Development of an EPIC-μCT imaging method for analyzing proteoglycan content in neocartilage constructs for the use in osteoarthritis therapeutics

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Abstract

Osteoarthritis (OA), characterized by debilitating joint pain, is a degenerative disease caused by the breakdown of cartilage in joints and adjacent bone. It is the most common type of arthritis, affecting 10-12% of the US adult population, and currently has no cure (Lawrence, et al.). Cartilage contains specialized molecules called proteoglycans (PGs). PGs are vital suppliers of swelling pressure that enable cartilage to withstand compressional forces applied by adjacent bones. A decrease in cartilage PG content can signal the subsequent onset of osteoarthritis. Current methods of OA therapy evaluation are destructive, however due to the degenerative nature of OA the longitudinal evaluation of therapies is crucial. EPIC- μCT (equilibrium partitioning of ionic contrasting agent – microcomputed tomography), is a volumetric, non-destructive imaging method. This method utilizes a contrast agent (Hexabrix 320 30%) that yields an equilibrium distribution that is inversely proportional to the density of PGs (Palmer, et al.) allowing for the quantification of PGs in neocartilage constructs. An EPIC- μCT method for analyzing proteoglycan content in neocartilage constructs would allow for the longitudinal evaluation of novel osteoarthritis therapies.

To develop this model, neocartilage constructs were created from bovine chondrocytes and cultured to 17 days in serum-free media. PG content was then assessed at 6, 12, and 17 days using the EPIC-uCT method. Results have shown that constructs were not affected by longitudinal scanning and reached equilibration in Hexabrix after 30 minutes of incubation. A negative correlation was also found to exist between neocartilage PG content and x-ray attenuation. Current research is being conducted to quantify the longitudinal degradation of PG content.

Introduction

Osteoarthritis (OA), characterized by debilitating joint pain, is a degenerative disease that is caused by the breakdown of cartilage in joints and adjacent bone. It is the most common type of arthritis, affecting 9.6% of men and 18% of women over 60 (Palazaro et al, 2014). Additionally, the total number of people afflicted with OA is projected to increase due to the aging population and the rise in obesity rates (Zhang et al, 2011). Although this disease affects this portion of the population and has been shown to be associated with increased mortality, there is currently no cure and only pain management treatments are available (Nuesch et al, 2011).

Proteoglycans (PGs) are well suited for diagnosing the onset of OA in cartilage and for evaluating cartilage repair by novel OA therapies (Palmer et al, 2006). PGs provide swelling pressure that enables the cartilage and therefore the joint to withstand compressional forces applied by the adjacent bones. A decrease in sulphated glycosaminoglycans (sGAGs), a negatively charged molecule linked to the PG backbone (Plaas, 1998), can denote a decrease in cartilage PG content from the extracellular matrix (ECM). Changes in the ECM composition have also shown to alter the biomechanical properties of the ECM (Setton, 1993) (Williamson, 2001), which can indicate the vitality of the articular cartilage and the progression of OA.

The ability to accurately quantify the morphologic changes in small animals like rats is crucial to the development and evaluation of new therapies for OA (Xie, 2009). Morphologic changes in cartilage ECM can be detected with histology or protein assays, but these methods are destructive. Nondestructive techniques like magnetic resonance imaging (MRI) and ultrasonography are adequate for measuring changes in humans, but do not provide adequate resolution for small animal models (Watrin et al, 2001) (Watrin-Pinzano, 2005). Optical coherence tomography (OCT) provides the needed resolution for rat cartilage but only produces two dimensional (2D) images (Jurvelin et al, 1995). EPIC- μCT (equilibrium partitioning of ionic contrasting agent – microcomputed tomography), is a volumetric, non-destructive method that allows for the longitudinal analysis of sGAG content in cartilage (Palmer et al, 2006). Because of the low radiopacity of soft tissues, μCT had previously not been used to analyze cartilage. However, EPIC- μCT uses a negatively charged contrast agent (Hexabrix 320 30%) that

yields an equilibrium distribution that is inversely proportional to the density of the negatively charged sGAGs (Palmer et al, 2006). EPIC- μCT is now used quantify and monitor changes in rat articular cartilage structure pre and post therapeutic exposure (Thote, 2013).

