DECELLUALARIZED CARTILAGE MICROCARRIERS AS A NOVEL PLATFORM FOR CHONDROGENIC EXPANSION

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DECELLULARIZED CARTILAGE MICROCARRIERS AS A NOVEL PLATFORM FOR CHONDROGENIC EXPANSION

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LIST OF SYMBOLS AND ABBREVIATIONS

ECM	Extracellular matrix
GAGs	glycosaminoglycans
OA	osteoarthritis
ACI	autologous chondrocyte implantation
MACI	matrix-induced autologous chondrocyte implantation
TCPS	tissue culture polystyrene
DPBS	Dulbecco's phosphate buffered saline
H&E	hematoxylin and eosin
SafO	Safranin-O
DMMB	dimethyl-methylene blue
DC	decellularized cartilage
CG	Cultispher®-G
µCs	Microcarriers

SUMMARY

Osteoarthritis is a degenerative disease associated with the degradation of cartilage. One of the few therapies aimed at regenerating cartilage, autologous chondrocyte implantation (ACI), involves *ex vivo* expansion and re-implantation of patient derived chondrocytes. Chondrocytes have relatively slow proliferation rates, however, and rapidly de-differentiate during the *ex vivo* culture performed with ACI. The objective of this study was to develop microcarriers (μCs) that provide a microenvironment that more closely mimics the complex extracellular matrix of native cartilage for chondrocyte expansion and retention of phenotype.

Porcine cartilage was isolated, lyophilized, milled, and sifted overnight to obtain μCs approximately 450 μm and 600 μm in diameter. Multiple decellularization procedures were tested and ultimately a series of chemical and enzymatic washes resulted in removal of more than 98% of the original DNA content. Preliminary seeding experiments performed with chondrogenic ATDC5 cells resulted in chondrogenic proliferation and viability over 7 days of culture on decellularized cartilage microcarriers (DC μCs).

Primary human chondrocytes were then cultured on the DC μCs, commercially available gelatin CultiSpher®-G (CG μCs), or tissue culture polystyrene over 14 days. Chondrocyte-laden DC μCs contained significantly more GAGs than the CG μCs or plated tissue culture polystyrene (TCPS) chondrocytes at all time points.

Ongoing experiments are evaluating changes in chondrogenic phenotype on μCs through histological, immunohistochemical, and gene expression analysis. DC μCs support chondrocyte proliferation, and although the rate of expansion is slower than CG μCs or TCPS, these microcarriers have GAG/DNA ratios more similar to that of native cartilage. This makes DC μCs a promising platform for chondrogenic expansion and suitable for direct implantation.

CHAPTER 1

BACKGROUND & LITERATURE REVIEW

CARTILAGE TISSUE: COMPOSITION AND MORPHOLOGY

Articular hyaline cartilage tissue is a specialized connective tissue found in joints of the body. Articular cartilage functions as a smooth frictionless surface for articulation of mobile joints during movement and also distributes the weight of biomechanical forces. This tissue type is unique in that it is both avascular and aneural, lacking both blood vessels and nerves [1]. Cartilage tissue has limited healing capacity due to its avascular nature, and therefore faces difficulty healing after events in which it is heavily damaged [1]. For this reason, damage to cartilage and degenerative diseases involving cartilage are linked to musculoskeletal morbidity [1].

Given that cartilage lacks vasculature and neural networks, the primary importance of its composition is due to the specialized cells it is composed of, known as chondrocytes, and the dense extracellular matrix (ECM) which the chondrocytes produce and in which they reside [1].

Chondrocytes

Chondrocytes are the primary cell type of articular cartilage tissue. They are sparsely distributed within the cartilage ECM, only making up 2% of the total volume of articular cartilage [1, 2]. Their functions involve the production, maintenance, and repair of the ECM. Chondrocytes maintain upkeep of the ECM by synthesizing various ECM components, including proteins and glycosaminoglycans (GAGs). As with most cells, chondrocyte phenotype and activity can be altered by their exposure or lack of exposure to certain biochemical and biomechanical factors. A meta-analysis demonstrated that multiple growth factors (members of the transforming growth factor-β superfamily, members of the fibroblast growth factor family,

insulin-like growth factor-1, and platelet-derived growth factor) are all potential targets as possible treatments to manage degenerative cartilage diseases such as arthritis due to growth factor influence on cartilage repair and chondrogenesis [3]. Most strategies are unable to recapitulate the complex milieu of growth factors involved in chondrocyte homeostasis.

Extracellular Matrix

ECM is the structure that surrounds and supports the chondrocytes within articular cartilage. ECM is majorly composed of tissue fluid, collagens, and proteoglycans. The majority of articular cartilage weight is made up of 65-80% tissue fluid, with the remaining 20-35% being mostly attributed to collagens and proteoglycans [1]. Molecules with a smaller presence in total ECM composition such as lipids, phospholipids, glycoproteins, and growth factors can also be found present in the ECM in trace amounts. One of the most prominent collagens in cartilage ECM is type II collagen (col II), making up 90 - 95% of the collagen in the ECM and providing major structural support in combination with proteoglycans [1]. Proteoglycans themselves account for 10-15% of articular cartilage in wet weight [1]. The most prevalent proteoglycan in cartilage is aggrecan. Within the cartilage tissue, aggrecan interacts with heavily sulfated glycosaminoglycan chains, which are covalently attached [1]. The sulfated glycosaminoglycan chains create a highly negative network that provides the ECM with osmotic properties which enable cartilage to resist compressive mechanical loading [1].

The continuous interactions between chondrocytes and cartilage ECM are what provide articular cartilage with the components necessary to promote a frictionless, load bearing connective tissue that is vital to the demanding biomechanical environment of diarthrodial joints. When this relationship is compromised, the damaging effects to the cartilage tissue as a whole propagate throughout the tissue, and cartilage's limited capacity for intrinsic healing results in a

disease pathology that is not easily corrected without intervention. This in turn can lead to degenerative joint diseases that involve chronic pain, inflammation, and degradation as the disease progresses throughout the joint [1].

OSTEOARTHRITIS: PREVALENCE AND TREATMENTS

Osteoarthritis

Osteoarthritis (OA) is a degenerative disease associated with the degradation of hyaline articular cartilage and affects nearly 27 million people in the US [4]. OA affects 13.9% of American adults aged 25-65 and 33.6% of adults over 65, and is marked by the degradation of cartilage in major joints such as the knee and hip [4]. OA involves major changes in cartilage function, including adversely affecting the load-bearing, stabilizing, and lubricating functions of healthy articular cartilage [5]. As OA pathology propagates, a single compositional change in one aspect of the ECM can have detrimental effects in other compositional aspects of the ECM. For example, a decrease in proteoglycans increases the likelihood of contact stresses. In turn, weakening of the collagen network due to enhanced contact stresses reduces osmotic and electrostatic forces that functionally allow cartilage tissue to redistribute loads [5]. As OA progresses, certain metabolic activities of the ECM cease to function, and synthesis of important structural molecules such as aggrecan and collagen ceases. Not only does OA lead to phenotypic changes in cartilage ECM, but it also has been linked to inflammatory responses within the joint. Inflammation of the joint adds to degradation of the cartilage and in turn activates the pain commonly associated with arthritis of the joint [5].

Current gold standard – joint arthroplasty

Common treatments for OA including pain relievers, anti-inflammatory drugs, cortisone shots, and lubrication injections are not regenerative and often necessitate total knee

replacement, 450,000 of which were performed annually in 2004 alone [6-8]. Although hip and knee replacement can improve quality of life, these major surgical procedures have multiple associated risks, and there is concern for long-term outcomes, especially in younger patients [9, 10].

