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PRINCIPAL INVESTIGATOR: Robert E. Guldberg

RECIPIENT: GEORGIA TECH RESEARCH CORPORATION
Atlanta, GA 30318

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Abstract

There are currently no treatment methods on the market for modifying OA – only drugs that help relieve pain and inflammation but do not address the underlying disease, allowing it to continue destroying joint tissues. When joint function becomes severely impaired and other management strategies are ineffective, arthroscopic or joint replacement surgeries become the only options for patients. More than 50% of people worldwide who are over 65 years of age show radiological evidence of OA. These patients and younger populations who are susceptible to OA onset due to sports injuries or other factors could significantly benefit from the development of strategies to mitigate disease progression. Furthermore, post-traumatic OA experienced by military personnel injured on the battlefield is a highly accelerated process, likely because these combat injuries are complicated by factors that are associated with greater risk including bone loss, surrounding soft tissue damage, and infection. In these combat-related cases, post-traumatic OA typically manifests less than 2 years after injury compared to post-traumatic OA resulting from sports injuries that takes about 10 years to develop.

The goals of this work are to test the ability of a novel therapeutic to hinder the progression of post-traumatic osteoarthritis. This debilitating joint condition more severely affects military service personnel who have sustained injuries in combat resulting from high energy impacts such as explosions, fragment projectiles, and gunshot wounds. The therapeutic we propose is derived from human amniotic membrane, which has shown promise in clinical studies for various regenerative applications. The objectives of the work are to characterize two formulations of the injectable therapeutic, determine its capacity for treating OA in a small animal model, then scale up the approach to a clinically relevant loading and disease progression timeframe in a large animal model in order to outline a pathway to human clinical trials of the treatment method.

Our central hypotheses are that joint retention time and therapeutic efficacy will be influenced by amniotic membrane particle size, treatment timing, and frequency of delivery in well-established small and large animal models of post-traumatic OA. These hypotheses will be tested via three Specific Aims: *Aim 1:* Evaluate the effects of human amniotic membrane (AM) particle size distribution on particle retention and progression of OA after 3 weeks in the rat medial meniscal transection (MMT) model. *Aim 2:* Assess differences in therapeutic efficacy of single and multiple post-injury particle injection treatments on OA progression during a 6 week time period. *Aim 3:* Evaluate therapeutic effects in an established ovine unilateral medial meniscectomy (MM) model of OA.

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1. INTRODUCTION:

Osteoarthritis (OA) is a debilitating articular joint condition resulting in functional impairment for nearly 27 million Americans at a cost of over \$128 billion per year for the U.S. economy. Post-traumatic OA (PTOA) associated with combat extremity injuries has a 26% greater incidence in active military service personnel 20-24 years of age and over twice the incidence after age 40 compared to the general population. There are no disease modifying OA therapeutics currently approved. The goal of this work is to develop a safe and effective long-term strategy, using intra-articular delivery of micronized dehydrated amnion/chorion membrane (dHACM), to inhibit OA disease progression following trauma. Factors we hypothesized would impact therapeutic efficacy of dHACM included particle size, timing of treatment, and frequency of delivery. Thus, the study aims involved varying and more fully characterizing particle size distribution, injecting at different time points, and utilizing single or multiple injections, in both the rat medial meniscus transection (MMT) model and the sheep medial meniscectomy (MM) model.

2. KEYWORDS:

Osteoarthritis therapeutic	Intra-articular injection	Amnion/chorion membrane	Rat medial meniscal transection	Sheep medial meniscectomy
Particle size distribution	therapeutic timepoints	EPIC- μ CT	Articular cartilage	Subchondral bone
Osteophytes	Proteoglycans			

3. OVERALL PROJECT SUMMARY:

In the first annual funding period (Sept 2014 – Sept 2015), significant progress was made in Specific Aims 1, 2, and 3; Major Tasks 1, 2, 3, and 4.

Specific Aim 1: Evaluate effects of micronized human amniotic membrane particle size distribution on particle retention and progression of OA after 3 weeks in the rat medial meniscal transection model

Major Task 1: Characterization of particle size distribution and retention.

Subtask 1: Characterize size distribution

- dHACM particles were embedded in histogel, cryosectioned, and stained with H&E. Protocols were developed to measure dimensions using light microscopy and image analysis (sample slide images and measurements shown in Figure 1).
- Size distribution histograms of the particles will be generated (ongoing work via H&E and SEM measurement techniques)

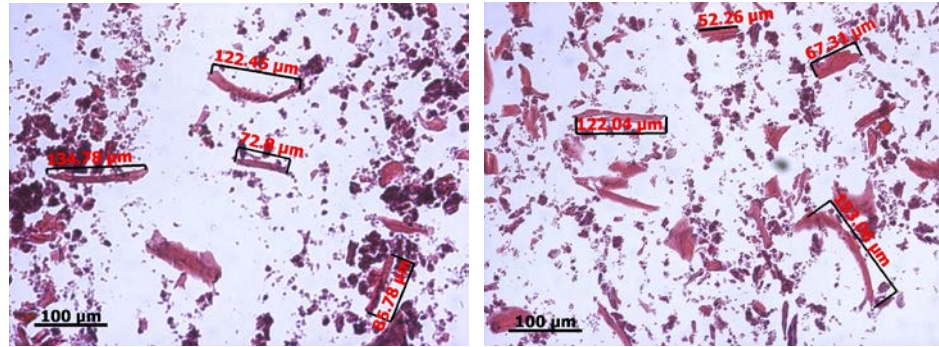


Figure 1. Histological sections stained with H&E depicting dHACM segments. Some of these segments were measured with calibrated visual tools, and dimensions were marked on the images.

Subtask 2: Submit documents for IACUC approval

- Georgia Tech IACUC protocol application was submitted, and approval for rat MMT model and intra-articular therapeutic delivery was received, 09-APR-2014.

Major Task 2: Assess 3 week therapeutic efficacy *in vivo* for two size distributions.

Subtask 1: Rat MMT surgical and treatment procedures for analyzing effectiveness of the two size distributions of dHACM after 3 weeks.

- Rat MMT surgeries were performed; animals received intra-articular injection of rehydrated dHACM (or saline) 24hrs after surgery.
- dHACM formulations were variations of the product AmnioFix® (MiMedx Group, Inc.) – standard formulation micronized dHACM = AF; super-micronized dHACM = SMAF.
- Animals were euthanized at 1 week and 3 weeks post-surgery, hindlimbs harvested and preserved for EPIC-μCT.

