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TITLE: Prevention of the Post-Traumatic Fibrotic Response in Joints: a Critical Preclinical Evaluation of an Antifibrotic Antibody

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14. ABSTRACT The main objective of this project is to help wounded soldiers to recover from injuries to their extremities by applying a therapeutic antibody to reduce posttraumatic joint stiffness. Since clinical interventions available today to limit post-traumatic joint stiffness are sparse and their outcomes are disappointing, our research is needed and timely. The soldiers are uniquely prone to suffer from joint injuries due to the risks associated with their military activities. One of the significant complications of trauma to the joints is post-traumatic joint stiffness that severely limits the range of motion, causes pain, and makes it impossible to resume military duties. Posttraumatic joint stiffness mainly results from the formation of scar tissue that alters the normal function of injured joints. Although post-traumatic joint stiffness may be partially reduced by surgery and lengthy physical therapies, the effectiveness of these interventions is not entirely satisfactory. To prevent joint stiffness, we developed a therapeutic antibody that blocks the formation of unwanted fibrotic scars responsible for the stiffening of damaged joints. Our research demonstrated this antibody's efficacy in reducing joint stiffness in a relevant animal model. Here, we moved our antibody-based inhibitor closer to clinical trials by defining this therapeutic agent's safety and efficacy parameters.					
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1. INTRODUCTION

The Expansion Study we performed during the last funding period moves forward our original research conducted in response to the request of the Department of Defense (DoD) to develop effective methods of reducing post-traumatic joint stiffness. This initial study, entitled: "Prevention of the Post-Traumatic Fibrotic Response in Joints," was funded by the Peer Reviewed Orthopaedic Research Program (PRORP) through the Translational Research Partnership Award mechanism. Our original study addressed the Focus Area: "Prevention or treatment of post-traumatic joint stiffness and contracture in the ankle, knee, and/or elbow, including physical-therapy approaches."

The DoD has defined this Focus Area as "critical for optimizing recovery and restoration of function for military personnel with orthopaedic injuries sustained in combat or combat-related duties." Studies show that traumatic joint injuries, including joint stiffness, not only limit the range of motion (ROM) but also constitute the major risk factors for developing osteoarthritis, a leading cause of disability and medical discharge in military personnel [1, 2].

The main objective of our study was to design and test effective methods to limit post-traumatic joint stiffness in military personnel by employing an antibody-based blocker of fibrotic scarring. We hypothesized that applying this therapeutic antibody (referred to as ACA) would reduce post-traumatic joint stiffness without causing any significant unwanted side effects. To test these hypotheses, we defined the following Specific Aims (SA): SA1: To determine the safety of the ACA applied short-term and long-term to reduce post-traumatic joint stiffness; SA2: To define the effects of the ACA on the healing of joint tissues.

2. KEYWORDS

arthrofibrosis; posttraumatic joint stiffness; therapeutic antibody; biologic therapeutics; orthopaedic trauma; scar tissue; wound healing

3. ACCOMPLISHMENTS

The Major Goals of the Project

The following points summarize the significant goals stated in the approved SOW:

Specific Aim 1

To determine the safety of the ACA applied short-term and long-term to reduce post-traumatic joint stiffness

Major Task 1

Production and purification of therapeutic antibodies (ACA).

Subtask 1. High-density cell culture to produce preparative-scale amounts of therapeutic ACA. In this subtask cell cultures will be carried out in a laboratory-scale bioreactor.

Subtask 2. Purification of the ACA. The ACA will be purified by affinity chromatography.

Subtask 3. Quality control of purified ACA. The structural integrity, purity, and affinity of purified ACA will be analyzed.

Milestones Achieved

Establishing sufficient quantities of functional, highly purified therapeutic antibody for animal-based tests.

Subtask 4. TJU IACUC and ACURO approvals.

Milestone Achieved

Securing the IACUC/ACURO approvals

Major Task 2

Concentration-dependent safety assays.

Subtask 1. Dose acceleration in short-term (1 week) tests.

Subtask 2. Safety studies in long-term (12 weeks).

Subtask 3. Assays of collagen metabolites in the context of the ACA treatment.

Milestones Achieved

Determined the safe ACA concentration and defined key biomarkers to study the effectiveness of the ACA treatment.

Specific Aim 2

To define the effects of the ACA on the healing of joint tissues.

Major Task 3

To determine the impact of the ACA treatment on the healing of joint tissues.

Subtask 1. Performed surgeries for rabbit-based contracture model.

Subtask 2. Biomechanical, biochemical, and histological assays of joint tissues. We studied the effects of the ACA applied to reduce post-traumatic joint contracture on the tendon, cartilage, and subchondral bone's biological, biochemical, and mechanical properties.

Subtask 3. Data analysis and statistical evaluation of results. Data were collected throughout the animal-based experiments and analyzed with the assistance of a statistician.

Milestones Achieved

We have established a clinically-relevant method to limit post-traumatic joint stiffness without altering the healing process. All experimental conditions and procedures have positioned our technology for preclinical tests in large animals.

We published a manuscript describing key findings (see Appendix). A book chapter entitled: “Beyond Drugs and Surgery: A Look at Orthobiologics” is now in press as a part of a book entitled: “Translational Sports Medicine”. This chapter includes a section devoted to our research on antibody-based prevention of posttraumatic joint contracture.

Key Accomplishments

The following paragraphs describe: (i) major activities; (ii) specific objectives; (iii) significant results and key outcomes; and (iv) other achievements.

For Specific Aim 1

To determine the safety of the ACA applied short-term and long-term to reduce post-traumatic joint stiffness

Major Task 1

Production and purification of therapeutic antibodies (ACA).

Subtask 1

High-density cell culture to produce preparative-scale amounts of therapeutic ACA. In this subtask cell cultures will be carried out in a laboratory-scale bioreactor.

Subtask 2

Purification of the ACA. The ACA will be purified by affinity chromatography.

Subtask 3

Quality control of purified ACA. The structural integrity, purity, and affinity of purified ACA will be analyzed.

Major Activities

In Major Task 1, we produced and purified the ACA needed for animal-based tests. In this task performed continuously for both specific aims, we utilized CHO cells engineered to produce the monoclonal ACA antibody, which was applied to reduce the fibrotic response of the injured knees of experimental rabbits. As a result, we generated the ACA that was available throughout the study.

Methods

Bioreactor Runs

Our original method of ACA production was based on using the CelliGen BLU 5 L Packed-Bed bioreactor (working volume of 3L; Eppendorf) controlled by the New Brunswick BioFlo/CelliGen 115 Benchtop Fermenter & Bioreactor (Eppendorf). CHO cells producing the ACA pre-conditioned to serum-free CD-CHO (Gibco) or Ham's F-12 (Corning) were grown in suspension to obtain 2×10^9 cells, the initial inoculum needed to seed the bioreactor. These cells were seeded into the bioreactor filled with porous discs. Consequently, the cells were grown attached to the bioreactor's discs.

A picture of the bioreactor vessel and control cabinet is seen in Figure 1. A schematic representation of the vessel is depicted in Figure 2. Bioreactor runs were performed using a modified controlled-fed perfusion format in which media was removed entirely from the vessel every two days, and 3 L of fresh media was added via inlet and outlet ports on the bioreactor. Based on tests, we found that CHO cells needed a dissolved oxygen content of 40% in order to ensure maximum cell viability and protein production.

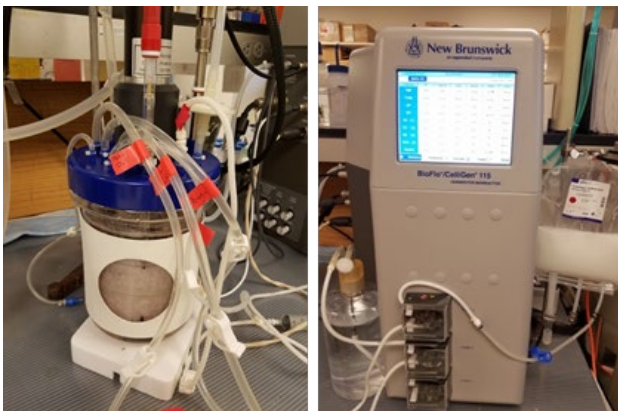


Figure 1: 5 L CelliGen BLU bioreactor and control cabinet. The vessel contains media inlet and outlet ports, a gas inlet, and a sample port. There is also a pH probe, DO probe, and a heating blanket. The control cabinet monitors and modulates all essential parameters.

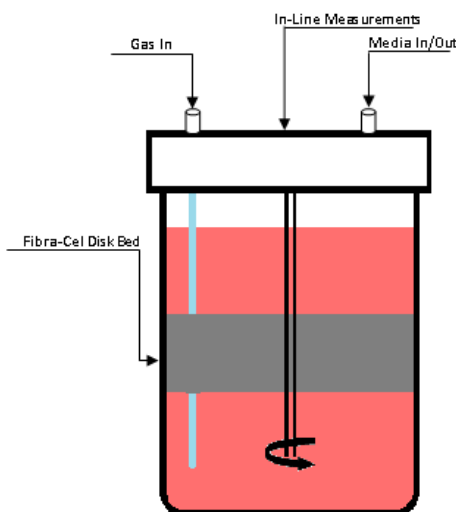


Figure 2. Schematic representation of PBR. The working volume of the vessel is 3.75 L, and the packed-bed contains 150 g Fibra-Cel disks. The vessel contains an impellor to circulate media throughout the vessel. Ports at the top of the vessel provide feed and harvest, as well as all in-line measurements such as dissolved oxygen, pH, and temperature. Glucose and lactate concentrations in cell culture media were monitored and regulated continuously with an in-line glucose/lactate monitor.

ACA Production Challenges Associated with the COVID-18 Pandemic

Although we managed to follow the plan described in the original SOW, we could not avoid significant roadblocks caused by the COVID-19 pandemic:

Inability to Purchase Bioreactor Vessels. We experienced a problem purchasing disposable bioreactor vessels (CelliGen BLU 5 L Packed-Bed bioreactor) needed to produce the ACA utilized in this study. In brief, the bioreactor producer (Eppendorf) has informed us that all vessels for growing surface-attached cells are shipped only to companies working on COVID-19 and cannot be sold to academia (Appendix #3).

Delay in Antibody Production. Since the beginning of our study, the system we developed to produce ACA depended on growing CHO cells anchored to the bioreactor's porous discs. Because the bioreactor vessels were unavailable for our purchase, we faced a delay in antibody production.

Changing Cell Culture Conditions. To circumvent the bioreactor problem, we had to change the anchorage-dependent cell culture conditions to grow the cells in suspension. Growing cells in suspension is an intrinsically slow process, which requires an investment to purchase specific cell culture media and pieces of hardware necessary to connect a cell-suspension vessel to a controller. Although we successfully changed the cell-culture conditions, this change cost us time and money.

Current Status of the ACA Production. Following adjustments to the cell culture conditions, we successfully produce the ACA in CHO cells grown in suspension. Crucial steps include: (i) seeding frozen stocks of CHO cells and expanding them in the spin flasks in the presence of selecting antibiotics (puromycin and G418); (ii) using the seed culture to inoculate a cell-suspension-culture bioreactor; (iii) maintaining cell culture in suspension in the seeded vessels; (iv) controlling crucial parameters, including glucose and lactate concentration, agitation, O₂, CO₂, pH, gas flow, and foaming; although crucial parameters are monitored and recorded continuously by in-line probes and sensors, periodic calibrations of these devices are also necessary; (v) collecting 2/3 volume of media for ACA purification, and (vi) adding new media to continue cell suspension culture.

Please see Appendix #1 with notes on crucial parameters of one of the cell suspension cultures and data depicting the regulation of glucose concentration (Appendix #1).

Purification and Characterization of the ACA

Each batch of collected media was filtered to remove CHO cells; the filtered media were frozen. This procedure was repeated with all batches of collected media.

Purity, Integrity, and Yields of the ACA. After collecting all media batches, they were concentrated using a tangential-flow ultrafiltration system (Sartorius). During this process, we exchanged the solvent from CD CHO medium to phosphate-buffered saline (PBS).

Subsequently, the media was run over a Protein-L affinity column (Fig. 3). Protein-L agarose (Genscript Inc.) is an affinity resin that binds specifically to the light kappa chain of human IgG, such as ACA.

The column-bound ACA was eluted with 0.1 M Glycine (pH=3.0). Gel electrophoresis (SDS-PAGE) was performed on samples reduced using 2-mercaptoethanol and the gels were stained with Coomassie blue (Bio-Rad) to assess the purity and structural integrity of eluted ACA. Protein yields were determined by a Bradford protein concentration assay (Bio-Rad). Finally, samples were concentrated using ultrafiltration on a 30 kDa-cut-off filter (Millipore).

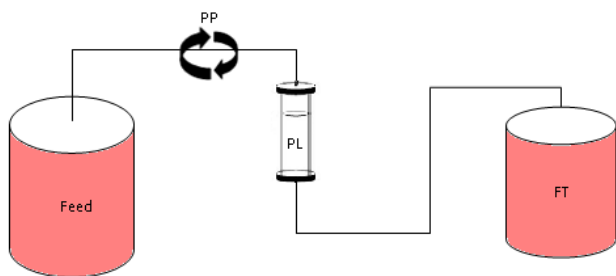


Figure 3. Schematic representation of protein-L agarose column. Collected media was processed before being pumped over a resin bed containing 5 mL of Protein-L agarose (PL) using a peristaltic pump (PP). Flow through media (FT) was also collected and analyzed to ensure all antibodies bound to the resin.

Size Exclusion Chromatography. The purified and concentrated ACA samples were tested using size exclusion chromatography HPLC (SEC-HPLC) to ensure that the protein does not form aggregates during either

the bioreactor run or after collection. A 15- μ L purified sample of the ACA from each bioreactor run at 2 mg/mL was run over a BioBasic SEC-300 size exclusion column (Thermo Scientific) using a Varian Prostar Model 210 HPLC system. The elution profiles were compared to a known molecular mass γ -globulin standard (Bio-Rad).

ACA-antigen binding assays. The binding kinetics of the combined pool of the ACA batches collected from each bioreactor run were evaluated by analyzing the binding affinity of the purified antibody to the procollagen I standard that contains the ACA-specific epitope. Binding assays were done using the Pioneer Biosensor (Sartorius).

Human procollagen I was bound to a COOH5 biosensor chip (Sartorius). The ACA that was solubilized in HEPES buffer (pH = 7.4) supplemented with 0.005% Tween-20 was run over the chip. In subsequent cycles that included association and dissociation events, the ACA was added at concentrations of 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, and 3.125 nM. Binding kinetics were analyzed using the Qdat software (Sartorius). Consequently, association rate constants (k_{on}), dissociation rate constants (k_{off}), and equilibrium dissociation constants (K_D) were determined from the data collected for each tested chIgG batch.

Results

Purity, structural integrity, and yields of ACA

After purification of the ACA, Coomassie-stained gels were analyzed to determine the purity of the eluted antibody. Electrophoretic separation of reduced ACA chains demonstrated the presence of expected γ and κ chains; no contaminating proteins were apparent, thereby indicating high purity of the ACA preparations (Fig. 4). Moreover, the absence of degraded γ and κ chains indicates proper structural integrity of the ACA preparations.

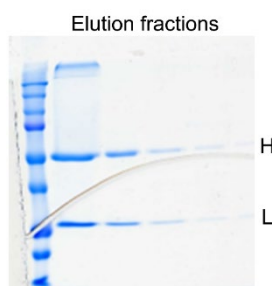


Figure 4. Coomassie-stained SDS-PAGE gel depicting elution fractions from protein L-agarose. The heavy gamma chain (H) and light kappa chain (L) of the ACA are indicated. Lane 1: Molecular mass marker is also depicted.

Size Exclusion Chromatography

Each ACA sample was eluted as one peak of expected mass with no apparent aggregate formation. Retention profiles for the peaks were similar to that of a known 158-kDa bovine γ -globulin standard (Bio-Rad) (Fig. 5).

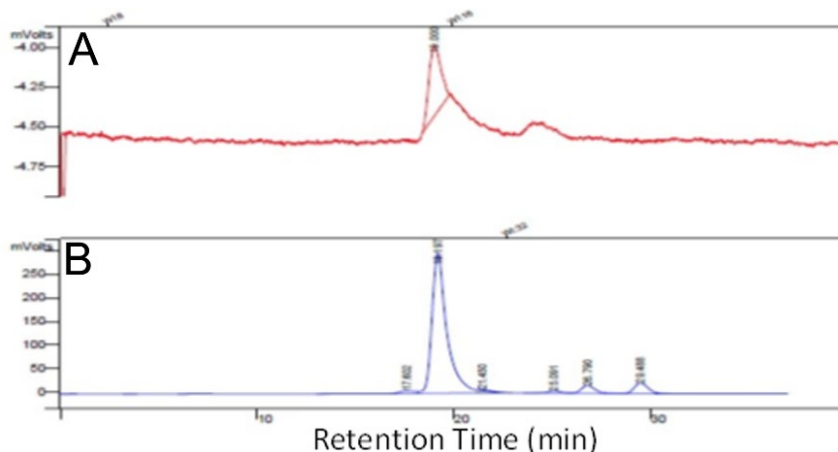


Figure 5. An example of chromatograms from size exclusion chromatography. A, peaks of the ACA produced in the CD-CHO medium; B, a 158-kDa γ -globulin standard.

ACA-antigen Binding Assays

Biosensor-based assays indicated a proper binding affinity of all ACA batches (data not shown)

ACA Yield

An average amount of purified and concentrated ACA was 1.3 g per entire bioreactor run. This amount was isolated from the medium volume of about 36 L collected during a typical 4-week cell culture batch.

Summary

Developing New Conditions for the ACA Production. Due to the COVID-19-related challenges described above, we had to change the ACA production method from our original one to the cell-suspension system. This adjustment required purchasing additional bioreactor hardware and “training” the CHO cells to grow in suspension.

Following these adjustments, we have developed a robust production method yielding significant ACA quantities suitable for animal studies. We plan to continue utilizing this method in all our studies on ACA-based anti-fibrotic therapies.

Milestones Achieved

In Major Task 1, we successfully developed a robust new method for producing the ACA.

Major Changes

No major changes were introduced.

Technical Problems

As indicated above, we encountered technical problems with COVID-19-related shortages of crucial components needed for our original ACA production method. We circumvented this problem by developing a new antibody production method with readily available supplies.

Major Task 2

Concentration-dependent safety assays.

Subtask 1

Dose acceleration in short-term (1 week) tests.

Subtask 2

Safety studies in the long-term (12 weeks).

Subtask 3

Assays of collagen metabolites in the context of the ACA treatment.

Major Activities

In Major Task 2, we studied ACA safety. Crucial parameters defining potential unspecific effects of this antibody on the rabbits included in-life observations, cellular and biochemical assays of the blood, and histological assays of various tissues and organs.

Moreover, we studied collagen metabolites associated with the scarring process to determine if analyzing them can monitor the ACA impact on the scar formation process.

Methods

A Rabbit-based Model of Arthrofibrosis

We employed an established rabbit model of posttraumatic joint contracture to study the potential side effects of the ACA and its impact on the healing of the joint tissues, including the posterior capsule (PC), subchondral bone (SB), and patellar tendon (PT) [3-7].

We used male and female New Zealand White rabbits, 8 to 12 months old (Charles River Laboratories, Wilmington, MA, USA). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University and a sponsoring agency's Animal Care and Use Review Office (ACURO).

Before applying general anesthesia, the rabbits received ketamine and xylazine. Subsequently, an analgesic was administered preoperatively and postoperatively to minimize the pain caused by surgery on the knee. At the end of the experiments, the rabbits were euthanized by intravascular or intracardiac bolus overdose of a euthanasia agent containing barbiturate sodium pentobarbital.

All animal experiments complied with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Since we were interested in posttraumatic arthrofibrosis, our model included damage to crucial elements of the knee joint, including the joint capsule, ligaments, tendons, cartilage, and subchondral bone. The model also included intraarticular bleeding. Necessary surgical procedures included the following steps: (i) An incision in the middle of the right knee was made, starting proximal to the patella and extending distally to the tibial tubercle and down the tibia. (ii) A lateral parapatellar arthrotomy was made, and the patella was dislocated medially. (iii) Following flexing the knee, the fat pad was released and retracted medially. (iv) The anterior crucial ligament (ACL) and the posterior crucial ligament (PCL) were transected, and the knee was hyperextended to 45° to disrupt the posterior capsule. (v) A full-thickness 3-mm osteochondral defect (OCD) was made in the medial femoral condyle at the weight-bearing apex. (vi) Beginning as far posteriorly in the notch as possible and exiting through the lateral condyle, a 2-mm hole was made for a catheter. (vii) A custom-made silicone catheter with a fixed retention bead at one end was passed through the hole. While the retention bead secured the catheter inside the operated knee, the opposite end was connected with a subcutaneous pump so as not to affect the knee area (iPRECIO SMP-200, Primetech Co., Tokyo, Japan) [7]. (viii) The operated right leg was placed back in extension with the patellar tendon in a normal position. (ix) A full-thickness, 3 mm-wide, 10 mm-long central portion of PT was resected with a surgical blade [8]. The defect location was marked with 5-0 nonabsorbable nylon sutures. Creating this standard defect enabled a comparison of the mechanical properties of the neotissue formed during the healing process. (x) The injured knee was fixed in the flexed position using a Kirschner (K)-wire, as described [6, 7]. (xi) Immediately after the surgery, the ACA was applied intraarticularly via a subcutaneous pump. (xii) Eight weeks later, the K-wires were removed, and the rabbits returned to their cages for 4 weeks. During the 4-week recovery period, the antibodies were not applied. In all rabbits, the left leg served as the uninjured control. Figure 6 illustrates crucial surgical steps; for additional information, please see our earlier reports [6, 7].

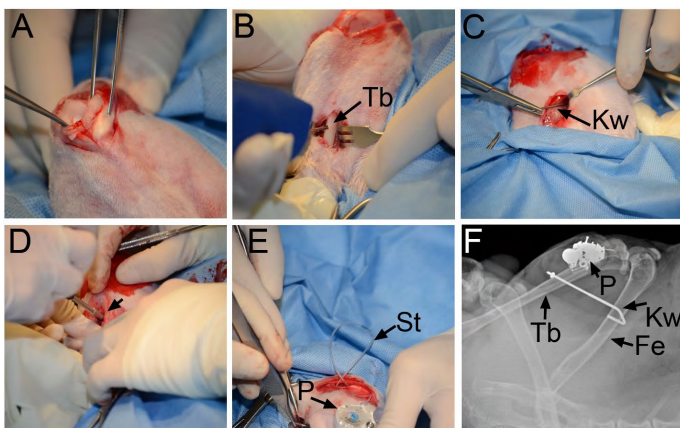


Figure 6. Crucial surgical steps to generate knee injury in a rabbit model of arthrofibrosis. A, Getting access to the knee cavity. B; Drilling a hole in the tibia (Tb) to install a Kirschner wire (Kw). C; Placing a loop of the Kw over a femur (Fe), D; Fixing the Kw with a nut placed over the tibia. E; Installing a pump (P) connected to the knee cavity via a silicone tube (St). F; An X-ray image of the operated leg immobilized in the flexed position with a Kw.

Analysis of Blood Parameters

We analyzed the ACA's effects on cellular and biochemical parameters of the blood and crucial collagen metabolites. The samples were collected at the same time of the day to minimize the possible impact of circadian rhythm on blood parameters and collagen metabolites.

Histology of Tissues and Organs

Following euthanasia, we collected samples of tissues and organs, including the brain, esophagus, thymus, aorta, heart, lung, liver, spleen, stomach, kidney, intestines, gonads, Achilles tendon, skeletal muscle, and sciatic nerve. The samples were processed for histology, stained with H&E, and then observed for any potential abnormalities associated with the ACA treatment, including structural alterations and an influx of inflammatory cells.

Assays of Production and Degradation of Collagen Molecules to Define Relevant Biomarkers

Monitoring the Degradation of Collagen Molecules During the Long-term Treatment. Fibril-incorporated collagen molecules that are chemically cross-linked form stable fibrillar assemblies. Their natural degradation/turnover in mature, healthy tissues is usually slow. In contrast, free collagen molecules are unstable *in vivo*, so they are readily degraded by proteolytic enzymes [9]. Since our ACA prevents free collagen I molecules from aggregating into fibrils, we predicted that the degradation of collagen I produced in response to joint injury would increase compared to control due to the ACA-mediated inhibition of fibrillogenesis. To measure this degradation, we analyzed the serum content of hydroxyproline, a collagen-specific amino acid, which remains within degraded collagen fragments (Fig. 7).

We also analyzed the fragments of collagen fibrils that include collagen cross-links (Fig. 7). Note that hydroxyproline assays provide information on the total amount of degraded collagen. Therefore, the hydroxyproline assays will reflect the amounts of both free collagen molecules and those derived from the collagen fibrils (Fig. 7). In contrast, assays of collagen I cross-links only provide information on the relative content of collagen derived from the fibrils because the formation of the cross-links occurs only after collagen fibrillogenesis. Consequently, we interpret the increase in the *hydroxyproline/cross-link* ratio as the increase in degradation of the free collagen monomers that are not incorporated into fibrils.

While chemical methods measured the hydroxyproline content, collagen I cross-links were analyzed by ELISA with commercially available antibodies that recognize the cross-linked C-telopeptides. The content of hydroxyproline and the collagen cross-links were measured in serum samples collected at defined time points.

Quantifying the Biosynthesis of Procollagen I in Response to Joint Trauma. Being able to approximate the extent of production of collagen I molecules in response to joint injury would provide a helpful tool not only to monitor the progress of the fibrotic process but also to gauge the amount of the ACA needed to block these molecules. Since frequent probing of the synovial space to assay newly-produced collagen I molecules within the joint cavity is associated with numerous risks, we analyzed the production of collagen I indirectly by measuring changes in the concentration of procollagen I propeptides present in serum (Fig. 7).

Collagen I molecules are secreted from cells as procollagen I containing the N propeptides and the C propeptides (Fig. 7) [10]. Upon secretion of procollagen I molecules from cells to the extracellular space, these propeptides are readily cleaved off to generate collagen I. Procollagen N proteinase and procollagen C proteinase catalyze this cleavage. Each of these enzymes recognizes a unique single site. Thus, following the cleavage, the removed propeptides remain primarily intact (Fig. 7). Consequently, procollagen propeptides are readily-detectable in serum, providing a practical, sensitive, and highly-specific method to detect changes in the content of propeptides in serum due to fibrosis [11].

Here, we applied commercially-available ELISA that targets the C propeptides of procollagen I. We applied this method to detect the C propeptides in serum samples collected periodically from the rabbits. We interpreted an increase in the concentration of the C propeptides as an indicator of enhanced production of new procollagen I in response to joint injury. We collected blood before performing knee surgery and treatment with the ACA to establish the baseline-levels of the analyzed parameters in each rabbit.

The above assays of collagen degradation expanded our understanding of the ACA-mediated mechanism of inhibiting fibrotic scarring of joint tissues. They helped to assess the efficacy of our antifibrotic approach. Moreover, we also measured serum hydroxyproline as a marker of total collagen degradation.

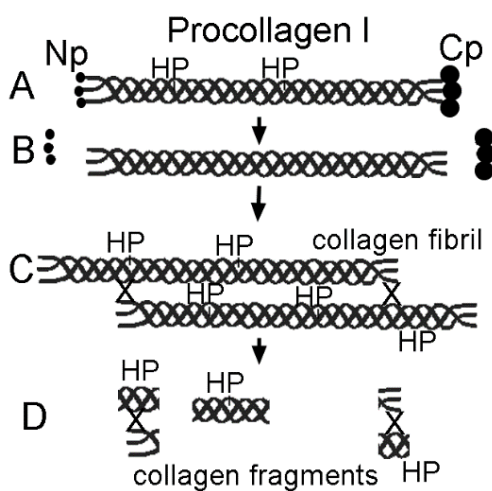


Figure 7. A schematic depicting the premises of assays of collagen fragments. A, A procollagen I molecule with intact N propeptides (Np) and C propeptides (Cp); hydroxyproline residues present in the triple-helical domain are also indicated (HP). B, a collagen I molecule generated by extracellular cleavage of the Np and the Cp; here, we will analyze the Cp of procollagen I. C, A typical staggered arrangement of collagen molecules incorporated into a fibril. Collagen cross-links that link these collagen molecules are indicated (X). D, Collagen fragments formed due to turnover/degradation of collagen fibrils. Note that these fragments will include HP, and some will retain collagen cross-links; these fragments will be specifically analyzed.

Results

A Rabbit Model of Arthrofibrosis

We performed 108 surgeries (K-wire installation and removal) on 54 rabbits. Overall, the surgeries were successful with no complications. However, two females (referred to as #1 and #2) died during pre-operative anesthesia. The third female (referred to as #3) broke the K-wire ten days after the first surgery. Subsequently, she died during applying anesthesia for the K-wire replacement surgery. Also, rabbit #3 demonstrated somewhat agitated behavior with signs of self-mutilation.

Histopathological evaluation of tissues and organs collected from these female rabbits was performed by IDEXX BioAnalytics (Columbia, MO, USA). Some underlying pathologies were noted. Female #1: heart: multifocal myocardial degeneration and fibrosis in the ventricles, hypertrophy of the tunica media, and reduced lumen diameter in one small cardiac artery; kidney: mild hyperplasia of the collecting duct epithelium; liver: small yellow-green nodules cytoplasmic granules in periportal hepatocytes. Female#2: lung: mildly underinflated, mild scattered alveolar macrophages, subpleural accumulation of heterophils with alveolar macrophages. Female #3: heart: degradation of cardiomyocytes with mild fibrosis and scattered mononuclear cell infiltrates; lung: mild to moderate heterophils within alveolar capillaries and more prominent vasculature. We observed no other physical or behavioral problems with the rest of the rabbits.

Blood Collection and Analysis

Blood samples were collected at defined time points, and their cellular and biochemical parameters were analyzed. Overall, the blood parameter values remained within the physiological ranges defined for the rabbits throughout the entire experiment (Appendix #2) [12].

Impact of the ACA on Tissues and Organs

Because of the dynamic exchange of macromolecules between the intraarticular space and the lymphatic and circulatory systems, we analyzed whether continuous administration of the ACA had any negative impact on distant tissues and organs. Histology of crucial tissues and organs collected from all rabbits treated with ACA for 8 weeks showed no structural abnormalities or excess inflammatory cells. Supplementary materials present histological images of assayed specimens from the rabbits treated with the ACA or control antibody at the highest concentration (Appendix #2).

Trajectories of Changes in the Concentration of the Serum Markers of Collagen Metabolism

We analyzed changes in the serum concentration of crucial collagen metabolites measured from the time of injury until the end of the 8-week antibody-application period. The serum concentration of hydroxyproline (HP), C-propeptide (CP), and cross-links (XL) at specific time points were analyzed by plotting the data, fitting linear curves, and comparing their slopes for the ACA group and the control (CA) group (Tab. 1). While the steep slopes (high numerical values) indicated relatively fast changes in measured parameters, more shallow slopes (low numerical values) signified relatively slow changes. Data indicate that the dynamics of changes in the HP and CP concentrations were similar in the ACA and control groups. In contrast, the rate of increase of the XL concentration in the ACA group was significantly faster than in the control group (Tab. 1).