Alternative method – Autologous chondrocyte implantation

An FDA approved cell therapy used to treat chondral defects but not OA involves a surgical procedure known as autologous chondrocyte implantation (ACI). ACI is one of the only methods used to regenerate cartilage [11]. In this procedure, a patient has a small sample of healthy hyaline cartilage resected from a non-weight bearing area of the affected joint [11]. The chondrocytes are then enzymatically isolated in a neutral buffer in preparation for expansion that usually takes 11-21 days [11]. The current procedure for chondrocyte expansion involves monolayer (2D) expansion in standard growth media and a polystyrene culture dish following typical cell culture procedures and enzymatic release of attached cells [11]. After expansion, these chondrocytes are transplanted into the defect area of the joint and secured using the periosteal flap [11]. Current drawbacks of ACI are the slow proliferation rates of isolated chondrocytes, as well as the tendency for chondrocytes to dedifferentiate during *ex vivo* expansion [12]. This dedifferentiation during *ex vivo* culture is a process by which the chondrocytes form a scar-like fibrocartilage instead of healthy hyaline cartilage [12]. The aforementioned dedifferentiation can be quantified by changes in collagen expression patterns, in which collagen II is downregulated and there is increased expression of collagen I [12]. This formation of fibrocartilage leads to calcification of the cartilage and promotes osteogenesis, which can lead to the growth of bone *in vivo* instead of replenishing the depleted hyaline cartilage, and results in additional pain and bone to bone contact in the affected joint [12]. Due to

these complications, the field investigating chondrocyte expansion and proliferation is pursuing solutions to address the current problems with ACI. In countries outside of the U.S., fibrocartilage formation is decreased by using matrix like materials in place of the periosteal flap to secure the transplanted chondrocytes in the defect in what is referred to as matrix-induced ACI (MACI) [13]. The current matrices approved for use are membranes derived from a porcine collagen I/III matrix [13]. Chondrocytes cultured *in vitro* on collagen I/III matrices produced more collagen II and GAGs in comparison to chondrocytes cultured on polystyrene, however, a separate study found high rates of apoptosis and lower cell proliferation on collagen I/III matrices when compared to collagen II matrices [13]. Collagen I and collagen III are less physiologically relevant collagens in articular cartilage, and there remains a gap in current MACI procedures to employ a cell delivery vehicle that better mimics the complex microenvironment found *in vivo* in healthy articular cartilage tissue.

CARTILAGE TISSUE ENGINEERING PLATFORMS

Decellularized cartilage

Xenogeneic and allogeneic implants often elicit an inflammatory response due to cellular antigens found in the tissues [14]. Often, extracellular proteins are largely conserved among species and cellular components can be removed through decellularization through a series of chemical and/or enzymatic washes while preserving ECM from the native tissue [14]. Decellularized tissues have shown potential for applications in various tissue types including heart valves, various other vasculature, connective tissues including tendons, ligaments and muscles, and even entire specialized organs such as the liver and bladder [15-21]. Decellularized cartilage has been shown to contain a variety of bioactive molecules that are found in native articular cartilage including proteoglycans, collagen II, and multiple chondrogenic growth factors [22-26]. Several studies have investigated the effect of chondrocyte culture on decellularized

articular cartilage [22-25]. When compared to tissue culture treated polystyrene (TCPS) monolayer expansion, culture on sheets of decellularized cartilage provided a greater retention of chondrogenic phenotype and limited the dedifferentiation normally experienced *ex vivo* [22-25]. Using decellularized cartilage for cellular expansion was beneficial for phenotypic retention, and decellularized cartilage is also biodegradable, which provides tremendous cell delivery applications [14]. The current drawback of using decellularized cartilage sheets to deliver cells is the inability to achieve successful tissue integration and cellular infiltration during scaffold delivery to chondral defects, as found by an ovine study evaluating meniscal scaffolds *in vivo* [27].

Microcarrier Expansion

Microcarriers are used as scalable culture platforms for a variety of cell lines. Microcarriers enhance proliferation when compared to monolayer culture due to their high surface area to volume ratio [28, 29]. In comparison to conventional monolayer culture, microcarrier culture supports improved cellular proliferation and yield with respect to time [30]. Manufactured microcarriers can be produced with many different materials, and chondrocyte expansion has been demonstrated on polystyrene microcarriers, glass microcarriers coated in polymer, and gelatin based microcarriers [30]. Though microcarriers outperform monolayer culture of chondrocytes in proliferation rate and efficiency, the synthetic microcarriers do not prevent dedifferentiation over extended culture periods [30]. Gelatin based microcarriers derived from multiple collagen types facilitated significant GAG production when compared to monolayer chondrogenic culture [30]. Cultured primary human chondrocytes on these gelatinous Cultispher®-G microcarriers resulted in significantly higher glycosaminoglycan content than T-flask cultured chondrocytes when the constructs were implanted subcutaneously in a mouse model after 14 days of *in vitro* culture on either microcarrier or monolayer platforms [30].

Though microcarriers have been shown to aid in retention of chondrogenic phenotype compared to monolayer culture, over the culture period chondrocytes gradually decrease synthesis of articular cartilage ECM [31]. These gelatin microcarriers are composed primarily of denatured collagen I and do not provide the same physiological relevance as collagen type II, found in high concentrations of healthy articular cartilage ECM and lack the complex milieu of other proteins, GAGs, proteoglycans, and growth factors found in a healthy cartilage microenvironment [14, 30]. Thus, there remains a need for a more physiologically relevant culture platform that maintains chondrogenic phenotype during expansion and supports cells with the appropriate microenvironment for direct implantation.

CHAPTER 2

A NOVEL MICROCARRIER PLATFORM FOR CHONDROGENIC EXPANSION

RATIONALE

Xenogeneic and even allogeneic tissue contains antigens that can trigger immune responses upon implantation, and regenerative therapies using native tissues should aim to reduce this potential inflammatory response. Decellularization is a technique that combines mechanical and chemical treatments to remove cellular contents from tissues, leaving an ECM containing growth factors, proteins, and structural morphologies found in the native tissue of the body [14]. These scaffolds are rich in bioactive factors and have been used previously for cell culture and phenotype retention, however, decellularized tissue sheets often do not result in successful tissue integration and cellular infiltration when used as a delivery mechanism *in vivo*. Microcarriers are a cell culture platform used in cell proliferation and expansion. Their surface area to volume ratio allows for culture of large amounts of cells with decreased requirements for space and nutrients. Microcarriers have also been used as delivery devices to deliver cells or compounds *in vivo*. Commercially available microcarriers are currently composed of single or dual molecules and do not recapitulate the complex microenvironment needed for chondrocytes to maintain healthy phenotype. Additionally, the materials they are manufactured from are often neither biodegradable, nor manufactured for direct use and often require enzymatic degradation before use [32].

OBJECTIVE

The **objective** of this study is to assess the efficacy of decellularized cartilage microcarriers as a cell culture and delivery platform for chondrogenic cells in order to improve

current cell therapies used to treat chondral defects. The **hypothesis** is that decellularized porcine articular cartilage microcarriers will support expansion while enhancing the retention of chondrogenic phenotype and ECM synthesis in comparison to gelatin microcarriers and standard monolayer culture on tissue culture polystyrene.

MATERIALS & METHODS

Cartilage Decellularization

Fresh porcine cartilage was isolated from the feet of eight market weight pigs obtained from a local meat processor. Extraneous tissue was removed to expose the articular cartilage surface and care was taken to avoid removing subchondral bone during isolation of the articular cartilage. The cartilage pieces were then washed with Dulbecco's phosphate-buffered saline, DPBS, and then aspirated before cartilage samples were stored at -80°C.