Subtask 3: Evaluation of therapeutic effects using EPIC-μCT and histology

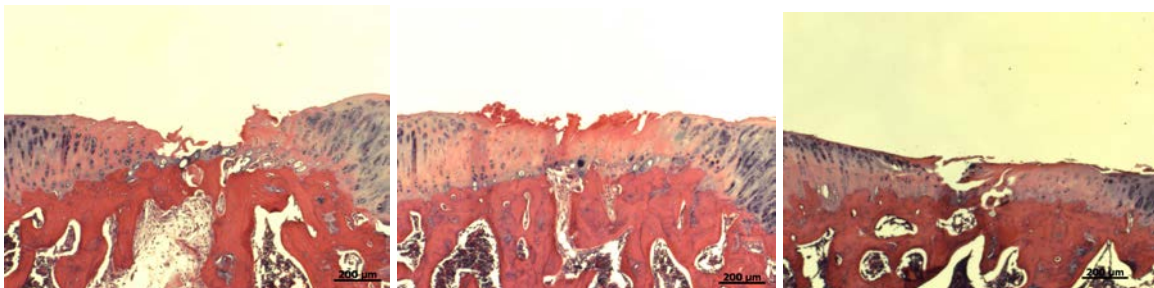


Figure 2. Paraffin-embedded histology sections of medial tibial articular surface stained with H&E. Saline injection (left) showing lesion and severe degradation, micronized dHACM injection (middle) showing fibrillated erosion of cartilage surface, super-micronized dHACM injection (right) showing lesion.

Quantitative microCT analyses were performed on medial tibial plateau regions separated into three volumes of interest (VOIs) – medial, central, and lateral thirds.

Articular cartilage analyses for average attenuation coefficient (with an inverse relationship to cartilage proteoglycan (PG) content), thickness, and surface roughness are shown in Figure 3. Lesion and marginal osteophyte occurrences were enumerated and are shown in Table 1.

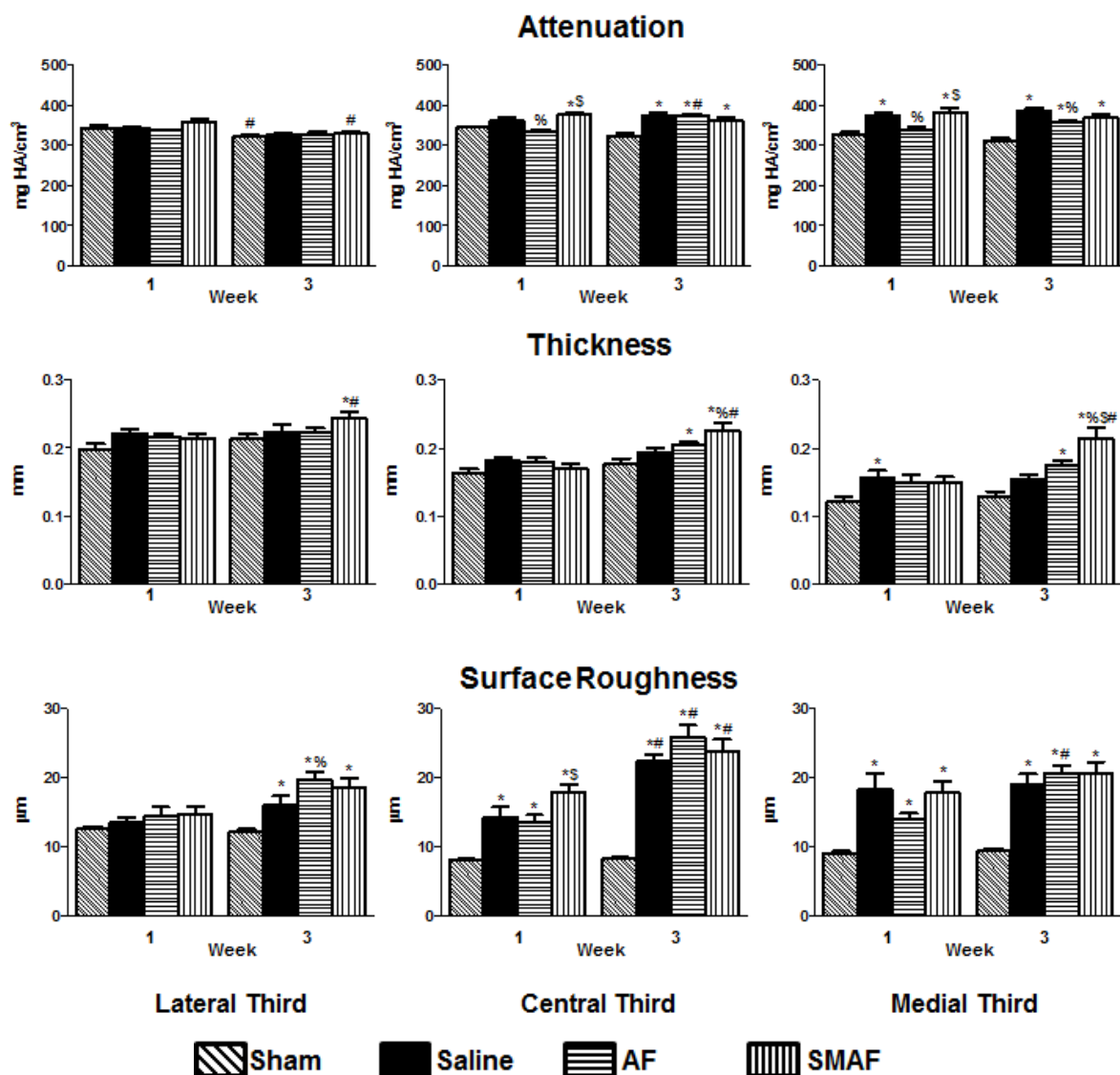


Figure 3. Quantification of medial tibial articular cartilage attenuation, thickness, and surface roughness using EPIC- μ CT. Cartilage attenuation increase due to MMT after 3 weeks in the medial third was reduced with AF but not with SMAF (high attenuation indicates low PG content). Higher surface roughness was detected in each VOI for each treatment type and at each endpoint. $n=7-8$, * $p<0.05$ vs sham at same endpoint, % $p<0.05$ vs saline-treated at same endpoint, # $p<0.05$ vs same treatment at Week 1, \$ $p<0.05$ vs AF-treated at same endpoint.

Table 1. Occurrence of lesions and osteophytes. Quantification of lesion volume, cartilage volume within osteophytes, and mineral volume within osteophytes have been computed for those specimens where lesions and osteophytes were detected, but no significant differences were found.