Please note that the GMR/wk presented in Table 1 represents the geometric mean ratio per week, i.e., the estimated geometric mean (GM) today divided by the estimated GM a week ago. For instance, for XL (Tab. 1) measured for the 0-8-week period, that is 1.10 for CA and 1.41 for the ACA group, i.e., every week, XL increased on average by 10% and 41%, respectively. The effect of ACA treatment is $1.41/1.1 = 1.28$, i.e., the ACA-associated increase per week is ~28% larger than the increase per week in the control group.

Table 1. A summary of differences in the trajectories of changes in serum hydroxyproline (HP), the C-terminal propeptide of procollagen I (CP), and cross-linked telopeptides (XL) measured between the ACA group and the CA group during the 8-week treatment period.

Parameter	Group	^a GMR/wk (slope of lineary fitted curves)	The effect of ACA treatment (ACA/CA) with confidence levels and <i>p</i> values	
HP (µg/ml)	CA	1.01	ACA/CA	1.00
	ACA	1.01	95% CI ^b	(0.98,1.02)
			<i>p</i> = 0.892	
CP (ng/ml)	CA	1.04	ACA/CA	1.01
	ACA	1.05	95% CI	(0.97,1.05)
			<i>p</i> = 0.559	
XL (ng/ml)	CA	1.10	ACA/CA	1.28
	ACA	1.41	95% CI	(1.12,1.46)
			<i>p</i> = 0.001	

^aGMR/wk, geometric mean ratios; ^bCI, confidence interval

Changes in the Serum Collagen Markers Across Various Time Points

To analyze changes in HP, CP, and XL within the ACA group and within the CA group across specific time intervals, we used repeated-measures ANOVA (Fig. 8, Tab. 1&2). As demonstrated in Table 2, there were no significant changes in the CA-treated rabbits. In contrast, statistically significant changes were observed in all analyzed parameters in the ACA-treated animals (Tab. 2).

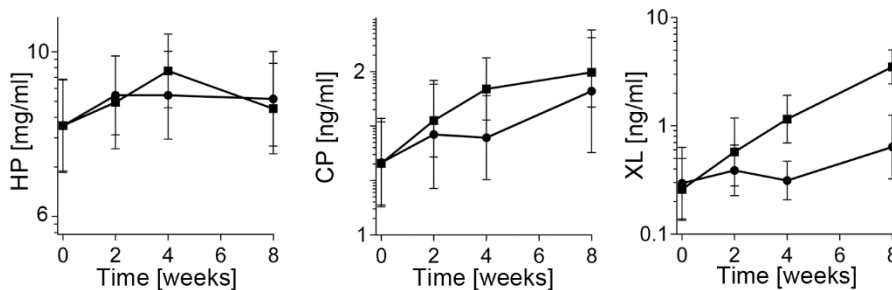


Figure 8. A graphic representation of the changes in the concentration of hydroxyproline (HP), the C-terminal propeptide (CP), and cross-linked telopeptides (XL) at indicated time points; the square symbols (■) represent the ACA-treated group, and the circle symbols (●) represent the control group. The geometric means (GMs) of analyzed parameters and 95% confidence intervals are presented.

represent the ACA-treated group, and the circle symbols (●) represent the control group. The geometric means (GMs) of analyzed parameters and 95% confidence intervals are presented.

Table 2. Significance of changes in serum concentrations of hydroxyproline (HP), the C-terminal propeptide of procollagen I (CP), and cross-linked telopeptides (XL). These changes were measured within the ACA and CA groups across various time points.

Marker	Treatment	Test within-subject effects (Greenhouse-Geisser)	Statistical significance of changes between specific time points (weeks)					
			0-2	0-4	0-8	2-4	2-8	4-8
HP	CA	$F(2.218, 28.837) = 3.085$, $p = 0.056$	n.s	n.s	n.s	n.s	n.s	n.s
	ACA	$F(1.840, 36.808) = 4.734$, $p = 0.017$	n.s	$p = 0.009$	n.s	n.s	n.s	$p = 0.038$
CP	CA	$F(1.436, 20.102) = 3.123$, $p = 0.08$	n.s	n.s	n.s	n.s	n.s	n.s
	ACA	$F(2.261, 42.966) = 12.439$, $p < 0.005$	$p = 0.039$	$p = 0.006$	$p = 0.002$	$p = 0.01$	n.s	n.s
XL	CA	$F(1.546, 13.914) = 1.844$, $p = 0.198$	n.s	n.s	n.s	n.s	n.s	n.s
	ACA	$F(2.148, 27.929) = 18.669$, $p < 0.005$	n.s	$p = 0.007$	$p < 0.005$	n.s	$p = 0.007$	n.s

n.s, not significant

Summary

In Major Task 2, we studied the potential side effects of the ACA treatment. Based on detailed biochemical and cellular blood parameters and histological assays, we determined the safety of the ACA at the highest concentration used in this study.

We also characterized crucial markers to follow changes in the biosynthesis of the collagen-rich matrix during the healing of injured joints.

Milestones Achieved

We determined authoritatively that the ACA offers a safe treatment option to reduce posttraumatic joint stiffness. This result provides a firm basis for future studies of the ACA utility in large animal models of joint injury. Furthermore, we established reliable tests to study the effects of the ACA treatment on the scar formation process.

Major Changes

No significant changes were introduced.

Technical Problems

No major technical problems were encountered.

For Specific Aim 2

To define the effects of the ACA on the healing of joint tissues.

Major Task 3

To determine the impact of the ACA treatment on the healing of joint tissues.

Subtask 1

Performed surgeries for rabbit-based contracture model.

Subtask 2

Biomechanical, biochemical, and histological assays of joint tissues. We studied the effects of the ACA applied to reduce post-traumatic joint contracture on the tendon, cartilage, and subchondral bone's biological, biochemical, and mechanical properties.

Subtask 3

Data analysis and statistical evaluation of results. Data were collected throughout the animal-based experiments and analyzed with the assistance of a statistician.

Major Activities

Since the normal healing process and excessive scar formation share the exact mechanisms, applying any anti-fibrotic intervention carries the risks of poor tissue repair. Consequently, in Major Task 3, we studied the impact of the ACA treatment on the healing of joint tissues, including PC, SB, and PT.

We used biomechanical, histological, spectroscopic, and micro-tomography assays to compare crucial properties of joint tissues from the ACA-treated and control groups.

Methods

Rabbit model of arthrofibrosis

We utilized a rabbit-based model of posttraumatic contracture (see above, Fig. 9). The rabbits were treated according to the following schematic:

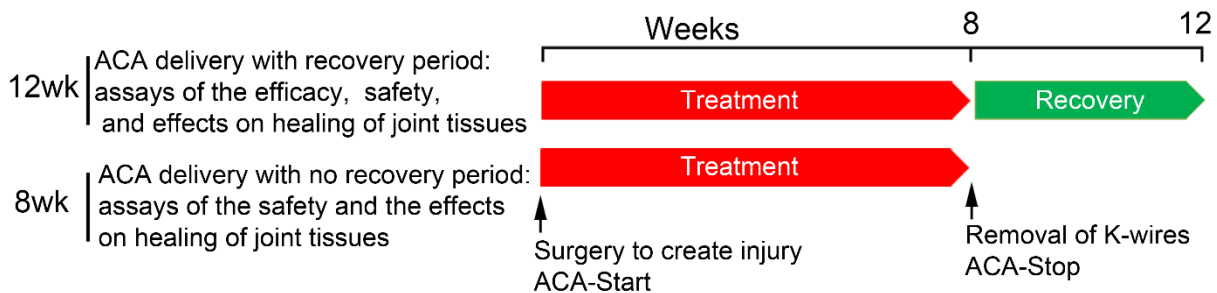


Figure 9. A schematic of the experimental design of the study.

Subsequently, the rabbits were euthanized and their joint tissues were studied in the context of healing quality.

Histological Assays of Collagen Fibrils

Since the scarring of the PCs is the main contributor to the knee stiffness in the rabbit model of arthrofibrosis employed here, the PCs were dissected and analyzed, as described [6, 7, 13]. In brief, the histological sections of the PCs were stained with collagen-specific picrosirius red dye. By combining this staining technique with polarized-light microscopy, we could approximate the thickness, organization, packing density, and to a certain degree, the collagen type-specific composition of the fibrils [6, 14-16]. Studies verified that as the thickness of fibers increases, their birefringence color changes under the polarized light microscope from green to yellow to orange to red, *i.e.*, from shorter to longer wavelengths [14, 17-20].

Employing a polarizing microscope (Eclipse LV100POL, Nikon Inc., Melville, NY) and the NIS Elements software (Nikon Inc.), the following groups of the birefringence colors were defined in captured images: (i) green birefringence (GB, identifies thin, loosely packed fibrils), (ii) yellow birefringence (YB, identifies intermediate-thickness fibrils), and (iii) red birefringence (RB, identifies thick, tightly packed fibrils) [6, 7].

All fibrils in captured viewing areas were analyzed, as described [6, 21]. In brief, the above birefringence colors were defined by applying the software's "color threshold" function. Subsequently, the software determined the areas occupied by pixels corresponding to the defined colors. Considering the sum of all pixels to be 100%, the percentage of each color group in the analyzed samples was calculated. A minimum of three histological

sections for control and three for injured PCs were analyzed per rabbit. The same threshold settings were applied to all images to ensure consistency in defining birefringence colors. Furthermore, automated calculations of all fibrils in the viewing areas eliminated potential bias. A one-way ANOVA was conducted to examine whether the percentage of subpopulations of collagen fibrils were different in the injured and uninjured capsules from the ACA-treated or the CA-treated groups (IBM SPSS Statistic for Windows, version 26, IBM Corp., Armonk, NY, USA).

Fourier Transformed Infrared Spectroscopy (FTIR)-based Assays of the Collagen Content and the Collagen Cross-Links

FTIR spectroscopy of tissue samples provides information about their composition and spatial distribution of analyzed macromolecules [22]. Here, we used this method to compare the relative collagen content in the scar tissues formed in the PCs and osteochondral defects (OCDs) of the ACA-treated and control rabbits. As references, we selected a protein-specific amide II peak and a peak corresponding to sulfated glycosaminoglycans (GAGs) (Tab. 3). Consequently, the sulfated GAGs peak/collagen peak ratios and amide II peak/collagen peak ratios were measured to determine the relative collagen content [23, 24]. Please note that the relation between the ratio values and collagen amount is negative.

We also analyzed the maturity of collagen cross-links by measuring the pyridinoline (PYR) peak/dehydro-dihydroxynorleucine (de-DHLNL) peak ratios [25, 26]. Hence, the maturity of collagen fibrils was defined as the relative content of the “mature” trivalent cross-links (PYR) vs. the “immature” divalent PYR precursor cross-links (de-DHLNL). Since the PYR/de-DHLNL ratio increases during the fibrotic process, assays of the cross-links provide a relevant parameter to evaluate the fibrotic status of collagen-rich deposits formed in the presence of the ACA [27-29].

All tissue samples were prepared as described earlier [30, 31]. In brief, paraffin-embedded 5- μ m thick tissue sections were deposited on the MirrIR low-e microscope slides (Kevley Technologies, Chesterland, OH). An FTIR spectrometer (Spotlight 400, Perkin Elmer, Waltman, MA) was used to analyze the regions of interest (ROIs) corresponding to the PC collagen-rich areas. The measurements were done in the 4000 cm^{-1} to 748 cm^{-1} wavenumbers spectral range, at a pixel resolution of 50 μ m, 8 scans per pixel, and a spectral resolution of 4 cm^{-1} . The Spectrum Image software generated co-added spectra from scanned ROIs (PerkinElmer, Inc.).

In all assays of the FTIR-derived spectra, overlapping peaks were deconvoluted and analyzed based on the second-order derivative spectra and pre-determined bell-type Gaussian peak fitting function using the OriginPro software (version 2021, OriginLab Corporation, Northampton, MA USA) (Fig. 10) [32, 33].

Table 3. Fourier-transform infrared (FTIR) spectroscopy parameters applied in collagen assays.

Target	Band position [cm^{-1}]	Bond vibrations	Purpose
Sulfated GAGs	1064	SO ⁻³ vibration	To provide a reference for collagen changes
Amide II	1549	N-H bending and the C-N stretching vibration	To provide a total protein reference for collagen changes
Collagen	1338	CH ₂ wagging vibration of proline side chains	To measure relative changes in collagen content
PYR	1660	A constituent of amide I peak	To measure mature pyridinoline cross-links
de-DHLNL	1690	A constituent of amide I peak	To measure immature dehydro-dihydroxynorleucine cross-links, an immature PYR precursor.

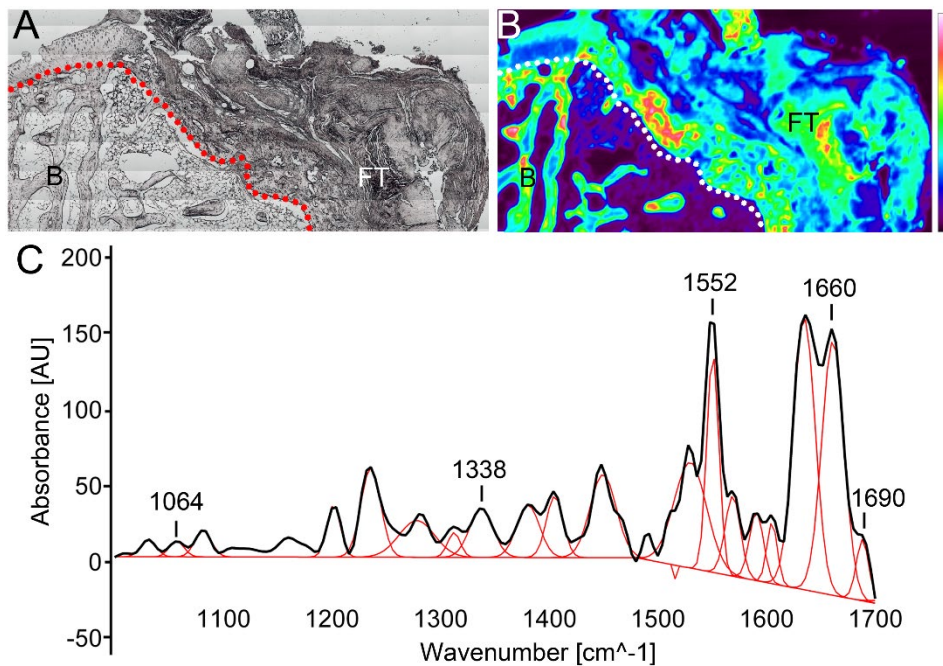


Figure 10. FTIR spectroscopy of the pannus-like scar tissue formed within OCDs. A, A microscopic image of a selected region of interest (ROI). A dotted line marks the border between the bone (B) and fibrotic pannus-like tissue (FT). B, A corresponding map of the average intensity of the FTIR signals. C, A representative FTIR spectrum (black line) with fitted peaks (red line). The peaks utilized in this study are indicated.

Assays of the Flexion Contracture

Assays of knee flexion contracture provide an overall readout for the ACA efficacy in reducing joint stiffness [6, 7]. These assays were done according to protocols developed for the model employed here using a custom-made instrument (Fig. 11) [4, 6, 7]. In brief, the injured or the contralateral uninjured leg was fixed in the instrument with the tibia and femur positioned at the right angle. This position was considered the starting point of zero degrees. Subsequently, applying the rate of loading set to 40°/min, an extension torque was applied to 0.2 Nm. Upon reaching the value of 0.2 Nm, the angle corresponding to the joint extension was recorded. (Fig. 11). The injured knee flexion contracture was then expressed as the uninjured control knee extension/the injured contralateral knee extension ratio [4, 6, 7]. Hence, relatively low ratio values indicated relatively mild flexion contracture, while large ratio values indicated relatively severe flexion contracture.

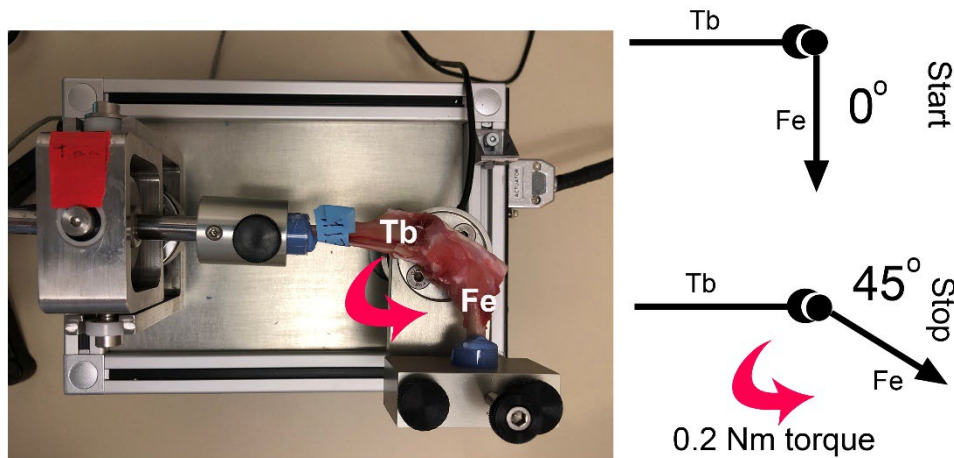


Figure 11. A custom-made device to measure the flexion contracture of the knee joints. Clamping of the femur (Fe) and tibia (Tb) is indicated. The arrow shows the movement of the femur.

Our main interest was evaluating the 12wk group that included the recovery period [6, 7]. The extent of the joint stiffness measured after the recovery period reflects the extent of the PC scarring due to injury. In contrast, the stiffness measured right after 8 weeks of immobilization results from the scarring of injured PCs and changes occurring strictly due to joint immobilization.

Assays of the Quality of Healed PTs

Because of the complexity of knee injury created here to cause posttraumatic joint stiffness, standardization of a model wound to study the ACA's impact on the quality of neotissue formed during the healing process is challenging. To circumvent this problem, we generated a uniform defect in the PTs to evaluate the healing quality by measuring the neotissue's mechanical properties.

Biomechanical assays of the neo-patellar tendons from the ACA-treated and the CA-treated rabbits were performed as described [34]. In brief, following the flexion contracture measurements and PC isolation, the remaining soft tissues were dissected, leaving a PT-tibia complex (Fig. 12). The neo-patellar tendon formed during the healing phase was isolated from the bulk of the remaining medial and lateral portions of the PT. A corresponding central portion of a PT from an uninjured leg was also prepared as a control.

The tensile behavior of the patellar tendon complexes from the ACA-treated and CA-treated rabbits was analyzed, as described [34]. First, the patellar and tibial ends of the tendon complex were secured in custom fixtures (Fig. 12). Next, tensile testing was performed using a material testing system (TA Instruments ElectroForce 3220 Series III) equipped with a 225 N load cell. The samples were pre-loaded (0.3 N), then conditioned for 10 cycles from 0 to 0.5 mm displacement at an elongation rate of 0.2 mm/s, similar to previous work [35, 36]. Finally, the tendons were loaded to failure using a displacement ramp with an elongation rate of 0.2 mm/s. Force and displacement were captured digitally, then converted to engineering stress and strain for analysis using custom software (GNU Octave). Outcomes included ultimate stress and Young's modulus.

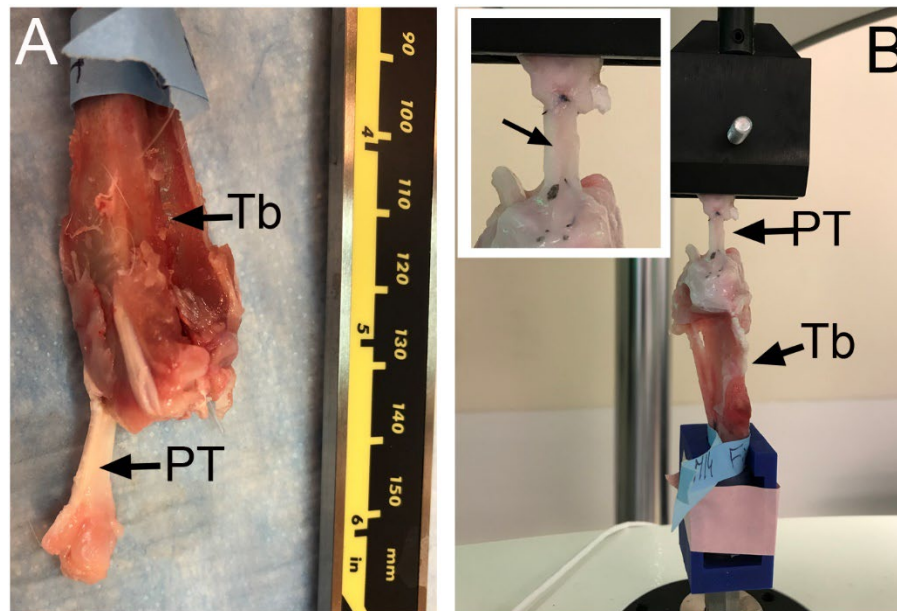


Figure 12. A. Isolation of the patellar tendon (PT)-tibia (Tb) complex. B. A setup of the complex seen in A for the mechanical tests. The insert depicts the PT in which the arrow indicates the healed defect created during the initial surgery.

Assays of OCD Mineralization and Scarring

We also studied the impact of the ACA on the healing of subchondral bone by measuring the calcification of the OCDs with a micro-computer tomography (μ CT) scanner (SkyScan, Bruker Inc., Carteret, NJ, USA), as

described [37]. Following the reconstruction of μ CT images, volumes of interest (VOIs) were selected in regions identified as the injury sites (Fig. 13). Corresponding VOIs from contralateral uninjured legs were also analyzed. Our primary focus was on the Bv/Tv (percent of bone volume to the total analyzed volume) and the BMD (bone mineral density).

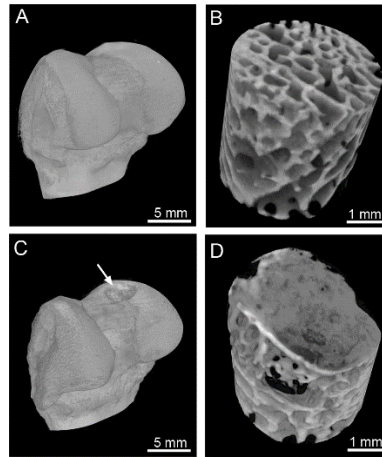


Figure 13. Representative computer tomography images of the uninjured (A) and injured (C, arrow) bones. Panels B (uninjured bone) and D (injured bone) indicate subchondral bone regions analyzed here to determine the extent of mineralization in the injured area (C&D).

Histology of New Soft Tissue Formed Within the OCDs

Following μ CT, we isolated the tissue plugs encompassing the OCDs (Fig. 14). The plugs were decalcified, processed for histology, then stained with hematoxylin and eosin (H&E) for general morphology and cellularity. The corresponding samples were also stained with picrosirius red to analyze the collagen-rich matrix of the newly formed tissue, as described above for the PC samples.

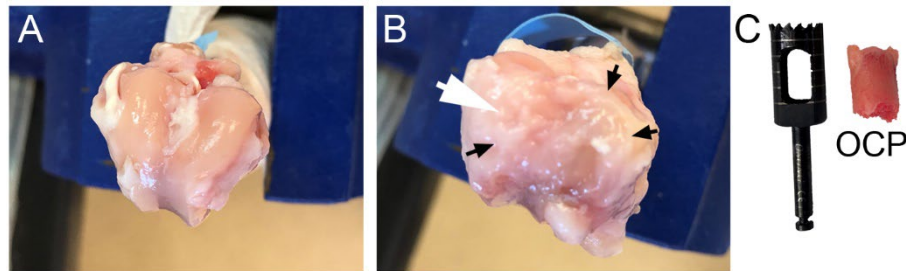


Figure 14. The appearance of the surface of uninjured (A) and injured (B) femoral condyles after twelve weeks of healing. The white arrow in B indicates the site of the osteochondral defect (OCD) made during the initial surgery. Please note the presence of the pannus-like tissue (black arrows) covering the femoral surface of the injured leg (B). C, An osteochondral plug (OCP) that encompasses the site of injury seen in panel B. A tool used to remove the plug is also indicated.

Data analysis

Due to the skewness of data, we applied the log transformation to minimize the skewness's impact on statistical analyses. Consequently, results of assays of the serum markers of collagen metabolism, joint tissues' mechanical properties, and FTIR-based analyses were expressed as geometric means (GM) and geometric mean ratios (GMR) instead of arithmetic means and mean differences.

Changes in the HP, CP, and XL concentrations in the sera of the ACA-treated group were compared to the CA-treated control. Linear fitted curves were generated to determine the kinetics of changes from time 0 to week

8, a period of the ACA administration. Subsequently, the curves' trajectories for the ACA and control groups were analyzed by comparing their slopes.

Furthermore, to analyze the HP, CP, and XL concentration changes across various time points within an analyzed group, we employed a within-group repeated-measures ANOVA with a Greenhouse-Geisser correction (IBM SPSS Statistics v. 26).

In all assays, statistical significance was defined as $p \leq 0.05$.

For the flexion contracture, we formulated the superiority hypothesis. We expected that the injured limb in the ACA-treated group would show reduced flexion contracture compared to the injured limb in the control group:

$$\mathbf{H1: \mu_1[ACA: injured] \neq \mu_0[CTR: injured]}$$

For the analyses, we estimated the mean difference between the ACA-treated and control groups ($D = \mu_1 - \mu_0$), along with the corresponding 2-sided 95% confidence limit and the p-value.

Also, it is often appropriate to test if a new experimental treatment is not unacceptably less efficacious than a control treatment. Hence, to study the effects of the ACA on the PT mechanical properties and calcification of the OCDs, we formulated the noninferiority hypothesis that the ACA treatment is not substantially worse than a control treatment. We computed the difference between each animal's injured and contralateral non-injured limbs. We then compared the average of this difference for the ACA-treated animals and the CA-treated control.

Regardless of the treatment, we expected the injured limb's values to be much lower than those for the uninjured leg. We aimed to show that this drop (from uninjured to injured limb) in the ACA-treated group is not meaningfully worse than in the control group (by a pre-specified margin):

$$\mathbf{H1: \mu_1[ACA: uninjured-injured] < M * \mu_0[CTR: uninjured-injured] \Rightarrow M * \mu_0 - \mu_1 > 0}$$

Here, M is the noninferiority margin. A relative margin of 20-33% (i.e., $M = 1.20$ to 1.33) is often appropriate since it is neither too broad (in which case it would be not very meaningful) nor too strict (in which case the study would require a considerable sample size). For example, a margin of 1.25 indicates that the worst-case scenario allowable for the ACA-treated group is an additional 25% beyond the decrease seen in the control group (always considering the difference between uninjured and injured limb).

Taking Young's modulus (MPa) of the PT as an example derived from a published similar rabbit model, if the mean in the control group is 800 (MPa) for the uninjured PT and 300 (MPa) for the injured PT, the mean difference in this group is 500 [8]. A margin of 25% means that we want to establish that the corresponding actual mean difference between uninjured and injured PTs in the treated group is no bigger than 625 ($1.25 * 500$), e.g., the mean of 800 in the uninjured PT vs. 175 or larger in the injured PT (and hence a difference of 625 or less).

For the analyses, we computed the quantity $D = M * \mu_0 - \mu_1$, along with the corresponding 1-sided (upper) 95% confidence limit and 1-tailed p-value (the non-inferiority hypothesis is inherently 1-sided).

Results

Collagen Matrix Formed in the PCs

We also examined the effects of the ACA and CA on the percentages of the green-birefringence (GB), the yellow-birefringence (YB), and the red-birefringence (RB) subpopulations of collagen fibrils present in the uninjured and injured PCs (Fig. 15). Table 4 summarizes the statistical significance of observed differences between analyzed groups.

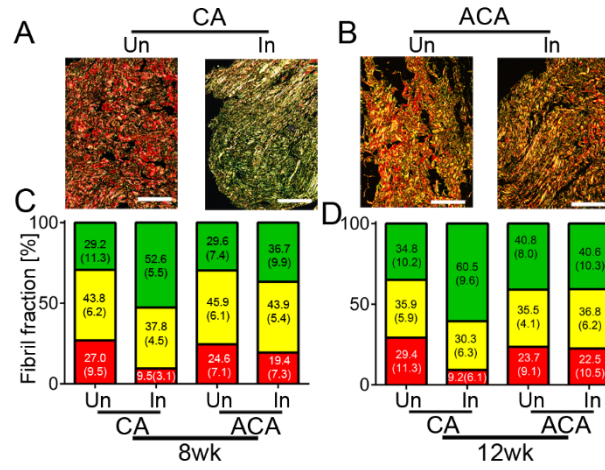


Figure 15. Histological quantification of various populations of picrosirius-stained collagen fibrils formed within injured posterior capsules (PCs). A, Samples of the PCs from uninjured (Un) and injured (In) knee joints of rabbits treated with control antibody (CA). B, Samples of the PCs from uninjured (Un) and injured (In) knee joints of the rabbits treated with the ACA. C&D, Corresponding graphs depict the percentages, with standard deviations in parentheses, of the green birefringence (GB), yellow birefringence (YB), and red birefringence (RB) subpopulations of collagen fibrils observed with the use of a polarized light microscope in the 8wk and 12wk groups. Bars = 100 μ m.

Table 4. Analysis of differences between specific groups of collagen fibrils, defined by specific birefringence, observed in uninjured and injured posterior capsules (PCs) isolated from the ACA-treated or control rabbits.

Group	Treatment	Fibril subpopulation		
		^a GB	^a YB	^a RB
8wk	CA	$F(1, 10) = 20.7$, $p = 0.001$	$F(1, 10) = 3.6$, $p = 0.088$	$F(1, 10) = 18.1$, $p = 0.002$
	ACA	$F(1, 14) = 2.6$, $p = 0.129$	$F(1, 14) = 0.447$, $p = 0.514$	$F(1, 14) = 2.05$, $p = 0.175$
12wk	CA	$F(1, 20) = 37.0$, $p < 0.0005$	$F(1, 20) = 4.5$, $p = 0.046$	$F(1, 20) = 27.1$, $p < 0.0005$
	ACA	$F(1, 26) = 0.004$, $p = 0.949$	$F(1, 26) = 0.459$, $p = 0.504$	$F(1, 26) = 0.091$, $p = 0.765$

^aGB, green birefringence (represents thin, loosely-packed fibrils); YB, yellow birefringence (represents intermediate-diameter fibrils); RB, red birefringence (represents thick, well-packed fibrils).

Collagen Matrix Formed in the OCDs

We also analyzed the ACA impact on the fibrillar composition of the pannus-like tissue formed around and within OCDs (Fig. 16). As adequate controls from the contralateral joints' uninjured sites are innately unavailable, we compared pannus-like tissues formed in the ACA and control groups. As indicated in Table 5, there were no statistically significant differences in the percentages of specific populations of fibrils present in the ACA-treated and CA-treated groups.