Multiple decellularization procedures were compared in order to determine an effective method for cellular removal with maximum retention of extracellular matrix components, namely glycosaminoglycans, or GAGs. Cartilage pieces were thawed at room temperature and washed on a rotary orbital shaker at 250RPM and 37°C with 500mL of deionized water, 1% Sodium dodecyl sulfate (SDS), or 1% TritonX-100 for 48 hours, or through a series of chemical and enzymatic washes based on a protocol used by the Badylak lab shown in Table 1 [33]. Samples were fixed in 10% neutral buffered formalin overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and/or with Safranin-O (Saf O) to show lack of visible nuclei and GAG content. In order to determine when GAGs were lost during the decellularization, samples were taken after multiple decellularization steps (items bolded in Table 1). Shorter trypsin washes (for 1.5 h and 4.5 hours) were also analyzed for GAG depletion and a trypsin-free decellularization was compared as well via SafO staining.

WASH SOLUTION	TIME
Trypsin	**6 h**
Deionized water	15 min, 3x
70% ethanol	**15 h**
3% hydrogen peroxide	15 min
Deionized water	15 min, twice
1% Triton X-100 in EDTA/Trizma	**6 h**
1% Triton X-100 in EDTA/Trizma	**15 h**
Deionized water	15 min
0.1% PAA/4% ethanol	**2 h**
Sterile DPBS	15 min, 3x

Table 1. Badylak Decellularization Protocol

Decellularized pieces were incubated with heparin sodium salt and chondroitin sulfate in order to determine if GAGs could be reincorporated into decellularized cartilage pieces. Chondroitin sulfate, a GAG found in cartilage extracellular matrix, and heparin sodium salt, a GAG nonspecific to cartilage, but with a higher negative charge density and therefore likely more reactive to binding with the decellularized ECM were added (100, 10, and 1% dry weight) to one milligram of decellularized cartilage pieces. The solution was placed in a sonicating bath for 15 minutes, incubated for twenty-four hours at 37°C, and washed in DPBS 3 times for 15 minutes each before staining with Safranin-O and imaging with a dissection scope.

Due to DNA being found in trypsin from multiple manufacturers, TrypLE Express, a purified, recombinant cell-dissociation enzyme was used in place of trypsin. A twenty-four hour DNase and RNase wash was also incorporated before the final DPBS wash. All decellularization solution was aspirated and decellularized cartilage was frozen at -80°C until further use. The final decellularization protocol is shown in Table 2.

Wash Solution	**Time**
TrypLE Express	6 h
Deionized water	15 min, 3x
70% ethanol	15 h
3% hydrogen peroxide	15 min
Deionized water	15 min, twice
1% Triton X-100 in EDTA/Trizma	6 h
1% Triton X-100 in EDTA/Trizma	15 h
Deionized water	15 min, 3x
14. DNase/RNase Solution	24 h
15. Deionized water	15 min, twice
16. Deionized water	15 min
17. 0.1% PAA/4% ethanol	2 h
18. Sterile DPBS	15 min, 3x

Table 2. Final Decellularization Protocol

In order to quantify decellularization, frozen cartilage pieces were subjected to lyophilization and digested in proteinase K according to the Quiagen® DNeasy protocol for tissues and DNA was quantified with PicoGreen® reagent and DNA gel electrophoresis. Histological samples were also stained with H&E and SafO to show lack of visible nuclei and GAG retention respectively.

Cartilage Microcarrier Formation

The decellularized cartilage pieces were lyophilized, flash-frozen in liquid nitrogen, and milled with a Thomas Wiley Mini-Mill™ using a 400μm mesh screen. The milled cartilage was then sieve sifted overnight to separate microcarriers by size in sieve meshes

ranging from 180 μm – 250 μm and 250 μm – 400 μm. The dry weight of cartilage samples after each step was recorded in order to determine yield efficiency. After sieving, the microcarriers were stained with Eosin for 15 minutes, washed five times with 0.1% TritonX-100 in D-PBS and imaged with a confocal microscope (Zeiss 700 LSM). Size was quantified in ImageJ® by determining the largest and smallest cross-sectional distances, approximate diameter, and aspect ratio of each microcarrier. Commercially available gelatin microcarriers, Cultispher®-G were also stained and used as a control for comparison.

Cell Seeding on Microcarriers

ATDC5 cells, a mouse teratocarcinoma cell line that mimics mesenchymal condensation and chondrogenic differentiation, was used for preliminary cell-loading experiments. ATDC5 cells were expanded in ATDC5 growth media consisting of DMEM/F-12 media with L-glutamine (Invitrogen) supplemented with 5% FBS (Atlanta biologics), 10 μg/mL transferrin (Invitrogen), and $3x10^{-8}$ M sodium selenite (Sigma-Aldrich) at 3,250 cells/cm^2. Cells were cultured at 37°C in 5% CO2 and fed every 2-3 days until 90% confluent.

After trypsinization, ATDC5 cells were seeded at $5x10^4$ cells/cm^2 or $5x10^5$ cells/cm^2 on the decellularized cartilage microcarriers through overnight incubation at a high cell density (500,000 cells/mL) in ATDC5 growth media in 50 mL conical tubes with 5 mL of agarose to provide a flat bottom for microcarriers to settle without clumping. Static, stirred, and intermittent stirring conditions were all compared for overnight seeding. After overnight incubation, the microcarriers were washed and stained using a Live/Dead viability assay (Calcein-AM and Ethidium homodimer-1) and imaged with confocal microscopy to determine an optimal seeding method. The media was collected after overnight seeding to determine seeding efficiency with a hemocytometer. The microcarriers were then transferred to 6-well

suspension plates and cultured on a rotary orbital shaker at 60 RPM for up to 7 days and samples were collected at Days 1, 4, and 7 for Live/Dead staining and histological analysis.

Human Chondrocyte Isolation

Human chondrocytes were isolated from the distal end of adult femurs of diabetic patients (n=4) undergoing above knee amputations. Briefly, articular cartilage was dissected and care was taken to exclude any areas of osteoarthritis. Cartilage was then cut into 2 mm x 2 mm pieces and sequentially digested with 1 mg/mL pronase (30 min) and 1mg/mL collagenase type 2 (overnight) at 37°C with slow agitation. The digested cartilage was then filtered through a 70 µm mesh, and any pieces larger than the mesh were resuspended in fresh 1 mg/mL collagenase type II and digested for an additional night. Isolated primary chondrocytes were washed in growth media consisting of DMEM/F-12, 10% fetal bovine serum, 50µg/mL L-ascorbic acid 2-phosphate, and penicillin-streptomycin-amphoteracin B (100 U/mL, 100 U/mL, and .25 µg/mL). Chondrocytes were frozen in 80% media, 10% FBS, 10% DMSO until microcarrier seeding except for a small portion from each donor that were seeded immediately on TCPS for endotoxin testing with the HEK-Blue LPS Detection Kit. Any vials from donors that tested positive for endotoxin were immediately disposed of (1 donor/4 donors total for a final n=3). Monolayer cultures on tissue culture treated polystyrene (TCPS) were used as a control for comparison with microcarrier culture and cells were seeded at $1x10^4$ cells/cm^2 in growth media and media changes were performed every 2-3 days. At day 7, one TCPS chondrocyte plate was passaged to result in TCPS P1 and P2 groups on day 14.