	Sham – One Week	Saline – One Week	AF – One Week	SMAF – One Week	Sham – Three Weeks	Saline – Three Weeks	AF – Three Weeks	SMAF – Three Weeks
Lesions	0/8	2/8	0/8	2/7	0/8	5/8	4/8	3/8
Osteophytes	0/8	2/8	3/8	2/7	0/8	8/8	8/8	8/8

Subchondral bone parameters of mineral density, porosity, and thickness were also quantified and are shown in Figure 4.

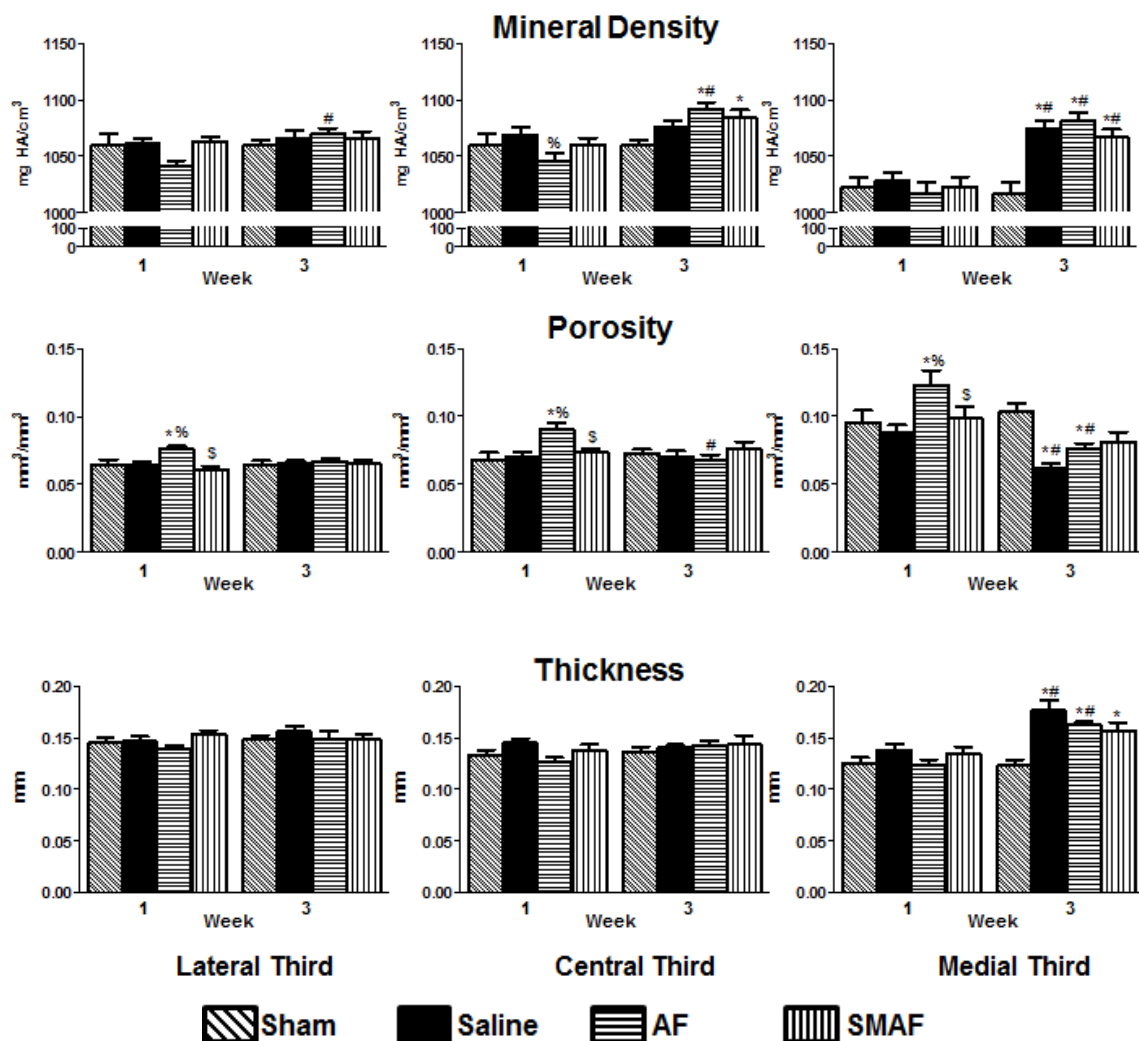


Figure 4. Quantification of subchondral bone mineral density, porosity, and thickness. Notably, these data demonstrated an increase in bone mineral density in the AF and SMAF groups at 3 weeks post-MMT and an increase in subchondral porosity in the AF group at 1 week post-MMT for all three VOIs analyzed. n=7-8, * p<0.05 vs sham at same endpoint,

% $p < 0.05$ vs saline-treated at same endpoint, # $p < 0.05$ vs same treatment at Week 1, \$ $p < 0.05$ vs AF-treated at same endpoint.

Specific Aim 1 Conclusions

Changes in articular cartilage and subchondral bone morphology and composition due to MMT surgery were able to be quantified 1 and 3 weeks after joint destabilization. Treatment with micronized dHACM (AF) demonstrated better preservation of cartilage proteoglycan (PG) content (inversely related to cartilage attenuation) at 1 and 3 weeks post-surgery. Significantly higher average cartilage thickness in the medial 1/3 VOI at 3 weeks post-surgery compared to AF-treated joints suggested greater degeneration after SMAF treatment. The majority of alterations to subchondral bone (density, thickness) were detected at 3 weeks (not at 1 week post-surgery) whereas cartilage attenuation and surface roughness differences were seen at only 1 week post-surgery. This suggests that in the rat MMT model, degenerative changes occur first in the articular cartilage, and subchondral bone changes follow.

A manuscript, including work summarized for Major Task 2 in this report, is in preparation.

Specific Aim 2: Assess differences in therapeutic efficacy of single and multiple post-injury particle injection treatments on OA progression during a 6 week time period.

Major Task 3: Assess therapeutic efficacy for single or multiple intra-articular injections.

Subtask 1: Surgical and treatment procedures for analyzing effectiveness of single or multiple injections of dHACM for up to 6 weeks.

- Medial meniscal transection (MMT) surgeries were performed to induce PTOA and assess therapeutic efficacy of dHACM, particularly after disease progression has begun, to emulate an already arthritic joint.
- MMT knees were treated with dHACM injection at 24 hrs (immediate treatment), 3 wks post-surgery (delayed treatment), and both 24 hrs and 3 wks post-surgery (multiple injection treatment). $n=6-8$ animals per group.
- Animals were euthanized at 6 wks post-surgery and harvested for quantitative EPIC- μ CT analyses and histology.

