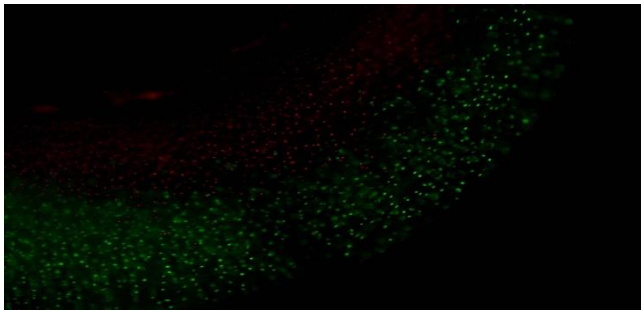


4°C Test

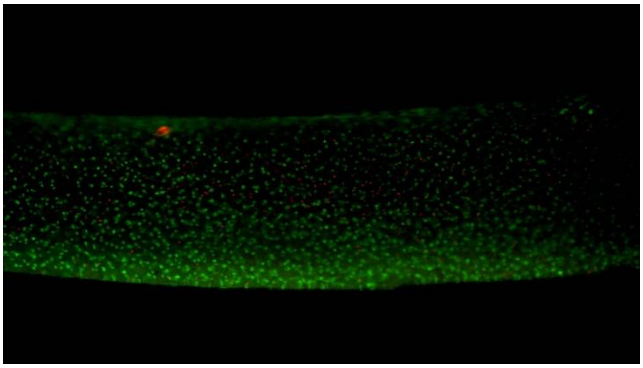


37°C Test

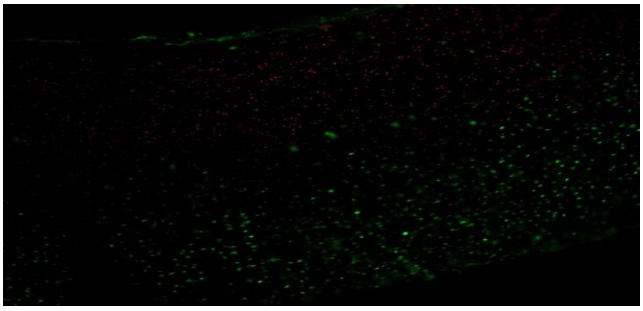
Figure 5: Representative images of femoral condyle chondrocyte viability from Day 56. (10X magnification)



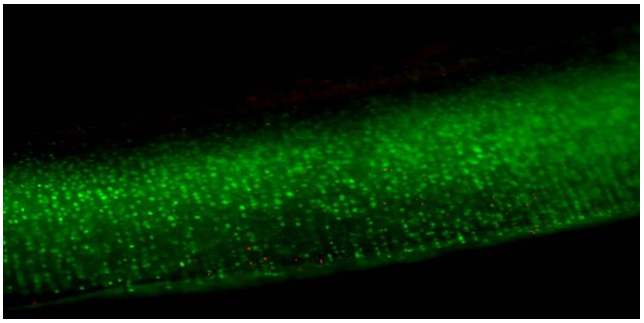
4°C Control



37°C Test

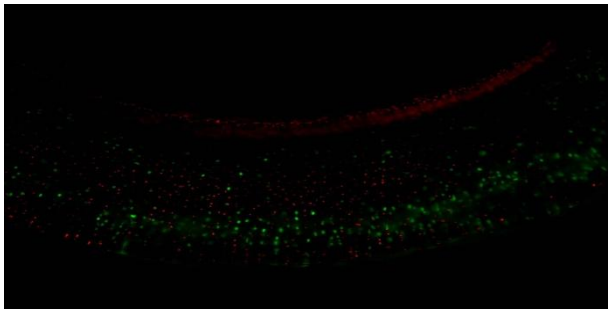


4°C Test

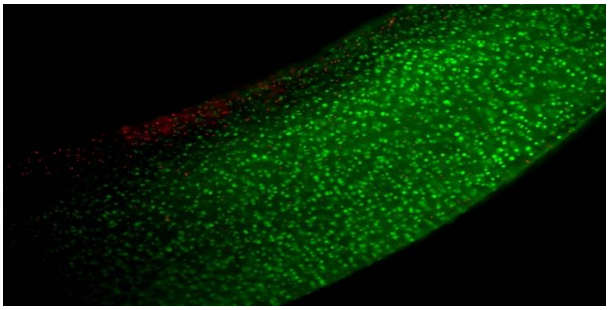


37°C Test

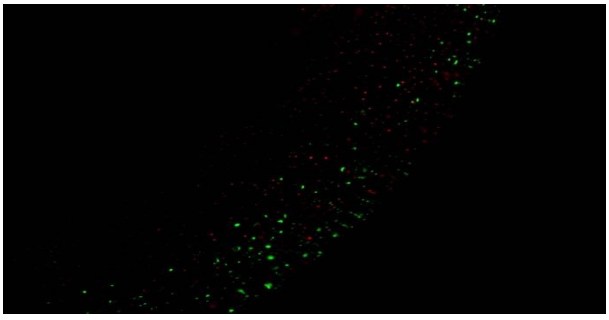
Figure 6: Representative images of tibial plateau chondrocyte viability at Day 28. (10X magnification)



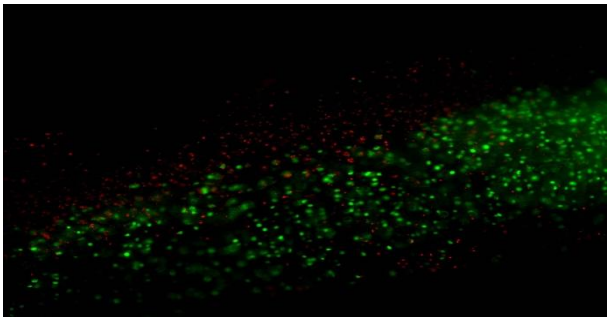
4°C Control



37°C Control



4°C Test



37°C Test

Figure 7: Representative images of tibial plateau chondrocyte viability at Day 56. (10X magnification)

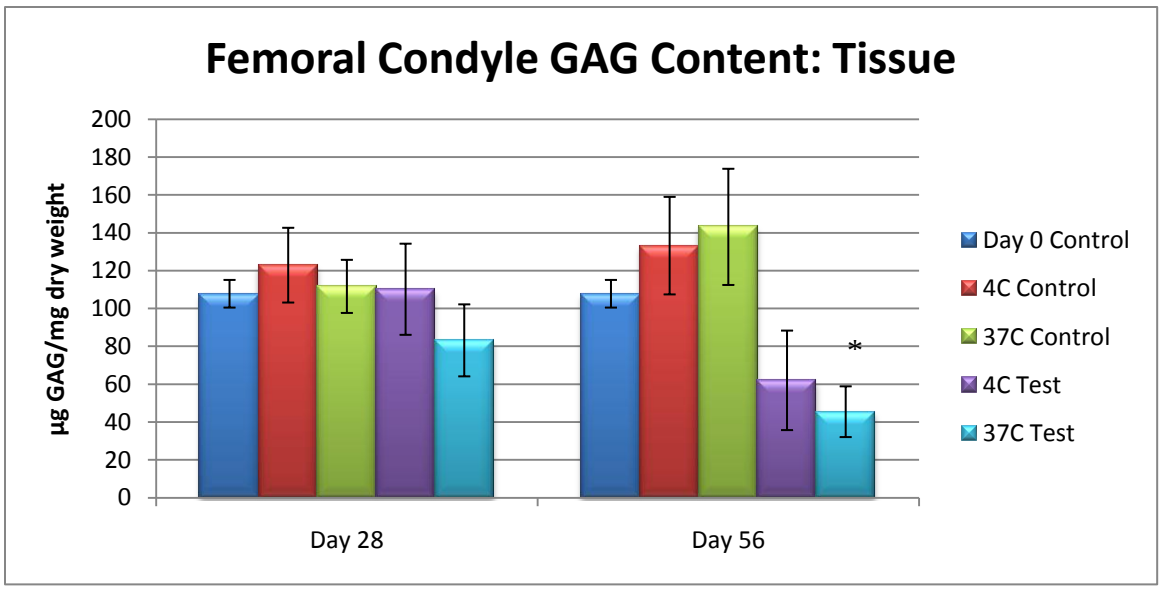


Figure 8: Tissue GAG content (±SEM) of femoral condyles. Values are reported as µg GAG/mg dry weight full thickness cartilage. At Day 56(*), OCAs stored in 37°C Test media had significantly less GAG than those stored in 4°C and 37°C Control media and Day 0 controls. ($p<0.05$)

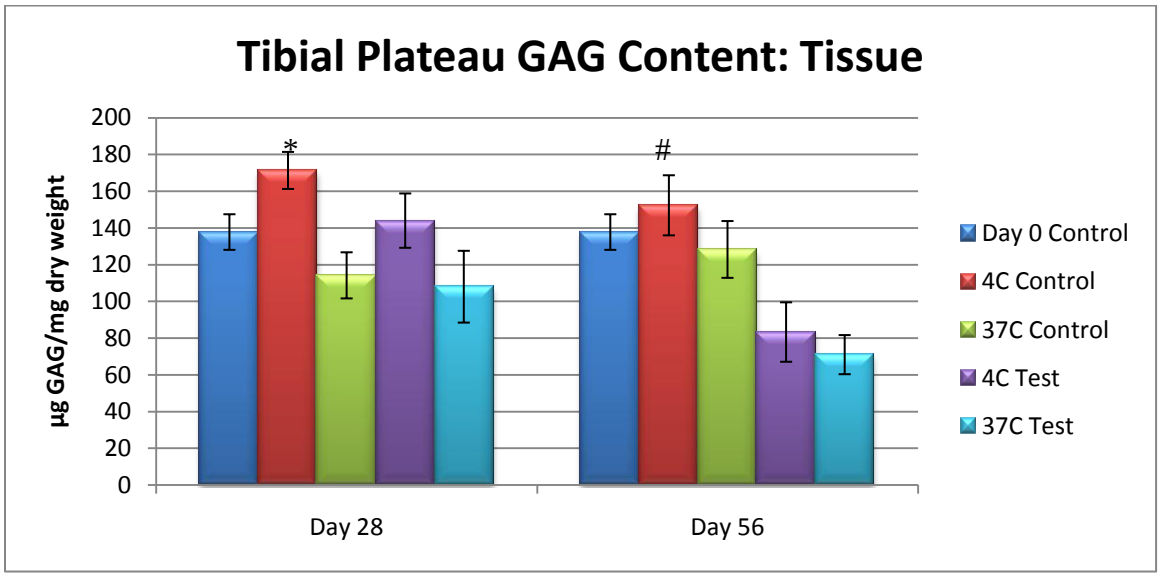


Figure 9: Tissue GAG content (±SEM) of tibial plateaus. Values are reported as µg GAG/mg dry weight full thickness cartilage. At Day 28(*), OCAs stored in 4°C Control media had significantly more GAG than both groups stored at 37°C. At Day 56(#), OCAs stored in 4°C Control media had significantly higher GAG content than those stored in 4°C Test and 37°C Test media, GAG content was significantly higher in 37°C Control than 37°C Test, and Day 0 levels of GAG were significantly higher than 4°C Test and 37°C Test media. ($p<0.05$)