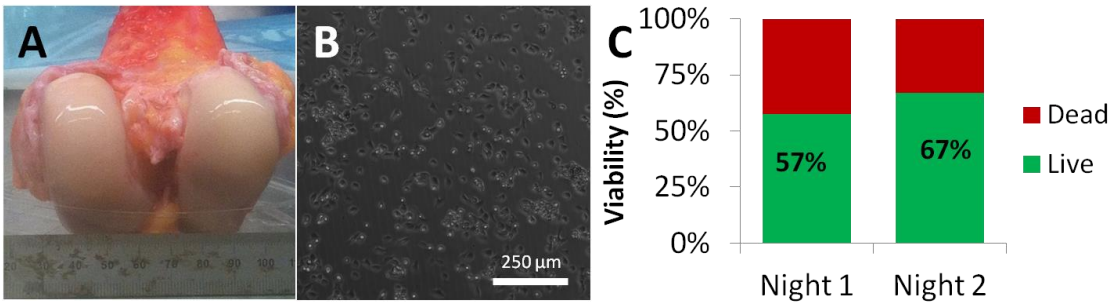

Figure 1. Primary Human Chondrocyte Isolation. Human chondrocytes isolated from the femurs (A) of diabetic patients were isolated (B) after digest in pronase and collagenase over 2 nights (B).

Chondrocyte expansion on microcarriers

The decellularized cartilage (180-250 micron sieve) and Cultispher®-G microcarriers were rehydrated in chondrocyte growth media for 30 minutes before cell loading. Primary human chondrocytes pooled from 3 donors were then seeded at 1x104 cells/cm2, mixed with gentle pipetting with a wide-bore pipette, and then split in 12-well Nunclon Sphera suspension plates. The monolayer and suspension microcarrier culture plates were placed in the same incubator at 37°C and 5% CO2. The microcarrier cell suspension was incubated under static conditions at 250,000 cells/mL overnight, after which time the microcarriers were again mixed with gentle pipetting, allowed to settle, and then media was taken off to assess seeding efficiency. The microcarriers were supplemented with 1 mL/well of chondrocyte growth media and placed on a rotary orbital shaker at 80 RPM for the duration of the experiment. Media changes were performed every 2-3 days and samples were collected at days 1, 7, and 14 in order to assess viability, proliferation, and ECM deposition. Histological samples were fixed in 10% neutral buffered formalin, manually processed for histology, and embedded in paraffin. Samples were then sectioned (5μm) for routine histology with hematoxylin and eosin (H&E) and safranin O/fast green (Saf O) staining. Samples collected for GAG deposition were washed in DPBS and then collected in lysis buffer (Quiagen), and frozen. At the conclusion of the experiment samples were digested with 20 μL Proteinase K overnight at 60°C for GAG quantification with a dimethyl-methylene blue (DMMB) assay and DNA quantification with PicoGreen® .

Statistical Analysis

Quantitative data was compared using GraphPad® Prism 5.0 for statistical significance using analysis of variance (ANOVA) with multiple comparisons and Bonferroni's correction (α=0.05) wherever appropriate. Otherwise Student's t-tests were performed with α=0.05 also using GraphPad® Prism 5.0.

RESULTS

Cartilage Decellularization

Histological comparisons of multiple decellularization protocols revealed only complete nuclei removal with a multi-step multi-wash protocol and 48 hours in deionized water, 1% SDS, or 1% Triton X-100 all yielded insufficient cellular removal (Figure 2). Although the Badylak decellularization did remove nuclei, Safranin-O staining also revealed GAG depletion after this process (Figure 3).Analysis of GAG retention after each decellularization step revealed GAG removal during the initial trypsin wash (Figure 4A) and shorter durations in trypsin still resulted in GAG depletion (Figure 4B). Removing trypsin wash completely from the protocol resulted in incomplete cell removal with visible nuclei present after the final wash (Figure 4C).

Efforts to redeposit glycosaminoglycans on decellularized cartilage microcarrier surfaces through charge interactions via overnight incubation were largely unsuccessful. There was a large amount of variability in the heparin sulfate incubated microcarriers with some positive staining in all groups and less variability in the microcarrier group with the highest concentration (100% weight matched) of heparin sulfate. There was no positive staining in the chondroitin sulfate incubated samples (Figure 5).

Because the protease wash of the protocol was responsible for both effective decellularization and glycosaminoglycan depletion in the cartilage sample, the final decellularization protocol was modified to include a TrypLE wash and DNase/RNase wash (Table 2). Effective decellularization was evidenced by the lack of visible nuclei in both H&E and Hoechst staining (Figure 6 A&B) and the absence of DNA band on gel electrophoresis (Figure 6 C). DNA quantification confirmed that the decellularized cartilage microcarriers contained less than 2% of original DNA content of the native cartilage (Figure 6 D). Decellularization also removed the majority of glycosaminoglycans, as evidenced by lack of safranin-O staining in comparison to native cartilage (Figure 6 E).

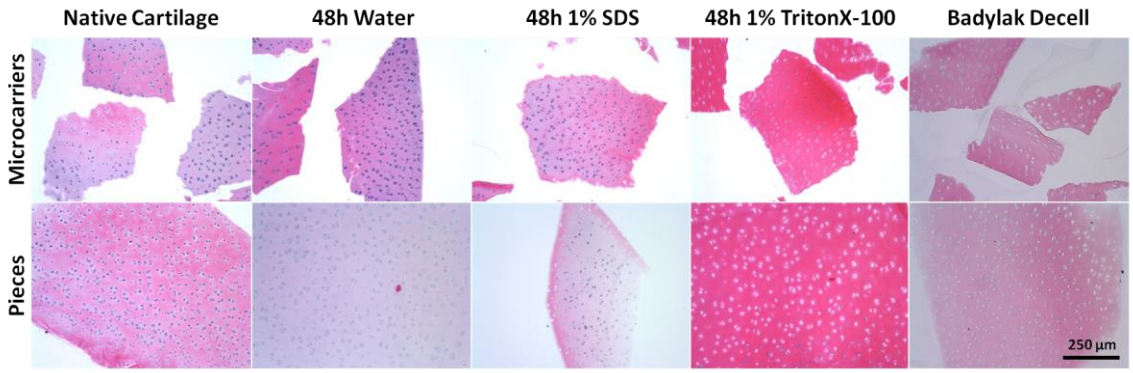

Figure 2. Histological comparison of various decellularization protocols on porcine articular cartilage. Hematoxylin and eosin (H&E) staining was performed on porcine cartilage pieces and microcarriers undergoing different decellularization protocols. Nuclei were stained dark brown and positive nuclei staining was visible following 48 hour washes in water, 1% SDS, or 1% TritonX-100 while no nuclei were present in microcarriers or pieces after the more complex Badylak decellularization protocol.

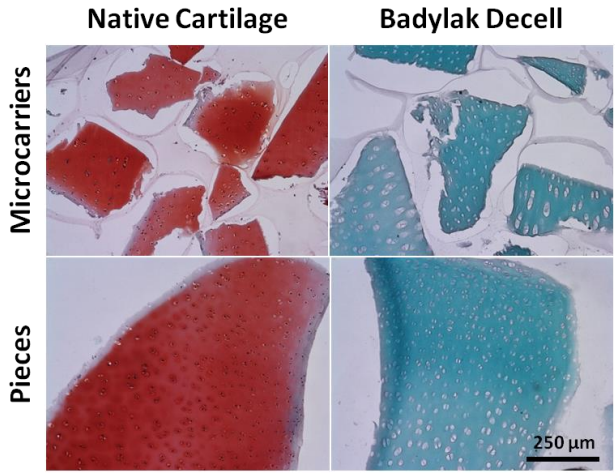

Figure 3. GAG depletion in decellularized porcine articular cartilage pieces. Porcine cartilage pieces and milled microcarriers were stained with Safranin-O before and after decellularization to assess glycosaminoglycan content.