Subtask 3: Evaluation of therapeutic effects using EPIC- μ CT and histology.

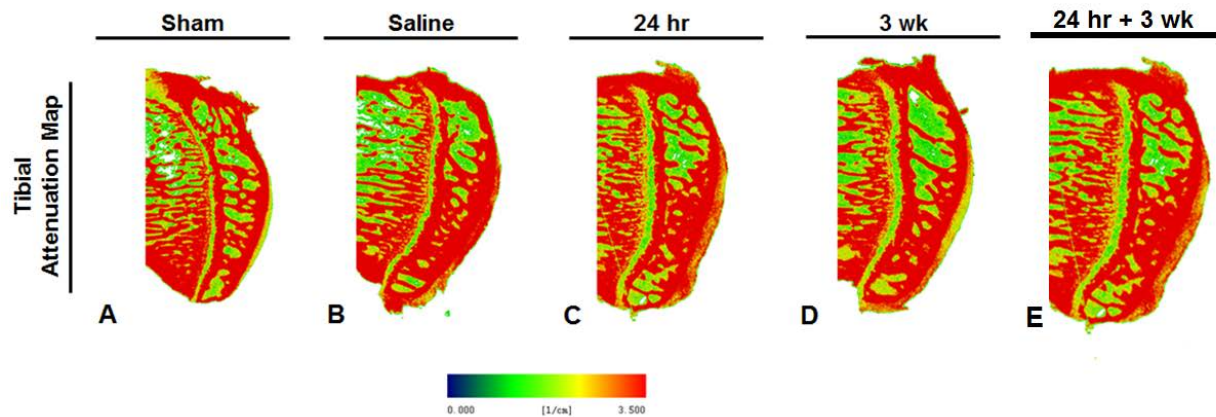


Figure 5. Medial tibial cartilage attenuation maps for sham (A), 24 hr saline injection (B), 24 hr dHACM injection (C), 3 wk dHACM injection (D), 24 hr + 3 wk dHACM injection (E) groups. Pseudocolor attenuation values – red indicates high attenuation (low PGs), green indicates low attenuation (high PGs). Qualitatively less cartilage degeneration for dHACM injection groups compared to saline – 24 hr dHACM injection group displayed more diffuse red (high attenuation / low PG) color compared to 3 wk and 24 hr + 3 wk dHACM injection groups; 3 wk dHACM injection group displayed high PG (green) central cartilage region.

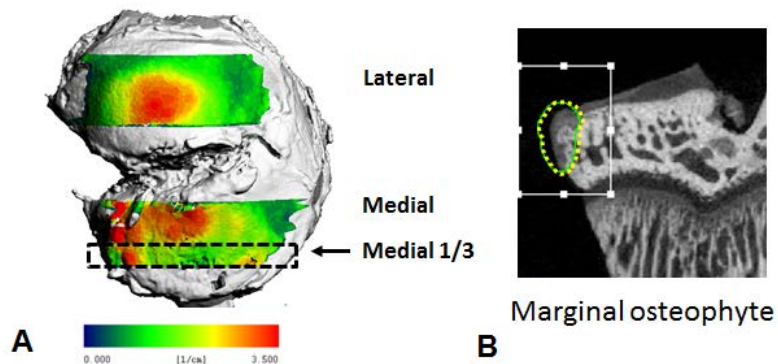


Figure 6. Depiction of medial tibial articular cartilage and subchondral bone quantification regions (medial 1/3 and medial marginal osteophyte).

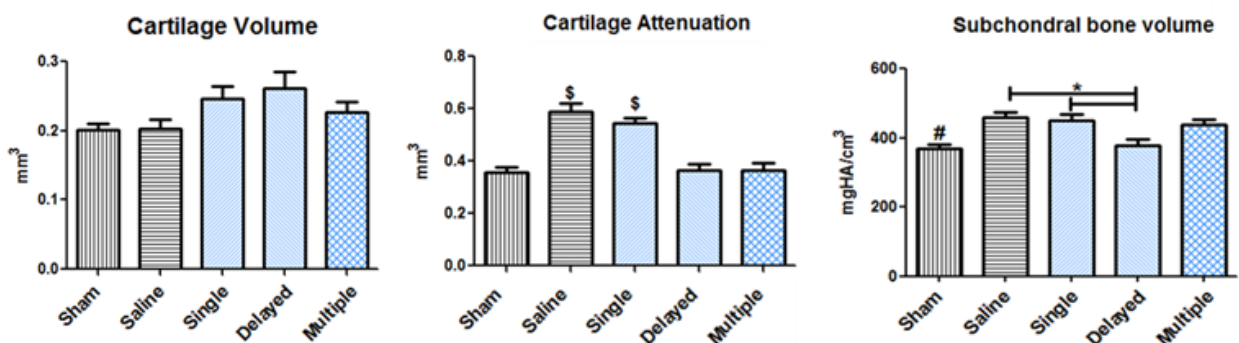


Figure 7. Quantitative cartilage and subchondral bone analyses of the medial 1/3 VOI. No differences were detected for cartilage volume analyses. Cartilage attenuation was

significantly higher in the saline and single 24 hr injection group compared to sham, delayed 3 wk and multiple 24 hr + 3 wk injection groups. Subchondral bone volume was significantly higher in the saline and single injection groups compared to the delayed injection group and lower in sham compared to saline, single and multiple injection groups. (\$ = $p < 0.05$ vs sham, delayed, multiple; # = $p < 0.05$ vs saline, single, multiple; n=6-8)