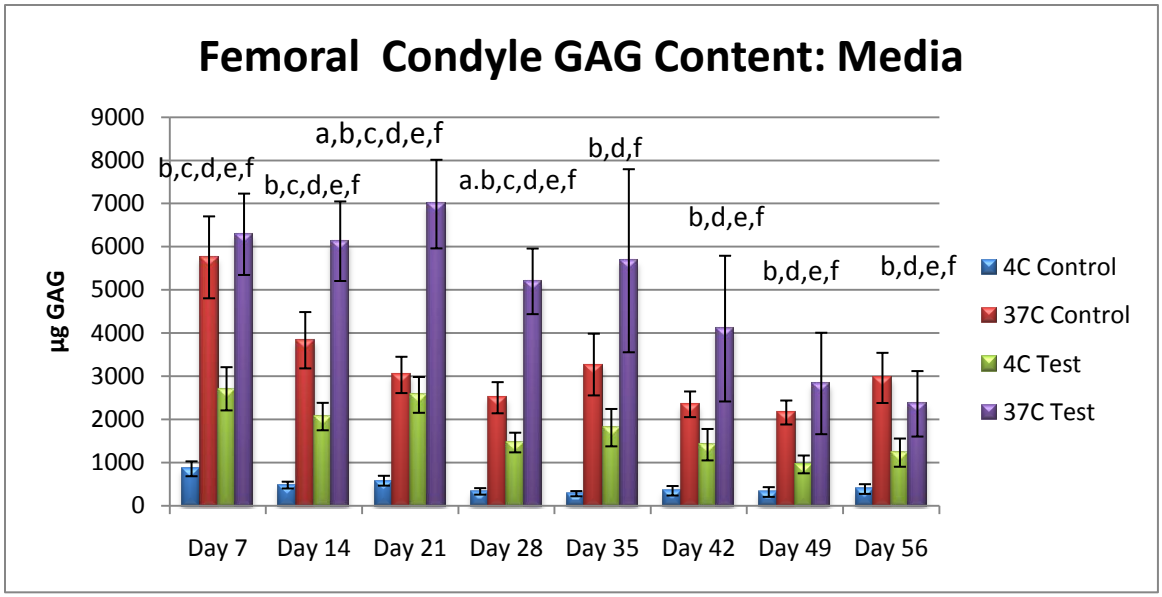


Figure 10: Media GAG release (±SEM) of femoral condyles. Values are reported as µg GAG and normalized to media volume. (a - 37°C Test>37°C Control, b - 37°C Test>4°C Control, c - 37°C Test>4°C Test, d - 37°C Control>4°C Control, e - 37°C Control>4°C Test, f - 4°C Test>4°C Control) $p<0.05$

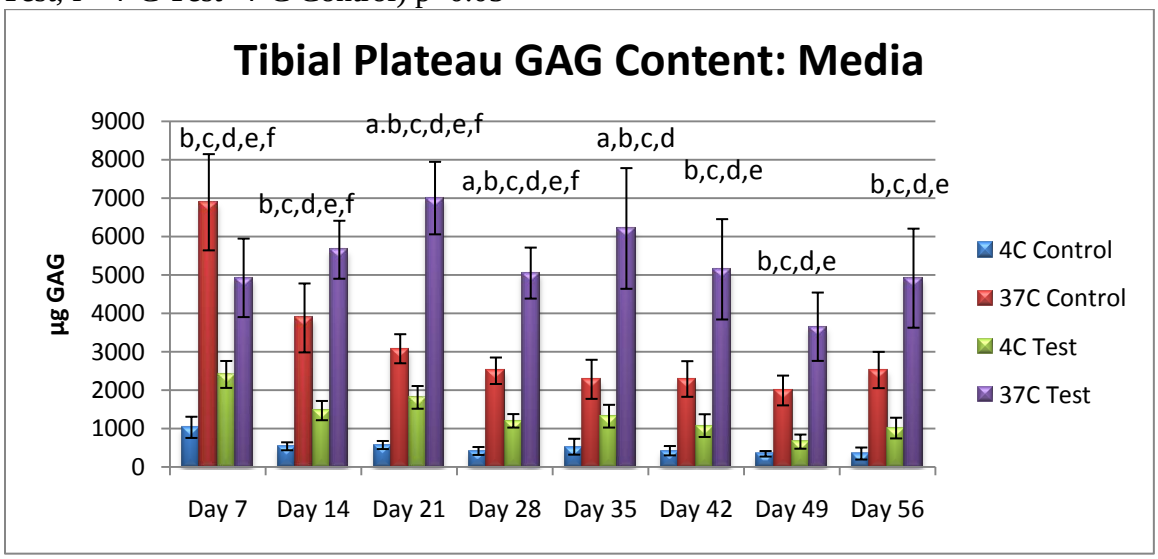


Figure 11: Media GAG release (±SEM) of tibial plateaus. Values are reported as µg GAG and normalized to media volume. (a - 37°C Test>37°C Control, b - 37°C Test>4°C Control, c - 37°C Test>4°C Test, d - 37°C Control>4°C Control, e - 37°C Control>4°C Test, f - 4°C Test>4°C Control) $p<0.05$

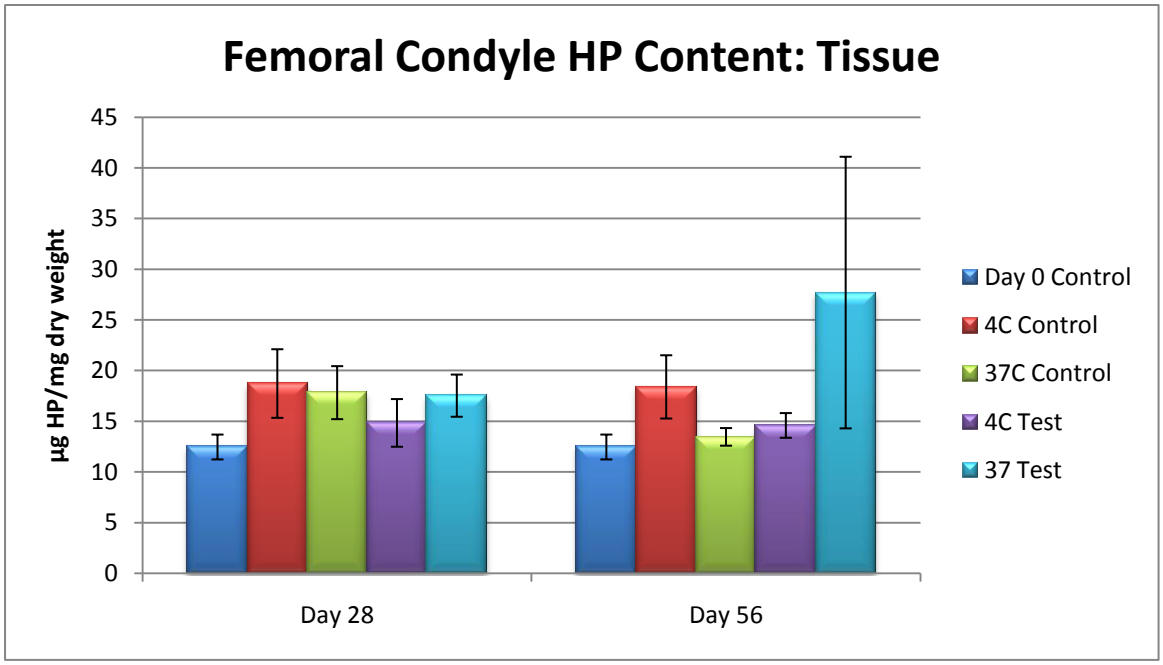


Figure 12: Total tissue HP content (±SEM) of femoral condyles. Values are reported as µg HP/mg dry weight of full thickness cartilage. No significant differences.

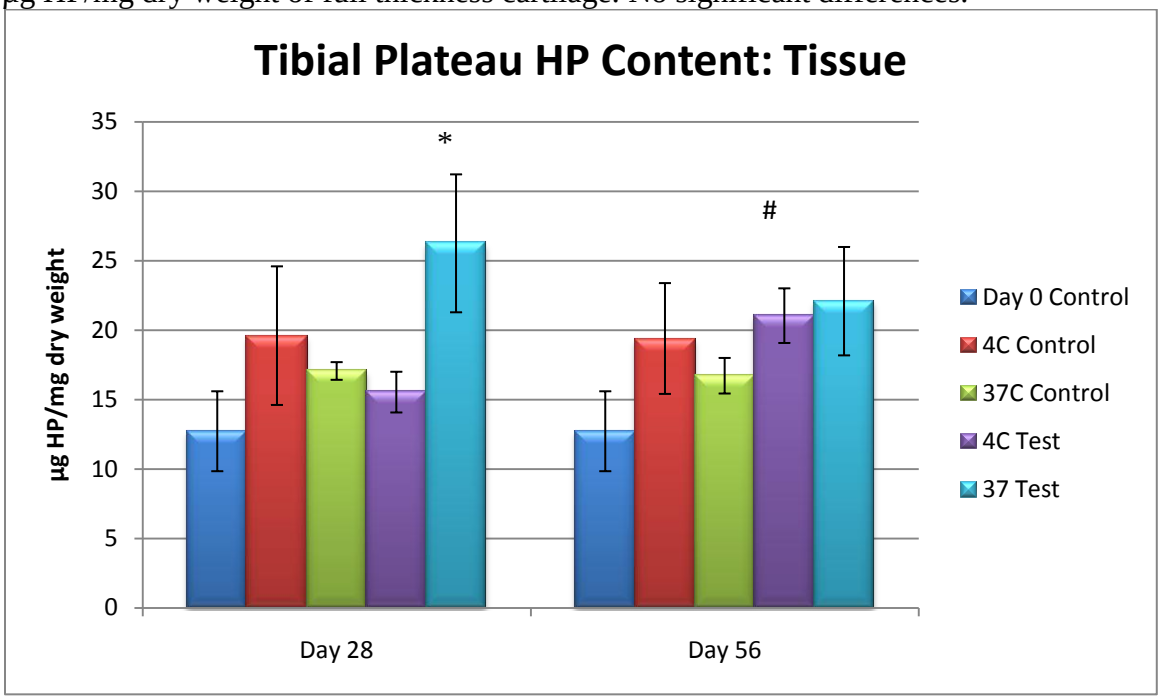


Figure 13: Total tissue HP content (±SEM) of tibial plateaus. Values are reported as µg HP/mg dry weight of full thickness cartilage. At Day 28(*), OCAs stored in 37°C Test media had significantly higher HP content than 37°C Control and Day 0. At Day 56(#), OCAs stored in 4°C Test media had significantly more HP than Day 0. ($p<0.05$)

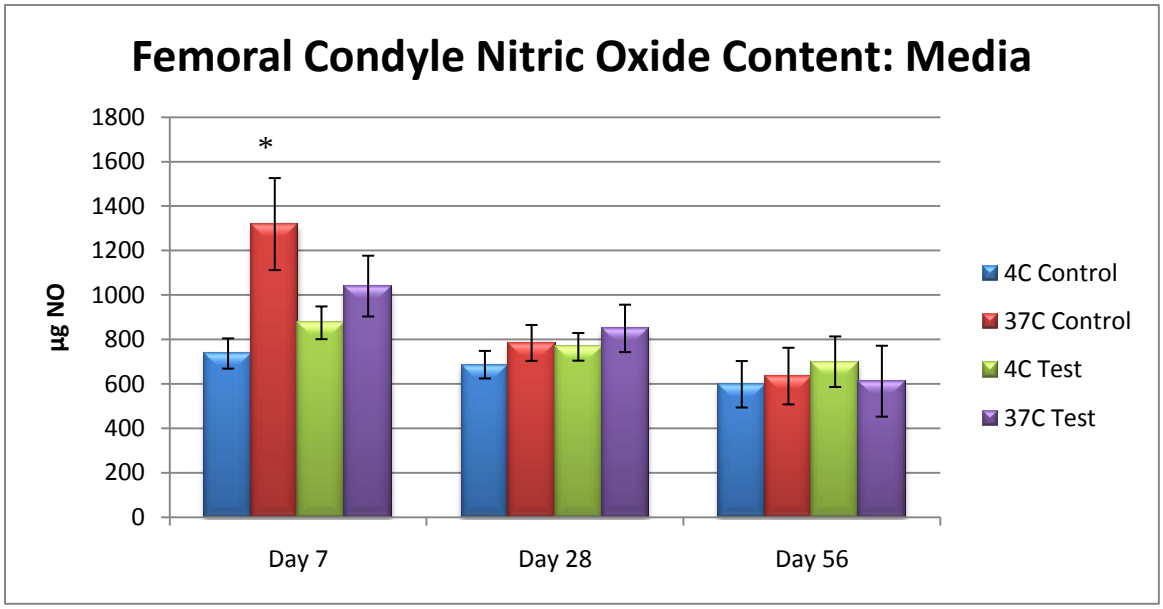


Figure 14:Total media nitric oxide release (±SEM) from femoral condyles. Values are reported as µg NO normalized to the media volume. At Day 7(*), OCAs stored in 37°C Control had significantly more NO release than both groups at 4°C. ($p<0.05$)