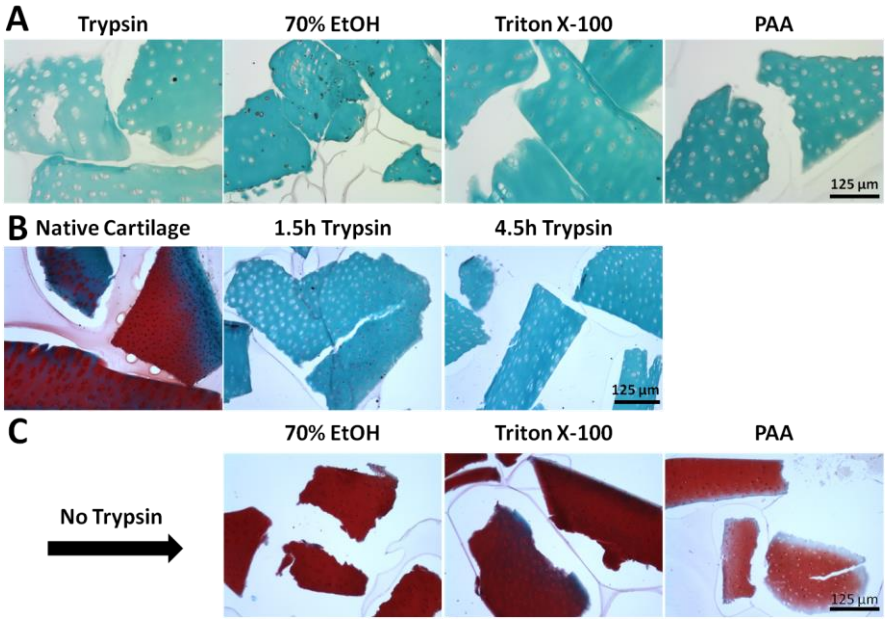

Figure 4. Comparison of glycosaminoglycan retention with various decellularization procedures with Safranin-O staining. Samples taken at multiple time points during Badylak decellularization indicate glycosaminoglycan loss after first trypsin wash (A). Shorter trypsin washes still resulted in glycosaminoglycan depletion (B) and decellularization procedures omitting trypsin resulted in inefficient decellularization with visible nuclei after the final PAA wash although glycosaminoglycans were largely retained (C).

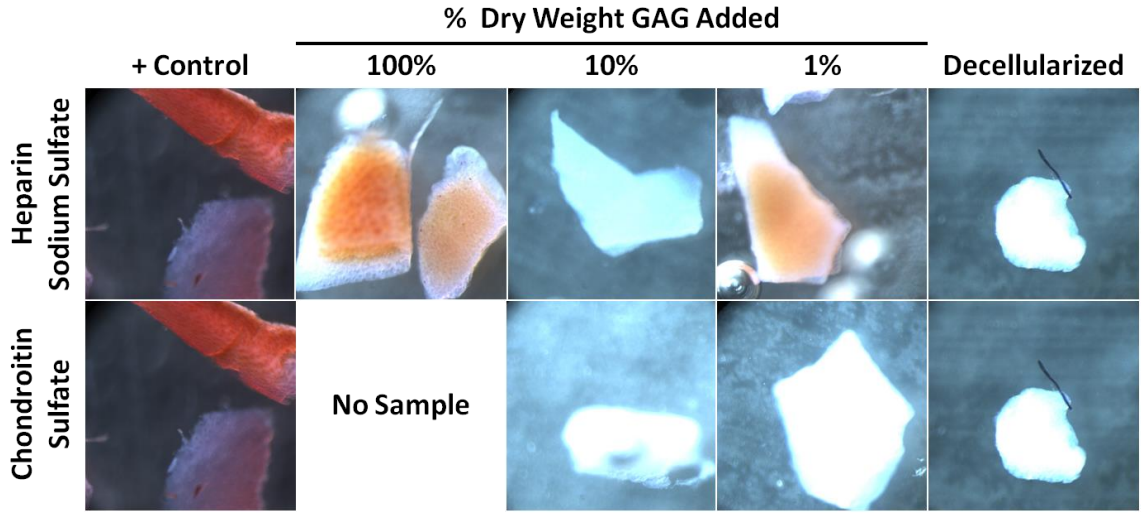

Figure 5. Assessing the deposition of glycosaminoglycans on cartilage microcarriers following decellularization. Safranin-O staining of decellularized cartilage samples incubated with heparin sulfate resulted in some positive staining for glycosaminoglycans with more positive staining at the highest concentration, but there was a large amount of sample variability in within all of the tested concentrations. Safranin-O staining on samples incubated with chondroitin sulfate resulted in no positive staining at any concentration.

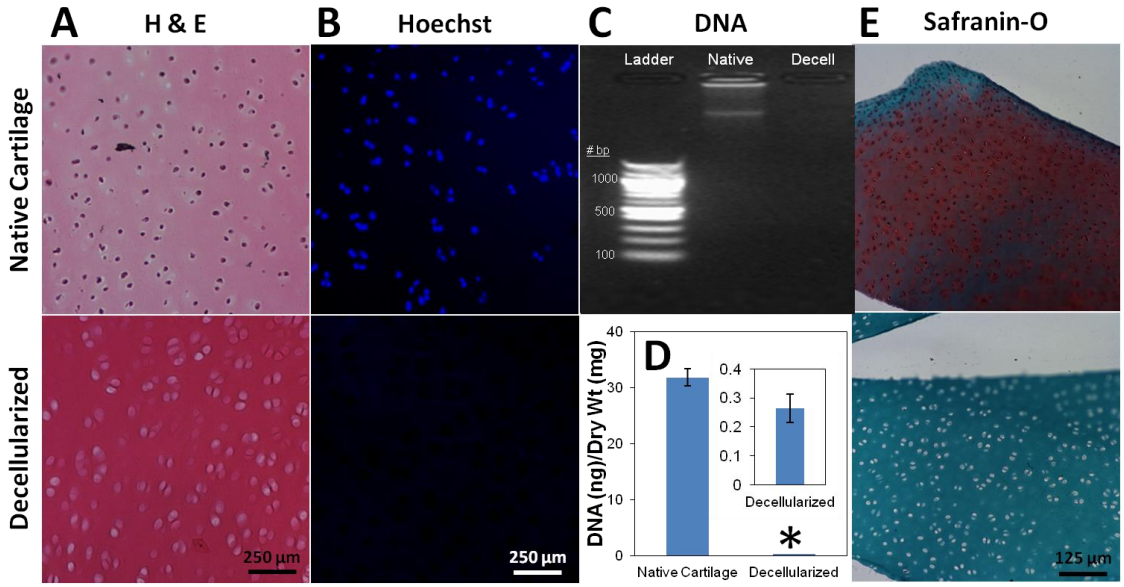

Figure 6. Characterization of decellularized cartilage microcarriers. Decellularization of porcine cartilage microcarriers was verified via lack of visible nuclei after hematoxylin & eosin (H&E) (A) & Hoechst staining (B), lack of visible DNA after gel electrophoresis (C), and 99% DNA removal quantified via PicoGreen® assay (D) (*t-test $p<0.05$ vs native cartilage). Safranin-O staining shows decellularization also removes glycosaminoglycans (E).