While small animal studies involving rats are the 'gold standard' of therapeutic evaluation, the use of in-vitro models prior provides preliminary results that can be assessed in less time and at a lesser cost. It is to this effect that a variety of in-vitro methods have been developed although with little regularity between researchers due to the need to tailor in-vitro models to reflect in-vivo disease (Johnson et al, 2016). While various methods of effective cartilage modeling have been used such as explants, neocartilage constructs created from functional articular chondrocytes of either bovine or human type, have been shown to display similar biomechanical properties of cartilage in-vivo (Huang, 2015) and are not limited by availability of explant harvests. The majority of in-vitro models can be categorized into models that use applied cytokines and models that use applied loads to induce OA symptoms. While applied load models are easily manipulated, they ultimately result in the release of inflammatory cytokines like interleukin (IL)-1β and tumor necrosis factor (TNF)-α after the integrin stimulated stress pathway is induced (Bader et al, 2011). Applied cytokine models skip to the end stage symptoms of OA ignoring potential early stage protective solutions. However, due to the OA-like biological changes that applied cytokines elicit, this model is an inexpensive and easily manipulated method for analyzing the temporal and concentration effects of novel therapies (Johnson et al, 2016). IL-1β and TNF-α are the mostly commonly used cytokines for inducing OA symptoms. Although other cytokines may play a role in inflammation caused by OA, they aren't as frequently used as they can be derived from IL-1β and TNF-α.

With the expense and significant investment it takes to implement rat models of OA, an accurate and efficient in-vitro method is needed that mimics the volumetric and non-destructive conditions of the EPIC- μCT method. An EPIC- μCT method for analyzing proteoglycan content in neocartilage constructs would allow for the longitudinal evaluation of novel osteoarthritis therapies. To demonstrate the potential of this approach to examine if neocartilage constructs can adequately capture the

longitudinal degradation of cartilage seen in OA patients, we first present data validating the ability of the constructs to produce PGs as they grow and to display a negative correlation between x-ray attenuation and sGAG content. Second, we present data illustrating the effects of exposing the neocartilage constructs to interleukins-beta (IL-1β), a known inflammatory cytokine, to stimulate the degradation of PGs in cartilage during the onset of OA. Lastly we will expose the constructs to a previously quantified therapeutic after exposure to IL-1β to then assess the efficacy of the method at detecting changes caused by treatments.

Methods and Materials

Media and Size

In phase I, bovine chondrocyte (Asarte Biologics LLC) pellets were created by centrifuging cells at 500G for 5 minutes. Pellets needed to be constructed and cultured in order to withstand the subsequent longitudinal scanning procedures. Pellets of 250k and 500k cells cultured for three weeks in media with and without fetal bovine serum (FBS) were compared **(Table 1)**. They were fed 3 times per week. Media with FBS contained Dulbecco's Modified Eagle Media (DMEM) without sodium pyruvate, FBS (10%), penicillin streptomycin (1%), nonessential amino acids (1%), gentamycin (0.1%), and L-ascorbic acid 2-phosphate (50 ng/mL). Media without FBS contained DMEM with sodium pyruvate, ITS+ (1%), nonessential amino acids (1%), gentamycin (0.1%), dexamethasone (100 mM), and L-ascorbic acid 2-phosphate (50 ng/mL).

	500k cells	250k cells
With Serum	4 pellets	4 pellets
Without Serum	4 pellets	4 pellets

Table 1

Equilibrium Time

To determine the average equilibration time needed for pellets in contrast agent (30% Hexabrix, PBS) six pellets were cultured for three weeks in serum free media and fed 3 times per week. Pellets were then incubated in contrast agent (30% Hexabrix, PBS) for additive time periods (15, 30, 45, 60, 90 minutes) and then scanned using a Scanco Medical uCT40.

Scanning Effects

Pellets were cultured for three weeks and fed 3 times per week. At each time point a set number of pellets were scanned and removed from culture to be frozen for assaying. A set number of pellets at each time point were fixed and saved for histology. 3 pellets were not scanned until the last time point in order to serve a control for the longitudinal effects of scanning.