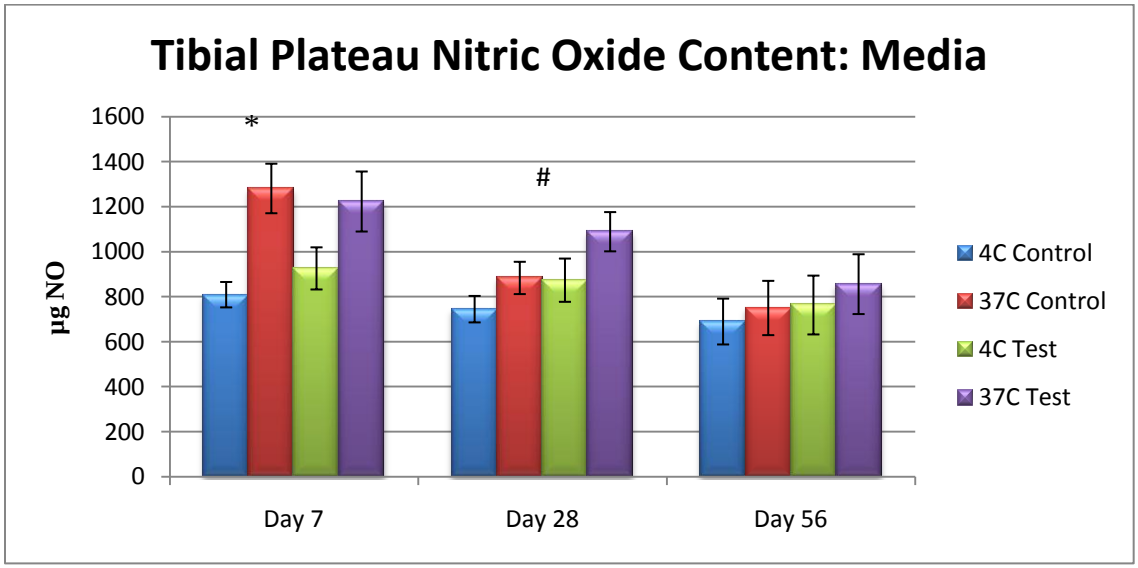


Figure 15: Total media nitric oxide release (±SEM) from tibial plateaus. Values are reported as µg NO normalized to the media volume. At Day 7(*), OCAs stored in 37°C Control media had significantly more NO release than both groups at 4°C and 37°C Test media had significantly more NO release than 4°C Control. At Day 28, the OCAs stored in 37°C Test media had significantly more NO release than those stored in 4°C Control. ($p<0.05$)

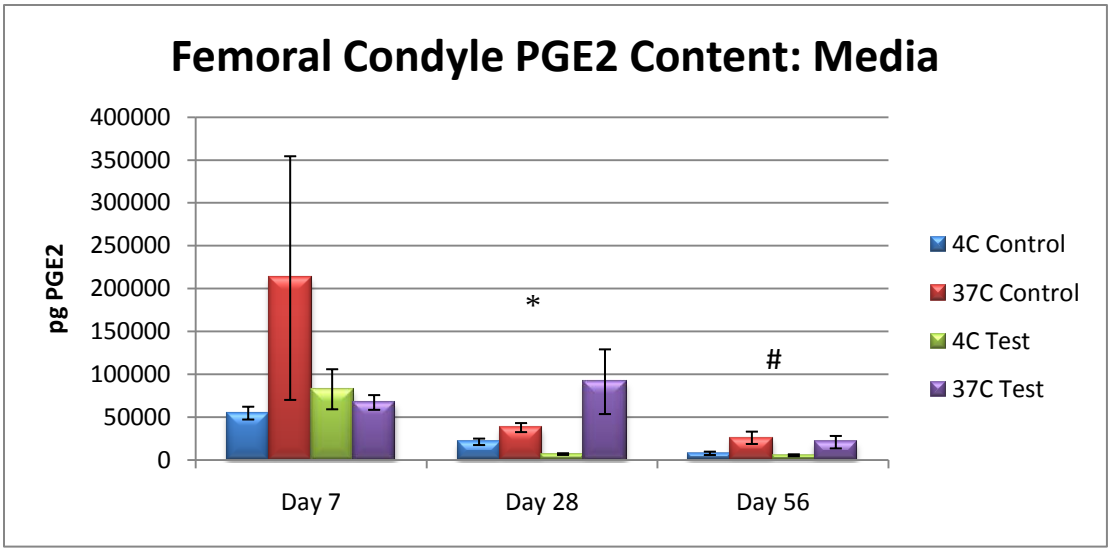


Figure 16: Total media PGE2 release (±SEM) from femoral condyles. Values are reported at pg PGE2 normalized to media volume. At Day 28(*), OCAs stored in 37°C Control had significantly higher PGE2 release than both 4°C groups. OCAs stored in 37°C Test media and 4°C Control media also had higher PGE2 release than 4°C Test media. At Day 56(#), OCAs stored in 37°C Control media had higher PGE2 release than both groups stored at 4°C. ($p<0.05$)

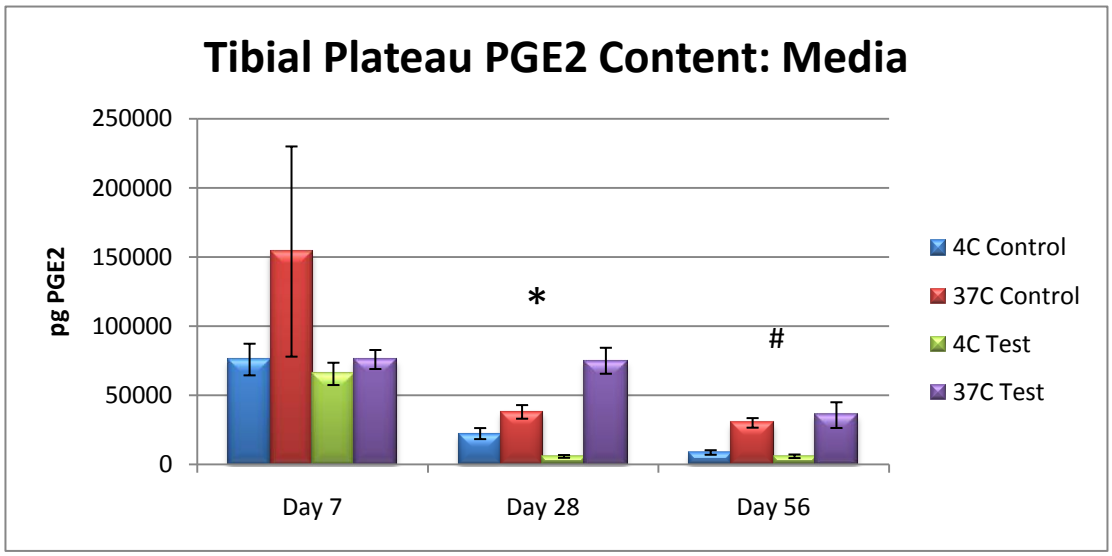
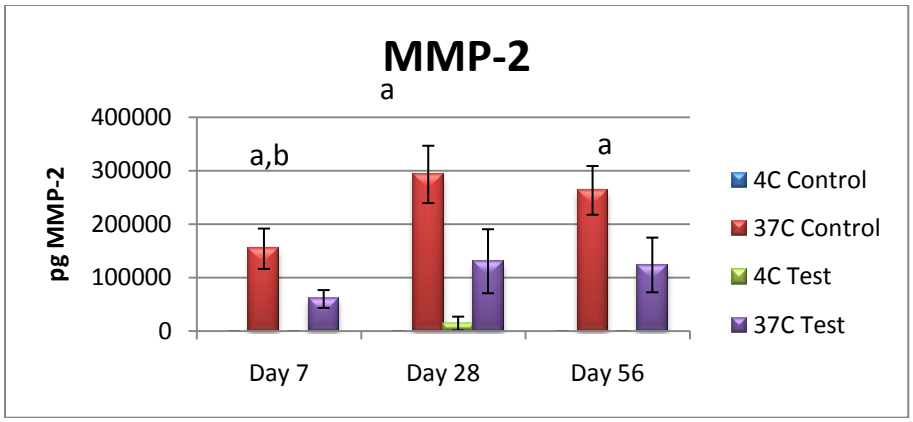
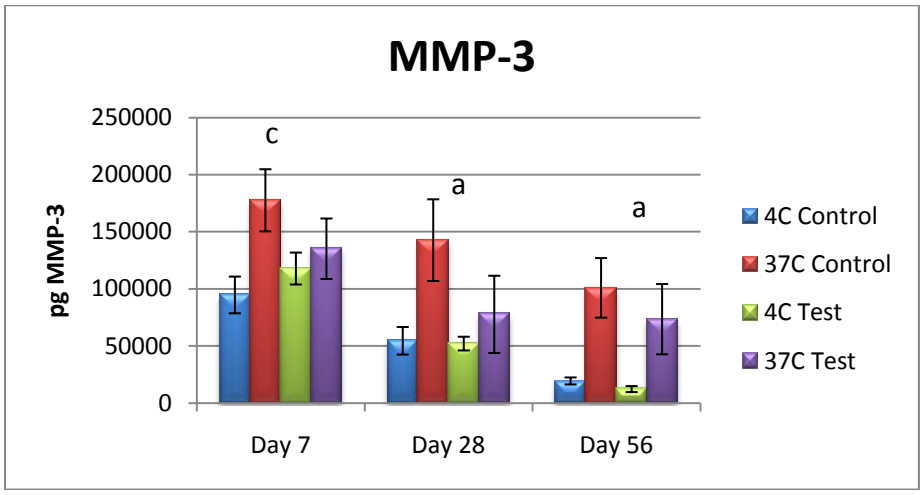


Figure 17: Total media PGE2 release (±SEM) from tibial plateaus. Values are reported at pg PGE2 normalized to media volume. At Day 28(*), OCAs stored in 37°C Test media had significantly higher PGE2 release than all other groups. Also, those stored in 37°C Control had significantly higher PGE2 release than both 4°C groups. Finally, release was statistically higher from OCAs in 4°C Control than 4°C Test media. At Day 56(#), OCAs stored in 37°C Control had significantly higher PGE2 release than both 4°C groups.





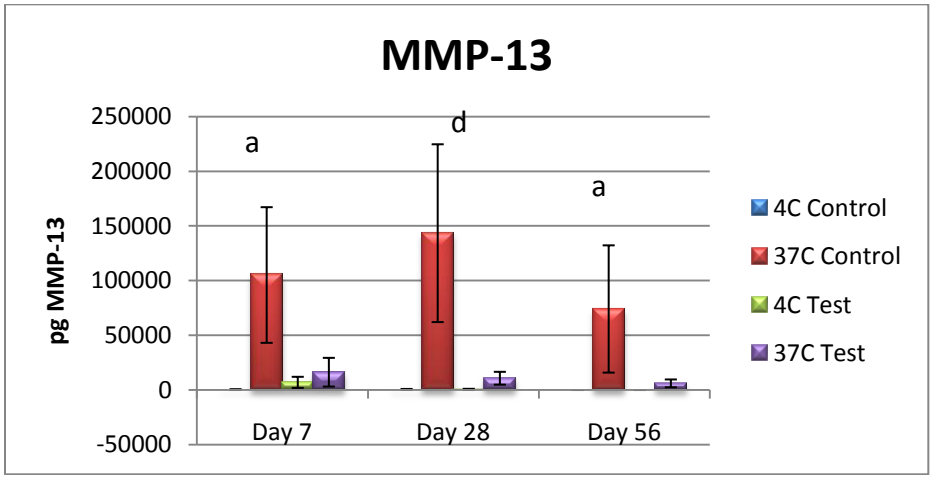


Figure 18: Media MMP release (±SEM) into media from femoral condyles. Values are reported at pg MMP normalized to media volume. (a – 37°C Control > 4°C groups, b - 37°C Test > 4°C groups, c - 37°C Control > 4°C Control, d - 37°C Control > all groups) $p<0.05$