Microcarrier characterization

Microcarrier yield was determined by quantifying the initial mass of the cartilage pieces, the mass after decellularization, and the mass of the carriers of each size range after sieving (Figure 7). The majority of microcarrier loss occurred during decellularization, though sample was lost during milling and sieving steps as well (Figure 7A). Size analysis was performed on DC and CG microcarriers using microscopy and image analysis (Figure 7 B-D). Hydrated CG carriers had a diameter around 250 µm while the 180-250 sieved DC microcarriers were around 400 µm as determined by confocal microscopy and image analysis with Image J (Figure 7 B). The CG carriers were more spherical with an aspect ratio around 1.2 while DC microcarrier aspect ratio was around 1.5 (Figure 7 C&D).

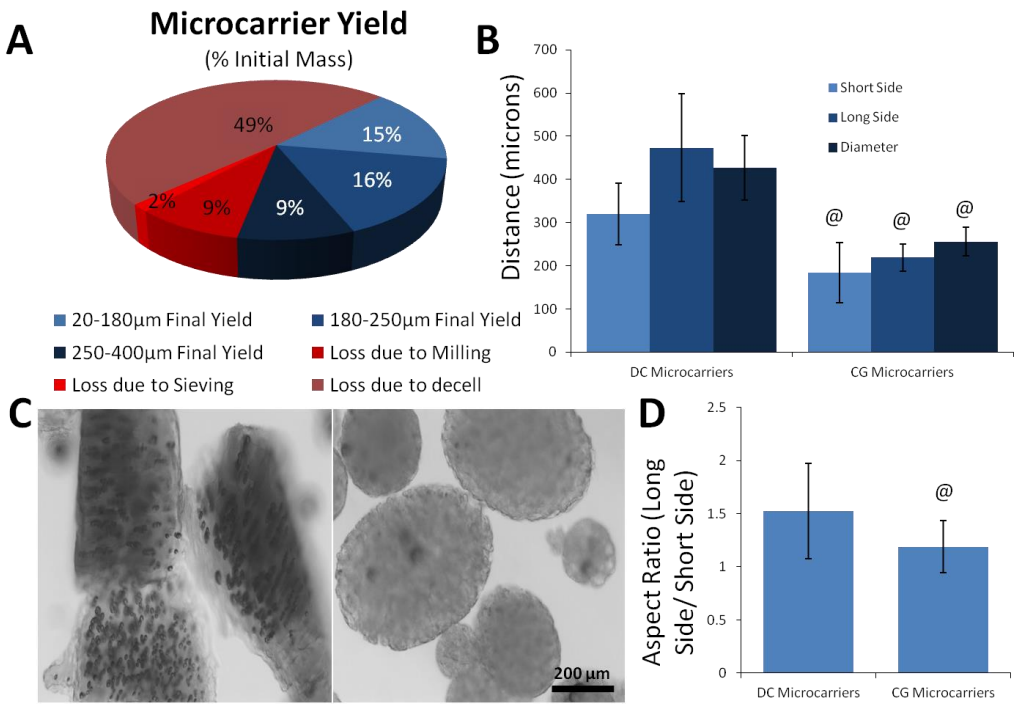

Figure 7. Microcarrier yield and size characterization. The final yield of the 180-250 and 250-400 micron sieved microcarriers was 15% and 16% of the initial weight of the cartilage pieces respectively and the majority of loss during processing was due to decellularization (A). The 180-250 micron sieved DC µCs were larger in comparison to commercially available CG µCs with hydrated diameters around 400 and 250 microns respectively based on ImageJ® analysis of the longest side, shortest side, and average diameters across µCs (n=105 and 97, respectively) @$p<0.05$ vs DC µCs ANOVA with Bonferroni correction (B). DC µCs and CG µCs both varied in size with some clumping even in the absence of cells (C). The CG µCs were more round than the DC µCs, resulting in an aspect ratio (distance of long side/short side) closer to 1 @ $p<0.05$ vs DC microcarriers for t-test (D).

ATDC5 Expansion on Microcarriers

In order to compare cell seeding protocols and loading efficiency on microcarriers, ATDC5 cells, a chondrogenic mouse teratocarcinoma cell line, were used for initial seeding studies. The size analyses were used to approximate microcarrier diameter and subsequent surface area in order to seed the DC and CG microcarriers at $5x10^4$ or $5x10^5$ cells/cm^2. The ATDC5 cells proliferated over the 7-day culture period and maintained a high viability (Figure 8 A&B). As expected, the cells did not infiltrate the microcarriers over this period and proliferated primarily on the surface/edges of the decellularized microcarriers as seen by positive nuclei staining with hematoxylin and eosin (Figure 8 C). Overall, there was little difference in the

seeding efficiency, viability, and expansion of ATDC5 cells on the microcarriers of different sizes, but most commonly used commercially available microcarriers are similar in size to the smaller microcarriers, thus only the 180-250 µm sieved microcarriers were chosen for further analysis with human chondrocyte culture.

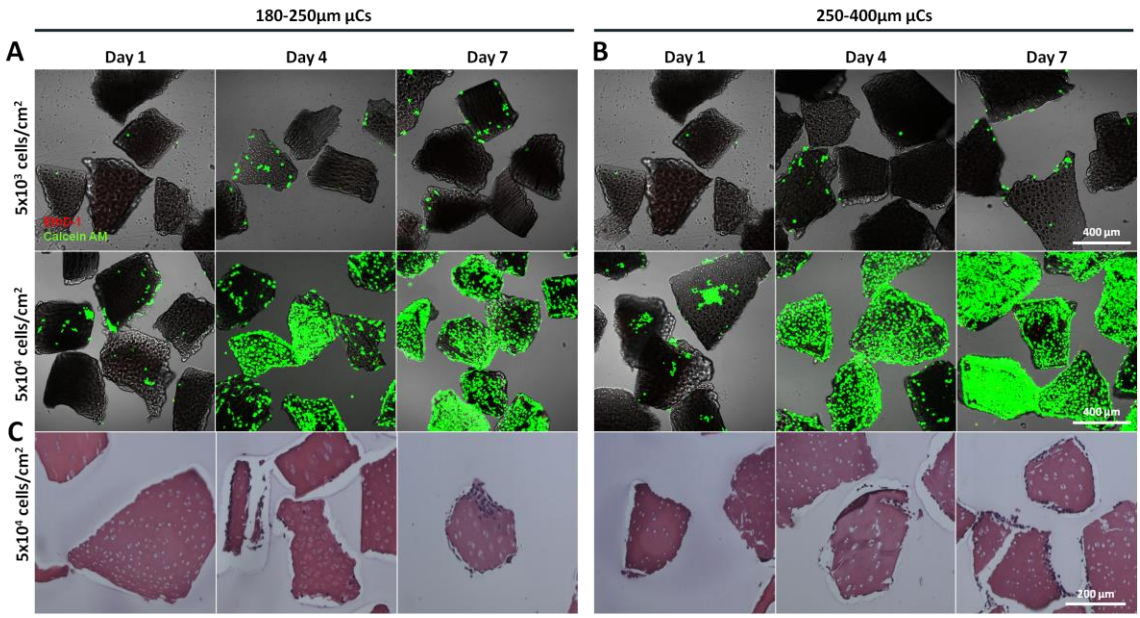

Figure 8. Chondrogenic ATDC5 cells attach to and proliferate on decellularized cartilage microcarriers and maintain a high viability over 7 days of culture. The chondrogenic mouse teratocarcinoma ATDC5 cell line maintained a high viability and proliferated up to 7 days on both 180- 250 micron sieved µCs (A) and 250-400 micron sieved µCs (B). The cells proliferated on the surfaces/edges of the decellularized microcarriers, as seen by positive nuclei staining with hematoxylin along the borders of the sectioned µCs for both sizes (C).