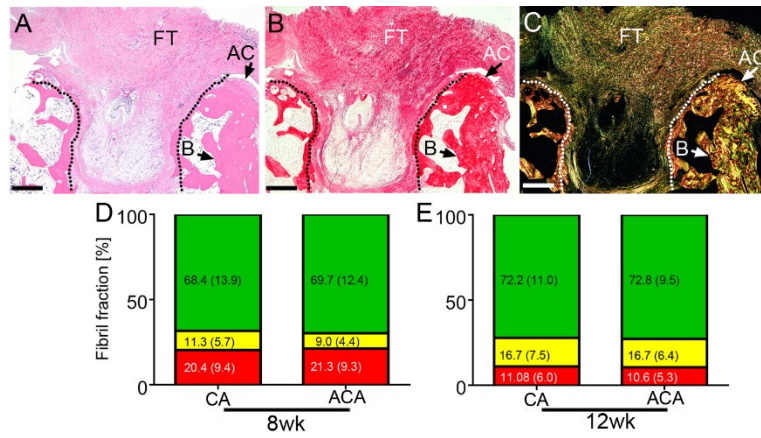


Figure 16. A histological assay of the pannus-like scar tissue formed in an osteochondral defect (delineated). The injury site was stained with H&E (A) and collagen-specific picrosirius red (B&C). The picrosirius red-stained samples were observed in normal light (B) and polarized light (C). Fibrotic tissue (FT), bone (B), and fragments of the articular cartilage (AC) are indicated. D&E, A summary of measurement of the percentages of the green birefringence (GB) fibrils, yellow birefringence (YB) fibrils, and red birefringence (RB) fibrils formed in the CA-treated and the ACA-treated rabbits. Corresponding segments of the bars include the means and standard deviations (in parentheses). Data for the 8wk and the 12wk groups are presented. Bars = 1 mm.

Table 5. Analysis of differences between the content of specific groups of collagen fibrils defined by the green (GB), yellow (YB), and red (RB) birefringence. The fibrils were observed in the osteochondral defects in the CA-treated and the ACA-treated rabbits.

Group	Fibril subpopulation		
	^a GB	^a YB	^a RB
8wk	$F(1, 48) = 0.12$, $p = 0.731$	$F(1, 48) = 2.3$, $p = 0.137$	$F(1, 48) = 0.117$, $p = 0.733$
12wk	$F(1, 142) = 0.102$, $p = 0.75$	$F(1, 142) = 0.001$, $p = 0.979$	$F(1, 142) = 0.3$, $p = 0.585$

^aGB, green birefringence (represents thin, loosely-packed fibrils); YB, yellow birefringence (represents intermediate-diameter fibrils); RB, red birefringence (represents thick, well-packed fibrils)

FTIR Assays of the Collagen Content in the PC and OCD Scars

We compared the relative collagen content in the PC scars formed in the presence of the ACA or CA. The amount of collagen was calculated concerning sulfated GAGs and the total protein content represented by the FTIR spectra amide II peak (Fig. 17). Similar assays were done for the OCD scars formed in the ACA-treated and CA-treated rabbits (Fig. 18). Table 6 summarizes tests to determine the statistical significance of observed differences between the analyzed groups.

FTIR Assays of Collagen Cross-Links in the PC and OCD Scars

We also measured the maturity of collagen cross-links formed in the PC scar tissue in the presence of the ACA or CA. The maturity was expressed as the ratio of the FTIR-derived peak representing the mature PYR cross-link and immature de-DHLNL cross-link (Fig. 17) [25]. Similar assays were done for the OCD scars formed in the ACA-treated and CA-treated rabbits (Fig. 18). Table 6 summarizes assays to determine the statistical significance of observed differences between the analyzed groups.

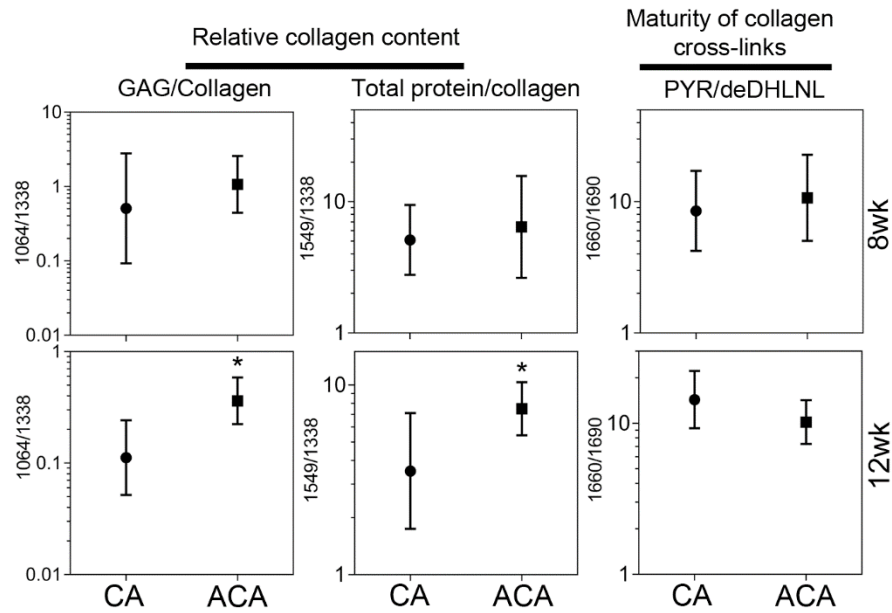


Figure 17. A graphic representation of the FTIR-based assays of the relative amount of collagen and the maturity of collagen cross-links in the scar tissue formed within injured posterior capsules of control (CA) and the ACA-treated rabbits. Results for the 8wk and the 12wk groups are presented. The graphs show geometric means (GMs) with 95% confidence intervals. Asterisks indicate groups with statistically significant differences of the means. The graph shows the relative collagen content calculated as the sulfated glycosaminoglycans (GAGs)/collagen and the total protein/collagen ratios. Consequently, the smaller the ratios, the higher the relative collagen content. The ratios were calculated based on the areas of the spectral peak corresponding to the sulfated GAGs (centered around 1064 cm^{-1}) and the spectral peak corresponding to collagen (centered around 1338 cm^{-1}). To corroborate these measurements, the relative amount of collagen was also calculated based on the ratios of the spectral peak corresponding to the total proteins, represented by the amide II peak (centered around 1549 cm^{-1}) and the spectral peak corresponding to collagen. The maturity of collagen cross-links was expressed as the ratio of the area of the spectral peak corresponding to the mature trivalent PYR cross-link (centered around 1660 cm^{-1}) and immature divalent deDHLNL cross-link (centered around 1690 cm^{-1}). Hence, the higher the PYR/deDHLNL ratio, the more mature collagen cross-links. Also, see Appendix #2.

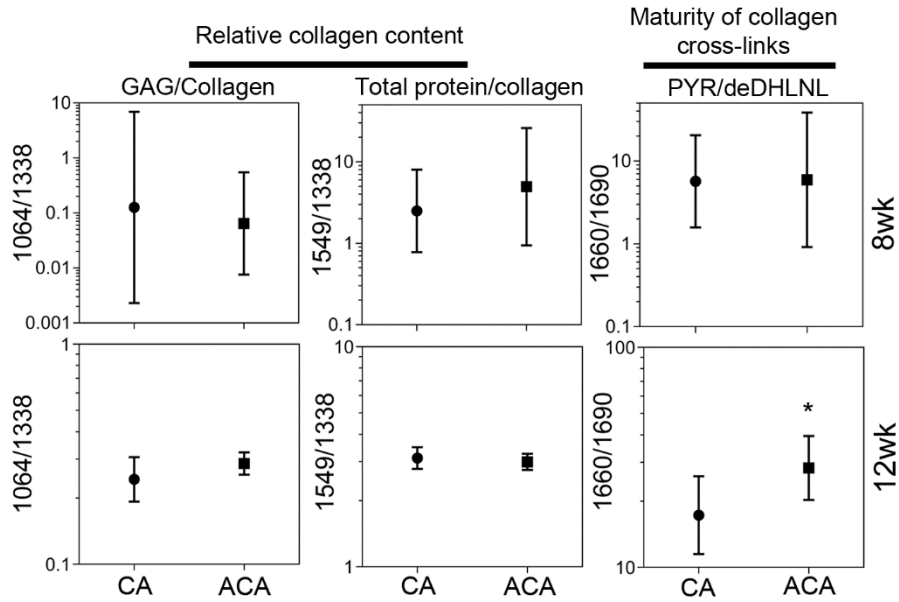


Figure 18. A graphic representation of the FTIR-based assays of the relative amount of collagen and the maturity of collagen cross-links in the scar tissue formed in the OCDs of the CA-treated and the ACA-treated rabbits. Results for the 8wk and the 12wk groups are presented. The graphs present GMs with 95% confidence intervals.

Asterisks indicate groups with statistically significant differences in means. The graph shows the relative collagen content calculated as the GAG/collagen and the total protein/collagen ratios. Hence, the smaller the ratios, the higher the relative collagen content. The ratios were calculated based on the areas of the spectral peak corresponding to the sulfated GAGs (centered around 1064 cm⁻¹) and the spectral peak corresponding to collagen (centered around 1338 cm⁻¹). To corroborate these measurements, the relative amount of collagen was also calculated based on the ratios of the spectral peak corresponding to the total proteins, represented by the amide II peak (centered around 1549 cm⁻¹) and the spectral peak corresponding to collagen. The maturity of collagen cross-links was expressed as the ratio of the area of the spectral peak corresponding to the mature trivalent PYR cross-link (centered around 1660 cm⁻¹) and immature divalent deDHLNL cross-link (centered around 1690 cm⁻¹). Thus, the higher the PYR/deDHLNL ratio, the more mature the collagen cross-links. See Appendix #2.

Table 6. Analysis of differences between relative collagen contents and the maturity of collagen-cross-links in scar tissues formed in the PCs and osteochondral defects (OCD) in the CA-treated ACA-treated rabbits.

Scar tissue	Group	FTIR ratios		
		1064 cm ⁻¹ /1338 cm ⁻¹ GAGs/collagen	1549 cm ⁻¹ /1338 cm ⁻¹ Amide II/collagen	1660 cm ⁻¹ /1690 cm ⁻¹ PYR/de-DHLNL
PC	8wk	$F(1, 9) = 0.02$, $p = 0.892$	$F(1, 9) = 0.159$, $p = 0.7$	$F(1, 9) = 0.003$, $p = 0.956$
	12wk	$F(1, 15) = 8.156$, $p = 0.012$	$F(1, 15) = 10.063$, $p = 0.006$	$F(1, 15) = 0.035$, $p = 0.854$
OCD	8wk	$F(1, 5) = 0.276$, $p = 0.621$	$F(1, 5) = 0.281$, $p = 0.619$	$F(1, 5) = 0.002$, $p = 0.965$
	12wk	$F(1, 22) = 1.043$, $p = 0.318$	$F(1, 22) = 0.407$, $p = 0.53$	$F(1, 22) = 4.331$, $p = 0.04$

Assays of the Flexion Contracture

Since the ultimate purpose of the anti-fibrotic treatment with the ACA is to reduce scarring-associated stiffness in the injured joints, we measured the extent of flexion contracture in the ACA-treated and control rabbits. Specifically focusing on the 12wk group, we performed the superiority test (see Data analysis above) to analyze the ACA impact on the knee joint flexion contracture (Tab. 7, Fig. 19). Our results indicate that the ACA effect is: $2.84/4.47 = 0.64$, and the 95% CI is 0.41, 0.99, $p = 0.047$. Thus, compared to the control, the ACA-treatment reduced the uninjured/injured ratio (contraction) or the gap between the flexion contractures measured in the uninjured and the injured knees; the reduction was significant ($p = 0.047$). In contrast, the 8wk group did not pass the superiority test. The ACA effect for this group was $9.82/8.6 = 1.14$, and 95% CI 0.63, 2.06, $p = 0.647$. (Tab. 7, Fig. 20).

Table 7. Summaries of tests to determine the ACA's effects on the flexion contracture and parameters defining the quality of healing of the PTs (stress and Young's modulus) and subchondral bone defects (Bv/Tv and BMD)^a.

Parameter	Duration	Group	GM	GM	GMR	The effect of ACA treatment (ACA/CA) with confidence levels and p values	
			Uninjured	Injured	Uni/Inj		
Stress (MPa)	12wk	CA	12.6	7.9	1.61	ACA/CA	0.98
		ACA	13.5	8.6	1.57	95% UCL	1.35
						$p = 0.103$	
	8wk	CA	15.7	3.9	4.03	ACA/CA	0.79
		ACA	13.5	4.2	3.20	95% UCL	1.21
						$p = 0.040$	
Young's modulus (MPa)	12wk	CA	134.8	53.5	2.52	ACA/CA	1.02
		ACA	163.0	63.2	2.58	95% UCL	1.81
						$p = 0.276$	

	8wk	CA	258.8	32.6	7.94	ACA/CA	0.81
		ACA	233.7	36.4	6.41	95% UCL	1.69
							$p = 0.161$
Extension at 0.2 Nm (deg)	12wk	CA	74.5	16.7	^b 4.47	ACA/CA	0.64
		ACA	75.7	26.6	^b 2.84	95% CI	(0.41, 0.99)
							$p = 0.047$
	8wk	CA	65.0	7.6	^b 8.60	ACA/CA	1.14
		ACA	65.8	6.7	^b 9.82	95% CI	(0.63, 2.06)
							$p = 0.647$
Bv/Tv (%)	12wk	CA	41.6	23.7	1.76	ACA/CA	0.97
		ACA	43.0	25.3	1.70	95% UCL	1.51
							$p = 0.167$
	8wk	CA	51.4	15.0	3.43	ACA/CA	1.11
		ACA	50.2	13.2	3.80	95% UCL	2.01
							$p = 0.366$
BMD [g/cm ³]	12wk	CA	0.70	0.45	1.55	ACA/CA	1.38
		ACA	0.68	0.32	2.14	95% UCL	2.27
							$p = 0.629$
	8wk	CA	1.08	0.47	2.30	ACA/CA	0.85
		ACA	0.87	0.44	1.95	95% UCL	1.66
							$p = 0.168$

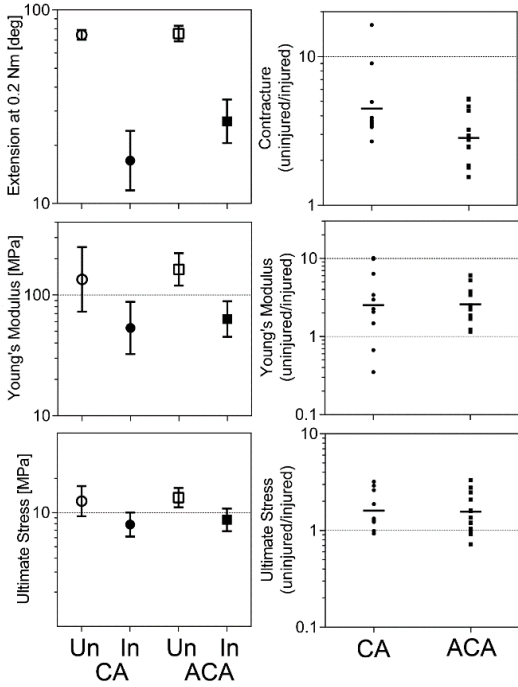
^a Presented parameters were measured in uninjured and injured legs of rabbits from the CA-treated and ACA-treated groups (GMs are presented). Within each group, potential changes of these parameters in the injured legs were expressed as the ratios of values calculated for the uninjured and the injured legs (GMR). Subsequently, the effect of the ACA on these ratios was evaluated (ACA/CA) and presented with p values.

^b Joint contracture values are defined as the ratio of knee extension of uninjured joint and that of the contralateral injured joint. Please note that the starting point to measure knee extension was positioning the femur and the tibia at the right angle (Fig. 11). At this starting position, the value of extension was set to 0°. Subsequently, the torque was applied to the value of 0.2Nm, and the extension angle (deg) was recorded. Non-stiff knees (uninjured) reached relatively high extension values, while stiff (injured) knees reached relatively low extension values. Hence, large uninjured/injured ratios of measured extensions indicate a relatively large contracture. In contrast, small uninjured/injured ratios indicate a relatively small contracture.

Impact of the ACA on the Quality of Healing of the PTs

We also measured the PT mechanical properties to determine their healing quality in the presence or absence of the ACA. Focusing on the PT ultimate stresses from the 12wk group as an example, we found, in brief, that the GMs for the ACA and CA groups' uninjured PTs were ~13 MPa, indicating proper randomization (Tab. 7, Fig. 19). In contrast, the GMs for injured PTs in both groups had lower values. The GM dropped from 12.6 to 7.9 in the CA group and from 13.5 to 8.6 in the ACA group. The corresponding GMRs were $12.6/7.9 = 1.61$ (CA) and $13.5/8.6 = 1.57$ (ACA) (Tab. 7).

Figure 19. A graphic representation of differences in mechanical parameters of the knee joints and PTs in the ACA-treated (□ and ■) and the CA-treated (○ and ●) rabbits from the 12wk group. The left column shows the results of measurements of uninjured (Un) and injured (In) legs. The geometric means (GMs) and 95% confidence intervals are indicated. The right panels show the Un/In ratios of analyzed parameters.



Considering the ratios for the CA group (1.61) and ACA group (1.57), in the CA group, the GM of the uninjured PT was 61% higher, on average, than the GM of the injured one. In the ACA group, the GM of the injured PT was 57% higher, on average, than the GM of the injured one. Thus, it seems that the ACA treatment reduced the "gap" between uninjured and injured tendons from 1.61 to 1.57. The formal treatment effect is $1.57/1.61 = 0.98$ (the smaller the value, the more significant the effect).

According to the hypothesis we presented in our original pre-experimental plan (see Data analysis), we are willing to accept up to 1.25 (25% increase of the "gap"). To formally test this non-inferiority hypothesis, we put a 95% one-sided (upper) confidence limit to the 0.98 value, resulting in a formal treatment effect of 1.35. So, even though our best estimate is that the ACA slightly reduces the uninjured-to-injured difference, we cannot exclude the possibility that it increases it up to 35%. Therefore, we have not proven non-inferiority (the associated p -value is 0.103, (Tab. 7). We reached a similar conclusion for the measurements of Young's modulus of the PTs from the 12wk group (Tab. 7, Fig. 19).

For the 8wk group (Tab. 7, Fig. 20), we have proven non-inferiority for the ultimate stress parameter ($p = 0.04$). Young's modulus did not pass the non-inferiority test ($p = 0.161$).

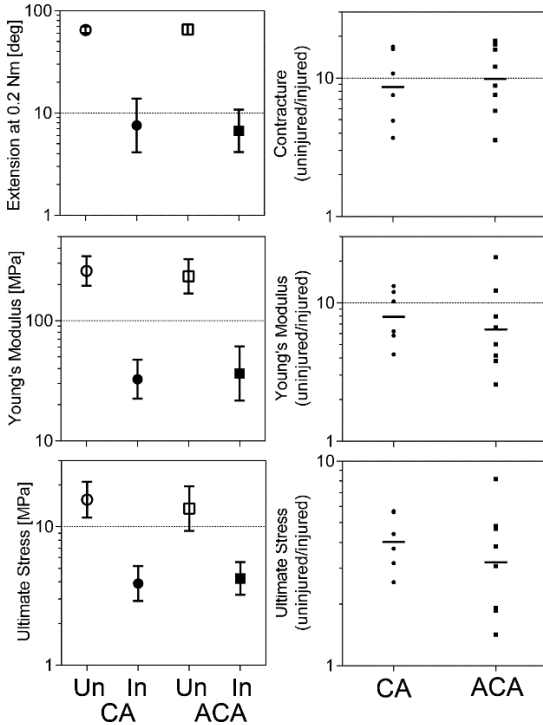


Figure 20. A graphic representation of differences in mechanical parameters of the knee joints and PTs in the ACA-treated ($\square$ and $\blacksquare$) and the CA-treated ($\circ$ and $\bullet$) rabbits from the 8wk group. The left column shows the results of measurements of uninjured (Un) and injured (In) legs. The GMs and 95% confidence intervals are indicated. The right panels show the Un/In ratios of analyzed parameters.

Biom mineralization in the Presence of the ACA

We also performed the non-inferiority test to compare the mineralization of subchondral bone in the ACA group versus the CA group. Assays of the Bv/Tv and the BMD parameters in the OCDs showed a drop in mineralization in both the ACA and CA groups (Tab. 7, Fig. 21 and 22). The Bv/Tv and BMD values for the ACA and CA groups' uninjured bones were similar, indicating good randomization. The ratios of parameters measured in the uninjured and injured sites were reasonably similar (Tab. 7, Fig. 21 and 22). Despite the close similarities, both Bv/Tv and BMD measured for the ACA group did not pass the non-inferiority tests (Tab. 7).

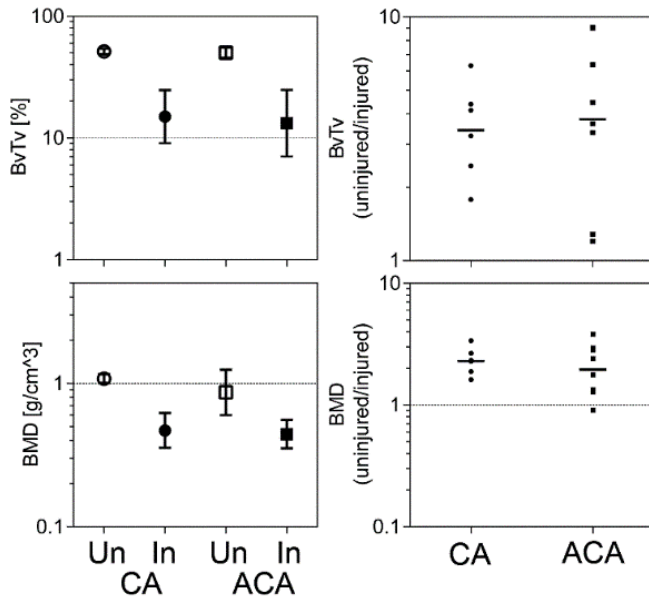


Figure 21. A graphic representation of differences in mineralization of osteochondral defects in the ACA-treated (□ and ■) and the CA-treated (○ and ●) rabbits from the 8wk group. The left column shows the results of measurements of uninjured (Un) and injured (In) legs. The GMs and 95% confidence intervals are indicated. The right panels show the Un/In ratios of analyzed parameters.

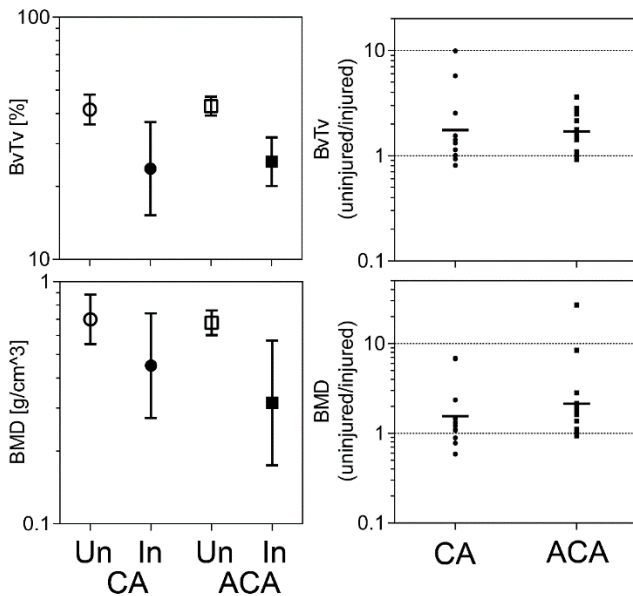


Figure 22. A graphic representation of differences in mineralization of osteochondral defects in the ACA-treated (□ and ■) and the CA-treated (○ and ●) rabbits from the 12wk group. The left column shows the results of measurements of uninjured (Un) and injured (In) legs. The GMs and 95% confidence intervals are indicated. The right panels show the Un/In ratios of analyzed parameters.

Summary

Here, we have utilized a clinically relevant arthrofibrosis model to study the broad mechanisms of the anti-scarring activity of this antibody. Moreover, we analyzed the effects of targeting collagen fibril formation on the quality of healed joint tissues, including the posterior capsule, patellar tendon, and subchondral bone. Our results show that blocking collagen fibrillogenesis reduces collagen content in the scar and accelerates the remodeling of healing tissues, and changes the collagen fibrils' cross-linking. In total, this study demonstrated that targeting

collagen fibrillogenesis to limit arthrofibrosis affects neither the quality of healing of the joint tissues nor disturbs vital tissues and organs.

Milestones Achieved

At this stage, we have established the efficacy of the method to limit post-traumatic joint stiffness without altering the healing process. All experimental conditions and procedures now position our technology for preclinical tests in large animals and future clinical tests. A manuscript describing key findings has been published.

Major Changes

No significant changes were made.

Technical Problems

No major technical problems were encountered.

Opportunities for training and professional development

This project offered great opportunities for training and professional development. Crucial opportunities include: (i) integrating basic scientists and clinicians to form a cohesive team focusing on posttraumatic arthrofibrosis, (ii) training participants in utilizing the FTIR technology to analyze chemical changes in analyzed tissues, (iii) integrating extracellular matrix biologists with the biomechanical research team to develop tests that allow monitoring the ACA-based treatment on crucial mechanical properties of joint tissues, and (iv) integrating veterinary and orthopaedic teams to create and master surgical techniques needed to study posttraumatic arthrofibrosis in the clinically-relevant environment.

Results dissemination to communities of interest

Results of this study were published in a peer-reviewed journal: Steplewski A, Fertala J, Tomlinson RE, Wang ML, Donahue A, Arnold WV, Rivlin M, Beredjiklian PK, Abboud JA, Namdari S, Fertala A: Mechanisms of reducing joint stiffness by blocking collagen fibrillogenesis in a rabbit model of posttraumatic arthrofibrosis. PLoS One 2021, 16:e0257147.

Moreover, elements of this study are a part of a chapter:

“Beyond Drugs and Surgery: A Look at Orthobiologics” will be published in the book entitled: “Translational Sports Medicine” (accepted in press).

During this project, in-person scientific conferences were eliminated mainly due to the COVID-19 pandemic.

Plans for the next reporting period

Nothing to report (This is the Final Report).

4. IMPACT

The Impact on the Development of the Principal Discipline(s) of the Project

We successfully tested *in vivo* the validity of the concept that blocking the formation of fibrotic scars limits posttraumatic joint stiffness. Specifically, we rationally engineered a therapeutic anti-collagen I antibody (referred to as ACA) that targets collagen I molecules and prevents their aggregation into scar tissue. Our research has validated our anti-fibrotic approach in a relevant animal model of posttraumatic joint stiffness. Consequently, we have moved our research from the proof-of-concept phase closer to clinical tests.

In the previous work, we have reached significant milestones that define the course of our proposed study. These milestones include: (i) verifying fibrotic scarring as a valid target to limit post-traumatic joint stiffness; (ii) engineering a clinically-relevant form of the ACA to inhibit fibrotic scarring; and (iii) validating *in vivo* the efficacy of the ACA to limit post-traumatic joint stiffness.

Here, we achieved the following goals: (i) we defined the concentration-dependent safety and efficacy of our ACA in the relevant *in vivo* model; (ii) we determined the effects of the ACA on the healing of joint tissues; and (iii) we defined clinically-relevant biomarkers to monitor the effects of the ACA-based treatment on injured joints. Achieving these goals provide a solid launching platform for future large-animal and clinical tests.

The immediate impact of our study is getting closer to implementing our method of arthrofibrosis prevention after injury. Consequently, if validated in clinical tests, this method will allow an earlier return to functional activities in patients with joint injuries.

Military personnel may return to duty or work sooner after sustaining an injury, may not need to change duties to accommodate a stiffened joint, and may demonstrate earlier return to duty and unit readiness after sustaining a musculoskeletal injury. Applying our therapeutic antibody may become the standard of care in patients who sustain orthopedic injuries or undergo orthopedic surgical procedures that may result in post-operative arthrofibrosis. With the simple delivery method of intraarticular injection, sustained biomaterial-based delivery, or other relevant methods, minimal anticipated patient care is necessary to restore function. In the short term, this may improve military personnel's quality of life.

In the long term, individuals recovering from traumatic orthopedic injuries may have higher patient satisfaction and increased work longevity if arthrofibrosis is prevented at the time of injury. The potential to restore function to baseline after an injury using the proposed antibody can change care, where applying the targeted antibody could become the standard of treatment for combat-related orthopaedic injuries or non-battle orthopaedic injuries. By providing long-term restoration of function, more military personnel may be able to return to work and duty for a longer time. Once this treatment modality is utilized in patients, new evidence-based policy changes may be made and can revolutionize how arthrofibrosis is managed after combat-related and non-combat-related orthopedic injuries.

Since the effects of post-traumatic joint stiffness and other scarring diseases in military personnel overlap with those of the general civilian population, the prospect for a broad impact of the proposed study is excellent.

The Impact on Other Disciplines

In a broad sense, by addressing excessive scarring and its outcome, fibrosis, our proposal addressed the general problem of improving the regeneration of all vital tissues and organs damaged due to accidental trauma or surgery but, in perspective, also of chronic fibrotic diseases, *e.g.*, idiopathic pulmonary fibrosis and liver cirrhosis. Our proposal was timely because, thus far, regenerative medicine has shown only limited progress in restoring the function of damaged tissues and organs due to fibrosis.

In many cases, clinicians can only manage some of the symptoms using medical devices or pharmacological compounds. These limited therapeutic approaches do not offer complete recovery of lost organ functions, presenting significant medical and socioeconomic problems. The latter is even more evident in the aging population, whose natural regenerative mechanisms are less effective than those in younger patients.

Improving tissue regeneration in fibrotic conditions requires a comprehensive approach that addresses a significant roadblock hampering this process. Hence, our ACA-based system offers a novel therapies to prevent or alleviate fibrosis conditions related to acute tissue injury or chronic diseases.

Posttraumatic scarring is recognized as the main obstacle to tissue regeneration [38]. Not only does the excessive scarring alter the architecture and function of injured sites, but it also negatively affects implants and cell-laden scaffolds used in therapies to regenerate damaged tissues. In one example, excessive scar formation impedes peripheral nerve regeneration by blocking the axons' outgrowth from the posterior stump of a damaged nerve. Furthermore, attempts to regenerate the skin of burn patients often fail due to the severe consequences of scar formation.

Unfortunately, the adverse effects of posttraumatic fibrotic scarring that hamper regenerative approaches are not limited to the above examples. Those negative effects affect the regeneration of all connective tissues and organs, including the eye, vocal folds, heart, muscle, lung, liver, tendon, and others.

Because scar tissue formation is a natural biological process needed to repair damaged sites, developing anti-scarring agents that limit excessive fibrotic scarring without blocking the repair process presents a challenging problem. Consequently, broadly active and safe anti-fibrotic agents do not exist on the market.

Our research team postulates that no regenerative, cell-based, transplant-based, biomaterial-based, or device-based approach can be fully effective without addressing the common underlying problem of excessive scarring. Considering that, except for fetal injuries, all accidental or surgical injuries in humans lead to scar formation, our postulate is well-justified and relevant to all tissues. Here, we addressed the problem of fibrotic scarring that hampers tissue regeneration by offering anti-fibrotic approaches that utilize novel targets and an advanced therapeutic compound.

In our proposal, we focused on blocking excessive fibrosis to enable the regeneration of the articular joints' tissues. Our choosing these targets emphasizes a significant unmet need to improve the outcomes of therapeutic approaches to regenerate these tissues.

Although we focused on these specific targets, our research will broadly impact the regeneration in all fibrotic conditions because our anti-fibrotic target is universal and does not depend on anatomical location or type of trauma.