Days	**Scan & Assay**	**Histology**	**Scan & Assay Only**
7	5	1	
14	5	1	
21	5	2	3

Table 2

Dimethylmethylene Blue Assay (DMMB)

Pellets saved for assaying were digested overnight at 60 degrees Celsius in 180 uL of proteinase K buffer (30 mM Tris Cl, 30 mM EDTA, 5% Tween 20, 0.5% triton x-100 and 800 mM GuHCl) and 20 uL of proteinase K. A DMMB assay was then conducted to determine the concentration of sulphated glycosaminoglycans (sGAG).

Results

Phase I

Average Equilibration Time of Neocartilage Constructs

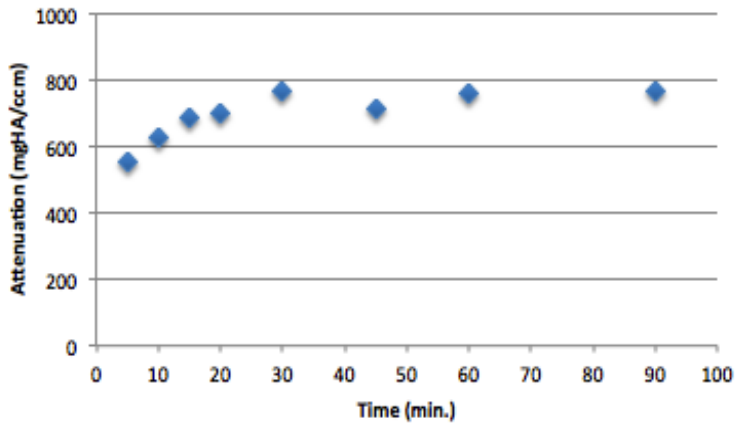

Figure 1. Average Equilibration Time of Neocartilage Constructs. After incubation in Hexabrix (30%) contrast agent, constructs made with 500,000 cells were found to have an average equilibration time of 30 minutes.

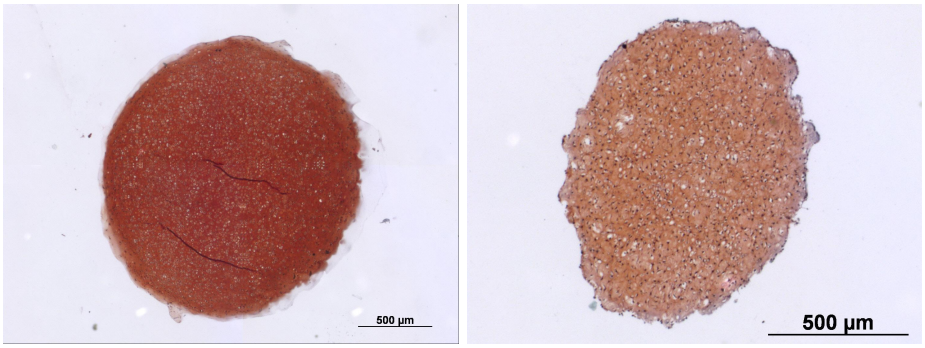

Figure 2. Phase I Histology. Saffarin-O histology was performed after 21 days of culturing on 500,000 cell constructs cultured in media without fetal bovine serum (FBS)(left) and with FBS (right) to determine ideal conditions for proteoglycan expression. Deep red indicates increased proteoglycan expression.

Phase II

Scanning Effect on sGAG Content

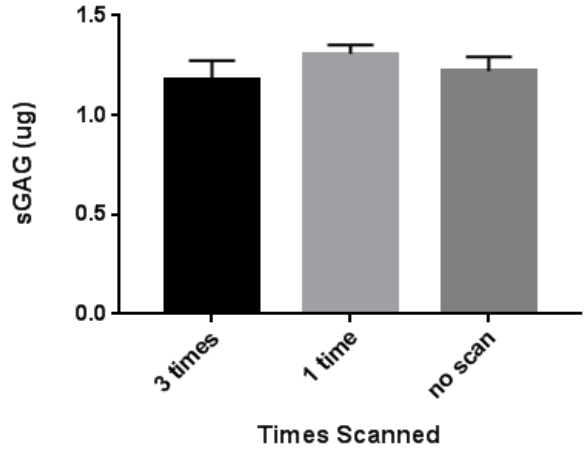

Figure 3. Scanning Effect on sGAG Content. A group of constructs was scanned at each time point for a total of three times. A second group was scanned at one time point, and a control group was not scanned during the culturing process. There was no significant difference between groups. N=4, one-way ANOVA used.