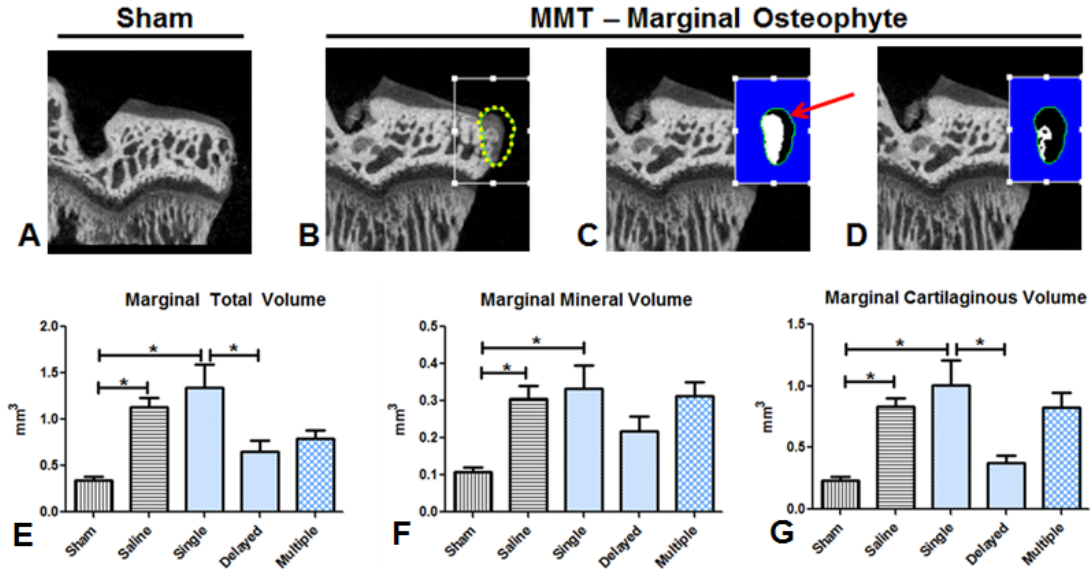


Figure 8. Segmentation and quantification of marginal osteophytes. Representative coronal EPIC-μCT sections for sham (A) and MMT (B,C,D) tibiae. When osteophyte was present: (B) representative segmentation region indicated by green/yellow contour, (C) total volume segmentation (white = used for total volume measurements), (D) mineralized volume segmentation (white = mineralized region only). Single saline and 24 hr dHACM treated groups demonstrated more marginal osteophyte development compared to sham: (E) Marginal total volume was significantly higher for saline and single 24 hr treatment groups compared to sham and delayed 3 wk treatment groups. (F) Marginal mineral volume was significantly higher for saline and single 24 hr treatment groups compared to sham. (G) Marginal cartilaginous volume was significantly higher for saline and single 24 hr treatment groups compared to sham and delayed 3 wk treatment groups. (* = $p < 0.05$; n=6-8)

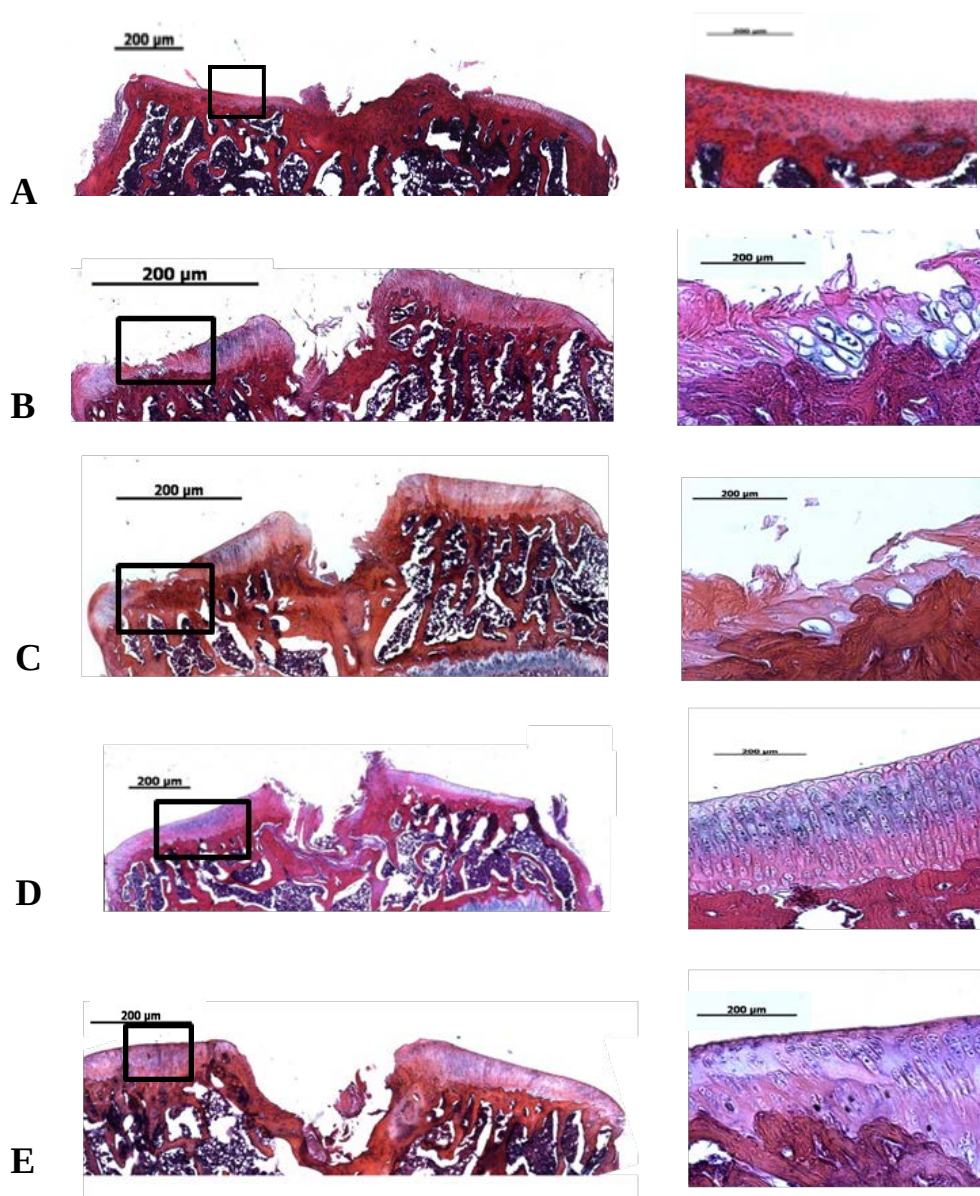


Figure 9. Representative coronal histology sections of tibiae at 6wks post-MMT surgery 4X H&E stained sections on the left, 10X image inserts on the right. Sham group (A) showed no cartilage damage, normal chondrocyte morphology. Saline injection group (B) and single 24 hr dHACM injection group showed cartilage damage with erosions and lesions. Delayed 3 wk dHACM injection group (D) showed no cartilage lesions. Dense columnar chondrocyte morphology was also observed in the 10X view. Multiple (24 hr + 3 wk) injection group (E) showed moderate inhibition of cartilage damage but not as extensively as in the delayed 3 wk injection group.