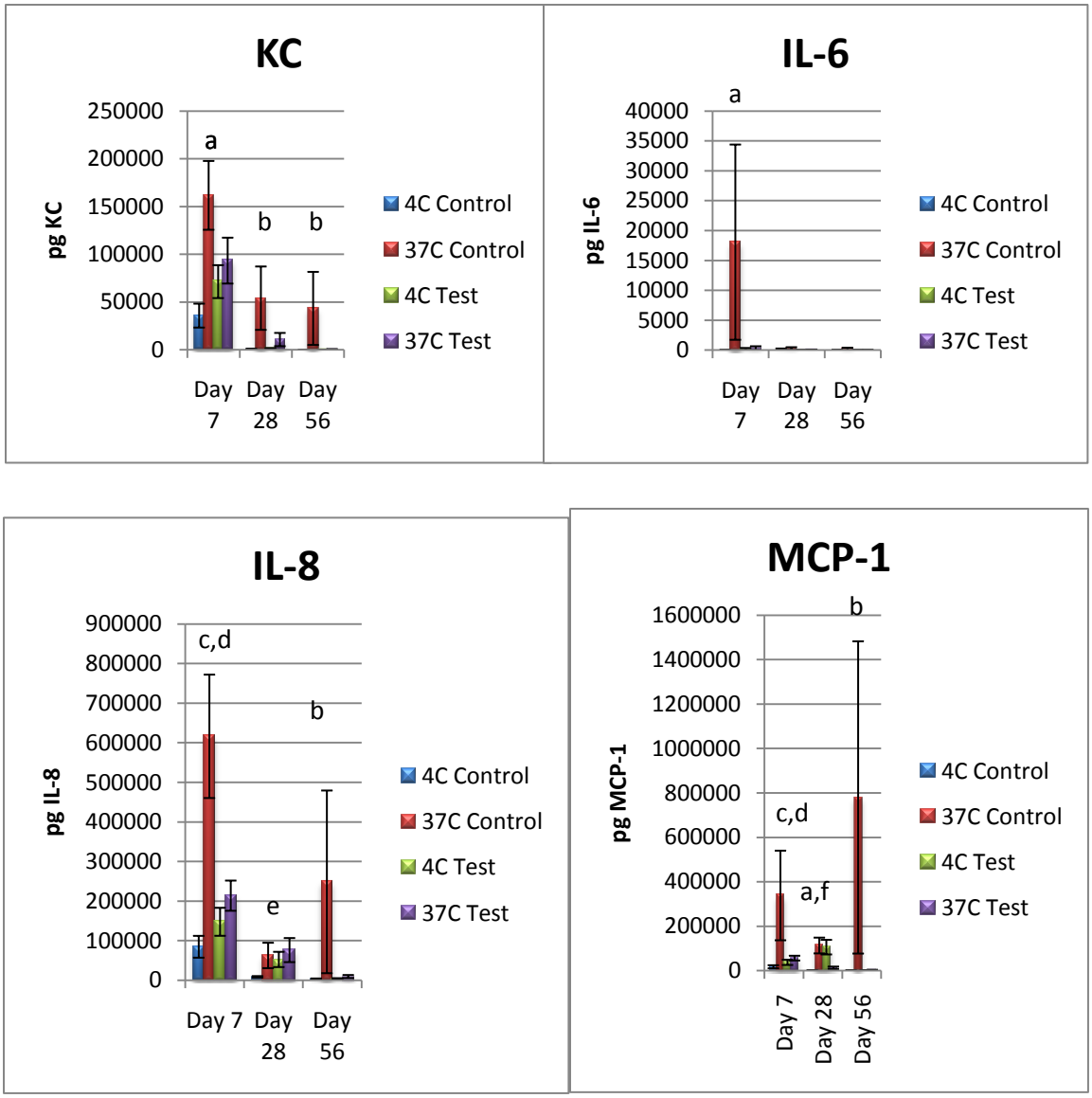


Figure 19: Media cytokine release (±SEM) from femoral condyles. Values are reported as pg cytokine normalized to media volume. (a – 37°C Control > 4°C Control, b - 37°C Control > 4°C groups, c - 37°C Control > all groups, d - 37°C Test > 4°C Control, e - 4°C Test > 4°C Control, f - 37°C Control > 37°C Test, g - 4°C Test > 37°C Test) $p<0.05$

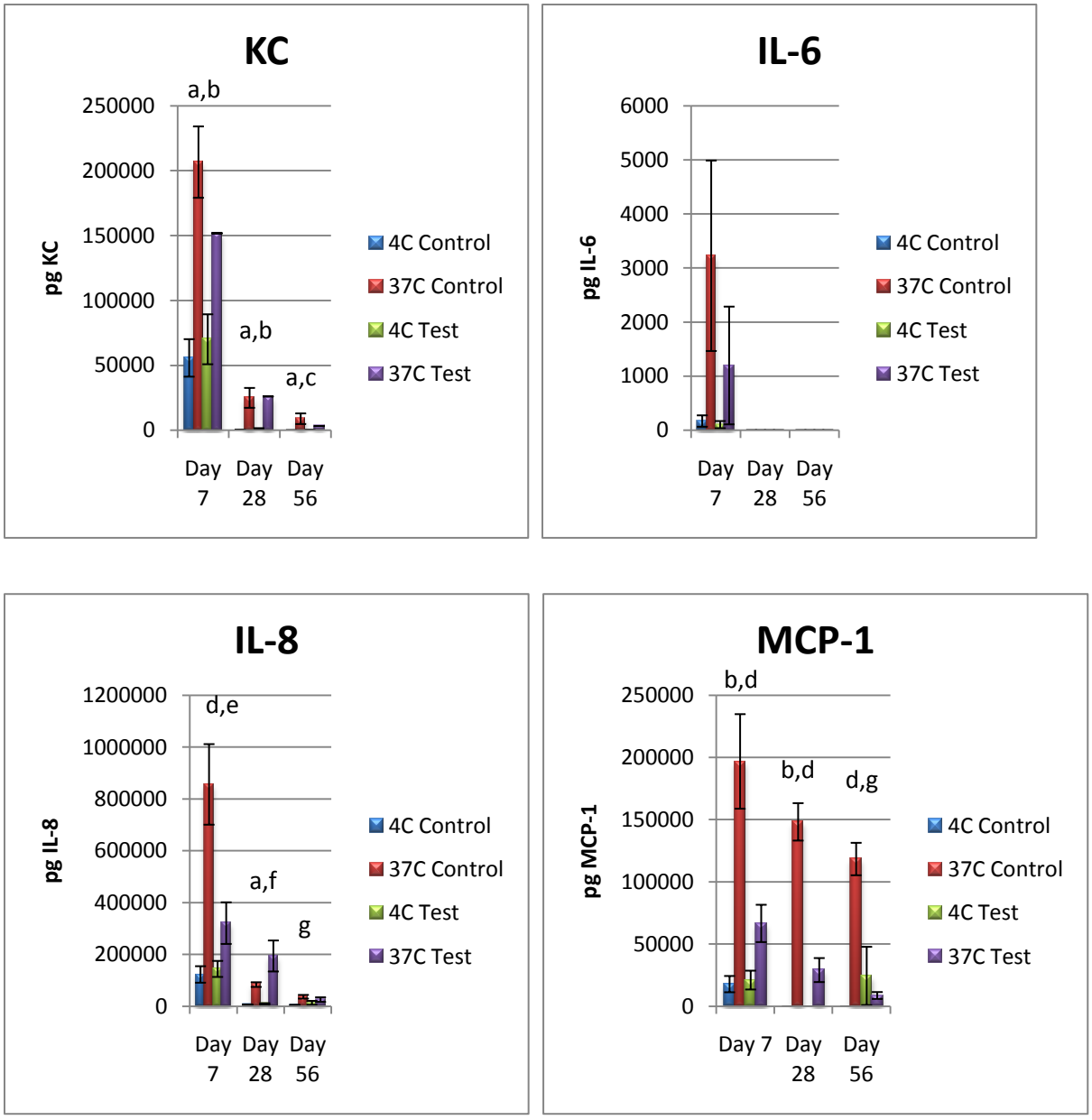


Figure 20: Media cytokine release (±SEM) from tibial plateaus. Values are reported as pg cytokine normalized to media volume. (a - 37°C Control > 4°C groups, b - 37°C Test > 4°C groups, c - 37°C Test > 4°C Control, d - 37°C Control > all groups, e - 37°C Test > 4°C Test, f - 37°C Test > all groups, g - 37°C Test > 4°C Control) $p<0.05$

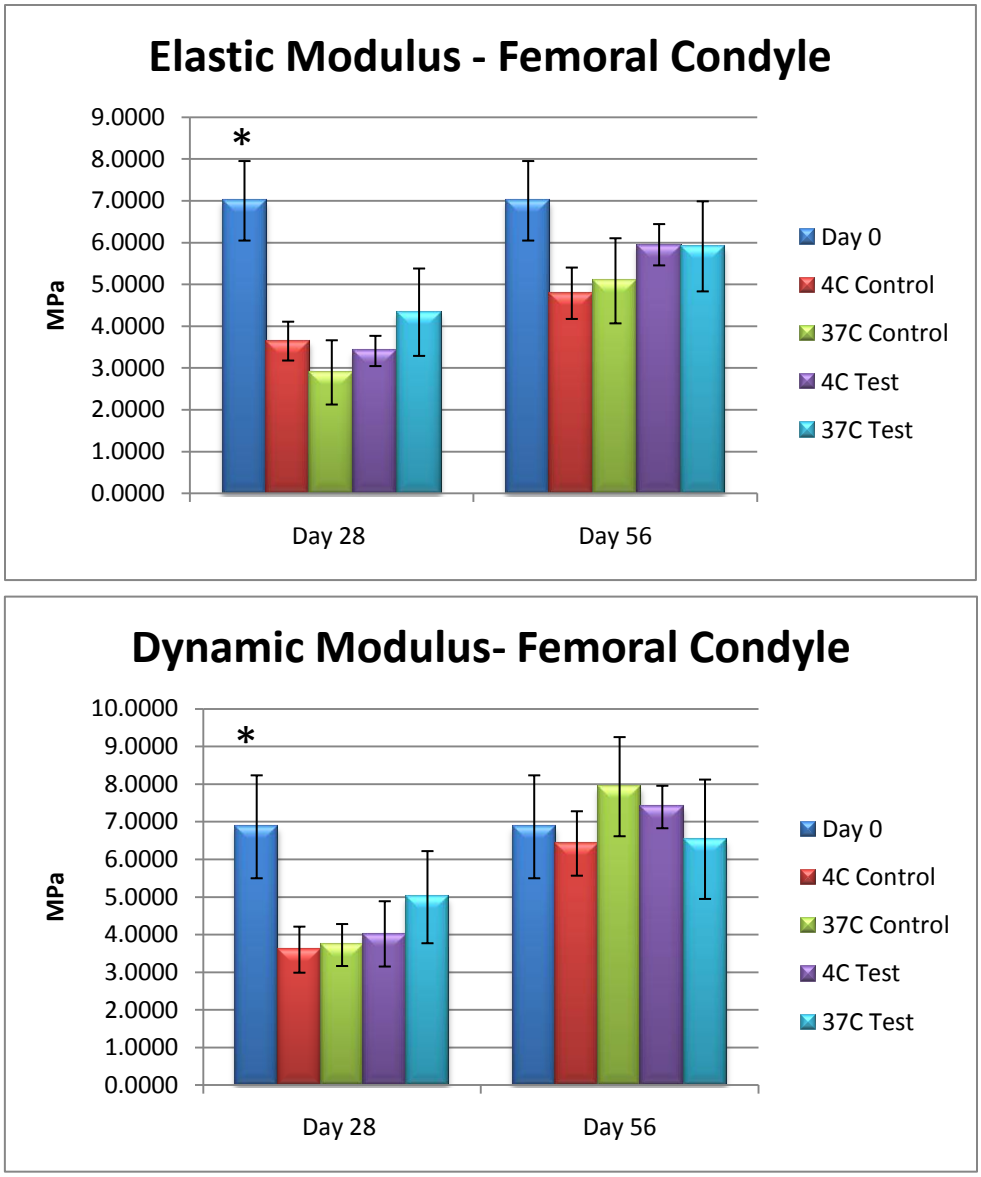


Figure 21: Biomechanical properties, elastic and dynamic moduli (±SEM), of femoral condyle full thickness cartilage from Day 0 Controls, 4°C Control, 37°C Control, 4°C Test, 37°C Test media at Day 28 and 56. Values reported as MPa. At Day 28(*), the Day 0 Control had significantly higher elastic modulus than OCAs stored in 4°C Control, 37°C Control, and 4°C Test media. Also at Day 28(*), the Day 0 Control had significantly higher dynamic modulus than OCAs stored in 37°C Control. ($p<0.05$)