Longitudinal sGAG Content in Neocartilage Constructs

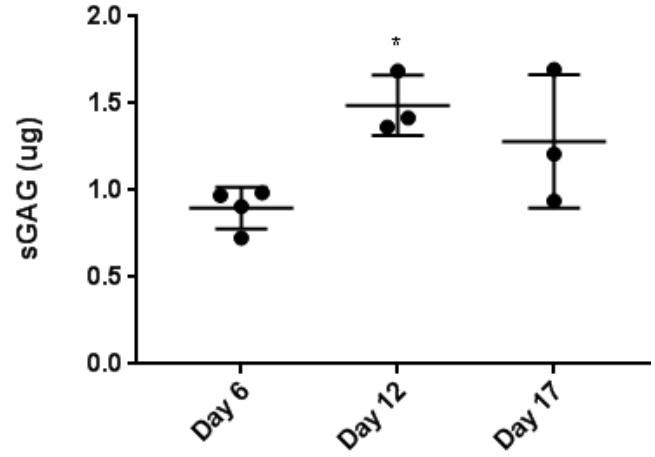

Figure 4. Longitudinal sGAG Content in Neocartilage Constructs. The results of a DMMB assay to quantify sGAG content in neocartilage constructs displays a significant difference between groups of pellets that were cultured to different time points. N=3,4, one-way ANOVA used. *statistically significant difference from 7-day treatment group.

Longitudinal uCT Attenuation in Neocartilage Constructs

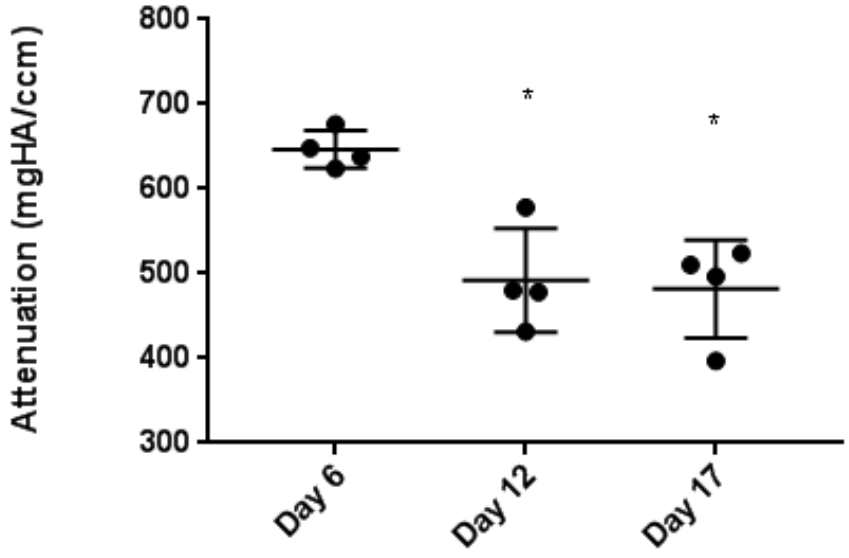

Figure 5. Longitudinal uCT Attenuation in Neocartilage Constructs. Neocartilage constructs that were cultured to and scanned at three different time points displayed a significant difference between 6 and 12 days and 6 and 17 days. N=3,4, one-way ANOVA used. *statistically significant difference from 7-day treatment group.