The Impact on Technology Transfer

Intellectual Property, Ownership, and Government Access

Our ACA is covered under Issued US patents 9,777,055, 11,208,472, and 10,501,533, which all go out to 2033. The progress of our current project prompted us to contemplate developing an even more advanced and clinically relevant humanized ACA variant. Subsequently, we attracted a partner experienced in developing therapeutic versions of antibodies. Consequently, they engineered and successfully tested the new ACA variant; we plan to patent it and use it in future studies.

To plan our collaboration on moving the ACA-based technology closer to clinical applications, we envision the following path:

Transition Plan and Regulatory Strategy

Jefferson Innovation

With a current portfolio of 280 issued patents and 400 pending patent applications, Jefferson has demonstrated a track record of successfully developing its inventions and discoveries, from standalone spinoffs and startups to licensing IP to industry leaders for further development. In the last five years, Jefferson Innovation facilitated over 100 licenses and 26 spinoffs, which have raised over \$500 million in capital funding in the past year alone.

Translation Plan, Milestones, Risk Assessment, and Partnership Plan

We plan to develop the Good Laboratory Practice (GLP)-level production of the anti-fibrotic antibody and design a sustained ACA delivery method for preclinical testing. We will ascertain the ACA production scalability by utilizing GLP methods and determining crucial ACA qualities. This process will enable us to produce uniform batches for large animal studies (i.e., sheep or pigs).

These methods will also ensure that the work done with the antibody hereunder will yield relevant quality data appropriate for some FDA submissions in the United States and may also demonstrate the scalability of the ACA into a product suitable for human use.

We plan to perform the above activities at the Jefferson Institute for Bioprocessing (JIB). This state-of-the-art contract development and manufacturing organization (CDMO) facility opened at Thomas Jefferson University in 2019. JIB is a 25,000-ft² ultra-modern facility equipped with end-to-end single-use equipment and technologies for process development, scale-up (2L-1000L), and monoclonal antibodies (mAbs) production. The facility provides a GLP environment for preclinical process development studies. It has significant analytical capability and the ISO 9001:2015 quality system management certification.

After completing the validation work done hereunder, the next stage of development for our ACA project would be to partner the ACA with an out-license to a third party with experience in developing therapeutic antibodies that would then take the ACA through regulatory development.

Going forward, we intend to have our licensee partner manage the development of the antibody through a new drug regulatory approval process, including determining the number and types of studies proposed to reach approval, licensure, or clearance. The license from TJU to our commercial partner would require the licensee to follow a rigorous timeline for taking the product to market. Below is a list of the minimum benchmark milestones that our technology transfer office would include in the ACA license:

- *Corroborating POC with cGMP product*
- *Pre-IND meeting with the FDA*
- *Dose range-finding study*
- *Suitable formulation*
- *14-day toxicology studies in relevant animals*
- *Filing of an IND*
- *Phase I clinical trial*
- *Phase II clinical trial*
- *Phase III clinical trial*
- *Submission of NDA*
- *Market approval of Licensed Product*

For now, we plan to partner the ACA technology under a license with Larix BioScience ("Larix") for the next stage of development. It should be noted that Larix has already committed significant resources to this project by generating the humanized version of the ACA that we are now scaling up and testing.

Larix is a spin-out company of a protein engineering and drug development company founded out of, and presently operating at, the Panorama Research Institute ("PRI"), a biotechnology R&D incubator employing approximately >20 scientists and technicians and occupying 42,000 sq. ft. of laboratory and office space in Sunnyvale, CA. PRI was founded in 1991 as a vehicle for the early-stage development of various commercially promising new technologies and products in cellular and molecular immunology, protein engineering, and medicinal chemistry. PRI has a successful track record of identifying promising technologies and pharmaceutical products with specific expertise in antibody discovery and development.

Many grant-supported projects have formed the basis of successful spin-out companies from PRI. While they also develop small molecule-based drugs, the core competency of Larix is antibody discovery and development.

Larix/PRI has developed over 30 products that have been out-licensed to strategic partners such as pharma or led to company formations, including nine antibody-based products. The aggregate equity value of SBIR-funded programs initiated at PRI is estimated to be more than 50-fold the value of the federal funds that supported their initial R&D. A pictorial view of this translation plan milestones, partnership plan, and the tentative timeline to sales are detailed in Figure 23.

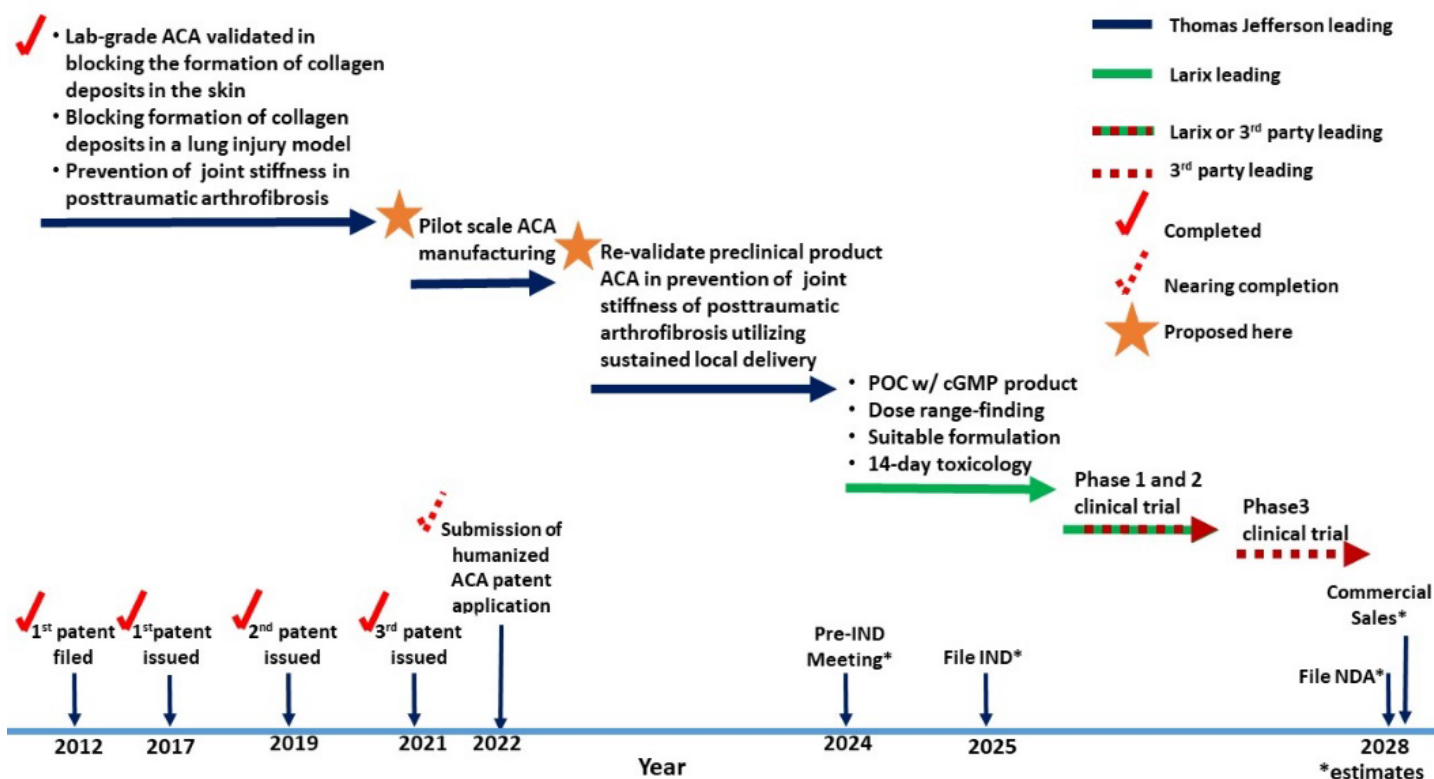


Figure 23. Milestones and Partnership Plan for Translation

The impact on society beyond science and technology

Scientists estimate that 45% of deaths worldwide can be attributed to fibrotic disorders [38, 39]. These disorders affect all tissues and organ systems. They include interstitial lung disease, liver cirrhosis, kidney disease, heart disease, diseases of the eye, peripheral and central nervous systems, and many others.

Apart from the scientific and technical aspects, the research we presented brings to attention the damaging consequences of posttraumatic fibrotic disorders, making society aware of them. Consequently, we expect society will be more tuned into the problem of patients and their families suffering from these incapacitating disorders. We also hope that increased public awareness will translate to increasing efforts to combat fibrotic diseases at the academic and industrial levels. Increasing these efforts is particularly important, considering that no anti-fibrotic drugs, tested thus far, can combat systemic and localized excessive scarring.

5. CHANGES/PROBLEMS

Nothing to report

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Except for some problems associated with the delivery of cell culture bioreactor components and rabbits due to the COVID-19 pandemic (see above), we did not experience any significant issues. Both delivery problems were solved by changing the ACA production conditions and choosing a different vendor for the rabbits (Appendix #3).

Changes that had a significant impact on expenditures

Because of the need to change the ACA production method and delays with the delivery of rabbits, the cost of our project increased. Consequently, we applied and obtained additional DoD funds to mitigate these problems (see Appendix #3)

Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and/or select agents

No changes were made.

Significant changes in the use or care of human subjects

Not applicable.

Significant changes in the use or care of vertebrate animals

Nothing to report.

Significant changes in the use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications.

Steplewski A, Fertala J, Tomlinson RE, Wang ML, Donahue A, Arnold WV, Rivlin M, Beredjiklian PK, Abboud JA, Namdari S, Fertala A: Mechanisms of reducing joint stiffness by blocking collagen fibrillogenesis in a rabbit model of posttraumatic arthrofibrosis. PLoS One 2021, 16:e0257147.

Acknowledgment of federal support: “Funding: Grants from the Department of Defense supported this research: W81XWH-18-1-0554 awarded to AF.”

Books or other non-periodical, one-time publications.

A chapter entitled: “Beyond Drugs and Surgery: A Look at Orthobiologics” is now in press as a part of a book entitled: “Translational Sports Medicine.” This chapter includes a section devoted to our research on antibody-based prevention of posttraumatic joint contracture.

Other publications, conference papers and presentations

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Production of the ACA in cell a cell suspension bioreactor.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

(i). Validation of a modified animal model of arthrofibrosis that includes patellar tendon injury.

(ii). Identification of collagen metabolism markers that potentially can be used to monitor the dynamics of scar formation and efficacy of anti-fibrotic therapies.

(iii) Collection of tissues associated with arthrofibrosis model. The collection includes all knee joint tissues, tissues, and organs from experimental and control groups of rabbits.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Andrzej Fertala
Project Role: PD/PI
Researcher Identifier ORCID ID 0000-0001-9153-1912

Nearest person month worked: 10

Contribution to Project: Dr. Fertala is an expert in the structure and function of extracellular fibrous proteins, focusing on collagenous proteins in health and disease. He developed the concept and then demonstrated its feasibility, that the pathological process of excessive deposition of collagenous material may be inhibited by blocking collagen fibril formation. He generated a clinically-relevant antibody characterized by the ability to block the formation of collagen-rich fibrotic deposits. Dr. Fertala directed all aspects of the project and interacted with other team members. Dr. Fertala monitored procedures for the preparative-scale production of the anti-fibrotic antibody.

Moreover, he collaborated closely with participating clinicians and Dr. Tomlinson. Dr. Fertala led all analyses to establish the safety and efficacy of the proposed approach to limit post-traumatic joint stiffness. In addition, Dr. Fertala was responsible for coordinating analyses of all data obtained from the proposed study. In these analyses, Dr. Fertala communicated with Dr. Daskalakis, the biostatistician assigned to this project. He also coordinated the preparation of manuscripts that describe the study. Dr. Fertala was also responsible for communicating with the DoD.

Funding Support: Nothing to report

Name: Constantine Daskalakis
Project Role: Biostatistician
Researcher Identifier: ORCID ID 0000-0001-9799-0060

Nearest person month worked: 2

Contribution to Project: Dr. Daskalakis is a biostatistician responsible for this study's statistical aspects. He interacted with the research team and provided guidance and consultation on selected aspects of experimental design and data analysis.

Funding Support: Nothing to report

Name: Jolanta Fertala
Project Role: Research Associate
Researcher Identifier: ORCID ID 0000-0001-9027-0917

Nearest person month worked: 21

Contribution to Project: Dr. J. Fertala has extensive experience in the physiology and pathology of skeletal connective tissues. She performed histological assays and microscopic quantification of collagen-rich deposits in tissues obtained from animal groups. Moreover, she studied the architecture of scar tissue formed in the presence of the therapeutic antibody. She was also responsible for the biochemical assays of the rabbit sera. These assays included processing crude samples, determining the concentration of collagen-specific hydroxyproline, and studying the composition of collagen-rich deposits.

Dr. Fertala also analyzed the results and prepared them for publication. She interacted closely with clinical partners to discuss the clinical relevancy of biochemical and morphological data.

Funding Support: Nothing to report

Name: Sunday Shoyele

Project Role:

Researcher Identifier: ORCID ID 0000-0002-7856-3538

Nearest person month worked: 1

Contribution to Project: Dr. Shoyele is an expert in the delivery of biological therapeutics with a particular focus on therapeutic antibodies and peptides. He participated in discussing ACA delivery and safety tests.

Funding Support: Nothing to report

Name: Andrzej Steplewski

Project Role: Research Associate

Researcher Identifier: ORCID ID 0000-0001-6869-3999

Nearest person month worked: 21

Contribution to Project: Dr. Steplewski is an expert in connective tissue research and has extensive experience in protein engineering and preparative-scale protein production. He was responsible for producing and characterizing recombinant antibodies needed for the proposed study. He also participated in animal surgeries by assisting an assigned surgeon. In collaboration with Dr. Tomlinson, Dr. Steplewski participated in biomechanical assays of joint tissues. Dr. Steplewski also performed micro-CT assays and FTIR-based measurements.

Funding Support:

Name: Ryan Tomlinson

Project Role: Co-Investigator

Researcher Identifier: ORCID ID 0000-0002-6713-6047

Nearest person month worked: 1

Contribution to Project: Dr. Tomlinson has a biomechanical and bioengineering scientific background. His research interest focuses on novel approaches to repairing skeletal tissues. In this study, he was responsible for biomechanical assays of joint tissues. In these studies, he collaborated with Dr. Steplewski. Moreover, he participated in data interpretation in the context of biomechanical properties of joint tissues as a function of the ACA-based anti-fibrotic treatment.

Funding Support: Nothing to report

Name: Mark Wang

Project Role: Co-Investigator

Researcher Identifier: NA

Nearest person month worked: 1

Contribution to Project: Dr. Wang is a practicing hand surgeon with a research interest in arthrofibrosis, osteoporosis, and tendon repair. He actively participated in animal studies using a rabbit-based arthrofibrosis model. Considering his bioengineering background, he also helped to interpret the results of the biomechanical tests performed in this project.

Funding Support: Nothing to report

A change in the active other support of the PD/PI(s) or senior/key personnel

Nothing to report.

Other organizations

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable.

QUAD CHARTS:

Not applicable

9. APPENDICES

Appendix #1: An example of parameters for establishing CHO cell culture and running a bioreactor to produce the antibody utilized in this study.

Appendix #2: A publication and corresponding supplementary materials: Steplewski A, Fertala J, Tomlinson RE, Wang ML, Donahue A, Arnold WV, Rivlin M, Beredjiklian PK, Abboud JA, Namdari S, Fertala A: Mechanisms of reducing joint stiffness by blocking collagen fibrillogenesis in a rabbit model of posttraumatic arthrofibrosis. PLoS One 2021, 16:e0257147.

Appendix #3: Documents associated with applying for additional funds needed due to COVID-19 related issues.

The Appendices are included at the end of this report.

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9. APPENDICES

Appendix #1: An example of parameters for establishing CHO cell culture and running a bioreactor to produce the antibody utilized in this study.

Appendix #2: A publication and corresponding supplementary materials: Steplewski A, Fertala J, Tomlinson RE, Wang ML, Donahue A, Arnold WV, Rivlin M, Beredjiklian PK, Abboud JA, Namdari S, Fertala A: Mechanisms of reducing joint stiffness by blocking collagen fibrillogenesis in a rabbit model of posttraumatic arthrofibrosis. PLoS One 2021, 16:e0257147.

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APPENDIX #1

Table. A summary of cell culture parameters of a bioreactor run.

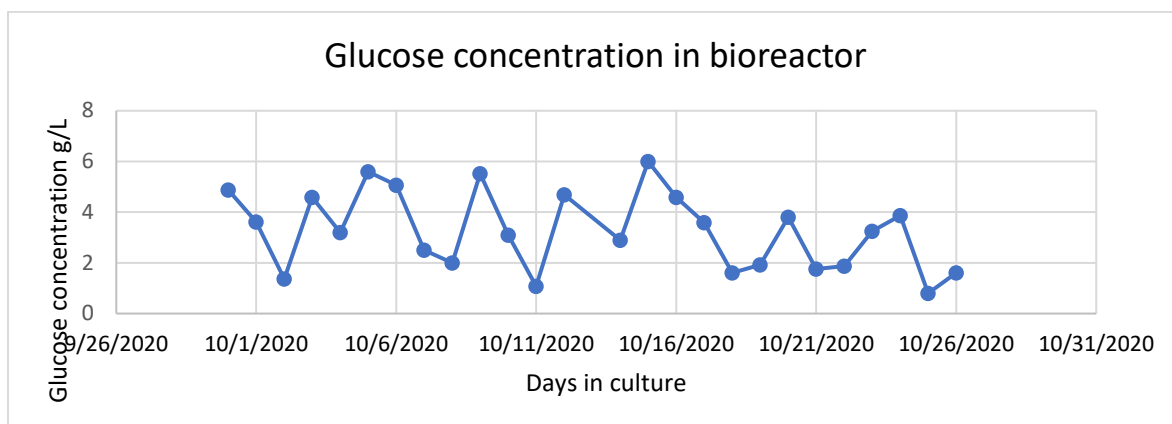
Number of seeding vials (Cell Bank Vial Description)	Media Volume	Live cells/ml	Dead cells/ml	Media used	Mixing speed	Date	Notes	
2 of CHO_44_42_2b AS_1_23_20_p1 1 of CHO_44_42_7_15_20_p 4	100 ml	660,000.00	200,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/11/2020	Puromycin added 200 uL stock and G418 2 ml stock to the final conc. of 10 ug/mL and 500 ug/mL, respectively.	PREPARING CELL INOCULUM
2 of CHO_44_42_2b AS_1_23_20_p1 1 of CHO_44_42_7_15_20_p 4	100 ml	240,000.00	600,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/14/2020	Many dead cells. Starting the second flask from frozen stock	
CHO_44_42_7_15_20_p 4	100 ml	420,000.00	360,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/15/2020		
2 of CHO_44_42_2b AS_2_5_20	75 ml	40,000.00	20,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/16/2020		
2 of CHO_44_42_2b AS_2_5_20	75 ml	80,000.00	10,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/18/2020		
2 of CHO_44_42_2b AS_2_5_20	75 ml	135,000.00	5,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/21/2020		
2 of CHO_44_42_2b AS_2_5_20	75 ml	240,000.00	0.00	CD Forti CHO P/S, L-Glut	110 rpm	9/22/2020		
2 of CHO_44_42_2b AS_2_5_20	75 ml	660,000.00	20,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/23/2020	75 ml of fresh medium added. Puro added 150 ul (stock 10 mg/ml). G418 1,5 ml (stock 5 mg/ml). Final conc. Puro 10 ug/ml and G418 500 ug/ml. Cells 330,000/ml	
2 of CHO_44_42_2b AS_2_5_20	150 ml	840,000.00	10,000.00	CD Forti CHO P/S, L-Glut	110 rpm	9/24/2020		
2 of CHO_44_42_2b AS_2_5_20	500 ml			CD Forti CHO P/S, L-Glut	80 rpm	9/24/2020	Cells were transferred into a 500 ml flask. Fresh 350 ml of medium added with 350 ul Puro and 3,5 ml G418	
2 of CHO_44_42_2b AS_2_5_20	500 ml	360,000.00	10,000.00	CD Forti CHO P/S, L-Glut	80 rpm	9/25/2020	Cells split into 500 ml flasks. 50/50. Fresh medium was added with Puro and G410. 500 ml in each flask.	
2 of CHO_44_42_2b AS_2_5_20	500 ml	320,000.00	40,000.00	CD Forti CHO P/S, L-Glut	80 rpm	9/26/2020	One flask is >500ml, while in the second flask is <500 ml of cultures	
2 of CHO_44_42_2b AS_2_5_20	500 ml	620,000.00	20,000.00	CD Forti CHO P/S, L-Glut	80 rpm	9/27/2020		BIOREACTOR CULTURE
2 of CHO_44_42_2b AS_2_5_20	2x500 ml	2,595,000.00	50,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.1 SLPM	9/28/2020	Bioreactor seeding	
2 of CHO_44_42_2b AS_2_5_20	3.5 L	750,000.00	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.2 SLPM	9/29/2020		
2 of CHO_44_42_2b AS_2_5_20	3.5 L	1,500,000.00	40,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	9/30/2020	4.88 g/L glucose, 1.87 g/L lactate (probe). External glucose 5.36 g/L Lactate 2.44 g/L; O2@40.2%; Gas delivery: O2 16% Air 84%	
2 of CHO_44_42_2b AS_2_5_20	3.5 L	4,480,000.00	20,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/1/2020	3.61 g/L glucose, 1.79 g/L lactate (probe). External glucose 3.69 g/L Lactate 2.1 g/L; O2@39.8%; Gas delivery: O2 38.7% Air 61.3%	

2 of CHO_44_42_2b AS_2_5_20	3.5 L	9280000, then diluted to about 700,000/ml	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/2/2020	1.37 g/L glucose, 1.96 g/L lactate (probe). External glucose 1.29 g/L Lactate 2.03 g/L; O2@40.3%; Gas delivery: O2 90% Air 10%. 3 L of medium removed. 3L of fresh medium added.
2 of CHO_44_42_2b AS_2_5_20	3.5 L	2,700,000.00	40,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/3/2020	4.59 g/L glucose, 1.74 g/L lactate (probe). External glucose 5.27 g/L Lactate 1.87 g/L; O2@39.5%; Gas delivery: O2 15% Air 85%
2 of CHO_44_42_2b AS_2_5_20	3.5 L	6,500,000.00	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/4/2020	3.2 g/L glucose, 2.21 lactate (probe). External glucose 3.3 g/L Lactate 2.58 g/L; O2@39%; Gas delivery: O2 41.4% Air 58.6%
2 of CHO_44_42_2b AS_2_5_20	3.5 L	12,500,000.0 0	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/5/2020	3.1 L of medium removed. Replaced with 3.0L of fresh medium
2 of CHO_44_42_2b AS_2_5_20	3.5 L	2,400,000.00	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/6/2020	5.06 g/L glucose, 1.92 g/L lactate (probe). External glucose 4.92 g/L Lactate 1.67 g/L; O2@41.3%; Gas delivery: O2 0.3% Air 92.4% CO2 7.33 pH 7.23
2 of CHO_44_42_2b AS_2_5_20	3.5 L	8,000,000.00	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/7/2020	2.5 g/L glucose, 2.11 g/L lactate (probe). External glucose 2.8 g/L Lactate 2.14 g/L; O2@39.4%; Gas delivery: O2 17.2% Air 82.8%
2 of CHO_44_42_2b AS_2_5_20	3.5 L	14,400,000.0 0	30,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/8/2020	2.0 g/L glucose, 2.43 g/L lactate (probe); O2@29.8%; Gas delivery: O2 100% Air 0%. 3.25 L of medium removed. Replaced with 3.25L fresh medium.
2 of CHO_44_42_2b AS_2_5_20	3.5 L	2,180,000.00	20,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/9/2020	5.52 g/L glucose, 2.28 g/L lactate (probe). External glucose 4.96 g/L Lactate 2.03 g/L; O2@39.5%; Gas delivery: O2 18.3% Air 81.7%
2 of CHO_44_42_2b AS_2_5_20	3.5 L	6,100,000.00	20,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/10/2020	3.1 g/L glucose, 2.4 g/L lactate (probe). External glucose 2.9 g/L Lactate 2.4 g/L; DO2@39.4%; Gas delivery: O2 50% Air 50%
2 of CHO_44_42_2b AS_2_5_20	3.5 L	12,400,000.0 0	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/11/2020	1.07 g/L glucose, 2.361 g/L lactate (probe). DO2@20%; Gas delivery: O2 100% Air 0%
2 of CHO_44_42_2b AS_2_5_20	3.5 L	2,700,000.00	40,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/12/2020	4.68 g/L glucose, 2.65 g/L lactate (probe). External glucose 4.74 g/L Lactate 2.51 g/L; O2@40.8%; Gas delivery: O2 0% Air 93.3% CO2 6.14 pH 7.21
2 of CHO_44_42_2b AS_2_5_20	3.5 L	14,400,000.0 0	40,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.4 SLPM	10/14/2020	2.9 g/L glucose, 2.65 g/L lactate (probe). External glucose 3.1 g/L Lactate 2.51 g/L; O2@50.8%; Gas delivery: O2 0% Air 50% CO2 6.14 pH 7.08
2 of CHO_44_42_2b AS_2_5_20	3.5 L	19,500,000.0 0	40,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.4 SLPM	10/15/2020	2.9 g/L glucose, 2.65 g/L lactate (probe). External glucose 3.1 g/L Lactate 2.51 g/L; O2@50.8%; Gas delivery: O2 0% Air 50% CO2 6.14 pH 7.08
2 of CHO_44_42_2b AS_2_5_20	3.5 L	1,000,000.00	40,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.4 SLPM	10/15/2020	6 g/L glucose, 2.65 g/L lactate (probe). O2@80%; Gas delivery: O2 0% Air 80% CO2 20.14 pH 7.2
2 of CHO_44_42_2b AS_2_5_20	3.5 L	1,800,000.00	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/16/2020	4.58 g/L glucose, (probe). External glucose 5.38 g/L; DO@38.2%; Gas delivery: O2 5.1% Air 88.8% CO2 6.14 pH 7.21

2 of CHO_44_42_2b AS_2_5_20	3.5 L	5,500,000.00	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.3 SLPM	10/17/2020	3.59 g/L glucose, 1.31 g/L lactate (probe). External glucose 3.94 g/L Lactate 1.68 g/L; O2@39%; Gas delivery: O2 21.5% Air 78.5% pH 7.2
2 of CHO_44_42_2b AS_2_5_20	3.5 L	8,800,000.00	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.4 SLPM	10/18/2020	1.6 g/L glucose, 1.7 g/L lactate (probe); O2@37.3%; Gas delivery: O2 42.2% Air 57.8% pH 7.09
2 of CHO_44_42_2b AS_2_5_20	3.5 L	10,000,000.0 0	10,000.00	CD Forti CHO P/S, L-Glut	Agit: 100 rpm/min; Air: 0.4 SLPM	10/19/2020	1.92 g/L glucose, 1.2 g/L lactate (probe); O2@39.2%; Gas delivery: O2 31.6% Air 68.4% pH 7.2
2 of CHO_44_42_2b AS_2_5_20	3.5 L	1,800,000	20,000	CD CHO P/S, L- Glut, HT supplemen t	Agit: 100 rpm/min; Air: 0.3 SLPM	10/20/2020	3.8 g/L glucose, 1.06 g/L lactate (probe). External glucose 5.28 g/L Lactate 1.05 g/L; O2@40%; Gas delivery: Air 100% pH 7.2
2 of CHO_44_42_2b AS_2_5_20	3.5 L	4,000,000.00	40,000.00	CD CHO P/S, L- Glut, HT supplemen t	Agit: 100 rpm/min; Air: 0.3 SLPM	10/21/2020	1.76 g/L glucose, 1.87 g/L lactate (probe). External g/L glucose 2.19 g/L G/L lactate 2.7 g/L; O2@21.7%; Gas delivery: Air 65.8%; O2 34.3 pH 7.02
2 of CHO_44_42_2b AS_2_5_20	3.5 L	8,700,000.00	80,000.00	CD CHO P/S, L- Glut, HT supplemen t	Agit: 100 rpm/min; Air: 0.4 SLPM	10/22/2020	1.87 g/L glucose, 3.77 g/L lactate (probe). External g/L glucose 2.33 g/L G/L lactate 4.78 g/L; DO@40.3%; Gas delivery: Air 68.6%; O2 31.4 pH 7.02. NaOH 46.7 added.
2 of CHO_44_42_2b AS_2_5_20	3.5 L	5,600,000.00	160,000.00	CD CHO P/S, L- Glut, HT supplemen t	Agit: 100 rpm/min; Air: 0.4 SLPM	10/23/2020	3.25 g/L glucose, 5.9 g/L lactate (probe); DO@40%; Gas delivery: Air 98.9%; O2 1.1 pH 7.18. NaOH 72.4 added.
2 of CHO_44_42_2b AS_2_5_20	3.5 L	1,060,000.00	10,000.00	CD CHO P/S, L- Glut, HT supplemen t	Agit: 100 rpm/min; Air: 0.3 SLPM	10/24/2020	3.87 g/L glucose, 2.34 g/L lactate (probe). External g/L glucose 5.11 g/L G/L lactate 2.48 g/L; DO@38.8%; Gas delivery: Air 98.4%; O2 1.6 pH 7.17.
2 of CHO_44_42_2b AS_2_5_20	3.5 L	1,780,000.00	10,000.00	CD CHO P/S, L- Glut, HT supplemen t	Agit: 100 rpm/min; Air: 0.3 SLPM	10/25/2020	0.8 g/L glucose, 2.5 g/L lactate (probe). External g/L glucose 1.8 g/L G/L lactate 2.5 g/L; DO@43%; Gas delivery: Air 100%; O2 0 pH 7.03. G/L glucose added for 15min @100%
2 of CHO_44_42_2b AS_2_5_20	3.5 L	2,640,000.00	60,000.00	CD CHO P/S, L- Glut, HT supplemen t	Agit: 100 rpm/min; Air: 0.3 SLPM	10/26/2020	1.6 g/L glucose, 3.87 g/L lactate (probe). External g/L glucose 2.44 g/L G/L lactate 5.59 g/L; DO@32.5%; Gas delivery: Air 76.1%; O2 23.9 pH 7.03. G/L glucose added for 20 min@100%

Abbreviations: P/S, penicillin/streptomycin mixture; CHO, Chinese hamster ovary cells; CD CHO; cell culture media to grow CHO cells; L-Glut, L-glutamine; Agit, culture agitation; DO, dissolved oxygen; rpm, rotations per minute; SLPM, Standard Liters Per Minute; Puro and G418, selection antibiotics; HT supplement, a cell culture supplement including hypoxanthine (10 mM) and thymidine (1.6 mM)

Figure. A representative graph showing regulation of glucose media within a set range of concentration.