Primary Human Chondrocyte Expansion on Microcarriers

The primary chondrocytes proliferated on all surfaces (DC µCs, CG µCs, and TCPS) over the 14-day culture period and maintained a high viability as represented by live/dead confocal imaging (Figure 9). PicoGreen® DNA analysis indicated better attachment to the DC microcarriers than the CG microcarriers or TCPS based on Day 1 DNA content (Figure 10 A). The cells proliferated on all three of the surfaces with significant increases on day 7 and day 14 from the previous time points (Figure 10 A). The Day 14 P2 samples that were passaged at day 7 were very similar in DNA content to the Day 7 P1 samples, indicating that the chondrocytes maintain their proliferative capacity in the second passage (Figure 10 A). Decellularized

cartilage and Cultispher®-G microcarriers both had a significantly higher amount of DNA than tissue culture polystyrene expanded groups on days 7 and 14 (Figure 10 A). Chondrocytes cultured on tissue culture polystyrene without passaging had significantly higher DNA content on day 14 than chondrocytes on their second passage (Figure 10 A). Dimethyl-methylene blue (DMMB) assay showed glycosaminoglycan content had no significant differences across time points for chondrocyte laden decellularized cartilage microcarriers other than in comparison to unloaded carriers (Figure 10 B). CG microcarriers and TCPS chondrocytes had significantly less GAG content than DC microcarriers at all timepoints or as unloaded microcarriers (Figure 11 B). GAG content increased on day 14 for both chondrocytes cultured on CG microcarriers and TCPS in comparison to days 1 and 7 (Figure 10 B). Day 14 passage 2 cells had less GAG content than day 14 passage 1 samples (Figure 10 B). The DC microcarrier cultured chondrocytes were the only group that had a significant increase in GAG content after just 1 day in comparison to the unloaded control DC microcarriers. When GAG content was normalized to DNA, the DC microcarrier group had significantly more GAG/DNA in comparison to native cartilage on day 1, but that ratio decreased at each time point as the number of cells increased with levels most similar to native cartilage occurring after 7 days of culture (Figure 10 C). The GAG/DNA content was higher in DC microcarrier groups at all time points than the CG microcarrier or TCPS groups (Figure 10 C). There was no significant difference in normalized GAG content over any time point for the CG microcarrier and TCPS groups (Figure 10 C).

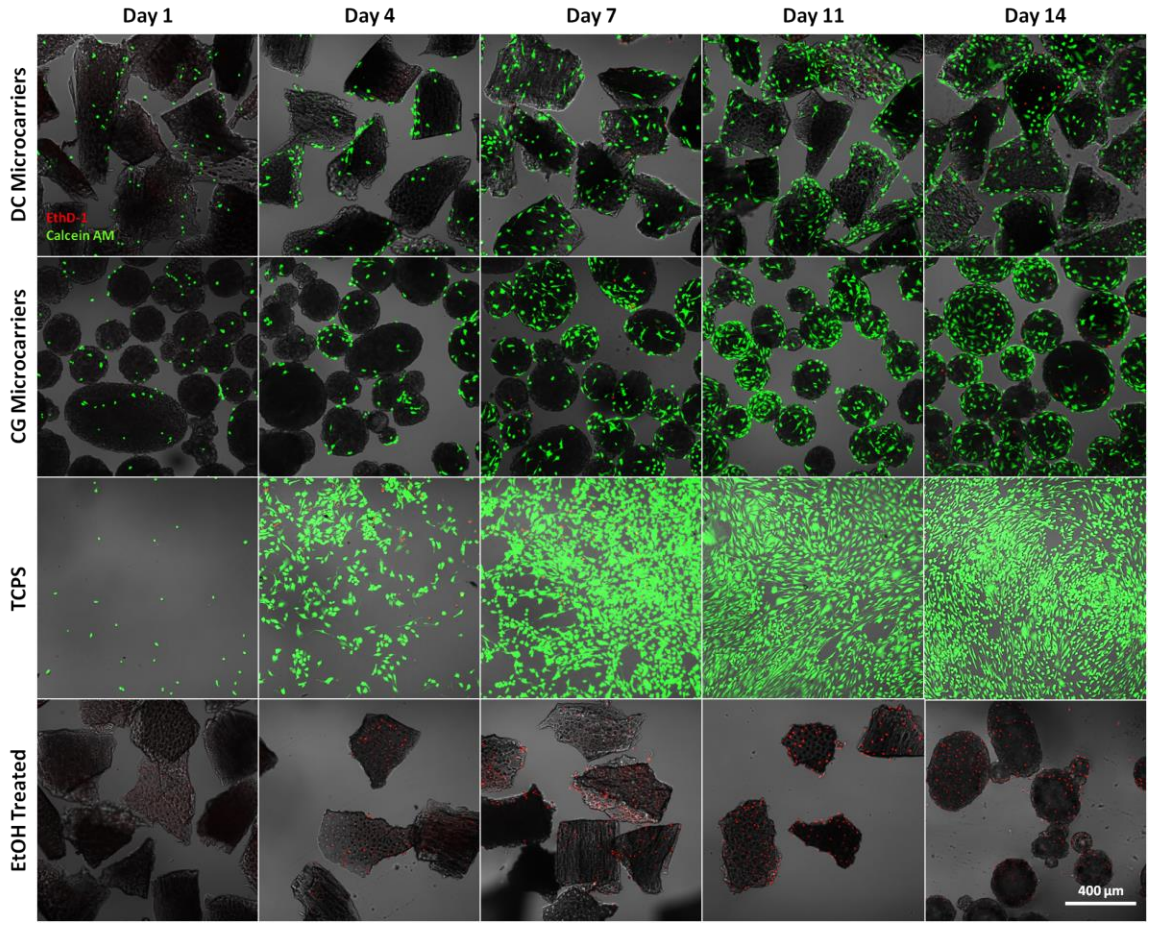

Figure 9. Primary human chondrocytes attach to and proliferate on ECM microcarriers over 14 days while maintaining high viability. Primary human chondocytes seeded on decellularized cartilage microcarriers (DC Microcarriers), Cultispher®-G Microcarriers (CG Microcarriers), and tissue culture polystyrene (TCPS) at $5x10^4$ cells/cm^2 maintained a high viability and proliferated up to 14 days as evidenced by confocal images after Live/Dead staining. Ethanol treated controls served as negative controls for each time point.