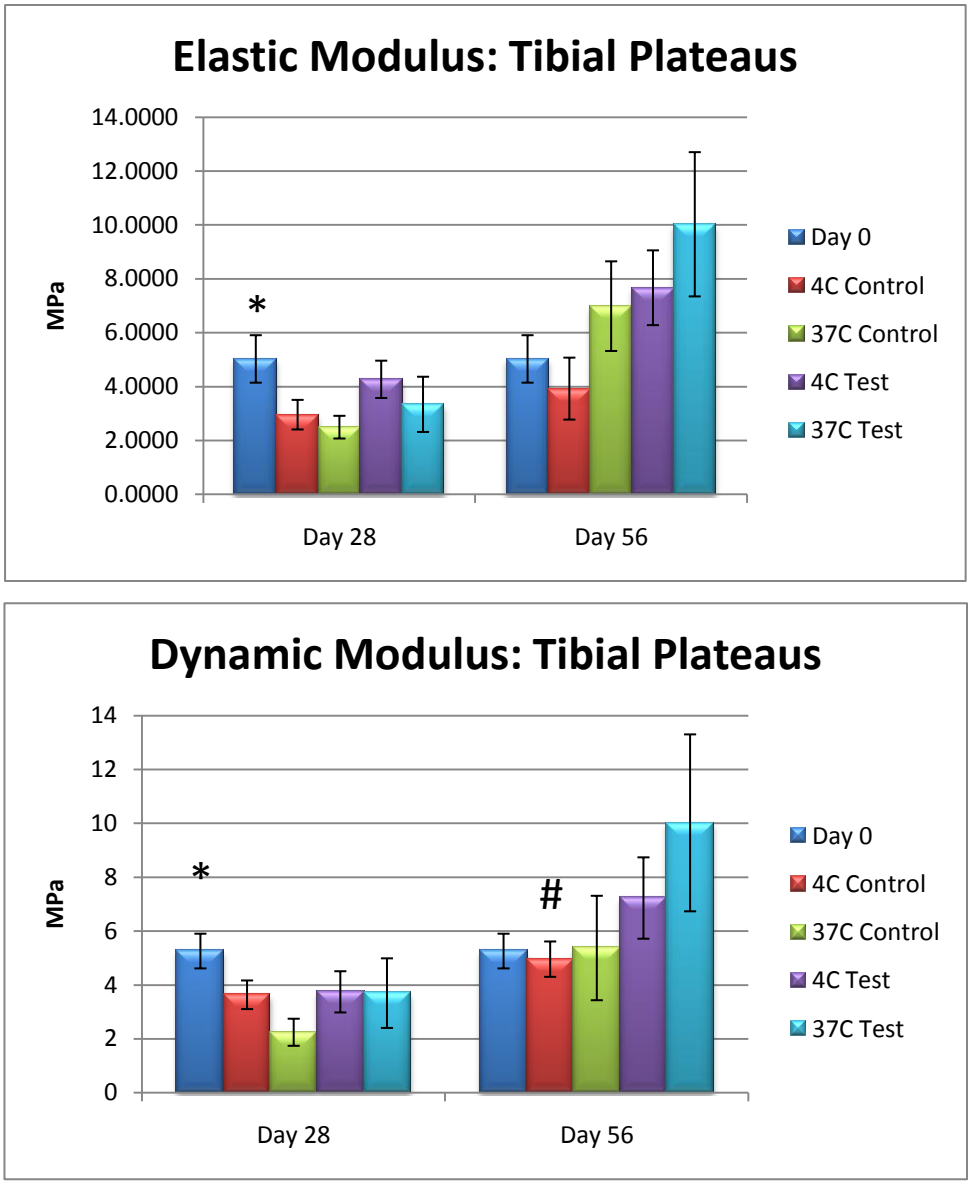
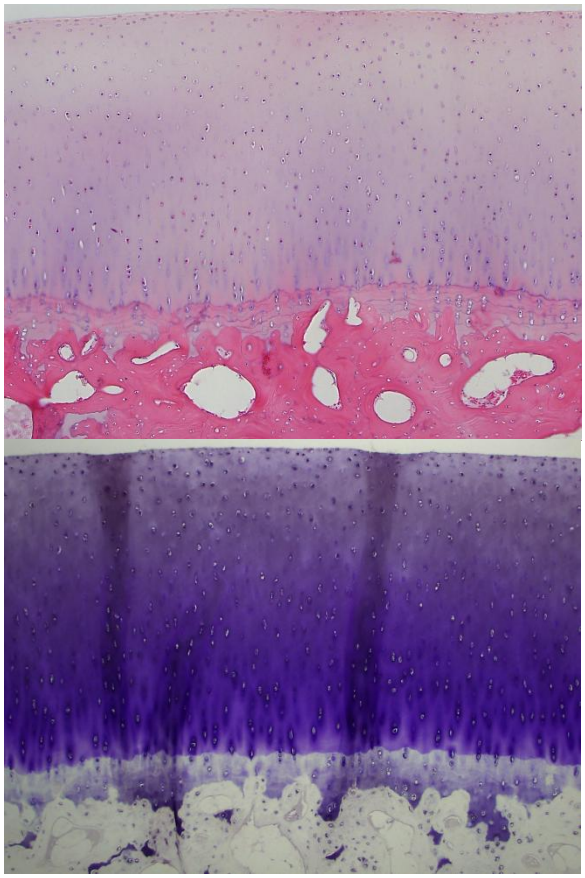
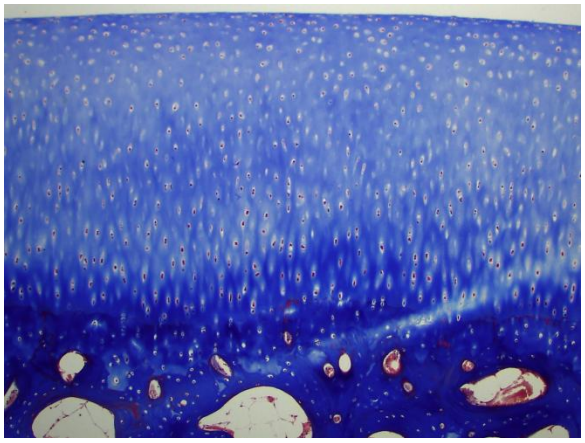


Figure 22: Biomechanical properties, elastic and dynamic moduli (±SEM), of tibial plateau full thickness cartilage from Day 0 Controls, 4°C Control, 37°C Control, 4°C Test, 37°C Test media at Day 28 and 56. Values are reported as MPa. At Day 28(*), the Day 0 Control had significantly higher elastic modulus than OCAs stored in 37°C Control media. Also at Day 28(*), the Day 0 Control had significantly higher dynamic modulus than OCAs stored in 37°C Control. At Day 56(#), the 37°C Control had significantly lower dynamic modulus than both 4°C Test and 37°C Test media. ($p<0.05$)



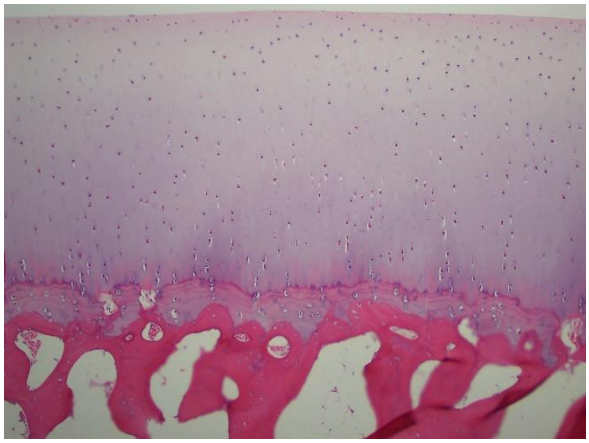
H&E stain

T-blue stain

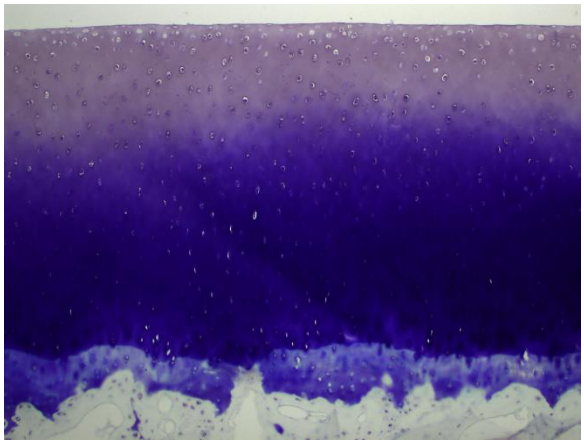


Trichrome stain

Figure 23: Representative images from Day 0 femoral condyles (10X magnification)



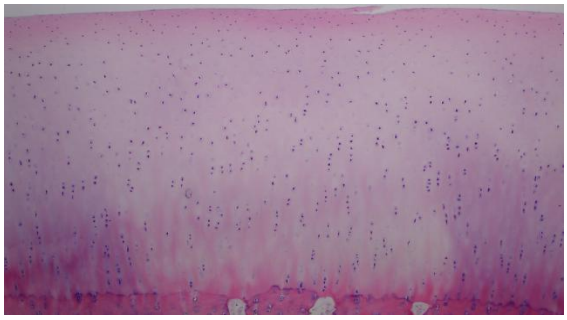
H&E stain



T-blue stain

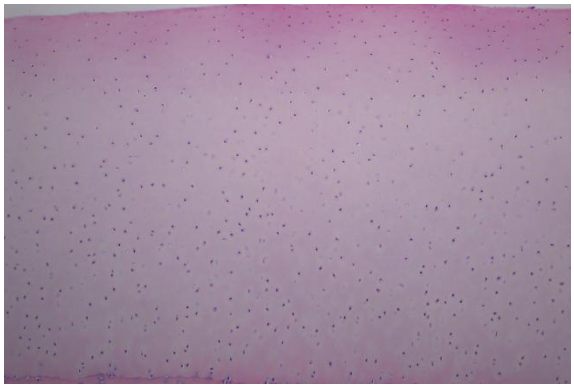
Trichrome stain

Figure 24: Representative images of Day 0 tibial plateaus (10X magnification)

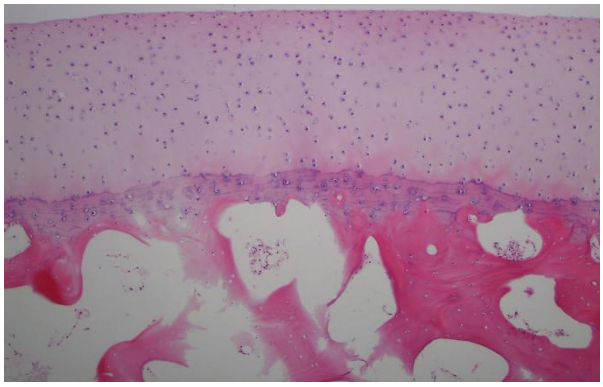


4°C Control

37°C Control



4°C Test



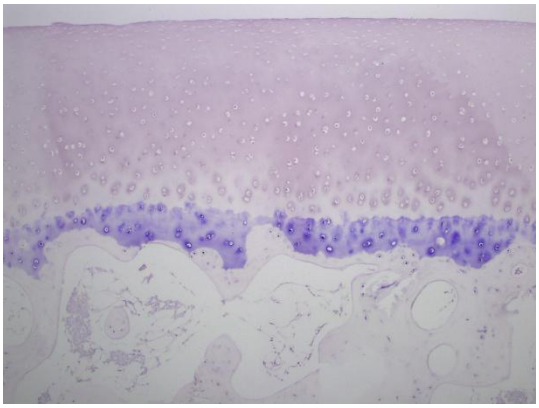
37°C Test

Figure 25: H&E stain of representative images of femoral condyles at Day 28 (10X magnification)