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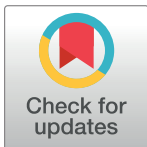
RESEARCH ARTICLE

Mechanisms of reducing joint stiffness by blocking collagen fibrillogenesis in a rabbit model of posttraumatic arthrofibrosis

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Abstract

Posttraumatic fibrotic scarring is a significant medical problem that alters the proper functioning of injured tissues. Current methods to reduce posttraumatic fibrosis rely on anti-inflammatory and anti-proliferative agents with broad intracellular targets. As a result, their use is not fully effective and may cause unwanted side effects. Our group previously demonstrated that extracellular collagen fibrillogenesis is a valid and specific target to reduce collagen-rich scar buildup. Our previous studies showed that a rationally designed antibody that binds the C-terminal telopeptide of the $\alpha 2(I)$ chain involved in the aggregation of collagen molecules limits fibril assembly *in vitro* and reduces scar formation *in vivo*. Here, we have utilized a clinically relevant arthrofibrosis model to study the broad mechanisms of the anti-scarring activity of this antibody. Moreover, we analyzed the effects of targeting collagen fibril formation on the quality of healed joint tissues, including the posterior capsule, patellar tendon, and subchondral bone. Our results show that blocking collagen fibrillogenesis not only reduces collagen content in the scar, but also accelerates the remodeling of healing tissues and changes the collagen fibrils' cross-linking. In total, this study demonstrated that targeting collagen fibrillogenesis to limit arthrofibrosis affects neither the quality of healing of the joint tissues nor disturbs vital tissues and organs.

Introduction

Balanced scar formation is crucial to natural wound healing [1]. However, when scarring is excessive, it negatively impacts the vital functions of the affected tissues. For example, fibrotic scars formed in muscles weaken them, and the scar tissue formed within and around injured joints causes arthrofibrosis [2, 3]. Arthrofibrosis, which can occur due to accidental trauma or surgery, stiffens the joints, restricts the ability to perform activities of daily living, and causes pain [4].

antibody for inhibition of fibrosis,” US Patent 9,777,055; 10,501,533.

Because mature scars comprise insoluble collagen fibrils, they are incredibly stable and persist in the injury sites for months, years, or even permanently [5, 6]. Surgical removal of established scar tissue often exacerbates the problem, and the available pharmacological methods to reduce posttraumatic fibrosis are neither safe nor effective. For instance, intralesional injection of steroids to treat excessive skin scarring may promote infection, cause hyperpigmentation and skin atrophy [7]. Long-term use of topical mitomycin-c and 5-fluorouracil causes substantial ocular toxicity [8, 9]. Intraarticular injections of steroids may reduce arthrofibrosis, but their effect is usually short-term, and frequent steroid administration carries the risk of osteoporosis and infection [3, 10].

Consequently, optimal anti-scarring efforts should prevent scar formation rather than remove it after it has been firmly established. Our research group has identified collagen fibril formation as a valid anti-fibrotic target [11]. The fundamental premise for targeting collagen fibrillogenesis is that collagen fibrils are the main component of mature scars, hence limiting their formation attenuates unwanted effects of excessive scarring [11, 12].

To reduce the number of collagen fibrils formed in response to injury, we developed a monoclonal anti-collagen antibody (ACA) that targets the C-terminal telopeptide of the $\alpha 2(I)$ chain ($\alpha 2Ct$), which is engaged in the aggregation of collagen monomers into fibrils [11, 13, 14]. Our studies with biologically and clinically relevant models of excessive scarring clearly demonstrated the ACA's ability to reduce collagen fibrillogenesis and limit scar formation [11, 13–16].

Since the fibrotic scar and neotissue formed during the healing process have nearly the same composition, achieving the proper balance between reducing unwanted scarring and maintaining proper healing is challenging. As all anti-fibrotic agents alter the collagen-based matrix formation needed to repair injury sites, testing any novel anti-fibrotic approach requires defining its anti-fibrotic utility and the impact on intrinsic healing [17].

Here, we studied the impact of the ACA on healing connective tissues, including the posterior joint capsule (PC), subchondral bone (SB), and the patellar tendon (PT). While we understand the primary mechanism of the ACA-dependent inhibition of collagen fibril assembly, the overall secondary effects of this interference are not known. Furthermore, we analyzed the safety of using ACA by measuring its impact on vital cellular and biochemical parameters of the blood of treated animals and studying histological features of distant tissues and organs that were not directly targeted for treatment.

We demonstrated that direct blocking of collagen fibrillogenesis changes the dynamics of collagen metabolism and impacts the architecture and composition of the collagen-rich scar tissues formed in response to injury. Although applying the ACA following knee injury reduced the flexion contracture of healed joints, the antibody neither altered the healing of injured joint tissues nor negatively impacted distant tissues and organs.

Overall, our study demonstrated that targeting the collagen-rich scar tissue assembly offers an effective and safe method to reduce joint tissues' posttraumatic fibrotic response. Moreover, discovering the secondary effects of ACA treatment suggests that blocking collagen fibrillogenesis has far-reaching beneficial consequences that enhance this antibody's anti-fibrotic potential.

Materials and methods

Production of the ACA

The ACA was produced in Chinese Hamster Ovary (CHO) cells cultured in a bioreactor (New Brunswick BioFlo 115, Eppendorf, Enfield, CT USA) in a protein-free medium (CD CHO, ThermoFisher Sci., Waltham, MA USA) [14]. The conditioned medium containing the ACA

was collected, then concentrated by ultrafiltration (ECO Cassette, Sartorius Stedim Biotech GmbH, Goettingen, Germany). Subsequently, the antibody was purified using the Protein-L resin (GenScript Biotech Corporation, Piscataway, NJ USA). Non-specific human IgG that served as a control antibody (CA) was purchased from a commercial supplier (Sigma-Aldrich, Allentown, PA USA). Each batch of CA was tested to exclude a possibility of its non-specific interaction with the $\alpha 2\text{Ct}$ epitope.

A rabbit model of arthrofibrosis

We employed an established rabbit model of posttraumatic joint contracture to study the effects of the ACA on the healing of the joint tissues, including the PC, SB, and PT [16, 18–21].

We utilized both male and female New Zealand White rabbits; 8 to 12 months old (Charles River Laboratories, Wilmington, MA USA). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University and the Animal Care and Use Review Office (ACURO) of a sponsoring agency.

Before applying general anesthesia, the rabbits received ketamine and xylazine. Subsequently, an analgesic was administered preoperatively and postoperatively to minimize the pain caused by surgery to the knee. At the end of experiments, the rabbits were euthanized by intravascular or intracardiac bolus overdose of a euthanasia agent containing barbiturate sodium pentobarbital.

All animal experiments complied with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Table 1 provides detailed information about the rabbits used in our study.

Since we were interested in posttraumatic arthrofibrosis, our model included damage to crucial elements of the knee joint, including the joint capsule, ligaments, tendons, cartilage, and subchondral bone. The model also included intraarticular bleeding. Necessary surgical procedures included the following steps: (i) An incision in the middle of the right knee was made starting proximal to the patella and extending distally to the tibial tubercle and down the tibia. (ii) A lateral parapatellar arthrotomy was made, and then the patella was dislocated medially. (iii) Following flexing the knee, the fat pad was released and retracted medially. (iv) The anterior crucial ligament (ACL) and the posterior crucial ligament (PCL) were transected, and the knee was hyperextended to 45° to disrupt the posterior capsule. (v) A full-thickness 3-mm

Table 1. A summary of experimental groups and the number of rabbits in the ACA and control groups.

^a Group (AB)	^b Number of rabbits (M, F)	^c Total AB delivered to an injured knee [mg]	^d Experimental design (weeks of AB delivery + recovery period)	^e Number of lost rabbits
12wk (+ACA)	8 (M), 7 (F)	4	8 + 4	1 (F)
12wk (+CA)	6 (M), 7 (F)	4	8 + 4	2 (F)
8wk (+ACA)	2 (M), 2 (F)	10	8 + 0	0
8wk (+CA)	2 (M), 2 (F)	10	8 + 0	0
8wk (+ACA)	2 (M), 2 (F)	60	8 + 0	0
8wk (+CA)	1 (M), 1 (F)	60	8 + 0	0

^aAB, Antibody used.

^bM, Males; F, Females.

^cThe amount of antibody received during the entire 8-week period.

^dThe 8-week period of antibody delivery and the 4-week period of recovery are indicated.

^eAll rabbits were lost while administering anesthesia (see below for autopsy results).

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osteochondral defect (OCD) was made in the medial femoral condyle at the weight-bearing apex. (vi) Beginning as far posteriorly in the notch as possible and exiting out through the lateral condyle, a 2-mm hole was made for a catheter. (vii) A custom-made silicone catheter with a fixed retention bead at one end was passed through the hole. While the retention bead secured the catheter inside the operated knee, the opposite end was connected with a subcutaneous pump placed so as not to affect the knee area (iPRECIO SMP-200, Primetech Co., Tokyo, Japan) [16]. (viii) The operated right leg was placed back in extension with the patellar tendon in a normal position. (ix) A full-thickness, 3 mm-wide, 10 mm-long central portion of PT was resected with a surgical blade [22]. The defect location was marked with 5–0 nonabsorbable nylon sutures. Creating this standard defect enabled a comparison of mechanical properties of the neotissue formed during the healing process. (x) The injured knee was fixed in the flexed position using a Kirschner (K)-wire, as described [16, 21]. (xi) Immediately after the surgery, the ACA was applied intraarticularly via a subcutaneous pump. (xii) Eight weeks later, the K-wires were removed, and the rabbits returned to their cages for 4 weeks. During the 4-week recovery period, the antibodies were not applied. In all rabbits, the left leg served as the uninjured control. S1 Fig in [S1 File](#) illustrates crucial surgical steps; for additional information please see our earlier reports [16, 21].

Experimental and control groups

Here, we were interested in two aspects of the ACA-based treatment: (i) its long-term impact on the healing process, and (ii) safety.

To address the first aspect, we created a group, referred to as the 12wk group, that was maintained for 12 weeks following the initial surgery ([Table 1](#) and S2 Fig in [S1 File](#)) [16, 21]. This group included 8 weeks of knee immobilization with the ACA delivery and 4 weeks of knee remobilization with no ACA delivery. The rabbits from the 12wk group received 0.5 mg/knee/week of the ACA, *i.e.*, in total 4 mg/knee. This specific dosage was consistent with that used in our earlier studies on the ACA's efficacy [16]. Based on these studies, we also calculated the number of rabbits needed for the 12wk group. Since the ACA decreases the amount of collagen (calculated as % of dry tissue mass) deposited in the PCs by 20% (with standard deviation SD = 16) compared to the control, we considered this reduction as the basis for the group size determination [16]. Consequently, 10 rabbits in each group have an 80% power to detect a difference between means of 21.26 with a significance level (alpha) of 0.05 (GraphPad StatMate version 2.00 for Windows, GraphPad Software, San Diego California USA).

Consistent with earlier studies, in the 12wk group we introduced the 4-week joint remobilization period to minimize the possible joint stiffening effect due to 8 weeks of immobilization [16, 20, 23]. Apart from the scarring due to the joint injury that was created surgically, the immobilization itself stiffens the joints due to, for example, rearrangement of collagen fibrils, lack of lubrication between collagen fibrils, and adaptive shortening of the joint capsule [24–26].

To fully utilize the 12wk group, we also employed it for the ACA safety assays as a part of dose escalation studies (see below).

To address the second aspect, we created the 8-week group to test the dose-dependent safety of the ACA. We applied a standard 4 + 4 design for the dose escalation study used commonly in phase I clinical tests [27].

In brief, a cohort of 4 rabbits (2 males and 2 females) is given the lowest dose ACA ([Table 1](#)). If none of the 4 rabbits exhibits a serious adverse effect, then the ACA is escalated to the next higher dose. If 1 or 2 of the first 4 rabbits exhibit a serious adverse effect, then another cohort of 4 rabbits is enrolled on the same dose. If <3 adverse effects are observed in the entire

group of 8 rabbits, the ACA's dose is again escalated to the higher dose. If more than 2 adverse effects ($>2/4$ or $>2/8$) are observed at any time, the dose is rendered unsafe.

The first 8wk group of 4 rabbits received 1.25 mg/knee/week of the ACA, *i.e.*, in total, 10 mg/knee. The second group received 7.5 mg/knee/week or 60 mg/knee during 8 weeks of the ACA administration. To define the safety of the ACA, we assayed crucial biochemical and cellular parameters of the blood and analyzed histopathological features of tissues and organs. To fully utilize these 8wk groups, we also employed them to study the ACA's action mechanisms and the scar tissue's morphology, composition, and mechanical characteristics.

Since the primary goal of forming the 8wk groups was to study the ACA's safety at different concentrations, organ histomorphology and blood parameters were analyzed separately for each subgroup. Other parameters were analyzed based on combined results from both 8wk subgroups.

Analysis of blood parameters

We analyzed the ACA's effects on cellular and biochemical parameters of the blood and crucial collagen metabolites. The samples were collected at the same time of the day to minimize the possible impact of circadian rhythm on blood parameters and collagen metabolites.

Degradation of collagen molecules

Cross-linked collagen molecules that form the fibrillar architecture of mature tissues are relatively resistant to proteolytic digestion. In contrast, free collagen molecules that have not been incorporated into mature fibrils degrade rapidly [28]. Since ACA prevents free collagen molecules from aggregating into fibrils, we hypothesized that the degradation of these molecules produced in response to joint injury would increase with ACA administration.

To test this hypothesis, we analyzed the serum concentration of hydroxyproline (HP), a collagen-specific amino acid (S3 Fig in [S1 File](#)). We also analyzed fibril-derived fragments that included the C-terminal telopeptides cross-linked by trivalent pyridinoline and deoxypyridinoline (XL; S3 Fig in [S1 File](#)). While serum HP assays measure both the HP residues derived from degraded free collagen molecules and those from degraded collagen fibrils, cross-linked collagen I telopeptide fragments arise solely from degraded fibrils and are not influenced by the degradation of newly synthesized collagen molecules or by dietary collagen intake [29].

HP was assayed by a chemical method (Sigma-Aldrich), and XL was assayed with the enzyme-linked immunosorbent assay (ELISA; G-Biosciences, St. Louis, MO USA). All assays were done in triplicate.

Biosynthesis of procollagen I

We also used ELISA to measure the serum concentration of the C propeptides (CP) of procollagen I as a marker of procollagen biosynthesis (MyBiosource, Inc., San Diego, CA USA) (S3 Fig in [S1 File](#)).

Histological assays of collagen fibrils

Since the scarring of the PCs is the main contributor to the knee stiffness in the rabbit model of arthrofibrosis employed here, the PCs were dissected and analyzed, as described [16, 21, 30]. In brief, the histological sections of the PCs were stained with collagen-specific picrosirius red dye. By combining this staining technique with polarized-light microscopy, we could approximate the thickness, the organization, packing density, and to a certain degree, the collagen type-specific composition of the fibrils [21, 31–33]. Studies verified that as the thickness of

fibers increases, their birefringence color changes under the polarized light microscope from green to yellow to orange to red, *i.e.*, from shorter to longer wavelengths [31, 34–37].

Employing a polarizing microscope (Eclipse LV100POL, Nikon Inc., Melville, NY) and the NIS Elements software (Nikon Inc.), the following groups of the birefringence colors were defined in captured images: (i) green birefringence (GB, identifies thin, loosely packed fibrils), (ii) yellow birefringence (YB, identifies intermediate-thickness fibrils), and (iii) red birefringence (RB, identifies thick, tightly packed fibrils) [16, 21].

All fibrils present in captured viewing areas were analyzed, as described [21, 38]. In brief, the above birefringence colors were defined by applying the software's "color threshold" function. Subsequently, the areas occupied by pixels corresponding to the defined colors were determined by the software. Considering the sum of all pixels to be 100%, the percentage of each color group in the analyzed samples was calculated. A minimum of three histological sections for control and three for injured PCs were analyzed per each rabbit. To ensure consistency in defining birefringence colors, the same threshold settings were applied to all images. Automated calculations of all fibrils in the viewing areas eliminated any potential bias. A one-way ANOVA was conducted to examine whether the percentage of subpopulations of collagen fibrils were different in the injured and uninjured capsules from the ACA-treated or the CA-treated groups (IBM SPSS Statistic for Windows, version 26, IBM Corp., Armonk, NY, USA).

Fourier Transformed Infrared Spectroscopy (FTIR)-based assays of the collagen content and the collagen cross-links

FTIR spectroscopy of tissue samples provides information about their composition and spatial distribution of analyzed macromolecules [39]. Here, we used this method to compare the relative collagen content in the scar tissues formed in the PCs and OCDs of the ACA-treated and CA-treated rabbits. As references, we selected a protein-specific amide II peak and a peak corresponding to sulfated glycosaminoglycans (GAGs). Consequently, the sulfated GAGs peak/collagen peak ratios and amide II peak/collagen peak ratios were measured to determine the relative collagen content (S1 Table in [S1 File](#)) [40, 41]. Please note that the relation between the ratio values and collagen amount is negative.

We also analyzed the maturity of collagen cross-links by measuring the pyridinoline (PYR) peak/dehydro-dihydroxynorleucine (de-DHLNL) peak ratios [42, 43]. Hence, the maturity of collagen fibrils was defined as the relative content of the "mature" trivalent cross-links (PYR) vs. the "immature" divalent PYR precursor cross-links (de-DHLNL). Since the PYR/de-DHLNL ratio increases during the fibrotic process, assays of the cross-links provide a relevant parameter to evaluate the fibrotic status of collagen-rich deposits formed in the presence of the ACA [44–46].

All tissue samples were prepared as described earlier [47, 48]. In brief, paraffin-embedded 5- μm thick tissue sections were deposited on the MirrIR low-e microscope slides (Kevley Technologies, Chesterland, OH). An FTIR spectrometer (Spotlight 400, Perkin Elmer, Waltham, MA) was used to analyze the regions of interest (ROIs) corresponding to the PC collagen-rich areas. The measurements were done in the 4000 cm^{-1} to 748 cm^{-1} wavenumbers spectral range, at a pixel resolution of $50\text{ }\mu\text{m}$, 8 scans per pixel, and a spectral resolution of 4 cm^{-1} . Co-added spectra from scanned ROIs were generated with the Spectrum Image software (PerkinElmer, Inc.).

In all assays of the FTIR-derived spectra, overlapping peaks were deconvoluted and analyzed based on the second-order derivative spectra and pre-determined bell-type Gaussian peak fitting function using the OriginPro software (version 2021, OriginLab Corporation, Northampton, MA USA) (S4 Fig in [S1 File](#)) [49, 50].

Assays of the flexion contracture

Assays of knee flexion contracture provide an overall readout for the ACA efficacy to reduce joint stiffness [16, 21]. These assays were done according to protocols developed for the model employed here using a custom-made instrument, (S5 Fig in S1 File) [16, 19, 21]. In brief, the injured or the contralateral uninjured leg was fixed in the instrument with the tibia and femur positioned at the right angle. This position was considered the starting point of zero degrees. Subsequently, applying the rate of loading set to 40°/min, an extension torque was applied to 0.2 Nm. Upon reaching the value of 0.2 Nm, the angle corresponding to the joint extension was recorded (S5 Fig in S1 File). The injured knee flexion contracture was then expressed as the uninjured control knee extension/the injured contralateral knee extension ratio [16, 19, 21]. Hence, relatively low ratio values indicated relatively mild flexion contracture, while large ratio values indicated relatively severe flexion contracture.

Our main interest was evaluating the 12wk group that included the recovery period [16, 21]. The extent of the joint stiffness measured after the recovery period reflects the extent of the PC scarring due to injury. In contrast, the stiffness measured right after 8 weeks of immobilization results from the scarring of injured PCs and changes occurring strictly due to joint immobilization.

Assays of the quality of healed PTs

Because of the complexity of knee injury created here to cause posttraumatic joint stiffness, standardization of a model wound to study the ACA's impact on the quality of neotissue formed during the healing process is challenging. To circumvent this problem, we generated a uniform defect in the PTs to evaluate the quality of healing by measuring the mechanical properties of neotissue.

Biomechanical assays of the neo-patellar tendons from the ACA-treated and the CA-treated rabbits were performed as described [51]. In brief, following the flexion contracture measurements and PC isolation, the remaining soft tissues were dissected, leaving a PT-tibia complex (S6 Fig in S1 File). The neo-patellar tendon formed during the healing phase was isolated from the bulk of the remaining medial and lateral portions of the PT. A corresponding central portion of a PT from an uninjured leg was also prepared as a control.

The tensile behavior of the patellar tendon complexes from the ACA-treated and CA-treated rabbits was analyzed, as described [51]. First, the patellar and tibial ends of the tendon complex were secured in custom fixtures (S6 Fig in S1 File). Next, tensile testing was performed using a material testing system (TA Instruments ElectroForce 3220 Series III) equipped with a 225 N load cell. The samples were pre-loaded (0.3 N), then conditioned for 10 cycles from 0 to 0.5 mm displacement at an elongation rate of 0.2 mm/s, similar to previous work [52, 53]. Finally, the tendons were loaded to failure using a displacement ramp with an elongation rate of 0.2 mm/s. Force and displacement were captured digitally, then converted to engineering stress and strain for analysis using custom software (GNU Octave). Outcomes included ultimate stress and Young's modulus.

Assays of OCD mineralization and scarring

We also studied the impact of the ACA on the healing of subchondral bone by measuring the calcification of the OCDs with a micro-computer tomography (μ CT) scanner (SkyScan, Bruker Inc., Carteret, NJ, USA), as described [54]. Following reconstruction of μ CT images, volumes of interest (VOIs) were selected in regions identified as the injury sites (Fig 1). Corresponding VOIs from contralateral uninjured legs were also analyzed. Our primary focus was on the Bv/Tv (percent of bone volume to the total analyzed volume) and the BMD (bone mineral density).

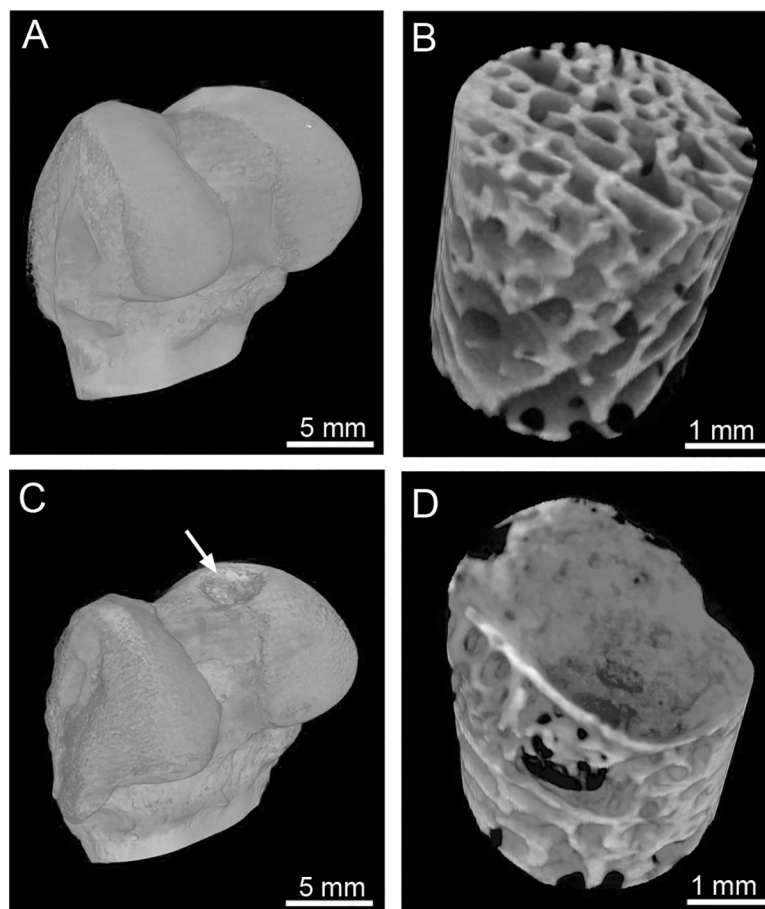


Fig 1. Representative computer tomography images of the uninjured (A) and injured (C, arrow) bones. Panels B (uninjured bone) and D (injured bone) indicate subchondral bone regions analyzed here to determine the extent of mineralization in the injured area (C&D).

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Histology of new soft tissue formed within the OCDs

Following μ CT, we isolated the tissue plugs encompassing the OCDs (Fig 2). The plugs were decalcified, processed for histology, then stained with hematoxylin and eosin (H&E) for general morphology and cellularity. The corresponding samples were also stained with picosirius

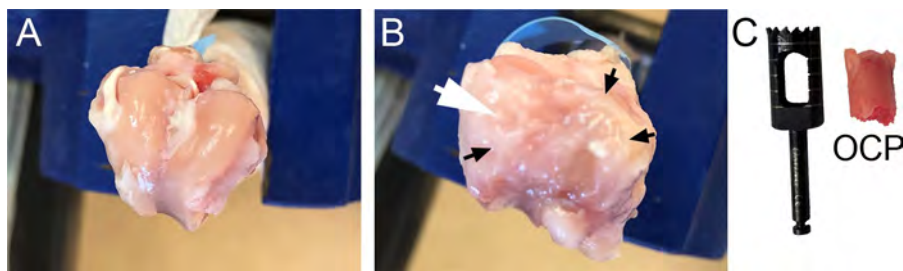


Fig 2. The appearance of the surface of uninjured (A) and injured (B) femoral condyles after twelve weeks of healing. The white arrow in B indicates the site of the osteochondral defect (OCD) made during the initial surgery. Please note the presence of the pannus-like tissue (black arrows) covering the femoral surface of the injured leg (B). C, An osteochondral plug (OCP) that encompasses the site of injury seen in panel B. A tool used to remove the plug is also indicated.

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red to analyze the collagen-rich matrix of the newly formed tissue, as described above for the PC samples.

Histology of tissues and organs

Following euthanasia, we collected samples of tissues and organs, including the brain, esophagus, thymus, aorta, heart, lung, liver, spleen, stomach, kidney, intestines, gonads, Achilles tendon, skeletal muscle, and the sciatic nerve. The samples were processed for histology, stained with H&E, and then observed for any abnormalities associated with the ACA treatment, including potential structural alterations and an influx of inflammatory cells.

Data analysis

Due to the skewness of data, we applied the log transformation to minimize the skewness's impact on statistical analyses. Consequently, results of assays of the serum markers of collagen metabolism, joint tissues' mechanical properties, and FTIR-based analyses were expressed as geometric means (GM) and geometric mean ratios (GMR) instead of arithmetic means and mean differences.

Changes in the HP, CP and XL concentrations in the sera of the ACA-treated group were compared to the CA-treated control. Linear fitted curves were generated to determine the kinetics of changes from time-0 to week-8, a period of the ACA administration. Subsequently, the curves' trajectories for the ACA and control groups were analyzed by comparing their slopes.

Furthermore, to analyze the HP, CP, and XL concentration changes across various time points within an analyzed group, we employed a within-group repeated-measures ANOVA with a Greenhouse-Geisser correction (IBM SPSS Statistics v. 26).

In all assays, statistical significance was defined as $p \leq 0.05$.

For the flexion contracture, we formulated the superiority hypothesis. We expected that the injured limb in the ACA-treated group would show reduced flexion contracture compared to the injured limb in the control group:

$$H1 : \mu_1[ACA : \text{injured}] \neq \mu_0[CTR : \text{injured}]$$

For the analyses, we estimated the mean difference between the ACA-treated and control groups ($D = \mu_1 - \mu_0$), along with the corresponding 2-sided 95% confidence limit and the p-value.

Also, it is often appropriate to test if a new experimental treatment is not unacceptably less efficacious than a control treatment. Hence, to study the effects of the ACA on the PT mechanical properties and calcification of the OCDs, we formulated the noninferiority hypothesis that the ACA treatment is not substantially worse than a control treatment. We computed the difference between the injured and contralateral non-injured limbs for each animal. We then compared the average of this difference for the ACA-treated animals and the CA-treated control.

Regardless of the treatment, we expected the injured limb's values to be much lower than those for the uninjured leg. We aimed to show that this drop (from uninjured to injured limb) in the ACA-treated group is not meaningfully worse than in the control group (by a pre-specified margin):

$$H1 : \mu_1[ACA : \text{uninjured} - \text{injured}] < M * \mu_0[CTR : \text{uninjured} - \text{injured}] \Rightarrow M * \mu_0 - \mu_1 > 0$$

Here, M is the noninferiority margin. A relative margin of 20–33% (i.e., $M = 1.20$ to 1.33) is

often appropriate since it is neither too broad (in which case it would be not very meaningful) nor too strict (in which case the study would require a considerable sample size). For example, a margin of 1.25 indicates that the worst-case scenario allowable for the ACA-treated group is an additional 25% beyond the decrease seen in the control group (always considering the difference between uninjured and injured limb).

Taking Young's modulus (MPa) of the PT as an example derived from a published similar rabbit model, if the mean in the control group is 800 (MPa) for the uninjured PT and 300 (MPa) for the injured PT, the mean difference in this group is 500 [22]. A margin of 25% means that we want to establish that the corresponding actual mean difference between uninjured and injured PTs in the treated group is no bigger than 625 (1.25×500), *e.g.*, the mean of 800 in the uninjured PT vs. 175 or larger in the injured PT (and hence a difference of 625 or less).

For the analyses, we computed the quantity $D = M^* \mu_0 - \mu_1$, along with the corresponding 1-sided (upper) 95% confidence limit and 1-tailed p-value (the non-inferiority hypothesis is inherently 1-sided).

Results

A rabbit model of arthrofibrosis

Out of 42 operated rabbits in this study, two females (referred to as #1 and #2) died during pre-operative anesthesia (Table 1). The third female (referred to as #3) broke the K-wire ten days after the first surgery and subsequently died during the application of anesthesia for the K-wire replacement surgery. Also, rabbit #3 demonstrated somewhat agitated behavior with signs of self-mutilation.

Histopathological evaluation of tissues and organs collected from these female rabbits was performed by IDEXX BioAnalytics (Columbia, MO USA). Some underlying pathologies were noted. Female #1: heart: multifocal myocardial degeneration and fibrosis in the ventricles, hypertrophy of the tunica media and reduced lumen diameter in one small cardiac artery; kidney: mild hyperplasia of the collecting duct epithelium; liver: small yellow-green nodules cytoplasmic granules in periportal hepatocytes. Female #2: lung: mildly underinflated, mild scattered alveolar macrophages, subpleural accumulation of heterophils with alveolar macrophages. Female #3: heart: degradation of cardiomyocytes with mild fibrosis and scattered mononuclear cell infiltrates; lung: mild to moderate heterophils within alveolar capillaries and larger vasculature. We observed no other physical or behavioral problems with the rest of the rabbits (Table 1); all of them were included in all assays.

Blood collection and analysis

Blood samples were collected at defined time points, and their cellular and biochemical parameters were analyzed. Overall, the blood parameters values remained within the physiological ranges defined for the rabbits throughout the entire experiment (S7–S10 Figs in S1 File) [55].

Trajectories of changes in the concentration of the serum markers of collagen metabolism

We analyzed changes in the serum concentration of crucial collagen metabolites measured from the time of injury until the end of the 8-week antibody-application period. The serum concentration of HP, CP, and XL at specific time points were analyzed by plotting the data, fitting linear curves, and comparing their slopes for the ACA group and the CA group (Table 2). While the steep slopes (high numerical values) indicated relatively fast changes in measured

Table 2. A summary of differences in the trajectories of changes in serum hydroxyproline (HP), the C-terminal propeptide of procollagen I (CP), and cross-linked telopeptides (XL) measured between the ACA group and the CA group during the 8-week treatment period.