Specific Aim 2 Conclusions

Articular cartilage composition, subchondral bone, and osteophyte data showed a beneficial effect of single dHACM injection at 3 wks post-MMT surgery, suggesting that after disease progression has begun, dHACM can provide protection against degradative effects. However, a single injection of dHACM 24 hrs post-MMT surgery did not provide a protective effect out to 6 wks post-surgery. Multiple injection dHACM treatment (at 24 hrs + 3 wks post-MMT surgery) demonstrated histological results suggesting moderate inhibition of cartilage damage; however, EPIC- μ CT quantification showed somewhat inconclusive results that may require further investigation.

A manuscript, including work summarized for Major Task 3 in this report, is in preparation.

Specific Aim 3: Evaluate therapeutic effects in an established ovine unilateral medial meniscectomy model of OA

Major Task 4: Produce and characterize ovine unilateral medial meniscectomy OA model.

Subtask 1: Submit documents for IACUC/ACURO and CRADA approvals

- SAMMC IACUC/ACURO documents were submitted for pilot study for model development, and approvals were received, 12-MAY-2014.
- IACUC/ACURO and CRADA documents for full study were submitted, required modifications, and have been resubmitted for approval (pending).

Subtask 2: Surgical procedures for ovine medial meniscectomy (MM).

Medial meniscectomy (MM) surgeries were completed for a pilot study at the San Antonio Military Medical Center (SAMMC) with Dr. Travis Burns and Dr. Ian Goodman according to the following Table 2. Left knees underwent MM; contralateral right knees were left unoperated as controls.

Table 2. Medial meniscectomy surgeries were performed on pilot sheep on 14-APR-2015 at SAMMC. Post-surgery animal takedown time points and animal numbers were as follows for this initial study.

<i>Surgical procedure</i>	<i>Timepoint</i>	<i>Number of sheep</i>
Medial meniscectomy	8 week	1
Medial meniscectomy	12 week	2
Medial meniscectomy	24 week	2
Arthrotomy alone	24 week	1

Example photographs of 12wk post-surgery proximal tibiae are shown in Figure 10.

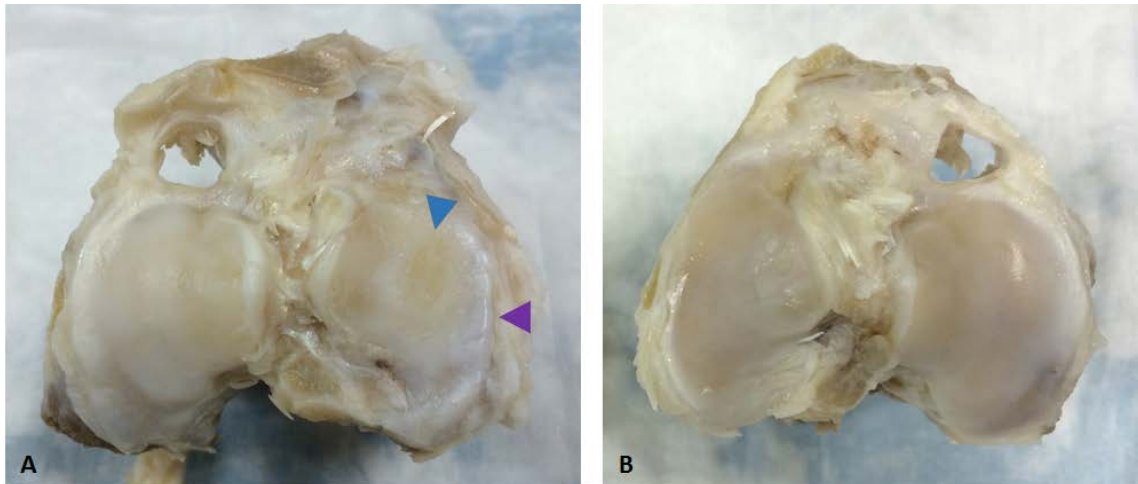


Figure 10. Left medial meniscectomy tibial plateau (A) and right contralateral control tibial plateau (B) are shown, 12 wks post-surgery. Damage can be seen visually in the form of cartilage degradation on the left medial plateau (blue arrow) as well as marginal alterations (purple arrow).

Subtask 3: Ovine model EPIC- μ CT protocol development.

For development of contrast-enhanced μ CT technique for this species and model, one sheep distal femur was sawed into three sections – medial femoral condyle, lateral femoral condyle, trochlear groove. Because femoral and tibial condyles are regions targeted for analyses in this model due to their potential for detectable OA damage, the trochlear groove was used for Hexabrix contrast agent concentration and incubation time testing.

Trochlear groove sample soaked for 30 mins at 30% Hexabrix concentration produced cartilage contrast enhancement at attenuation levels too close to bone. At 20% Hexabrix concentration, the sample was soaked for 30 mins in 100ml contrast agent solution at 37°C, then scanned with a Scanco μ CT50.

Further incubation times involved incremental addition of 30 mins in 100ml of 20% Hexabrix solution until a cumulative time of 3 hrs was reached. Figure 11 shows the average X-ray linear attenuation coefficient at the incremental time points of incubation.

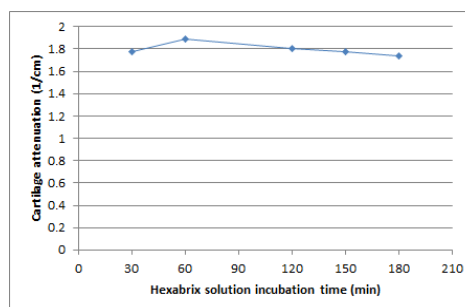


Figure 11. Average cartilage linear attenuation coefficient of femoral trochlear groove cartilage as a function of Hexabrix incubation time (20% Hexabrix in PBS). Unfortunately, the 90 minute cumulative incubation scan contained some errors and could not be reported.

The 30 minute incubation time produced an average cartilage attenuation value of over 90% of the maximum value (seen at 60 minutes of incubation time). Average attenuation at the subsequent incubation time points out to 180 minutes declined toward a similar average value seen at 30 minutes. Long incubation times are not ideal for throughput considerations, and extended durations at 37°C have the potential to produce deleterious effects on cartilage composition. In this test, incubation times out to 3 hours demonstrated little benefit (similar average cartilage attenuation as 30-minute incubation). For subsequent analyses, a 30-minute incubation was deemed appropriate.

Subtask 4: Ovine cartilage and subchondral bone characterization.

For full characterization of pilot study ovine joints, 20% Hexabrix solution was initially used; however, this concentration produced average linear attenuation levels in cartilage that were too similar to bone attenuation levels, preventing adequate segmentation of the tissues. 10% Hexabrix solution (maintaining 30 minute incubation) demonstrated good segmentation between cartilage and bone. Thus, further analyses for the pilot study would be performed at 10% Hexabrix concentration.