4°C Control

37°C Control

4°C Test



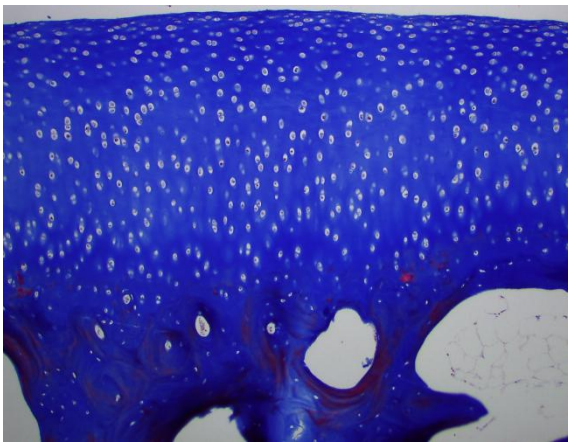
37°C Test

Figure 26: T-blue stain of representative images of femoral condyles at Day 28 (10X magnification)

4°C Control

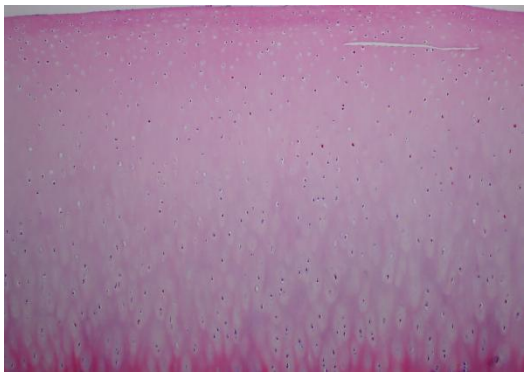
37°C Control

4°C Test

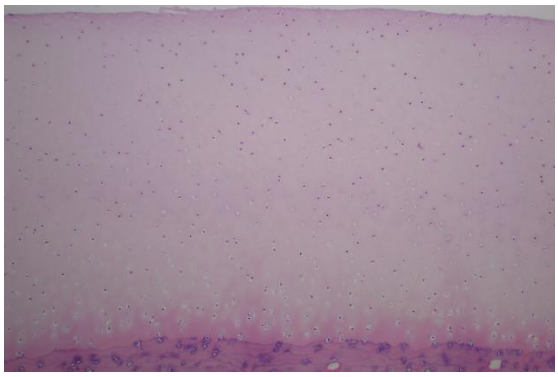


37°C Test

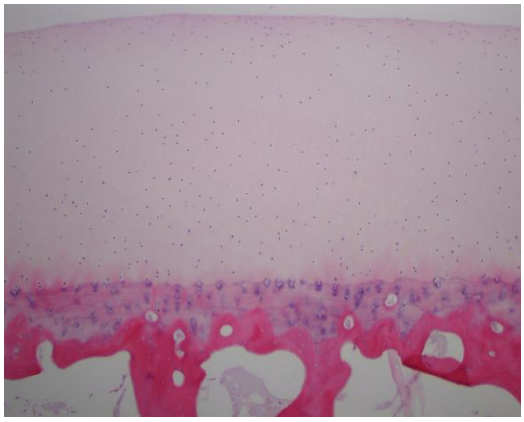
Figure 27: Trichrome stain of representative images of femoral condyles at Day 28 (10X magnification)



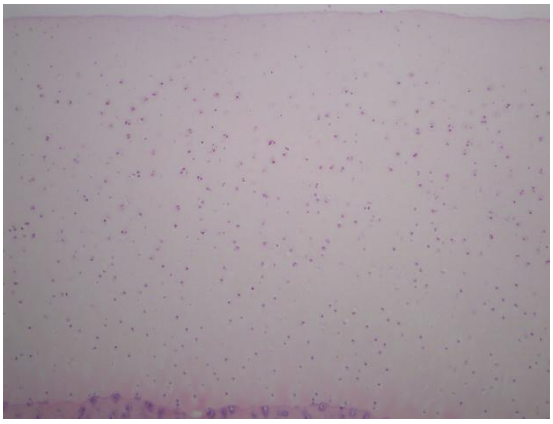
4°C Control



37°C Control

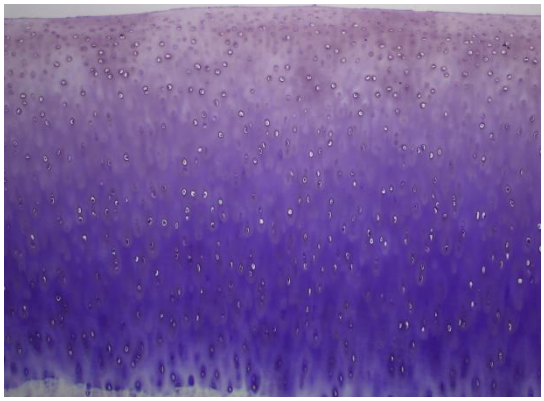


4°C Test

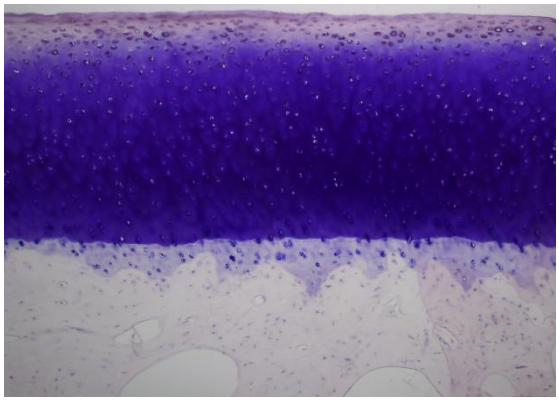


37°C Test

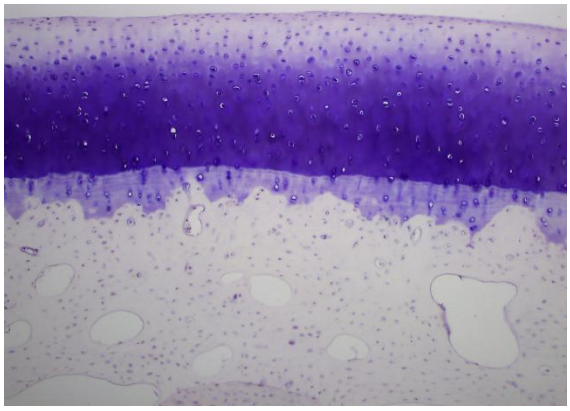
Figure 28: H&E stain of representative images of femoral condyles at Day 56 (10X magnification)



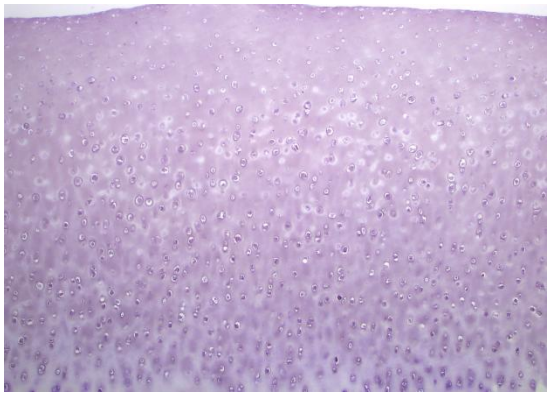
4°C Control



37°C Control



4°C Test

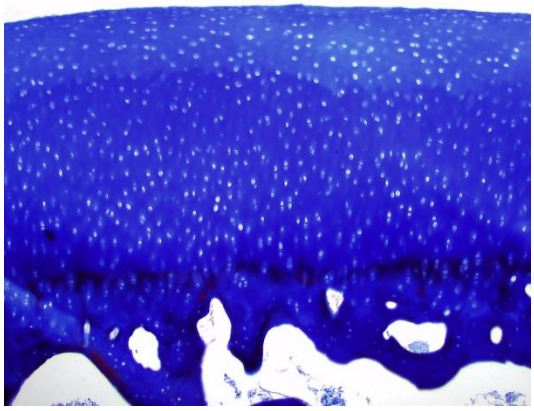


37°C Test

Figure 29: T-blue stain of representative images of femoral condyles at Day 56 (10X magnification)

4°C Control

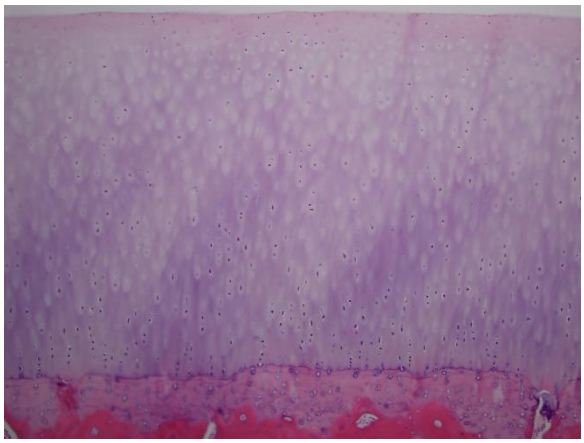
37°C Control



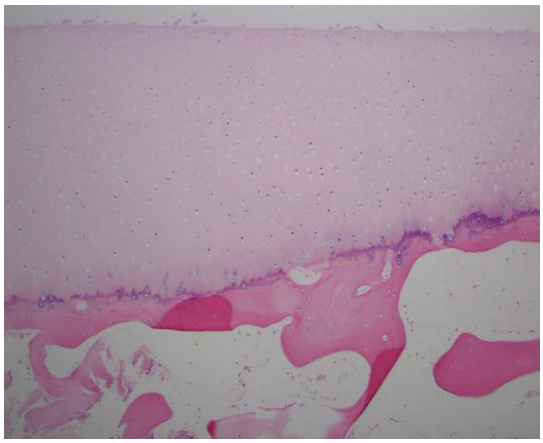
4°C Test

37°C Test

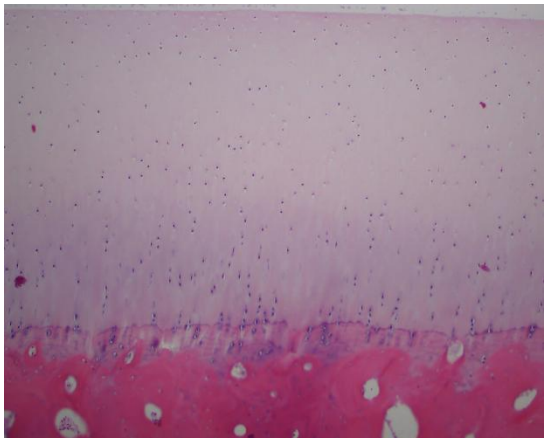
Figure 30: Trichrome stain of representative images of femoral condyles at Day 56 (10X magnification)



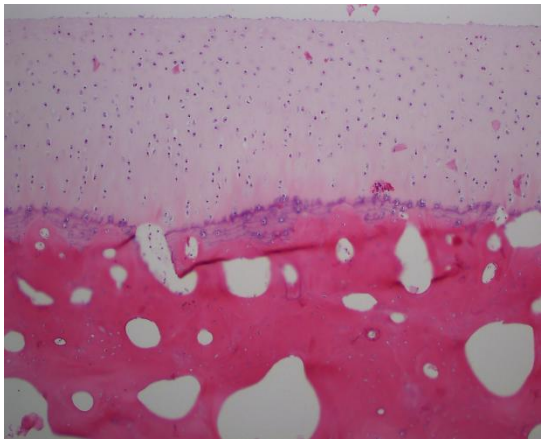
4°C Control



37°C Control



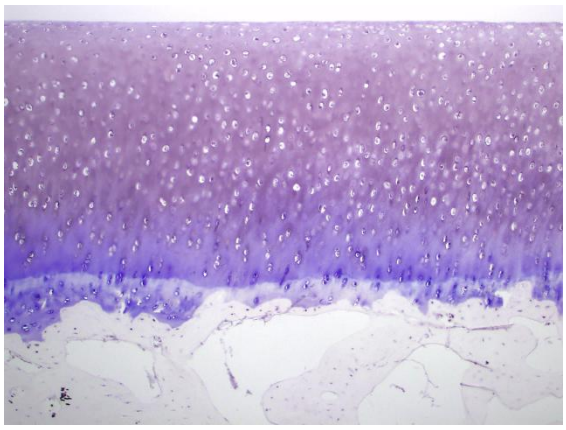
4°C Test



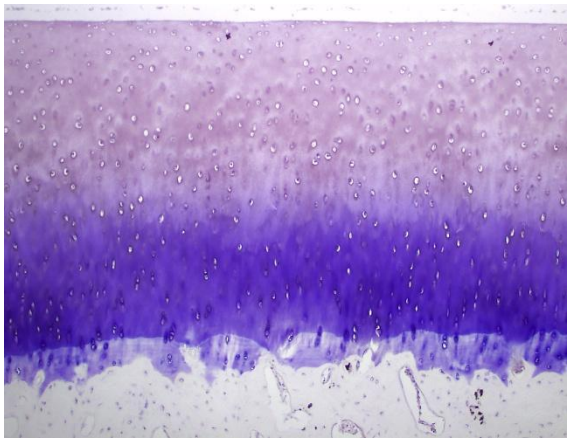
37°C Test

Figure 31: Representative images of H&E stained tibial plateaus at Day 28 (10X magnification)