Parameter	Group	^a GMR/wk (slope of lineary fitted curves)	The effect of ACA treatment (ACA/CA) with confidence levels and <i>p</i> values	
HP (μg/ml)	CA	1.01	ACA/CA	1.00
	ACA	1.01	95% CI ^b	(0.98,1.02)
			<i>p</i> = 0.892	
CP (ng/ml)	CA	1.04	ACA/CA	1.01
	ACA	1.05	95% CI	(0.97,1.05)
			<i>p</i> = 0.559	
XL (ng/ml)	CA	1.10	ACA/CA	1.28
	ACA	1.41	95% CI	(1.12,1.46)
			<i>p</i> = 0.001	

^aGMR/wk, geometric mean ratios.

^bCI, confidence interval.

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parameters, more shallow slopes (low numerical values) signified relatively slow changes. Data indicate that the dynamics of changes in the HP concentration and the CP concentration were similar in the ACA and control groups. In contrast, the rate of increase in the XL concentration in the ACA group was significantly faster than in the control group (Table 2).

Please note that GMR/wk presented in Table 2 represent the geometric mean ratio per week, i.e., the estimated GM today divided by the estimated GM a week ago. For instance, for XL (Table 2) measured for the 0-8-week period, that is 1.10 for CA and 1.41 for the ACA group, i.e., every week, XL increased on average by 10% and 41%, respectively. The effect of ACA treatment is $1.41/1.1 = 1.28$, i.e., the ACA-associated increase per week is ~28% larger than the increase per week in the control group.

Changes in the serum collagen markers across various time points

To analyze changes in HP, CP, and XL within the ACA group and within the CA group across specific time intervals, we utilized the repeated-measures ANOVA (Fig 3, Table 3). As demonstrated in Table 3, there were no significant changes in the CA-treated rabbits. In contrast, statistically significant changes were observed in all analyzed parameters in the ACA-treated animals (Table 3).

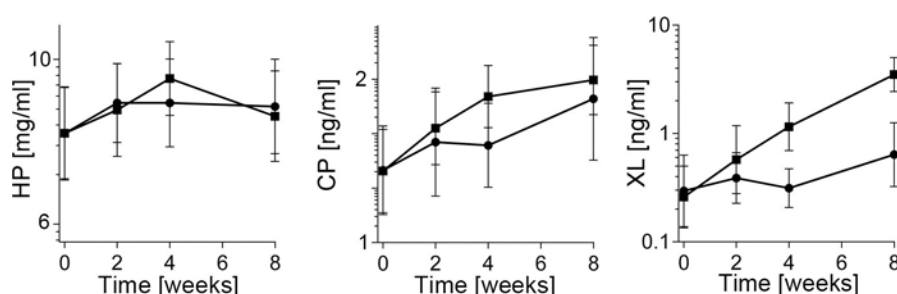


Fig 3. A graphic representation of the changes in the concentration of hydroxyproline (HP), the C-terminal propeptide (CP), and cross-linked telopeptides (XL) at indicated time points; the square symbols (■) represent the ACA-treated group, and the circle symbols (●) represent the control group. The geometric means (GMs) of analyzed parameters and 95% confidence intervals are presented.

<https://doi.org/10.1371/journal.pone.0257147.g003>

Table 3. Significance of changes in serum concentrations of hydroxyproline (HP), the C-terminal propeptide of procollagen I (CP), and cross-linked telopeptides (XL). These changes were measured within the ACA group and within the CA group across various time points.

Marker	Treatment	Test within-subject effects (Greenhouse-Geisser)	Statistical significance of changes between specific time points (weeks)					
			0–2	0–4	0–8	2–4	2–8	4–8
HP	CA	$F(2.218, 28.837) = 3.085, p = 0.056$	n.s	n.s	n.s	n.s	n.s	n.s
	ACA	$F(1.840, 36.808) = 4.734, p = 0.017$	n.s	$p = 0.009$	n.s	n.s	n.s	$p = 0.038$
CP	CA	$F(1.436, 20.102) = 3.123, p = 0.08$	n.s	n.s	n.s	n.s	n.s	n.s
	ACA	$F(2.261, 42.966) = 12.439, p < 0.005$	$p = 0.039$	$p = 0.006$	$p = 0.002$	$p = 0.01$	n.s	n.s
XL	CA	$F(1.546, 13.914) = 1.844, p = 0.198$	n.s	n.s	n.s	n.s	n.s	n.s
	ACA	$F(2.148, 27.929) = 18.669, p < 0.005$	n.s	$p = 0.007$	$p < 0.005$	n.s	$p = 0.007$	n.s

n.s, not significant.

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Collagen matrix formed in the PCs

We also examined the effects of the ACA and CA on the percentages of the green-birefringence (GB), the yellow-birefringence (YB), and the red-birefringence (RB) subpopulations of collagen fibrils present in the uninjured and injured PCs (Fig 4). Table 4 summarizes one-way ANOVA tests to determine the statistical significance of observed differences between analyzed groups.

Collagen matrix formed in the OCDs

We also analyzed the ACA impact on the fibrillar composition of the pannus-like tissue formed around and within OCDs (Fig 5). As adequate controls from the contralateral joints' uninjured sites are innately unavailable, we compared pannus-like tissues formed in the ACA and control groups. As indicated in Table 5, there were no statistically significant differences

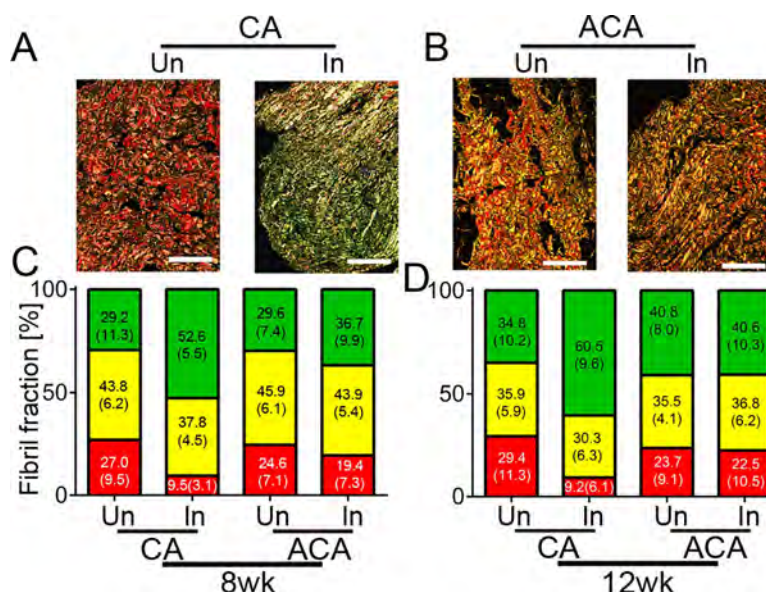


Fig 4. Histological quantification of various populations of picrosirius-stained collagen fibrils formed within injured posterior capsules (PCs). A, Samples of the PCs from uninjured (Un) and injured (In) knee joints of rabbits treated with control antibody (CA). B, Samples of the PCs from uninjured (Un) and injured (In) knee joints of the rabbits treated with the ACA. C&D, Corresponding graphs depict the percentages, with standard deviations in parentheses, of the green birefringence (GB), yellow birefringence (YB), and red birefringence (RB) subpopulations of collagen fibrils observed with the use of a polarized light microscope in the 8wk and 12wk groups. Bars = 100 μ m.

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Table 4. Analysis of differences between specific groups of collagen fibrils, defined by specific birefringence, observed in uninjured and injured posterior capsules (PCs) isolated from the ACA-treated or control rabbits.

Group	Treatment	Fibril subpopulation		
		^a GB	^a YB	^a RB
8wk	CA	$F(1, 10) = 20.7, p = 0.001$	$F(1, 10) = 3.6, p = 0.088$	$F(1, 10) = 18.1, p = 0.002$
	ACA	$F(1, 14) = 2.6, p = 0.129$	$F(1, 14) = 0.447, p = 0.514$	$F(1, 14) = 2.05, p = 0.175$
12wk	CA	$F(1, 20) = 37.0, p < 0.0005$	$F(1, 20) = 4.5, p = 0.046$	$F(1, 20) = 27.1, p < 0.0005$
	ACA	$F(1, 26) = 0.004, p = 0.949$	$F(1, 26) = 0.459, p = 0.504$	$F(1, 26) = 0.091, p = 0.765$

^aGB, green birefringence (represents thin, loosely-packed fibrils); YB, yellow birefringence (represents intermediate-diameter fibrils); RB, red birefringence (represents thick, well-packed fibrils).

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in the percentages of specific populations of fibrils present in the ACA-treated and CA-treated groups.

FTIR assays of the collagen content in the PC and OCD scars

We compared the relative collagen content in the PC scars formed in the presence of the ACA or CA. The amount of collagen was calculated with respect to sulfated GAGs and with respect to the total protein content represented by the FTIR spectra' amide II peak (S4 Fig in [S1 File](#), [Fig 6](#)). Similar assays were done for the OCD scars formed in the ACA-treated and CA-treated rabbits ([Fig 7](#)). [Table 6](#) summarizes one-way ANOVA tests to determine the statistical significance of observed differences between the analyzed groups.

FTIR assays of collagen cross-links in the PC and OCD scars

We also measured the maturity of collagen cross-links formed in the PC scar tissue in the presence of the ACA or CA. This was expressed as the FTIR-derived peaks' ratio representing the

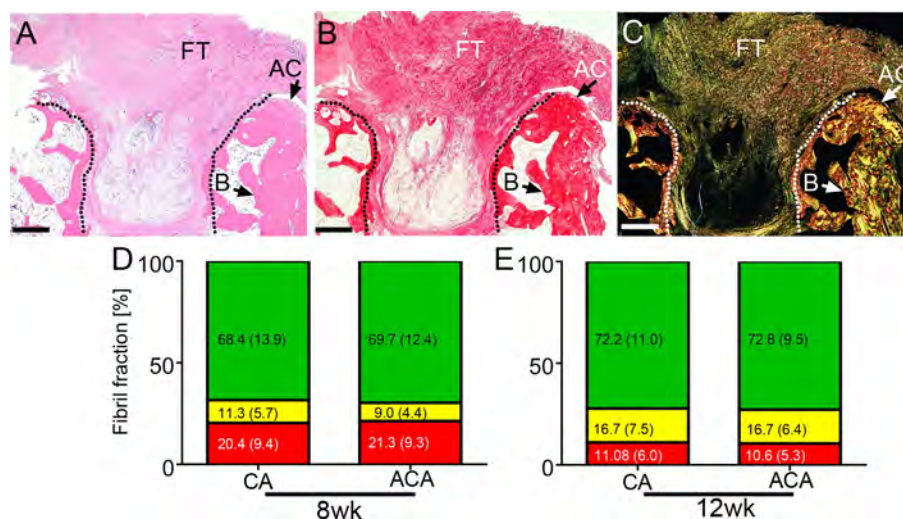


Fig 5. A histological assay of the pannus-like scar tissue formed in an osteochondral defect (delineated). The site of injury was stained with H&E (A) and with collagen-specific picrosirius red (B&C). The picrosirius red-stained samples were observed in normal light (B) and polarized light (C). Fibrotic tissue (FT), bone (B), and fragments of the articular cartilage (AC) are indicated. D&E, A summary of measurement of the percentages of the green birefringence (GB) fibrils, yellow birefringence (YB) fibrils, and red birefringence (RB) fibrils formed in the CA-treated and the ACA-treated rabbits. Corresponding segments of the bars include the means and standard deviations (in parentheses). Data for the 8wk and the 12wk groups are presented. Bars = 1 mm.

<https://doi.org/10.1371/journal.pone.0257147.g005>

Table 5. Significance of differences between the content of specific groups of collagen fibrils defined by the green (GB), yellow (YB), and red (RB) birefringence. The fibrils were observed in the osteochondral defects in the CA-treated and the ACA-treated rabbits.

Group	Fibril subpopulation		
	^a GB	^a YB	^a RB
8wk	$F(1, 48) = 0.12, p = 0.731$	$F(1, 48) = 2.3, p = 0.137$	$F(1, 48) = 0.117, p = 0.733$
12wk	$F(1, 142) = 0.102, p = 0.75$	$F(1, 142) = 0.001, p = 0.979$	$F(1, 142) = 0.3, p = 0.585$

^aGB, green birefringence (represents thin, loosely-packed fibrils); YB, yellow birefringence (represents intermediate-diameter fibrils); RB, red birefringence (represents thick, well-packed fibrils).

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mature PYR cross-link and immature de-DHLNL cross-link (Fig 6) [42]. Similar assays were done for the OCD scars formed in the ACA-treated and CA-treated rabbits (Fig 7). Table 6 summarizes one-way ANOVA tests to determine the statistical significance of observed differences between the analyzed groups.

Assays of the flexion contracture

Since the ultimate purpose of the anti-fibrotic treatment with the ACA is to reduce scarring-associated stiffness in the injured joints, we measured the extent of flexion contracture in the

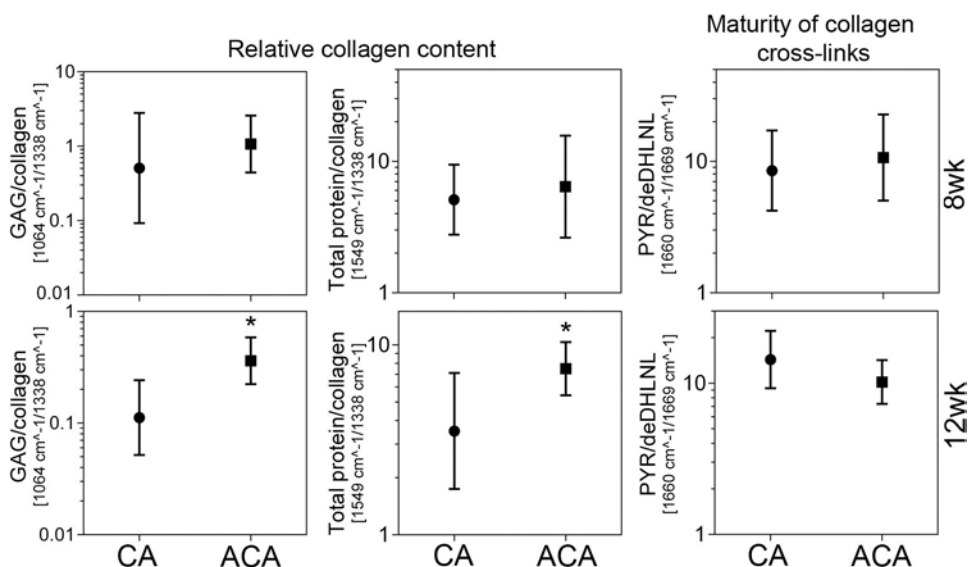


Fig 6. A graphic representation of the FTIR-based assays of the relative amount of collagen and the maturity of collagen cross-links in the scar tissue formed within injured posterior capsules of control (CA) and the ACA-treated rabbits. Results for the 8wk and the 12wk groups are presented. The graphs show geometric means (GMs) with 95% confidence intervals. Asterisks indicate groups with statistically significant differences of the means. The graph shows the relative collagen content calculated as the sulfated glycosaminoglycans (GAGs)/collagen and the total protein/collagen ratios. Consequently, the smaller the ratios, the higher relative collagen content. The ratios were calculated based on the areas of the spectral peak corresponding to the sulphated GAGs (centered around 1064 cm^{-1}) and the spectral peak corresponding to collagen (centered around 1338 cm^{-1}). To corroborate these measurements, the relative amount of collagen was also calculated based on the ratios of the spectral peak corresponding to the total proteins, represented by the amide II peak (centered around 1549 cm^{-1}) and the spectral peak corresponding to collagen. The maturity of collagen cross-links was expressed as the ratio of the area of the spectral peak corresponding to the mature trivalent PYR cross-link (centered around 1660 cm^{-1}) and immature divalent deDHLNL cross-link (centered around 1690 cm^{-1}). Hence, the higher the PYR/deDHLNL ratio the more mature collagen cross-links. Also, see S1 Table in S1 File.

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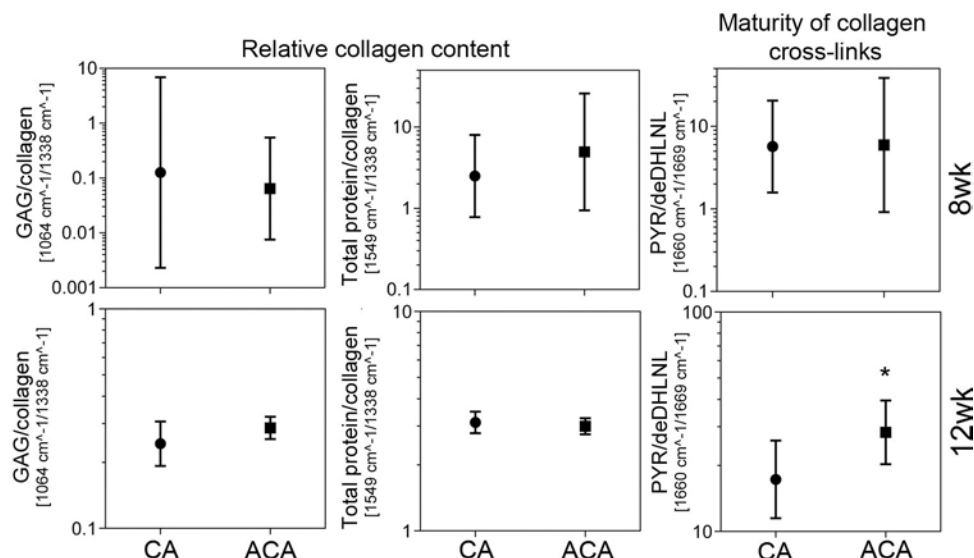


Fig 7. A graphic representation of the FTIR-based assays of the relative amount of collagen and the maturity of collagen cross-links in the scar tissue formed in the OCDs of the CA-treated and the ACA-treated rabbits. Results for the 8wk and the 12wk groups are presented. The graphs present GMs with 95% confidence intervals. Asterisks indicate groups with statistically significant differences of means. The graph shows the relative collagen content calculated as the GAG/collagen and the total protein/collagen ratios. Hence, the smaller the ratios, the higher relative collagen content. The ratios were calculated based on the areas of the spectral peak corresponding to the sulphated GAGs (centered around 1064 cm^{-1}) and the spectral peak corresponding to collagen (centered around 1338 cm^{-1}). To corroborate these measurements, the relative amount of collagen was also calculated based on the ratios of the spectral peak corresponding to the total proteins, represented by the amide II peak (centered around 1549 cm^{-1}) and the spectral peak corresponding to collagen. The maturity of collagen cross-links was expressed as the ratio of the area of the spectral peak corresponding to the mature trivalent PYR cross-link (centered around 1660 cm^{-1}) and immature divalent deDHLNL cross-link (centered around 1690 cm^{-1}). Thus, the higher the PYR/deDHLNL ratio, the more mature the collagen cross-links. See also S1 Table in S1 File.

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ACA-treated and control rabbits. Specifically focusing on the 12wk group, we performed the superiority test (see Data analysis above) to analyze the ACA impact on the knee joint flexion contracture (Table 7, S11 Fig in S1 File). Our results indicate that the ACA effect is: $2.84/4.47 = 0.64$, and the 95% CI is 0.41, 0.99, $p = 0.047$. Thus, compared to the control, the ACA-treatment reduced the uninjured/injured ratio (contraction), or the gap between the flexion contractures measured in the uninjured and the injured knees, and the reduction was significant ($p = 0.047$). In contrast, the 8wk group did not pass the superiority test. The ACA effect for this group was $9.82/8.6 = 1.14$, and 95% CI 0.63, 2.06, $p = 0.647$ (Table 7, S12 Fig in S1 File).

Impact of the ACA on the quality of healing of the PTs

We also measured the PT mechanical properties to determine the quality of their healing in the presence or absence of the ACA. Focusing on the PT ultimate stresses from the 12wk

Table 6. Analysis of differences between relative collagen contents and the maturity of collagen-cross-links in scar tissues formed in the PCs and osteochondral defects (OCD) in the CA-treated ACA-treated rabbits.

Scar tissue	Group	FTIR ratios		
		$1064\text{ cm}^{-1}/1338\text{ cm}^{-1}$ GAGs/collagen	$1549\text{ cm}^{-1}/1338\text{ cm}^{-1}$ Amide II/collagen	$1660\text{ cm}^{-1}/1690\text{ cm}^{-1}$ PYR/de-DHLNL
PC	8wk	$F(1, 9) = 0.02, p = 0.892$	$F(1, 9) = 0.159, p = 0.7$	$F(1, 9) = 0.003, p = 0.956$
	12wk	$F(1, 15) = 8.156, p = 0.012$	$F(1, 15) = 10.063, p = 0.006$	$F(1, 15) = 0.035, p = 0.854$
OCD	8wk	$F(1, 5) = 0.276, p = 0.621$	$F(1, 5) = 0.281, p = 0.619$	$F(1, 5) = 0.002, p = 0.965$
	12wk	$F(1, 22) = 1.043, p = 0.318$	$F(1, 22) = 0.407, p = 0.53$	$F(1, 22) = 4.331, p = 0.04$

<https://doi.org/10.1371/journal.pone.0257147.t006>

Table 7. Summaries of tests to determine the ACA's effects on the flexion contracture and parameters defining the quality of healing of the PTs (stress and Young's modulus) and subchondral bone defects (Bv/Tv and BMD)^a.

^a Parameter	Duration	Group	GM	GM	GMR	The effect of ACA treatment (ACA/CA) with confidence levels and <i>p</i> values	
			Uninjured	Injured	Uni/Inj		
Stress (MPa)	12wk	CA	12.6	7.9	1.61	ACA/CA	0.98
		ACA	13.5	8.6	1.57	95% UCL	1.35
						<i>p</i> = 0.103	
	8wk	CA	15.7	3.9	4.03	ACA/CA	0.79
		ACA	13.5	4.2	3.20	95% UCL	1.21
						<i>p</i> = 0.040	
Young's modulus (MPa)	12wk	CA	134.8	53.5	2.52	ACA/CA	1.02
		ACA	163.0	63.2	2.58	95% UCL	1.81
						<i>p</i> = 0.276	
	8wk	CA	258.8	32.6	7.94	ACA/CA	0.81
		ACA	233.7	36.4	6.41	95% UCL	1.69
						<i>p</i> = 0.161	
Extension at 0.2 Nm (deg)	12wk	CA	74.5	16.7	^b 4.47	ACA/CA	0.64
		ACA	75.7	26.6	^b 2.84	95% CI	(0.41, 0.99)
						<i>p</i> = 0.047	
	8wk	CA	65.0	7.6	^b 8.60	ACA/CA	1.14
		ACA	65.8	6.7	^b 9.82	95% CI	(0.63, 2.06)
						<i>p</i> = 0.647	
Bv/Tv (%)	12wk	CA	41.6	23.7	1.76	ACA/CA	0.97
		ACA	43.0	25.3	1.70	95% UCL	1.51
						<i>p</i> = 0.167	
	8wk	CA	51.4	15.0	3.43	ACA/CA	1.11
		ACA	50.2	13.2	3.80	95% UCL	2.01
						<i>p</i> = 0.366	
BMD [g/cm ³]	12wk	CA	0.70	0.45	1.55	ACA/CA	1.38
		ACA	0.68	0.32	2.14	95% UCL	2.27
						<i>p</i> = 0.629	
	8wk	CA	1.08	0.47	2.30	ACA/CA	0.85
		ACA	0.87	0.44	1.95	95% UCL	1.66
						<i>p</i> = 0.168	

^a Presented parameters were measured in uninjured and injured legs of rabbits from the CA-treated and ACA-treated groups (GMs are presented). Within each group, potential changes of these parameters in the injured legs were expressed as the ratios of values calculated for the uninjured and the injured legs (GMR). Subsequently, the effect of the ACA on these ratios was evaluated (ACA/CA) and presented with *p* values.

^b Joint contracture values defined as the ratio of knee extension of uninjured joint and that of contralateral injured joint. Please note that the starting point to measure knee extension was positioning the femur and the tibia at right angle (S5 Fig in [S1 File](#)). At this starting position, the value of extension was set to 0°. Subsequently, the torque was applied to the value of 0.2Nm and the extension angle (deg) was recorded. Non-stiff knees (uninjured) reached relatively high extension values while stiff (injured) knees reached relatively low extension values. Hence, large uninjured/injured ratios of measured extensions indicate a relatively large contracture while small uninjured/injured ratios of measured extensions indicate a relatively small contracture.

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group as an example, we found, in brief, that the GMs for the ACA and CA groups' uninjured PTs were ~13 MPa, indicating proper randomization ([Table 7](#), S11 Fig in [S1 File](#)). In contrast, the GMs for injured PTs in both groups had lower values. The GM dropped from 12.6 to 7.9 in the CA group and from 13.5 to 8.6 in the ACA group. The corresponding GMRs were 12.6/7.9 = 1.61 (CA) and 13.5/8.6 = 1.57 (ACA) ([Table 7](#), S11 Fig in [S1 File](#)).

Considering the ratios for the CA group (1.61) and ACA group (1.57), in the CA group, the GM of the uninjured PT was 61% higher, on average, than the GM of the injured one; and in the ACA group, the GM of the injured PT was 57% higher, on average, than the GM of the injured one. Thus, it seems that the ACA treatment reduced the "gap" between uninjured and injured tendons from 1.61 to 1.57. The formal treatment effect is $1.57/1.61 = 0.98$ (the smaller the value, the more significant the effect).

According to the hypothesis we presented in our original pre-experimental plan (see Data analysis), we are willing to accept up to 1.25 (25% increase of the "gap"). To formally test this non-inferiority hypothesis, we put a 95% one-sided (upper) confidence limit to the 0.98 value, resulting in a formal treatment effect of 1.35. So, even though our best estimate is that the ACA slightly reduces the uninjured-to-injured difference, we cannot exclude the possibility that it increases it up to 35%. Therefore, we have not proven non-inferiority (the associated *p*-value is 0.103, [Table 7](#) and S11 Fig in [S1 File](#)). We reached a similar conclusion for the results of measurements of Young's modulus of the PTs from the 12wk group ([Table 7](#), S11 Fig in [S1 File](#)).

For the 8wk group ([Table 7](#), S12 Fig in [S1 File](#)), we have proven non-inferiority for the ultimate stress parameter (*p* = 0.04). The Young's modulus, however, did not pass the non-inferiority test (*p* = 0.161).

Biom mineralization in the presence of the ACA

We also performed the non-inferiority test to compare the mineralization of subchondral bone in the ACA group versus the CA group. Assays of the Bv/Tv and the BMD parameters in the OCDs showed a drop in mineralization in both the ACA and CA groups ([Table 7](#), S13 and S14 Figs in [S1 File](#)). The Bv/Tv and BMD values for the ACA and CA groups' uninjured bones were similar, indicating good randomization. The ratios of parameters measured in the uninjured and injured sites were reasonably similar ([Table 7](#), S13 and S14 Figs in [S1 File](#)). Despite the close similarities, both Bv/Tv and BMD measured for the ACA group did not pass the non-inferiority tests ([Table 7](#)).

Impact of the ACA on internal tissues and organs

Because of the dynamic exchange of macromolecules between the intraarticular space and the lymphatic and circulatory systems, we analyzed whether continuous administration of the ACA had any negative impact on distant tissues and organs. Histology of crucial tissues and organs collected from all rabbits from the 8wk and the 12wk groups did not show any structural abnormalities or excess inflammatory cells. Supplementary figures present histological images of assayed specimens from the 8wk group treated with the ACA or CA highest concentration (S15–S22 Figs in [S1 File](#)).

Discussion

Surgical and accidental injuries to articular joints may lead to arthrofibrosis. Because of the significant burden of arthrofibrosis, therapeutic approaches are needed to effectively and safely reduce excessive posttraumatic scarring [56, 57]. Our earlier research demonstrated that targeting collagen fibrillogenesis with a novel ACA reduces scar formation and decreases joint stiffness [16]. While we have previously demonstrated the primary mechanism of the ACA-dependent inhibition of collagen fibril formation, the overall effects of applying this antibody have not been studied thus far. Hence, studies presented here aim to define the crucial effects of blocking collagen fibrillogenesis in the posttraumatic model of arthrofibrosis.

Impact of the ACA on collagen turnover

Our results on serum concentration of HP measured across various stages of the healing process indicate that it increased significantly between time-0 and week-4 in the ACA-treated group. We associate this increase, in part, with the ACA-mediated upsurge of free collagen molecules that have not been incorporated into collagen fibrils.

While assays of serum HP provide information on the degradation of collagenous proteins, concentration of serum CP reflects the biosynthesis of procollagen I [58]. In contrast to the control, we determined that the CP serum concentration in the ACA group changed significantly across selected time points. This observation was somewhat unexpected because the ACA does not affect the production of procollagen I. However, it is essential to point out that in normal conditions procollagen I molecules, with their C-terminal propeptides, associate transiently with matrix-bound collagen microfibrils. Studies have demonstrated that CPs present on the surfaces of such fibrils regulate their shape [59]. Thus, it is conceivable that the lack of significant changes in the serum concentration of the CPs across measured time points in control group was due to the trapping of the procollagen I molecules via their binding with tissue microfibrils. Hence, we hypothesize that the ACA reduces the amount of microfibril-bound procollagen I, thereby accelerating its degradation and increasing the CP's serum concentration. Our recent study result showing that the ACA may bind its epitope in the microfibrils, in addition to binding free collagen molecules, supports this hypothesis [11, 60].

In the ACA group, the CP's serum concentration peak and the HP's concentration peak coincided. These data suggest that the first four post-surgery weeks represent a dynamic phase of collagen biosynthesis and degradation in the model of arthrofibrosis utilized here. These data are consistent with collagen metabolism kinetics measured after injuries to the bone, tendon, and skin [61–63].

Unlike HP and CP, cross-linked collagen peptides present in the sera are derived solely from collagen fibrils. Thus, assays of collagen I-derived XLs provided an opportunity to measure the dynamics of matrix remodeling in the collagen I-rich joint tissues in the presence of the ACA. We demonstrated that the increase of the XL serum concentration was significantly higher in the ACA-treated group than in control. We predict that this rapid increase in the ACA-treated rabbits reflects a robust remodeling phase of the wound healing process. Due to the ACA-dependent reduction of the new collagen fibrils that stabilize the scar tissue, the remodeling phase likely had an earlier onset. In support of this postulation, a study of skin wound healing demonstrated that new collagen fibrils appeared in the wounded site three days post-injury [64]. Hence, we suggest that the ACA-dependent acceleration of tissue remodeling may represent an additional mechanism by which this antibody reduces the scar formation and improves joint mobility after traumatic injuries.

The ACA-dependent reduction of scar collagen

Histological and FTIR assays of collagen matrices in the scar tissues formed in the PCs and within the OCDs support the ACA-dependent reduction of collagen fibrils. Histological assays of the PCs from the control group's injured joints demonstrated a significant increase in the thin fibrils formed in the injury sites [16, 21]. In contrast, we observed no significant increase in the thin fibrils in the injured PCs treated with the ACA, most likely due to the ACA-dependent attenuation of collagen fibrillogenesis [16].