Knee joints from two 12wk post-surgery animals and one 8wk post-surgery animal have been evaluated. Representative images from one of the 12wk post-surgery animals are shown in Figure 12.

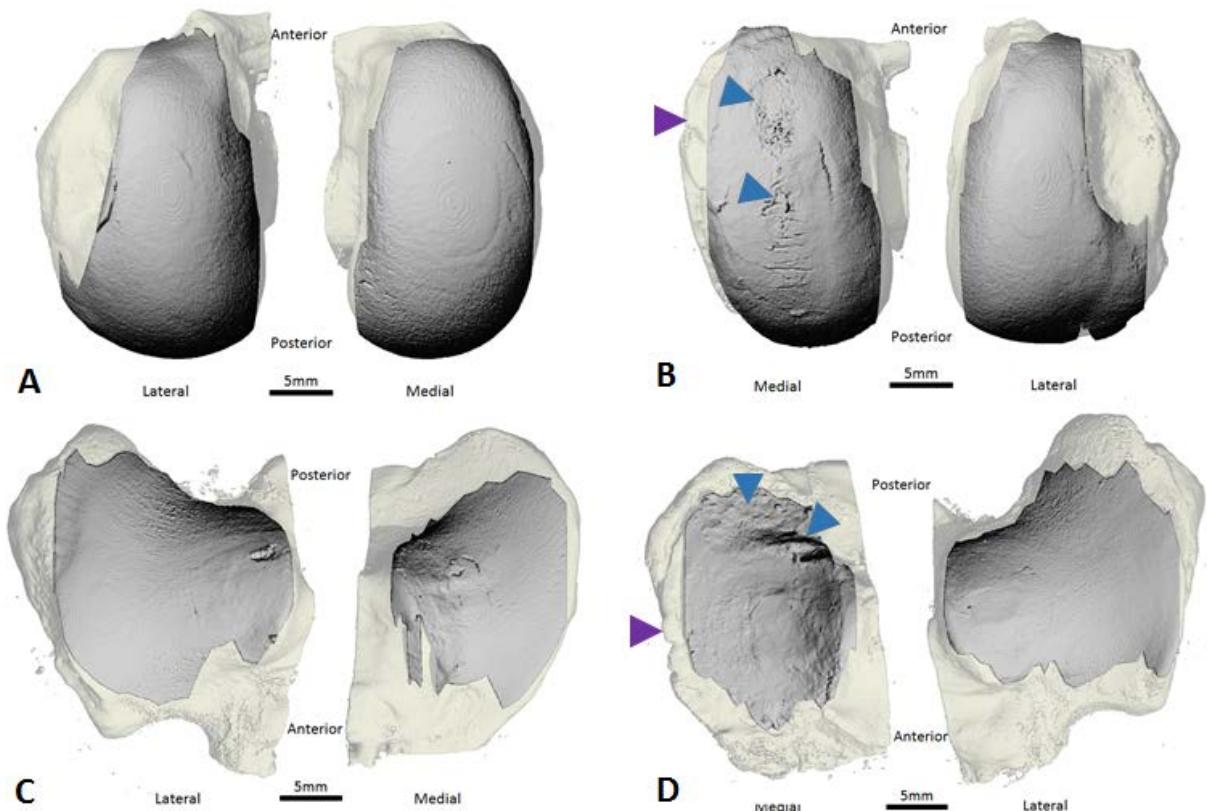


Figure 12. Example cartilage-bone overlay images for 12wk post-surgery femur and tibia after Hexabrix contrast-enhancement (A,C = Right contralateral control femur and tibia, respectively; B,D = Left MM femur and tibia, respectively). Qualitative differences were seen in left medial compartments compared to right - articular cartilage not as smooth (blue arrows), visible areas of cartilage wear (blue arrows), cartilage margins more difficult to discern, marginal bone remodeling (purple arrows).

Average 3D articular cartilage thickness, volume, and attenuation were quantified separately for the medial femoral condyle, lateral femoral condyle, medial tibial plateau, and lateral tibial plateau. Data from 12wk post-surgery volumes of interest are shown in Figure 13.

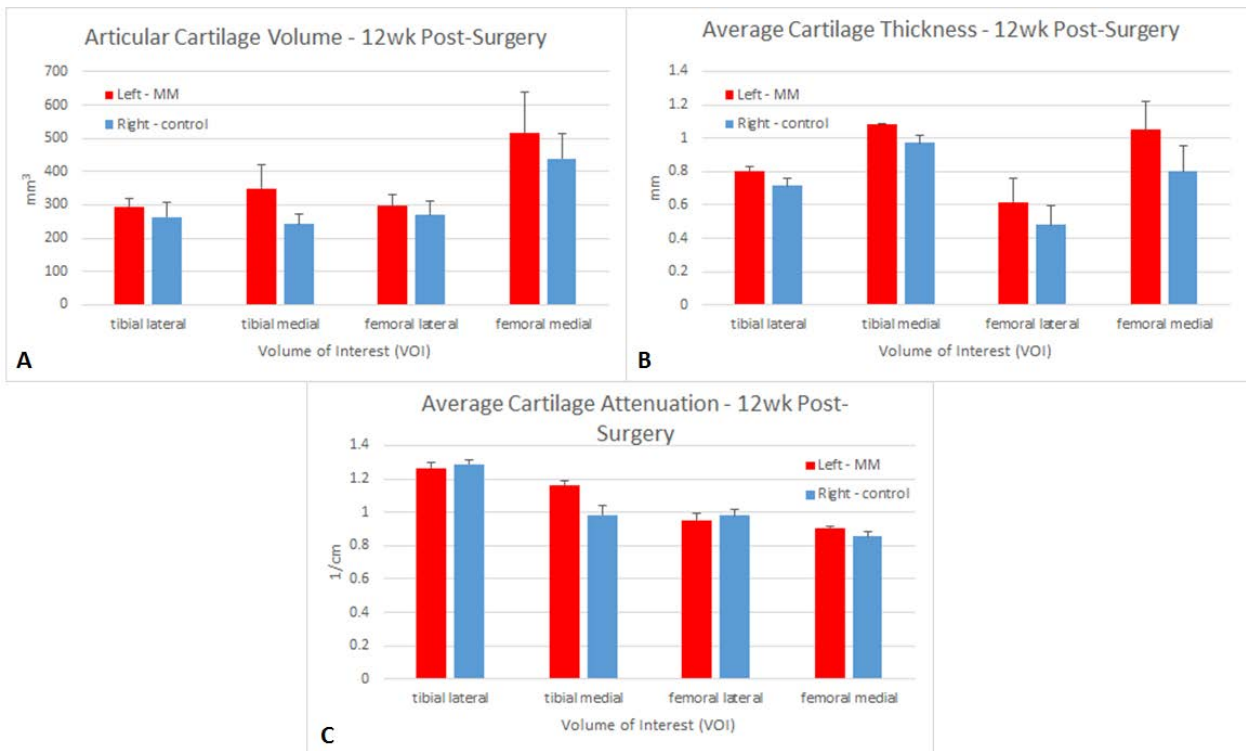


Figure 13. Articular cartilage volume, average thickness, and average attenuation were quantified for lateral tibia, medial tibia, lateral femur, medial femur VOIs (n=2). Left knees underwent medial meniscectomy (MM, red); right knees = contralateral control (blue).