4°C Control



37°C Control



4°C Test

37°C Test

Figure 32: Representative T-blue stained tibial plateaus at Day 28 (10X magnification)

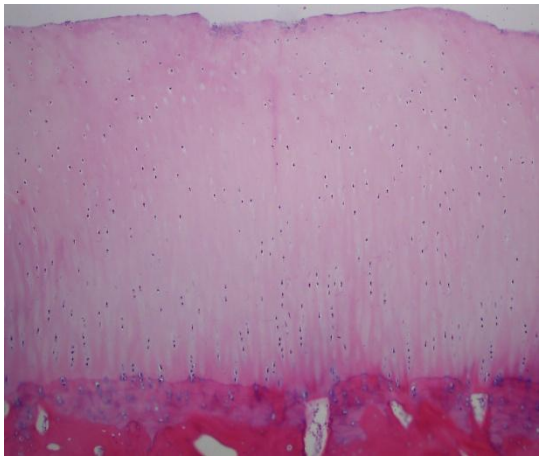
4°C Control

37°C Control

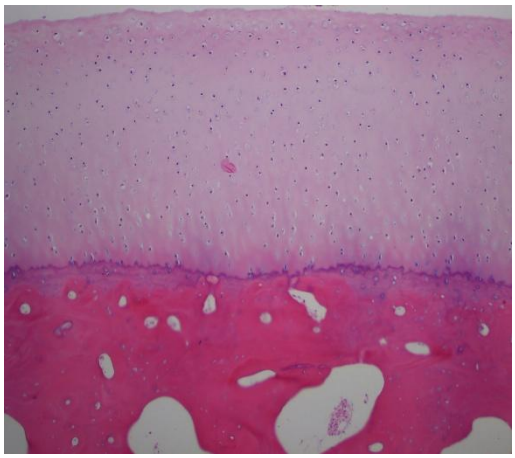
4°C Test

37°C Test

Figure 33: Representative images of Trichrome stained tibial plateaus at Day 28 (10X magnification)

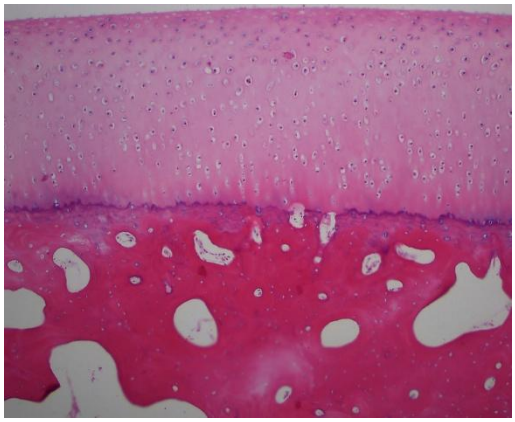


4°C Control



37°C Control

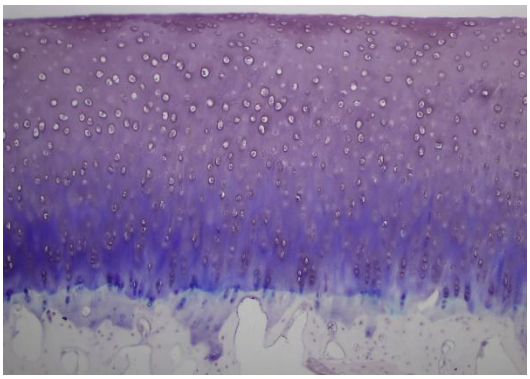
4°C Test



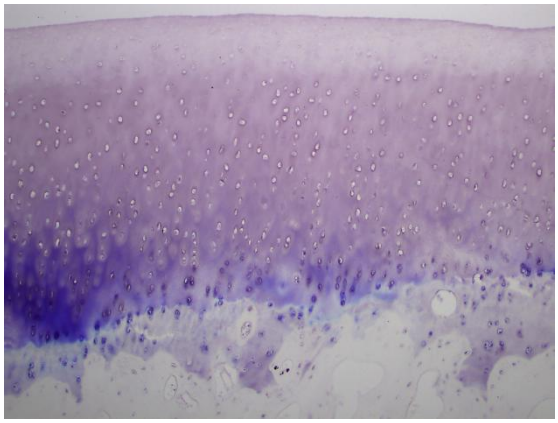
37°C Test

Figure 34: Representative images of H&E stained tibial plateaus at 56 days. (10X magnification)

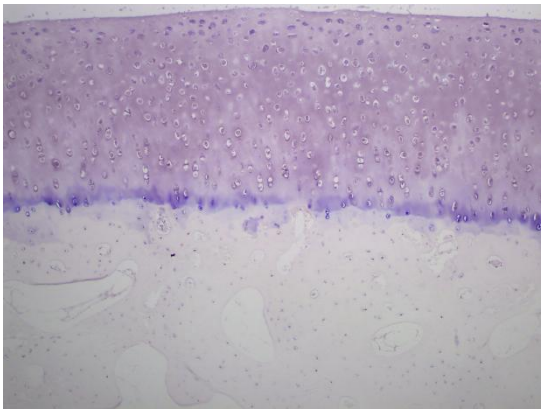
4°C Control



37°C Control

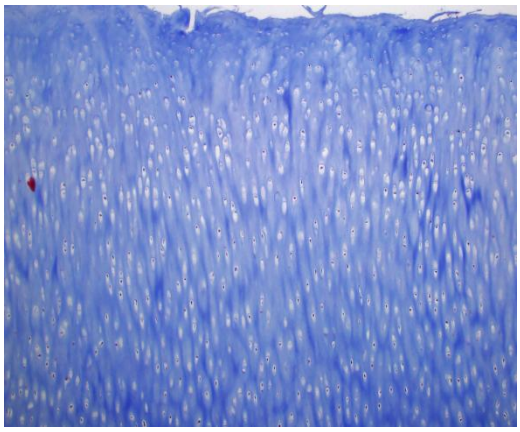


4°C Test



37°C Test

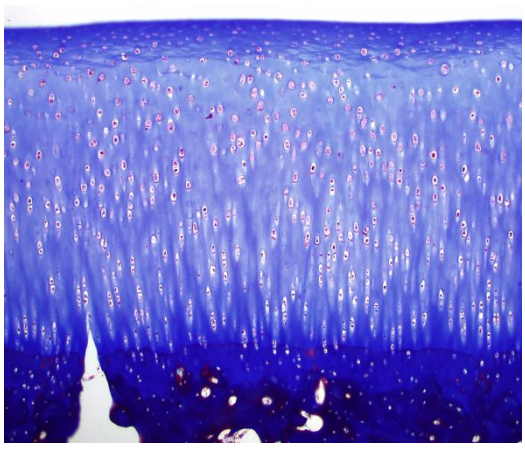
Figure 35: Representative images of T-blue stained tibial plateaus at Day 56 (10X magnification)



4°C Control

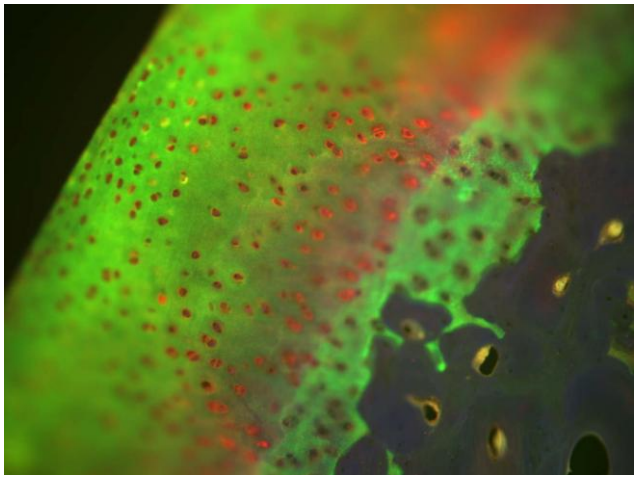
37°C Control

4°C Test

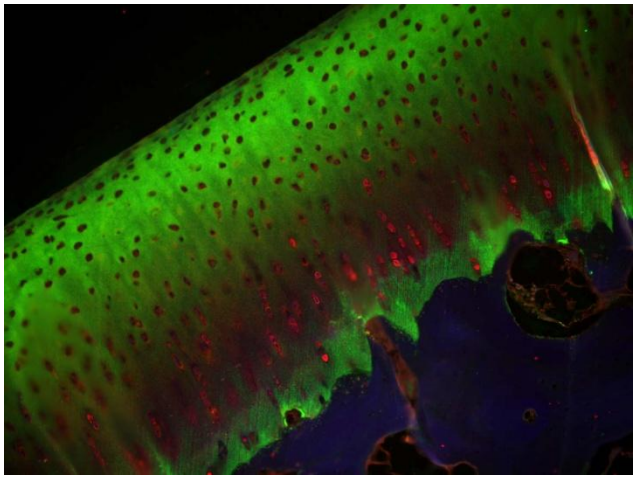


37°C Test

Figure 36: Representative images of Trichrome stained tibial plateaus at Day 56. (10X magnification)

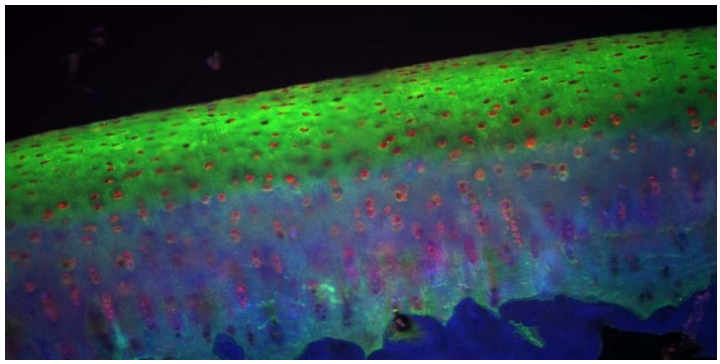


Femoral Condyle

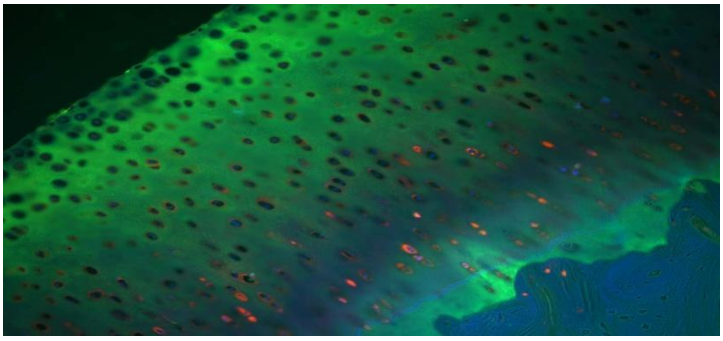


Tibial Plateau

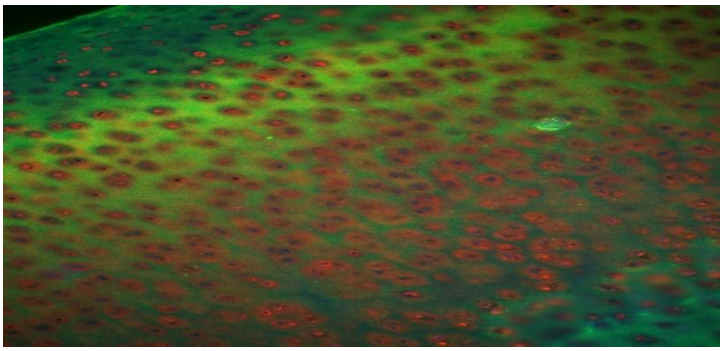
Figure 37: Representative IHC images of Day 0 Controls (20X magnification)