Unlike in the PCs, in the pannus-like tissue formed around and within the OCDs, we observed no differences in the relative contents of corresponding collagen subpopulations, with different birefringence, in the ACA and control groups. To explain this dissimilarity, we point out that while in the PCs the scar tissue is mainly formed by local fibroblasts that

produce collagen I, in the OCD, the pannus-like scar tissue is formed *de novo* with potential involvement of chondrocyte-like cells [16, 21, 65]. As such, this scar includes collagen II fibrils, in addition to collagen I [66, 67]. Since collagen II fibrils are relatively thin, they demonstrate green birefringence when stained with picosirius red and observed under polarized light [68]. Because the ACA does not interact with collagen II, it does not interfere with collagen II fibrillogenesis. Therefore, we postulate that the ACA impact on pannus-like structure formation is less prominent than on strictly collagen I-based matrices.

ACA-associated changes in collagen cross-links

We also studied the ACA effects on collagen fibrils cross-links. The FTIR-based studies showed no effect of the ACA on the mature PYR/immature de-DHLNL cross-link ratio in injured PCs. In contrast, we observed a statistically significant increase in this ratio in the fibrotic tissue formed in the pannus-like tissue formed within the OCDs of the ACA-treated 12wk group.

The increase of this ratio could reflect the decrease of the denominator, i.e., the immature cross-links, due to the ACA-dependent reduction of new collagen I-based fibrils that contain de-DHLNL cross-links [69]. However, we cannot exclude a possibility that the numerator increase, i.e., the PYR cross-links, could have raised the PYR/de-DHLNL ratio. Since we observed this rise in the 12wk group but not in the 8wk group, we postulate that mechanical stimulation enabled by joint remobilization could have partially increased the PYR cross-links content [70]. Considering the dynamic nature of changes in developing scar tissues, the observed increase in the PYR/de-DHLNL ratio represented, most likely, the net changes in their numerator and denominator components.

Changes in joint stiffness

Since the excessive formation of collagen-rich scar tissue stiffens the knee joint structures, most notably the PC, we analyzed whether the ACA-mediated changes in matrix remodeling and the collagen content reduce joint stiffness. Utilizing the superiority test, we confirmed our earlier observations and demonstrated that the ACA reduces the flexion contracture significantly in the 12wk group [16].

We did not see significant differences between the ACA-treated and the CA-treated rabbits from the 8wk group analyzed immediately after removing the K-wires. As indicated earlier, the immobilization itself stiffens the joints by mechanisms other than scarring, and a recovery period is needed to reduce this impact [24–26, 71]. Utilizing the same animal model, Hildebrand *et al.* showed that even without any intervention, the flexion contracture decreases gradually following the K-wire removal and plateaus after about 16 weeks without reaching a typical value of an uninjured knee [71]. Consequently, significantly reduced stiffness in the 12wk group treated with the ACA, but not with CA, suggests that this antibody accelerates the recovery process compared to the CA-treated control. Considering faster remodeling and decreased amounts of collagen material in the PCs, we propose that the ACA-mediated inhibition of collagen fibrillogenesis is a linchpin of the accelerated recovery.

The quality of healing in the presence of ACA

Although reduced flexion contracture in the ACA group is a positive therapeutic outcome, the quality of joint tissue healing needed to be assessed since the ability of ACA to reduce fibrillogenesis could negatively impact the quality of healed tissues.

We hypothesized that the ACA treatment is not substantially worse than a control treatment. Since the joint tissue mechanical properties define their quality, we measured the

ultimate stress and Young's moduli of healed PTs. We also measured the mineralization of the OCDs as a parameter defining the healing of the bone defects. Our results indicate that the mechanical and mineralization parameters were similar in the ACA and control groups, albeit we did not prove non-inferiority of the ACA treatment. Future experiments with larger animal groups will clarify the non-inferiority issue.

Effects of the ACA administration on distant organs

A crucial requirement of any antibody-based therapy is a lack of significant off-target cross-reactivity and any significant negative impact on vital tissues and organs. Our earlier studies demonstrated that the ACA does not cross-react with uninjured collagen-rich tissues undergoing no active fibrillogenesis [14]. Here, the dose escalation studies demonstrated that a relatively high concentration of the ACA does not cause any unwanted changes in crucial tissues and organs, despite long-term treatment. In particular, the lack of the ACA effects on collagen I-rich tissues, including tendon, sciatic nerve, muscle, lung, and others, indicates that this antibody has target-specific properties. Normal cellular and biochemical parameters of the blood of the ACA-treated rabbits further indicate the safety of the ACA treatment.

Our study presented here is the first report that defines crucial mechanisms for reducing posttraumatic arthrofibrosis by directly targeting collagen fibrillogenesis. Detailed assays of the collagen metabolism and structure of collagen-rich matrices demonstrate the high specificity and safety of the ACA. Consequently, this study paves the way to move the anti-fibrotic technology presented here toward clinical application.

Limitations of the study

This study has a few limitations that should be considered when interpreting the results. First, the arthrofibrosis model we employed here does not represent all injury types caused by trauma to the joints. For instance, we did not introduce bone fractures, which frequently occurs due to joint injury. As healing of bone fractures may impact the healing process of joint tissues, future models should include them to study the utility of anti-fibrotic agents. Second, we realize that although the number of animals in the 8wk groups was appropriate for the dose escalation studies, the number was not adequate for mechanical and biochemical assays of scar tissues. Consequently, results of these assays should be interpreted considering this limitation. Third, although a pump-based system provided a reliable and controllable ACA-delivery platform, we realize its limits for clinical use. Hence, in the future, clinically-relevant biomaterials should be evaluated for sustained delivery of the ACA.

Supporting information

S1 File.
(PDF)

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Supplementary Materials

Mechanisms of reducing joint stiffness by blocking collagen fibrillogenesis in a rabbit model of posttraumatic arthrofibrosis.

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Supplementary Table S1. Fourier-transform infrared (FTIR) spectroscopy parameters applied in collagen assays.

Target	Band position [cm ⁻¹]	Bond vibrations	Purpose
Sulfated GAGs	1064	SO ⁻³ vibration	To provide a reference for collagen changes
Protein-specific amide II peak	1549	N-H bending and the C-N stretching vibration	To provide a total protein reference for collagen changes
Collagen	1338	CH ₂ wagging vibration of proline side chains	To measure relative changes in collagen content
PYR cross-link	1660	A constituent of amide I peak	To measure mature pyridinoline cross-links
de-DHLNL cross-link	1690	A constituent of amide I peak	To measure immature dehydro-dihydroxynorleucine cross-links, an immature PYR precursor.

Supplementary Figures:

Supplementary Figure S1. Crucial surgical steps to generate knee injury in a rabbit model of arthrofibrosis. A, Getting an access to the knee cavity. B, Drilling a hole in the tibia (Tb) to install a Kirschner wire (Kw). C, Placing a loop of the Kw over a femur (Fe), D, Fixing the Kw with a nut placed over the tibia. E, Installing a pump (P) connected to the knee cavity via a silicone tube (St). F, An X-ray image of the operated leg immobilized in the flexed position with a Kw.

Supplementary Figure S2. A schematic of the experimental design of the study.

Supplementary Figure S3. A schematic depicting the premises of assays of collagen metabolites. A, A procollagen I molecule with intact N propeptides (NP) and C propeptides (CP); hydroxyproline residues present in the triple-helical domain are also indicated (HP). B, A collagen I molecule generated by extracellular cleavage of the NP and the CP; here, we will analyze the CP of procollagen I. C, A typical staggered arrangement of collagen molecules incorporated into a fibril. Collagen cross-links (XL) that link these collagen molecules are indicated with X. D, Collagen fragments formed due to turnover/degradation of collagen fibrils. Note that all collagen triple helix-derived fragments will include HP, and some will retain the collagen cross-links.

Supplementary Figure S4. FTIR spectroscopy of the pannus-like scar tissue formed within OCDs. A, A microscopic image of a selected ROI. A dotted line marks the border between the bone (B) and fibrotic pannus-like tissue (FT). B, A corresponding map of the average intensity of the FTIR signals. C, A representative FTIR spectrum (black line) with fitted peaks (red line). The peaks utilized in this study are indicated.

Supplementary Figure S5. A custom-made device to measure the flexion contracture of the knee joints. Clamping of the femur (Fe) and tibia (Tb) is indicated. The arrow shows the movement of the femur.

Supplementary Figure S6. A. Isolation of the patellar tendon (PT)-tibia (Tb) complex. B. A setup of the complex seen in A for the mechanical tests. The insert depicts the PT in which the arrow indicates the healed defect created during the initial surgery.

Supplementary Figure S7. Cell parameters of the blood samples collected at indicated time points from the 8wk ACA-treated group (red lines) and control (black lines).

Supplementary Figure S8. Biochemical parameters of the blood samples collected at selected time points from the 8 wk ACA-treated group (red lines) and control (black lines).

Supplementary Figure S9. Cell parameters of the blood samples collected at various time points from rabbits maintained for 12 weeks after surgery in the presence (red lines) or the absence (black lines) of the ACA.

Supplementary Figure S10. Biochemical parameters of the blood samples collected at various time points from rabbits maintained for 12 weeks after surgery in the presence (red lines) or the absence (black lines) of the ACA.

Supplementary Figure S11. A graphic representation of differences in mechanical parameters of the knee joints and PTs in the ACA-treated ($\square$ and $\blacksquare$) and the CA-treated ($\circ$ and $\bullet$) rabbits from the 12wk group. The left column shows results of measurements of uninjured (Un) and injured (In) legs. The GMs and 95% confidence intervals are indicated. The left panels show the Un/In ratios of analyzed parameters.

Supplementary Figure S12. A graphic representation of differences in mechanical parameters of the knee joints and PTs in the ACA-treated ($\square$ and $\blacksquare$) and the CA-treated ($\circ$ and $\bullet$) rabbits from the 8wk group. The left column shows results of measurements of uninjured (Un) and injured (In) legs. The GMs and 95% confidence intervals are indicated. The left panels show the Un/In ratios of analyzed parameters.

Supplementary Figure S13. A graphic representation of differences in mineralization of osteochondral defects in the ACA-treated ($\square$ and $\blacksquare$) and the CA-treated ($\circ$ and $\bullet$) rabbits from the 8wk group. The left column shows results of measurements of uninjured (Un) and injured (In) legs. The GMs and 95% confidence intervals are indicated. The left panels show the Un/In ratios of analyzed parameters.

Supplementary Figure S14. A graphic representation of differences in mineralization of osteochondral defects in the ACA-treated ($\square$ and $\blacksquare$) and the CA-treated ($\circ$ and $\bullet$) rabbits from the 12wk group. The left column shows results of measurements of uninjured (Un) and injured (In) legs. The GMs and 95% confidence intervals are indicated. The left panels show the Un/In ratios of analyzed parameters.

Supplementary Figure S15. Histology of brain tissues. Symbols: ScWm; subcortical white matter, Odg; oligodendroglia, Pc; Purkinje cells.

Supplementary Figure S16. Histology of stomach and esophagus. Symbols: Mm; mucous membrane, Sm; submucosa, Lp; lamia propia, Sse-k; stratified squamous epithelium-keratinized.

Supplementary Figure S17. Histology of aorta and heart muscle. Symbols: Tum; tunica media, Myc; myocardium.

Supplementary Figure S18. Histology of the lung tissue and intestines. Symbols: Ig; intestinal glands. Because the lungs were not inflated prior to the fixation, the lung tissue appears compact.

Supplementary Figure S19. Histology of liver and kidney. Symbols: Hl; hepatic lobule, Rc; renal cortex.

Supplementary Figure S20. Histology of spleen and thymus. Symbols: Cr-Tc; cortex thymic cells, Ln; lymphoid nodule.

Supplementary Figure S21. Histology of the sciatic nerve and the Achilles tendon. Symbols: Nf; neural filaments, Cf; collagen fibers.

Supplementary Figure S22. Histology of testis and ovaries. Symbols: CST; convoluted seminiferous tubules, Grn; granulosa cells, Ti; theca interna, Oc; oocyte.

SUPPLEMENTARY FIGURES

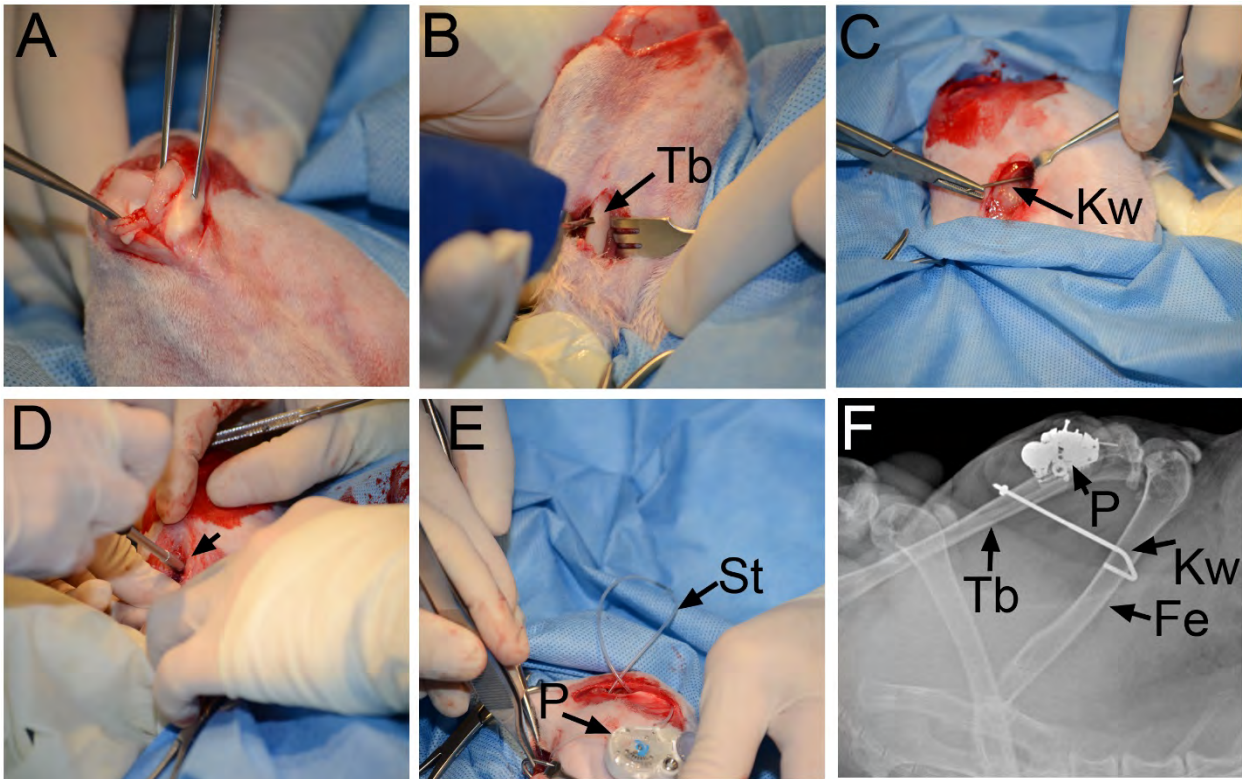


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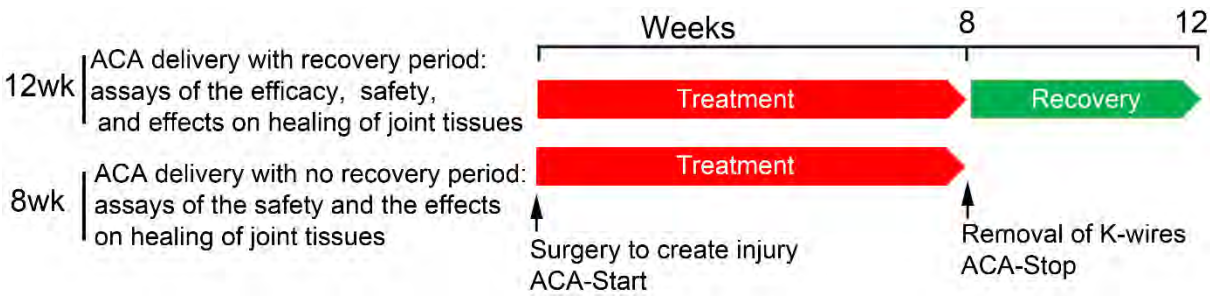


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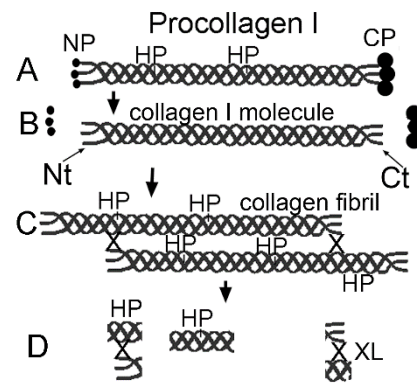


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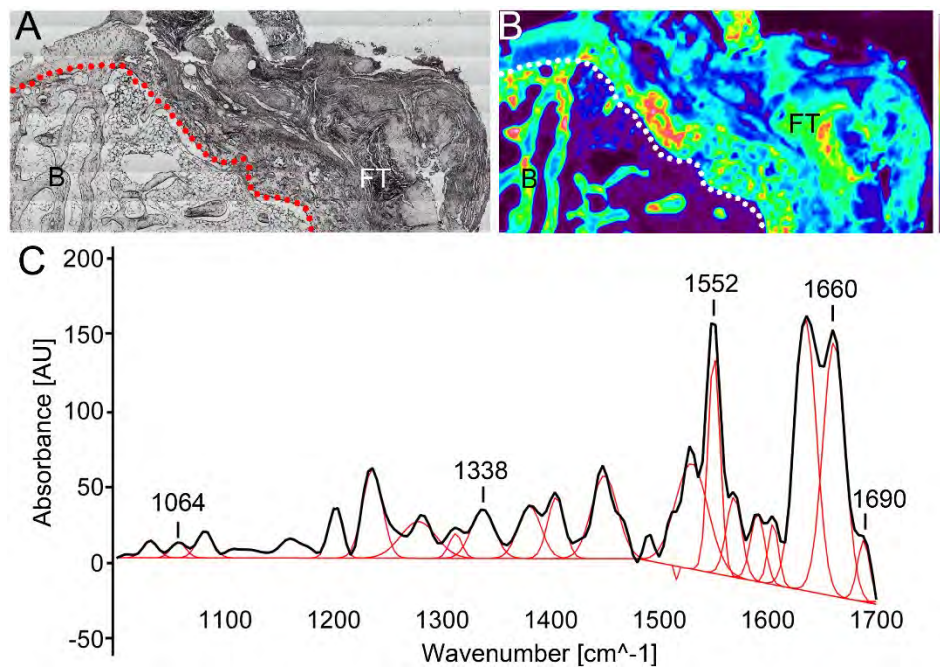


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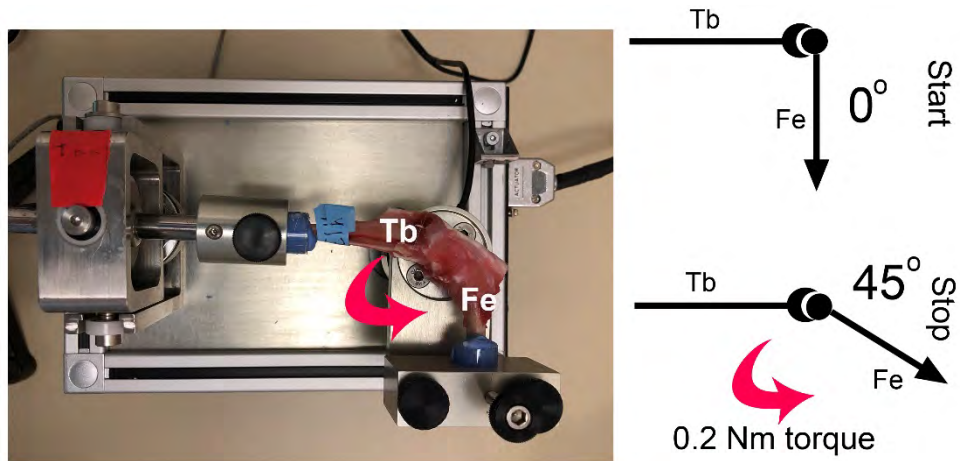


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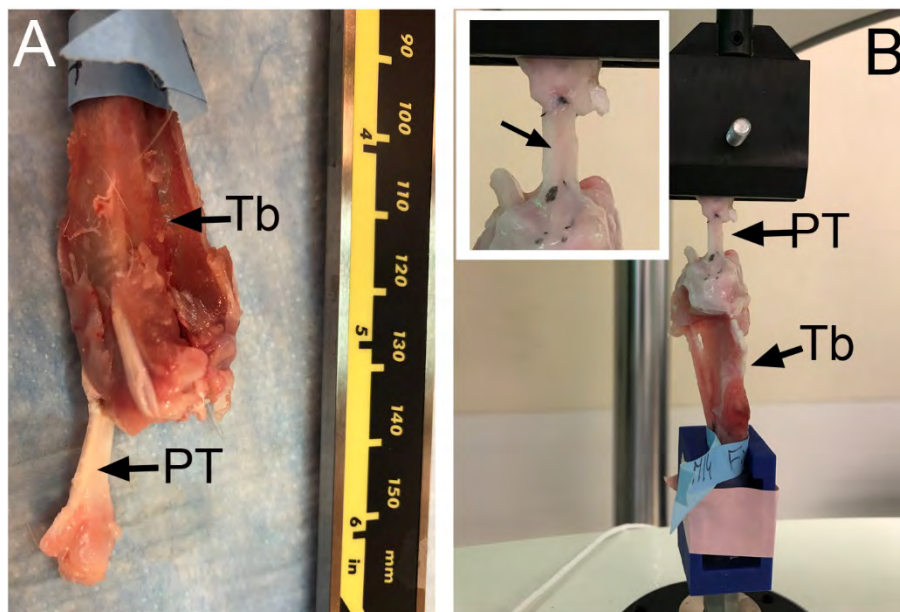


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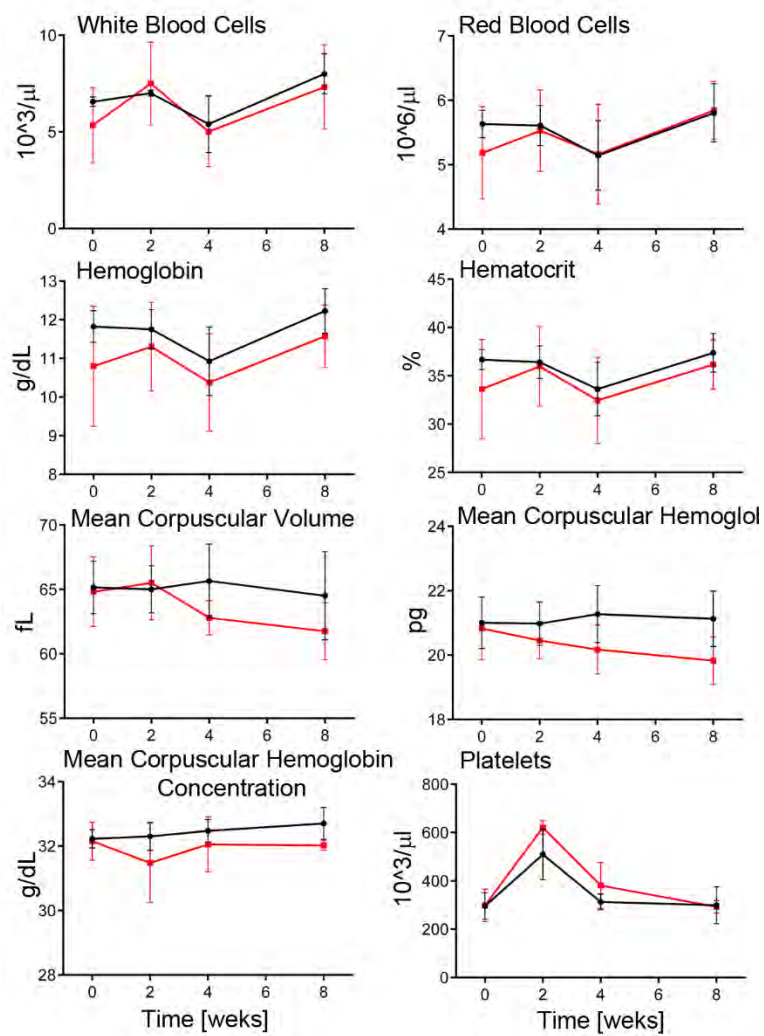


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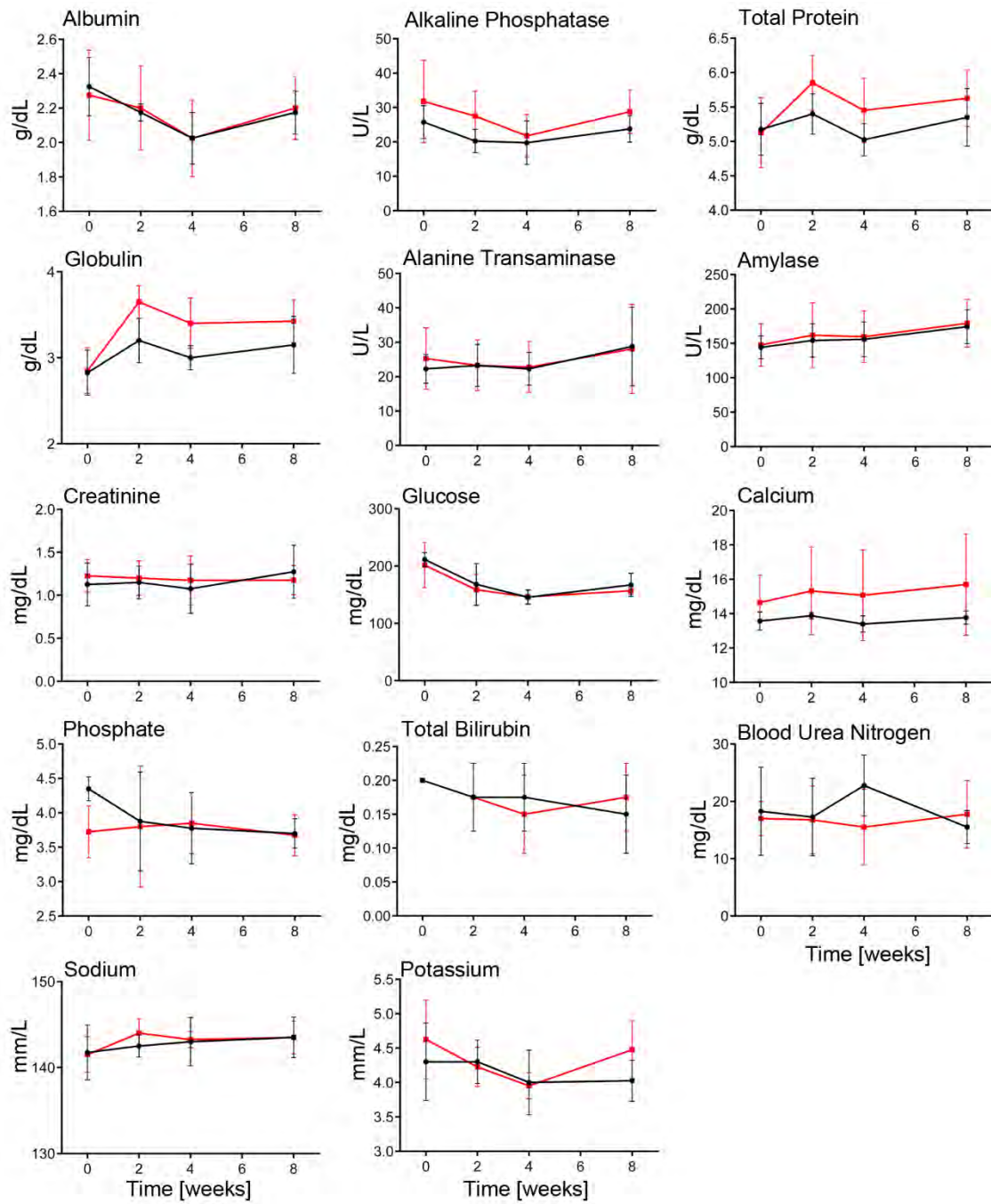


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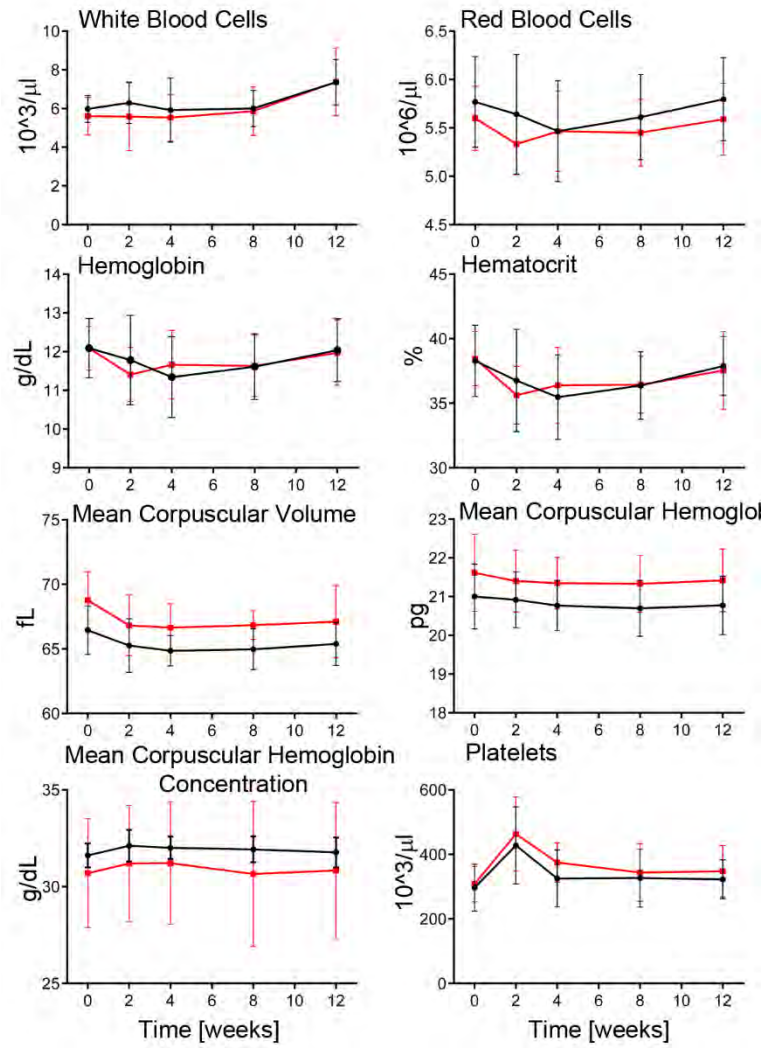