Specific Aim 3 Conclusions

These preliminary results suggest possible increases in cartilage volume, thickness, and attenuation in left (MM) medial tibiae compared to right (control). Articular cartilage in the medial third of the medial tibial plateau of affected joints in the rat medial meniscal transection (MMT) model of OA have been shown to exhibit degenerative changes that are consistent with these potential changes. This indicates that the MM surgical techniques show promise for inducing detectable degenerative changes at 12 wks post-surgery

potentially without the need to limit analysis regions to smaller VOIs, as was necessary in the rat MMT model in order to detect degenerative changes.

Current and Anticipated Problems

There have been a few issues that have resulted in minor adjustments in study timelines, and potential future approaches have been considered, as listed below:

1. *dHACM particle fluorescent labeling & in vivo retention profile analyses:* (Specific Aim 1, Major Task 1, Subtasks 3 & 4)

Problems have been encountered with fluorescence signal detection of dHACM particles after exposure to fluorochromes *in vitro*. Potential solutions to be explored include using a new system that we have acquired, an IVIS Spectrum CT, with higher sensitivity to detect small changes in fluorescence. We have also considered exploring alternative methods of dHACM particle labeling such as using a human-specific collagen IX antibody.

2. *Inflammatory response evaluation in the rat MMT model:* (Specific Aim 1, Major Task 2, Subtask 2; Specific Aim 2, Major Task 3, Subtask 3)

The originally proposed methodology was to extract synovial fluid from rat joints via knee joint lavage technique. However, this technique proved to be inconsistent in acquiring enough fluid to analyze. A potential alternative method we will explore is to quantify gene expression and inflammatory markers using qPCR (Fluidigm BioMark Genetic Analysis Platform).

3. *Assessing therapeutic efficacy of dHACM treatment in ovine MM model:* (Specific Aim 3, Major Task 5)

IACUC approval for the therapeutic efficacy study in the ovine MM model has been delayed. Original CRADA had been approved but required revision and re-approval, and we are currently waiting for this to occur. IACUC approval at SAMMC is pending completion of the CRADA approval. At the current time, this delay is not impeding progress. We are still characterizing the MM model with the pilot study (Specific Aim 3, Major Task 4), and the therapeutic efficacy study is scheduled for the end of Year 2 through Year 3 according to the proposed SOW. However, GT will not release subcontract funding prior to receiving IACUC approval documents from SAMMC.

4. KEY RESEARCH ACCOMPLISHMENTS:

1. We determined through work in Specific Aim 2 that intra-articular treatment with dHACM after the onset of OA changes can provide protective effects against disease progression (evaluation of effects at 6 weeks post-MMT surgery when dHACM treatment was administered at 3 wks post-surgery).

2. Evaluation of disease progression at 1 and 3 weeks post-MMT surgery in the rat model (from Specific Aim 1) suggested that degenerative changes in articular cartilage (attenuation and surface roughness) were detectable prior to those of subchondral bone (mineral density and thickness). This addresses a major question in the field on the sequential pathology of post-traumatic OA.

5. CONCLUSION:

The combined effects of post-traumatic and degenerative OA on active military personnel and veterans are the cause of substantial disability, medical discharge, and long-term costs. Assuming success of this work and further clinical studies to demonstrate safety and effectiveness of the proposed treatment method, the injectable nature of the therapeutic could facilitate accessibility of treatment in the field. Because OA disease progression is accelerated for soldiers who experience high energy combat injuries, one potential course of action would be to administer the therapeutic immediately post-operatively in an effort to prevent the accelerated response and reduce the loss of personnel and costs associated with disability.

An important consideration for injectable OA therapeutics is the ability to be retained in the joint and therefore sustain beneficial effects and minimize the frequency of injections. This work will provide necessary preclinical data including safety and immune response, retention, and long-term effectiveness of the particle injections. If successful, this project will present a readily translatable, low risk, minimally invasive treatment method that modifies OA disease progression in a clinically relevant model, leading to human clinical trials as the clear next step.

Much progress has been made thus far on the study aims. Two particle size distributions of dHACM were assessed for their therapeutic effects after MMT in the rat, and the micronized formulation of dHACM (AmnioFix®) resulted in better preservation of articular cartilage PG content after 1 and 3 weeks. Effects of single early, single delayed, and multiple dHACM intra-articular treatments were assessed in the rat MMT model, and the single delayed injection (emulating treatment after disease progression has begun) provided a protective effect against degradative changes in the joint. Development and characterization of the large animal model (sheep MM) has begun, and degenerative changes have been observed at 12 weeks post-surgery.

Two critical focus areas that remain to be completed for the small animal model are more mechanistic in nature - immune response within the joint post-injection and dHACM retention profile analyses. For the large animal model, characterization of disease progression after medial meniscectomy is ongoing, and evaluation of the therapeutic in this model has yet to be initiated. The full large animal study is expected to begin in calendar year 2016.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Presentations:

Type	Description
Conference	Reece, D.S., Sridaran, S., Thote, T., Stevens, H., Lin, A., Willett, N.J.,

presentation / poster	Guldborg, R.E. Effects of Micronized Dehydrated Human Amnion Chorion Membrane on Post-Traumatic Osteoarthritis. Military Health System Research Symposium (MHSRS), August 2015.
Conference presentation / poster	Reece, D.S., Thote, T., Lin, A., Willett, N.J., Guldborg, R.E. Cartilage Surface Roughness Measurements: Utility in Model Characterization and Therapeutic Evaluation. 61st Annual Meeting of the Orthopaedic Research Society, March 2015.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: Nothing to report.

9. OTHER ACHIEVEMENTS: Nothing to report.