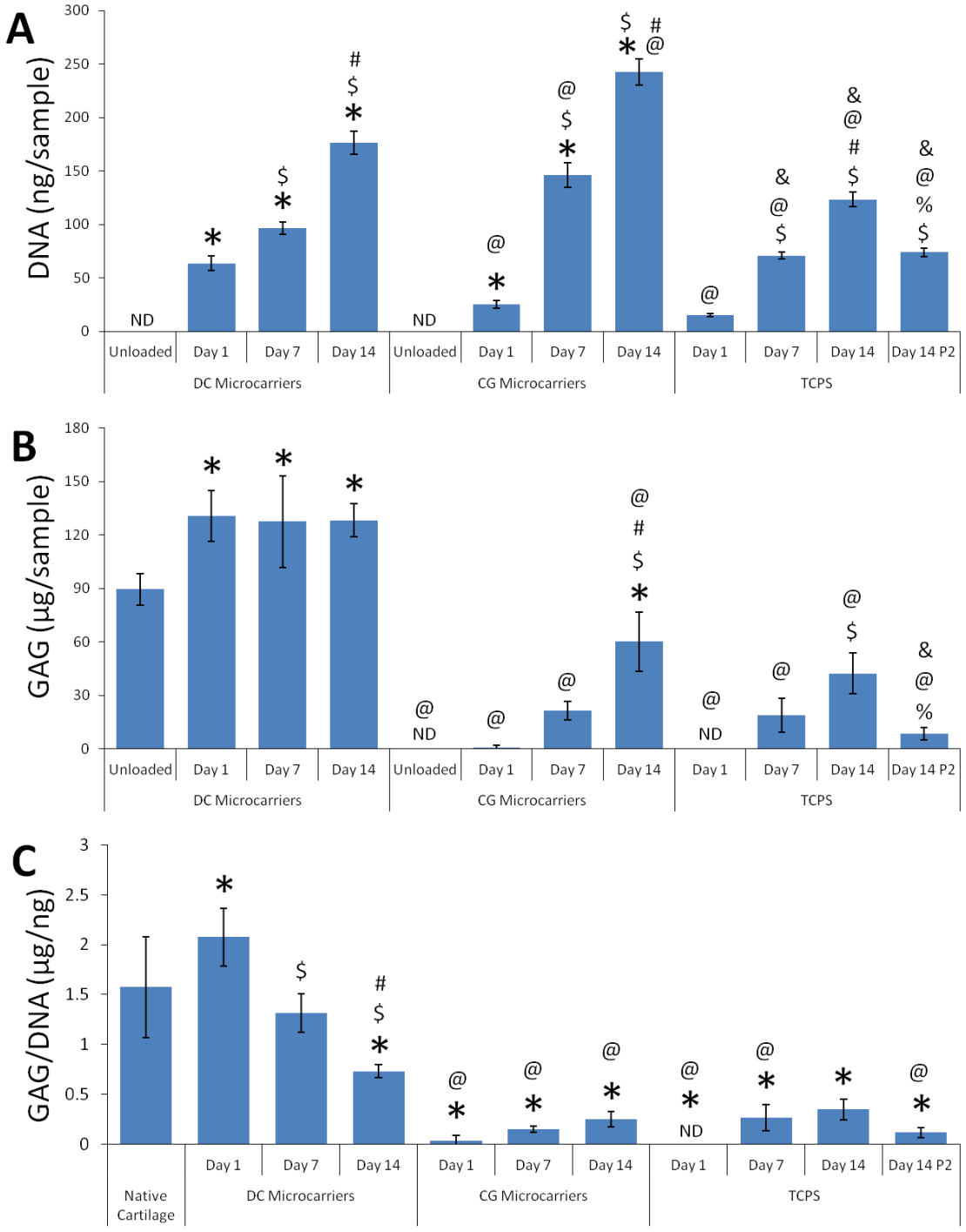

Figure 10. Primary human chondrocytes proliferate on ECM microcarriers faster than TCPS and DC µC culture maintains higher GAG/DNA content than CG µCs or TCPS. DNA was quantified with PicoGreen® Assay for unloaded and chondrocyte-loaded microcarriers and TCPS cultured chondrocytes over 14 days (A). GAG content was quantified with dimethyl-methylene blue assay (B) and normalized to DNA and compared to native cartilage (C). $p<0.05$ ANOVA with multiple comparisons * vs unloaded microcarriers from same group (A & B) or native cartilage (C), $ vs Day 1 same group, # vs day 7 same group, % vs day 14 P1 same group, @ vs DC same time point, & vs CG same time point.

DISCUSSION

This study aimed to develop a novel cartilage ECM-derived microcarrier platform to promote the retention of the chondrogenic phenotype and reduce chondrocyte dedifferentiation during *in vitro* expansion. Previous studies have separately focused on the expansion of chondrocytes on microcarriers or decellularized tissue, but both have had drawbacks. Current microcarrier platforms do not provide the rich environment of bioactive factors the native cartilage ECM is composed of, and decellularized cartilage sheets failed when implanted *in vivo* due to their limited integration with chondral defects and low cellularity [14, 27, 29, 30]. The aim of this study was to combine microcarrier expansion and decellularized cartilage tissue to create a novel microcarrier platform that would promote chondrogenic expansion and act as a biocompatible delivery vessel as a solution to the current drawbacks of cell-based therapies used to treat chondral defects.

Decellularization of porcine articular cartilage was achieved through modification of a previous protocol [33]. The successful removal of cellular contents was associated with the reduction of glycosaminoglycans, molecules which add to the osmotic properties and therefore mechanical load distribution of cartilage ECM [1]. Attempts to redeposit glycosaminoglycans on the surface of decellularized microcarriers through simple incubations were unsuccessful. While decellularization removed most of the native GAGs, the DC microcarriers retained some GAGs and had similar proportions of GAG to DNA as native articular cartilage after 7 days of chondrocyte culture, which was significantly better than expansion on CG microcarriers or tissue culture polystyrene. Interestingly, the chondrocytes were unable to produce enough GAG to retain the same GAG/DNA ratio over 14 days. One hypothesis for this is that secreted GAGs could have been released into the media rather than becoming incorporated into the decellularized ECM. GAG deposition in media has previously been quantified in plated primary human chondrocytes over-expressing SOX9 and IGF-I in conditioned media rather than cell sample digests, suggesting that chondrocyte synthesis of GAGs might be present in conditioned media in addition to cell scaffolds [34]. Conditioned media collected from our samples will be

used for a DMMB analysis and total GAG (secreted and cell/ECM-bound) will be compared between each group in addition to the cell/ECM bound digests we have already analyzed.

The underlying collagen network provides a structure for GAG incorporation and attachment, and therefore further analysis of the collagen structure may lend an explanation to this lack of increase in GAG deposition [35]. While the relatively high GAG/DNA ratio of DC microcarrier cultured chondrocytes is encouraging, the ratio of collagen type II to collagen type I is the most common method of analyzing dedifferentiation [34]. Ongoing studies will analyze the col II/col I ratio by quantifying RNA of the cultured chondrocytes with Fluidigm. Samples were already collected and other chondrogenic (Sox9, aggrecan, cartilage oligomeric matrix protein), and hypertrophic (collagen type X) markers will be assessed and normalized to housekeeping genes (GAPDH and RPS-18) [36]. Immunohistochemical staining of Collagens I, II, and X will also be used to validate these findings.

CONCLUSION

Cell based therapies used to treat osteoarthritis are currently handicapped by the inability to expand chondrocytes *ex vivo* without loss of phenotype and resulting dedifferentiation. A cell culture platform that will successfully stimulate proliferation and chondrogenesis over lengthy expansions *ex vivo* is required in order to allow for the advancement of cell based therapies aimed at treating degenerative joint diseases such as osteoarthritis. By utilizing decellularized articular cartilage tissue, this study has better captured the biological microenvironment chondrocytes are exposed to in native cartilage ECM, enhancing the potential for retention of a chondrogenic phenotype. The utilization of a microcarrier platform may better allow for not only cellular expansion, but also improved tissue integration and more rapid regeneration with the use of this combinatorial delivery chondrocyte and ECM to cartilage defects *in vivo*. In order to confirm the hypothesis that decellularized porcine articular cartilage microcarriers will support expansion while enhancing the retention of a chondrogenic phenotype and ECM synthesis in

comparison to gelatin microcarriers and standard monolayer culture on tissue culture polystyrene, the Col II/Col I ratio must be quantified and analyzed for all aforementioned groups.

In conclusion, this study has shown the potential for a novel decellularized cartilage derived substrate that could serve as both a cell expansion platform, as well as a cell delivery method in future cell based therapies for degenerative joint diseases.

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