Neocartilage Construct sGAG Content vs. uCT Attenuation

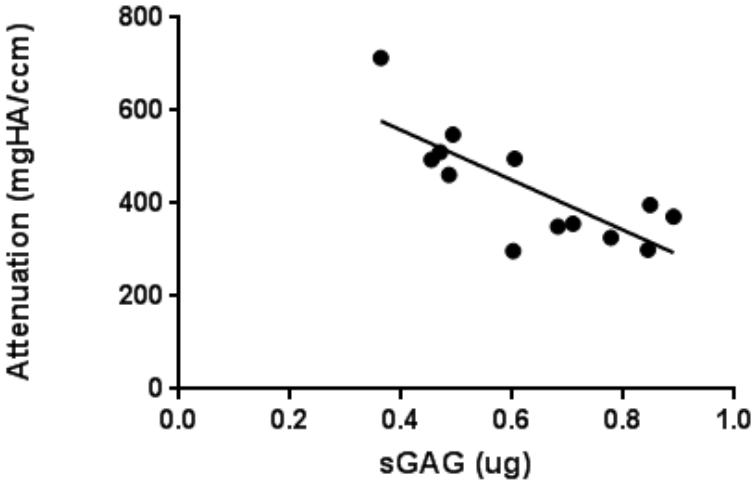

Figure 6. Neocartilage Construct sGAG Content vs. uCT Attenuation. The uCT attenuation of each construct scanned was plotted against its sGAG determined by a DMMB assay. $R^2 = 0.608$

Discussion

Phase I

There are two common types of culture media for articular bovine chondrocytes, one containing fetal bovine serum (FBS) and one that does not. FBS contains a complex array of growth factors and proteins intended to aid cell growth. However, it was found that this uncharacterized array of growth factors could have contributed to the suppression of proteoglycans in cells cultured in media with FBS. This is evidenced by the deep red of proteoglycan expression in pellets cultured in media without FBS as seen in Figure 2. As proteoglycans are essential for cartilage vitality, media without FBS was chosen for the remainder of experiments.

The average construct equilibration time was determined from the average attenuation coefficient for each construct. This component of phase I was necessary to fully saturate the pellet with contrast agent in order to ensure a reliable correlation between uCT attenuation and sGAG content. The average construct equilibration time was found to be 30 minutes, which seems long as previous experiments conducted using full rabbit tibias also used an equilibration time of 30 minutes (Palmer et al, 2006). However, despite the seemingly extended equilibration time, 30 minutes was chosen for the remainder of the experiments to ensure full saturation and over saturation is not a concern.

Phase II

After comparing the sGAG content of groups of constructs that were scanned at differing time points to a control group that was not scanned, no statistically significant difference was seen between groups. Similar results that show scanning does not have a significant effect on proteoglycan content has also been seen in in-vivo studies (Palmer et al, 2006).

The results of a DMMB assay to quantify sGAG content of neocartilage constructs showed an increasing trend, yet did not yield statistically significant results

between groups. This is problematic as it's assumed the volume of pellets, acquired by culturing overtime, should display a positive correlation to sGAG and therefore proteoglycan content.

When uCT attenuation results of neocartilage constructs were analyzed, a statistically significant difference was found between groups scanned at day 7 and 14, and 7 and 21. This suggests that pellets are growing, however based on aforementioned DMMB assay results the contents of the pellet growth cannot be determined. In order to determine the contents of the pellet growth, the DMMB assay should be refined and repeated. When uCT attenuation is plotted against the sGAG content of constructs, a weak negative correlation is observed. This could be improved by a more sensitive DMMB assay to capture a comprehensive quantification of construct sGAG content.

Conclusion

Quality neocartilage constructs that have the ability to withstand longitudinal scanning procedures can be cultured in serum-less media, while equilibration time occurs after 30 minutes of incubation in the EPIC-uCT contrast agent, Hexabrix (30%). In addition, scanning does not have a longitudinal effect on proteoglycan content in neocartilage constructs. Neocartilage constructs displayed a decreased attenuation over time, suggesting an increase in sGAG content. However, results from the DMMB assay do not support this hypothesis and show that there was no statistically significant difference in sGAG conent of pellets cultured to and scanned at increasing time points. Although a weak negative correlation does exist between sGAG content and attenuation experiments should be repeated to ensure that sGAG quantification methods are sensitive enough to pick up of nuanced differences between increasing time points.

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