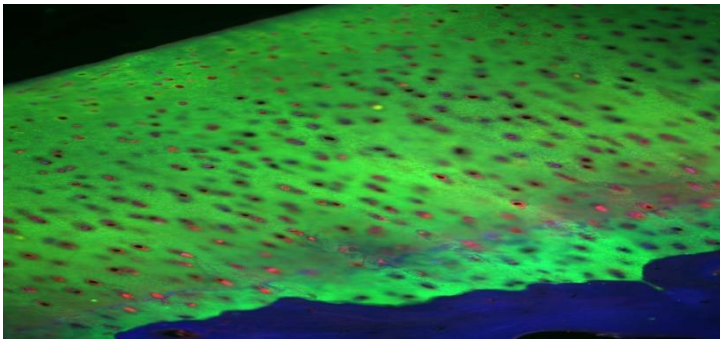
4°C Control



37°C Control

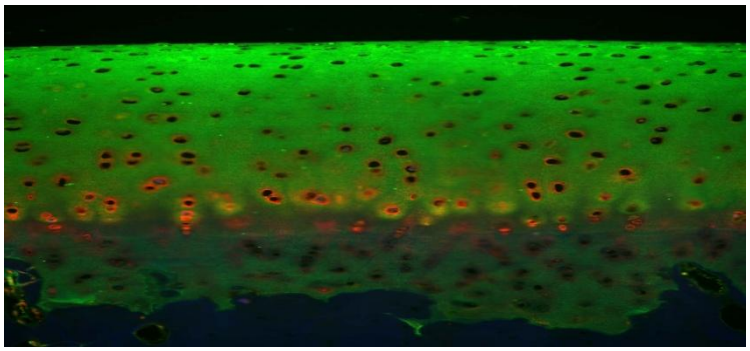


4°C Test

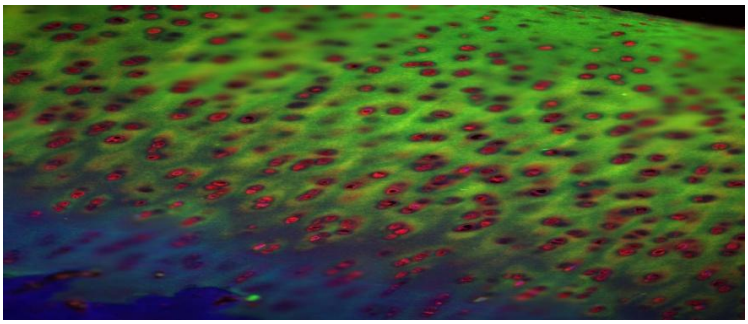


37°C Test

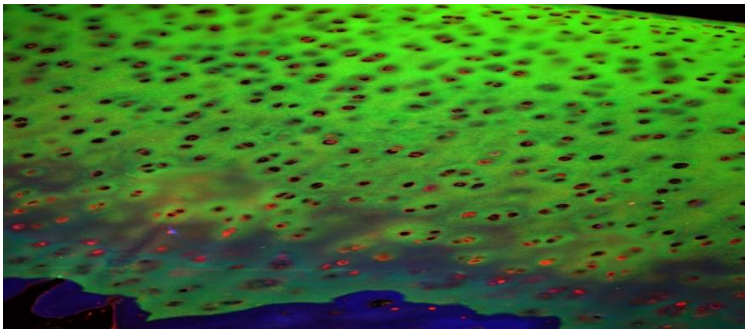
Figure 38: Representative IHC images of femoral condyles at Day 28 (20X magnification)



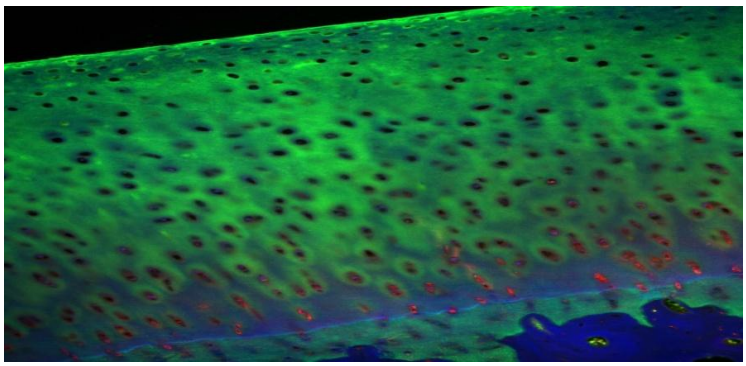
4°C Control



37°C Control

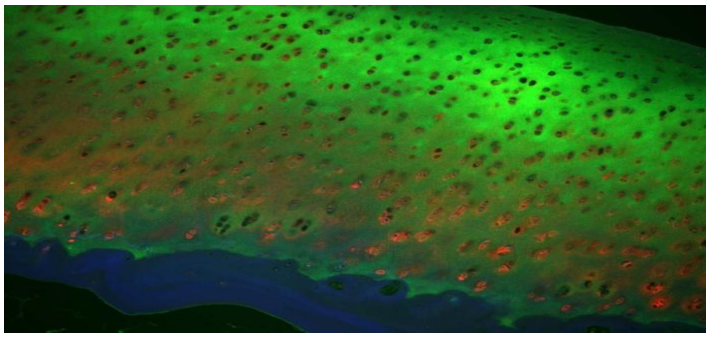


4°C Test

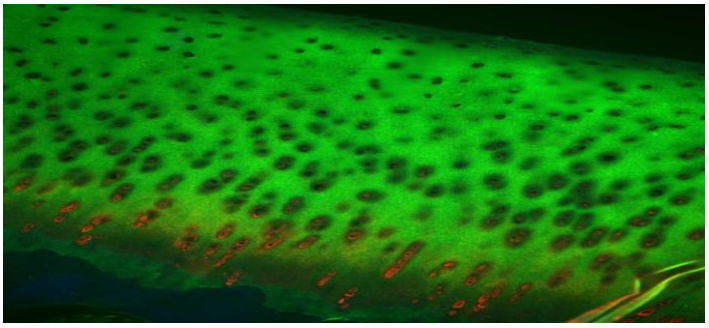


37°C Test

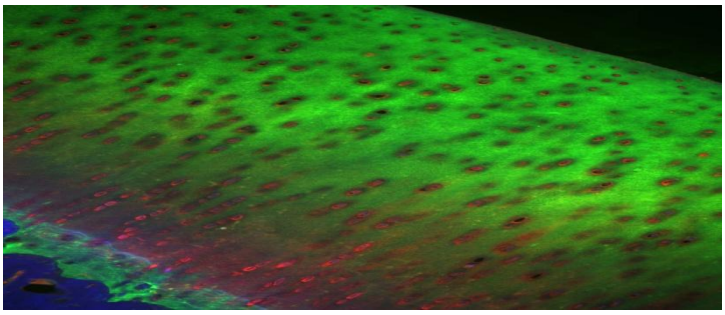
Figure 39: Representative IHC images of femoral condyles at Day 56 (20X magnification)



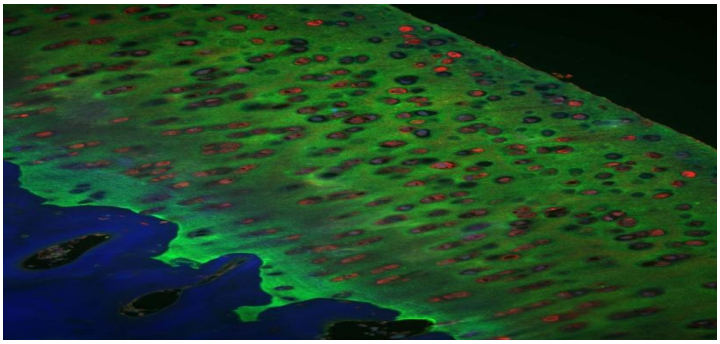
4°C Control



37°C Control

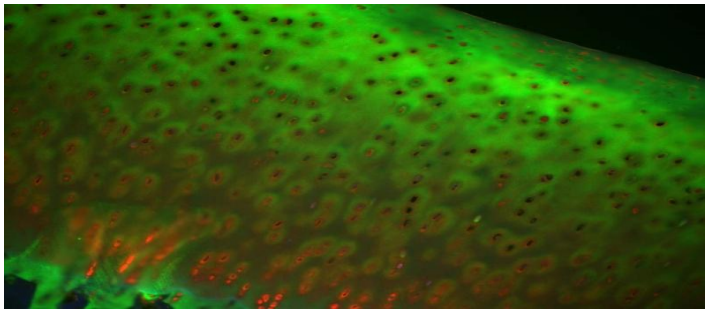


4°C Test

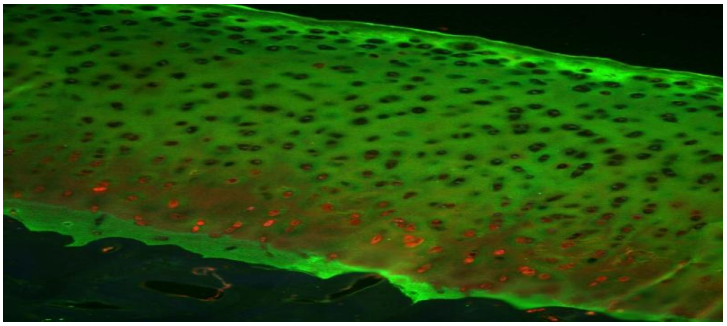


37°C Test

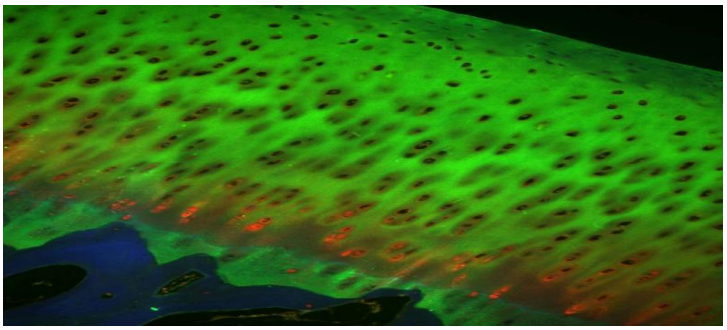
Figure 40: Representative IHC images of tibial plateaus at Day 28 (20X magnification)



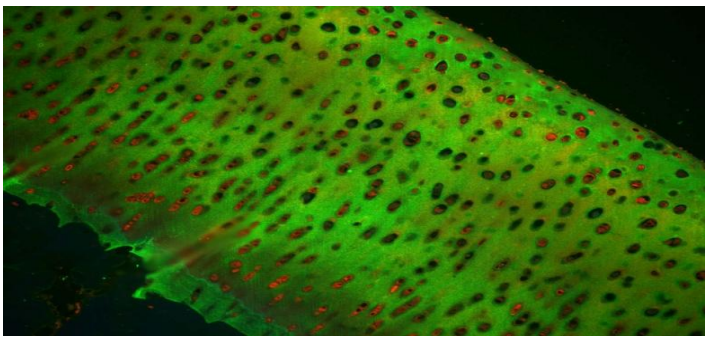
4°C Control



37°C Control



4°C Test



37°C Test

Figure 41: Representative IHC images of tibial plateaus at Day 56 (20X magnification)

VITA

Joseph Thomas Garrity was born on August 14, 1985 in Moline, Illinois. He graduated from Orion High School in 2004. From there he decided to attend the University of Missouri to continue wrestling and pursue a Bachelor of Science in Biological Sciences which he received in 2008. Upon graduation he received the Dr. Prentice Gautt Postgraduate Scholarship from the Big XII Conference and chose to use this to pursue a Master of Science in Biomedical Sciences with an emphasis on Pathobiology. He is currently a second year medical student at the University of Missouri School of Medicine.

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