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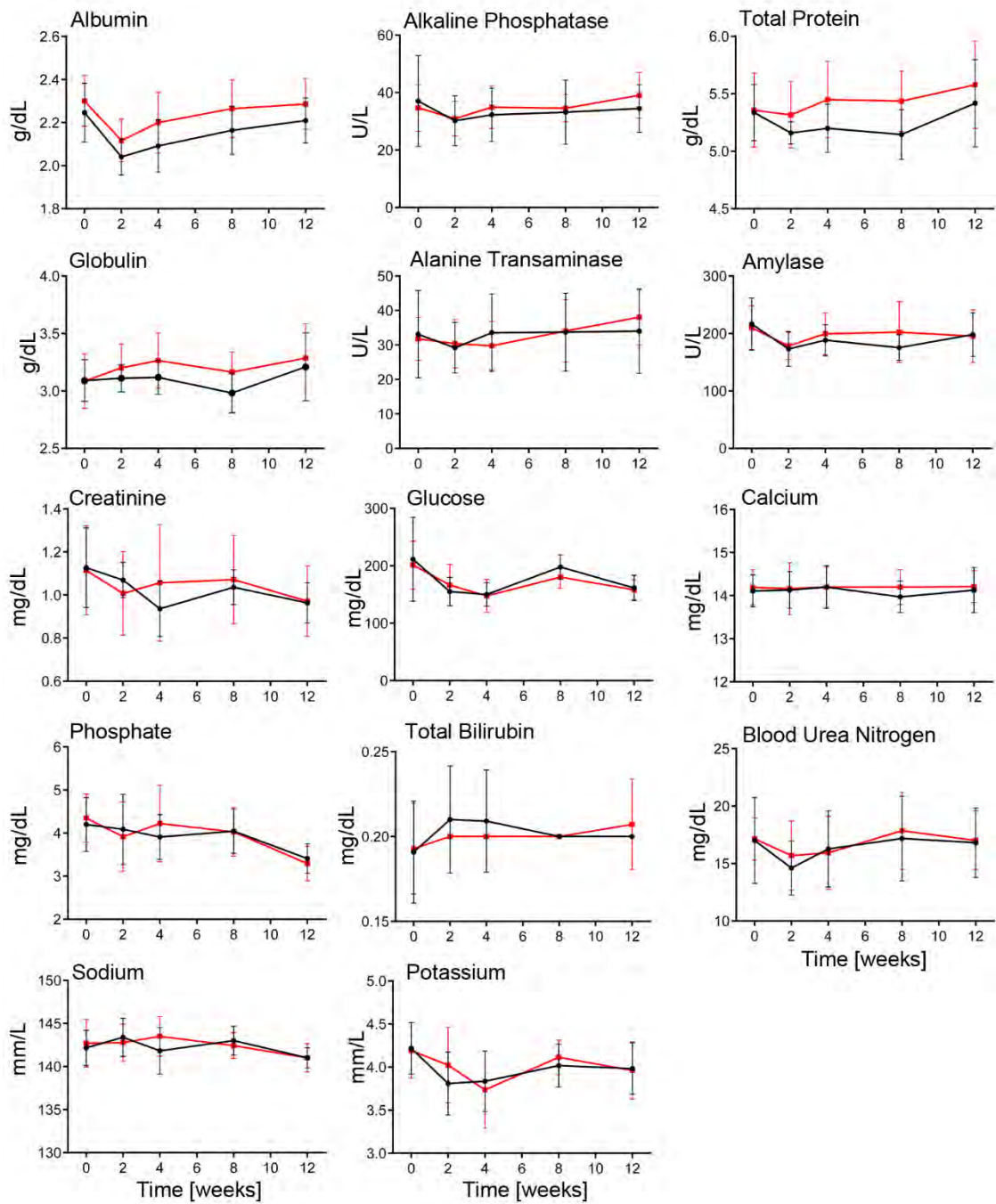


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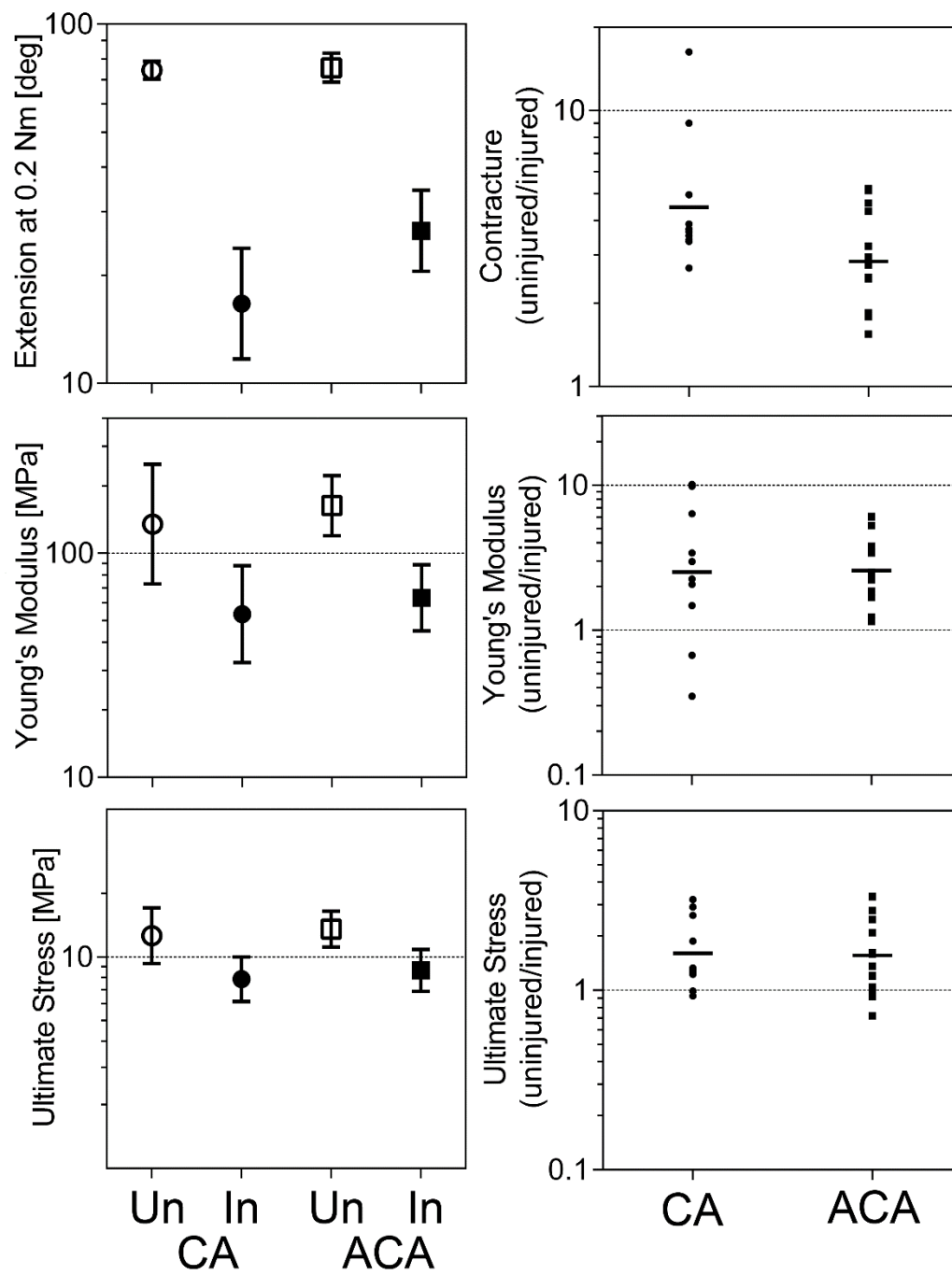


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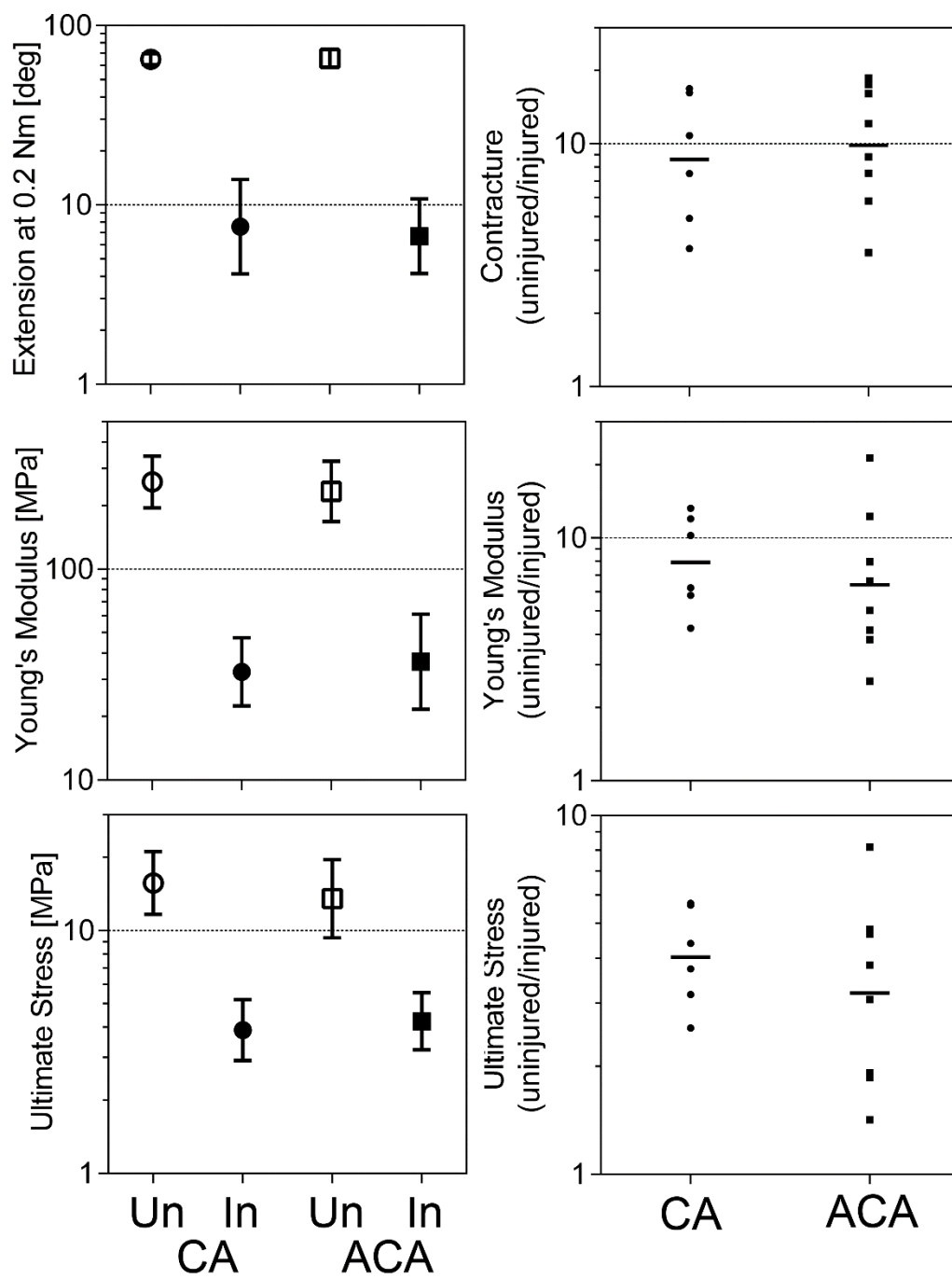


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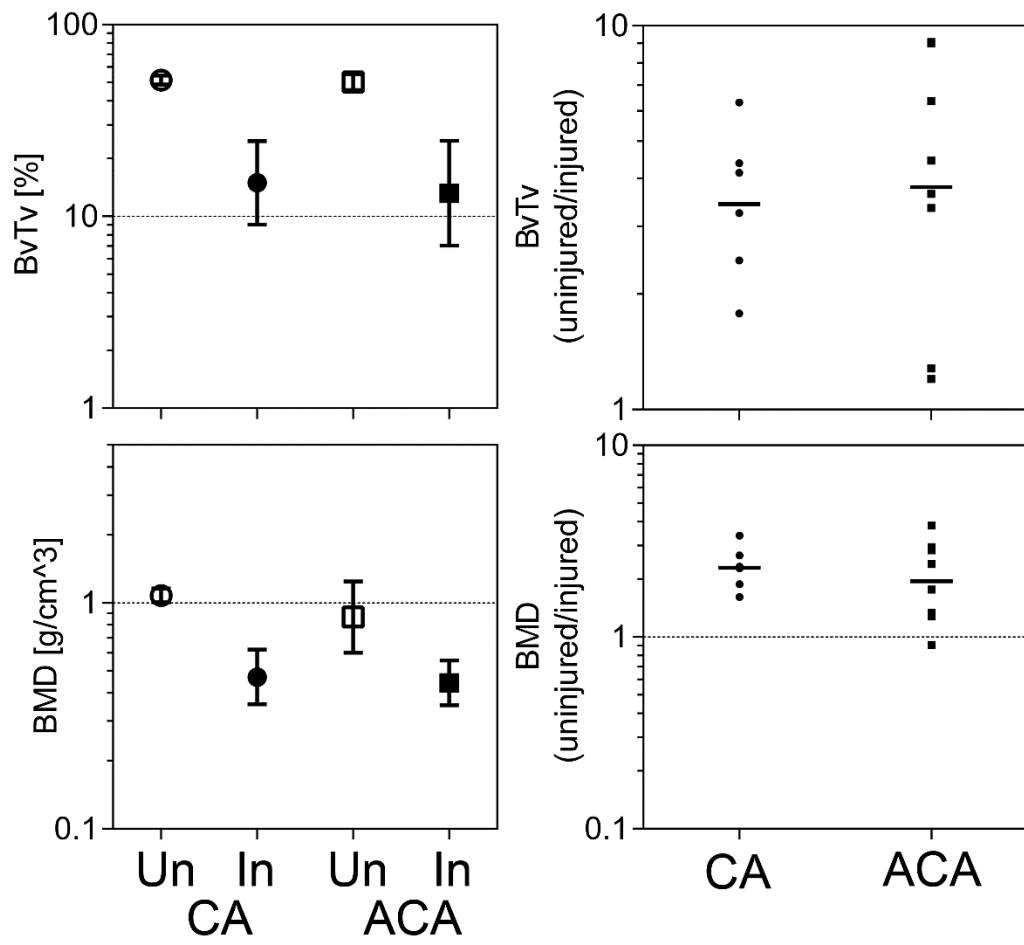


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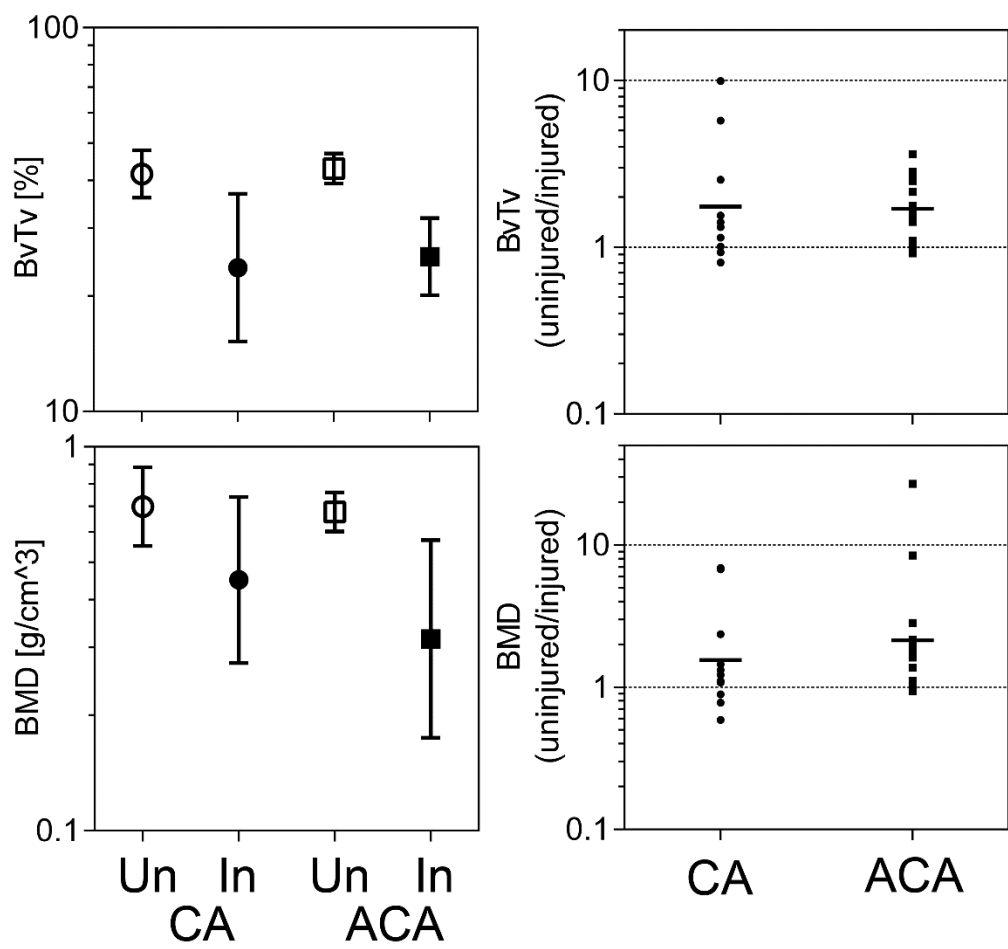


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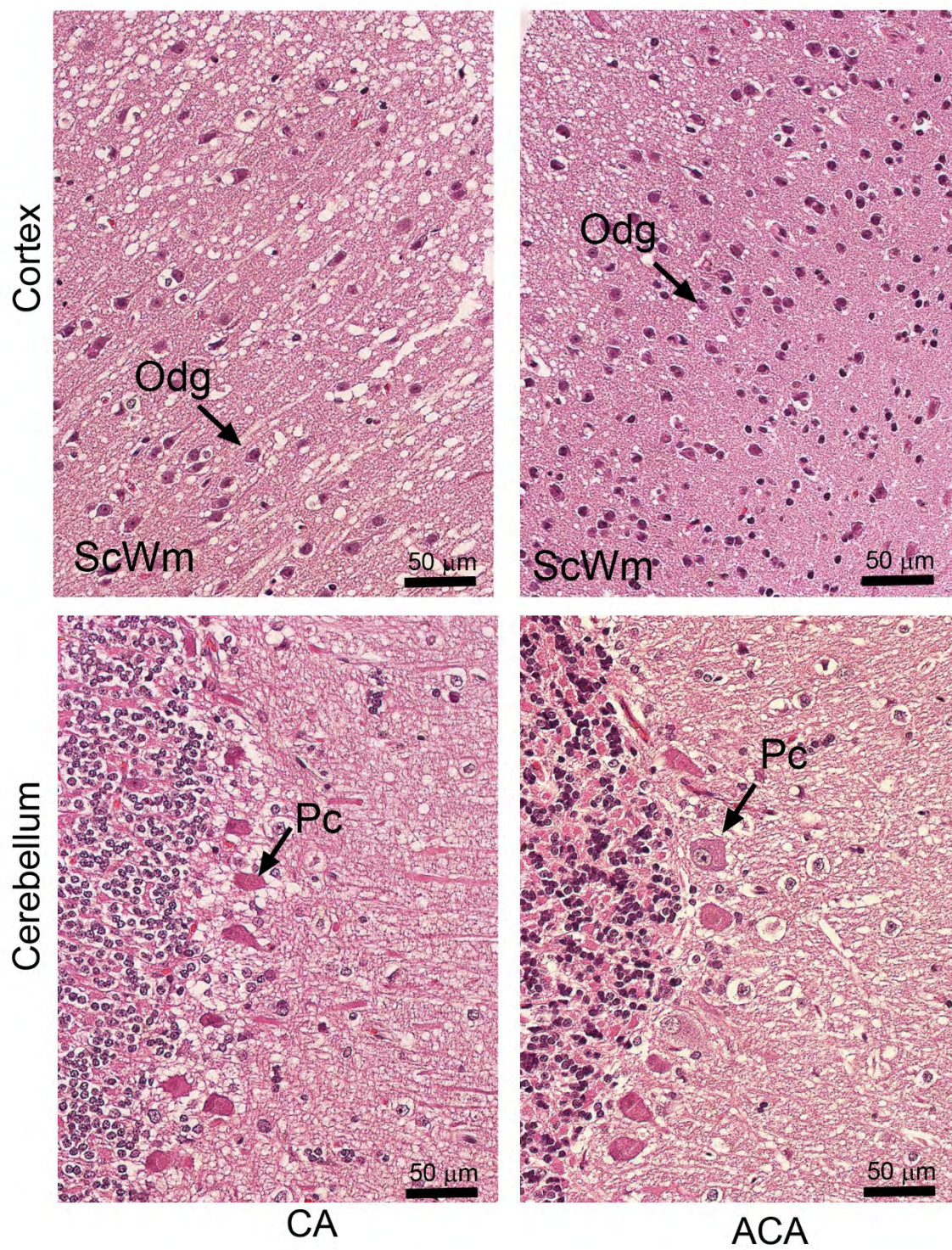


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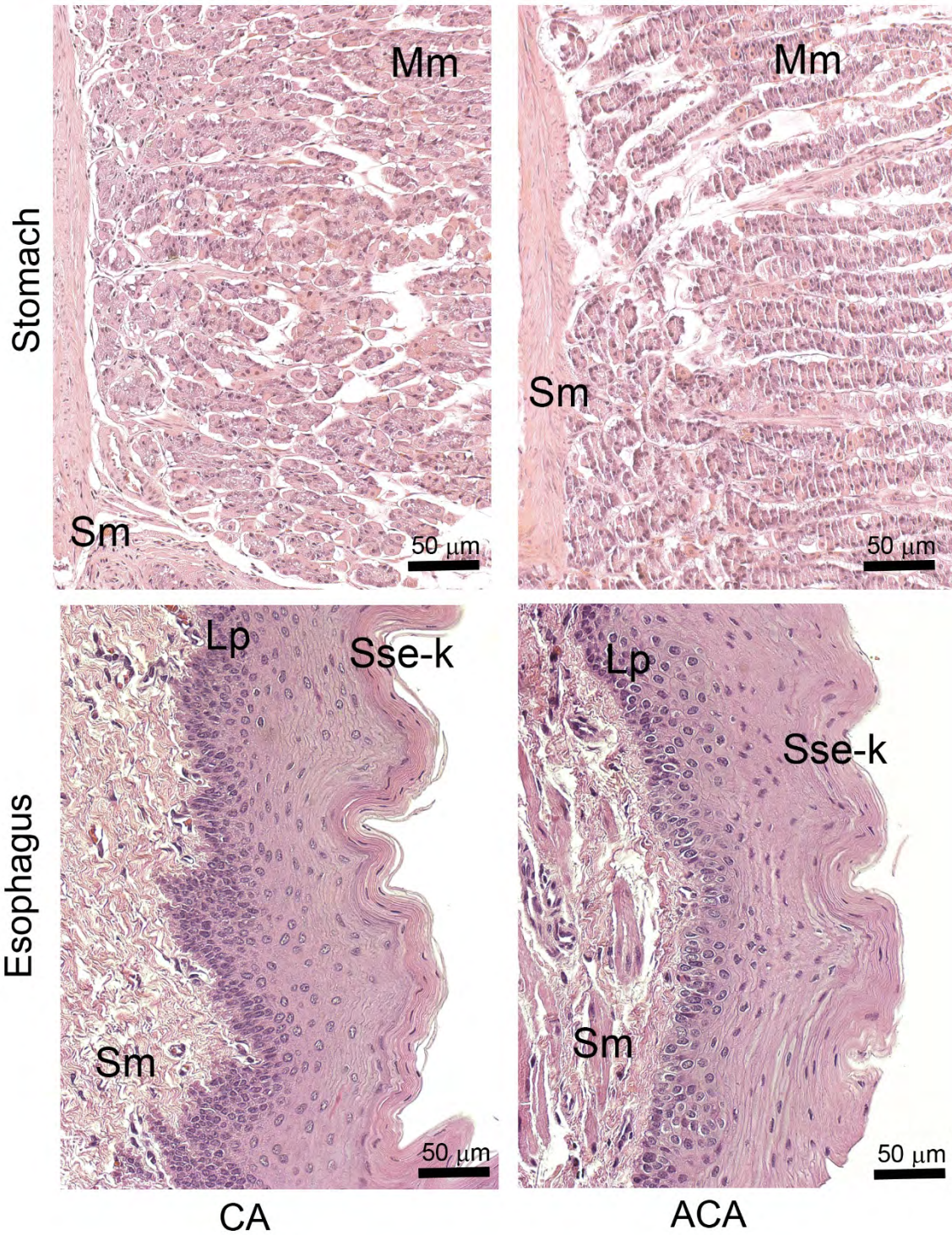


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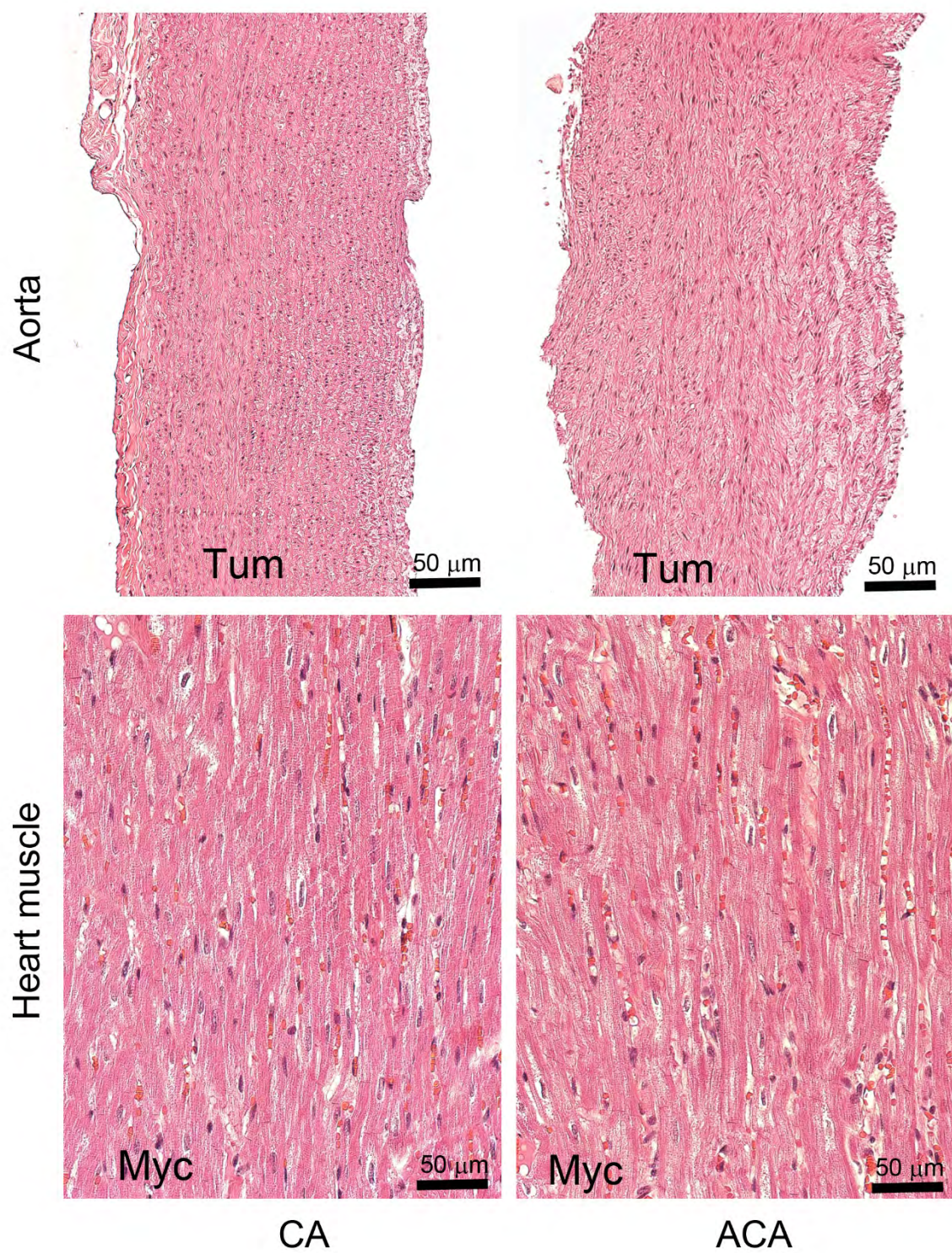


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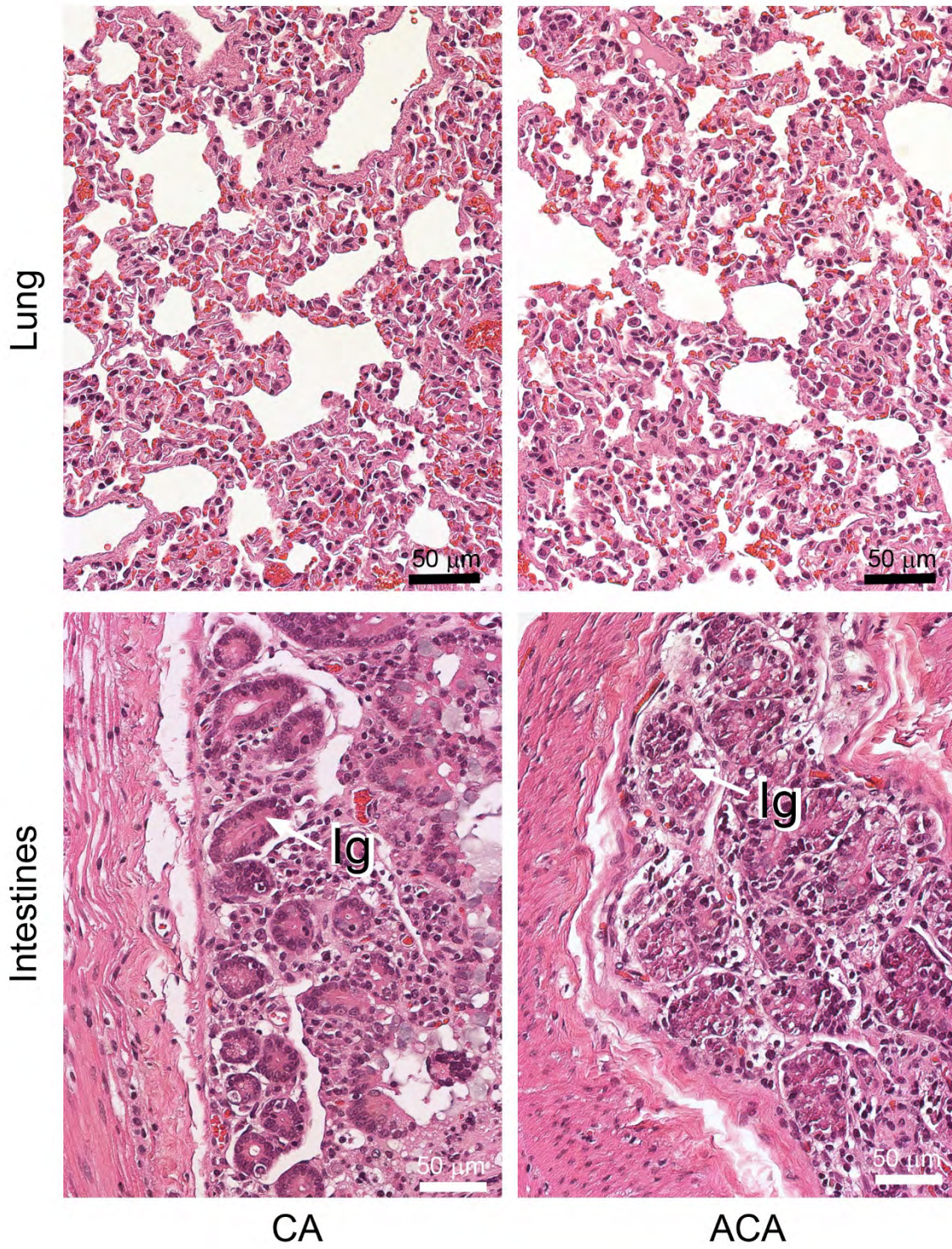


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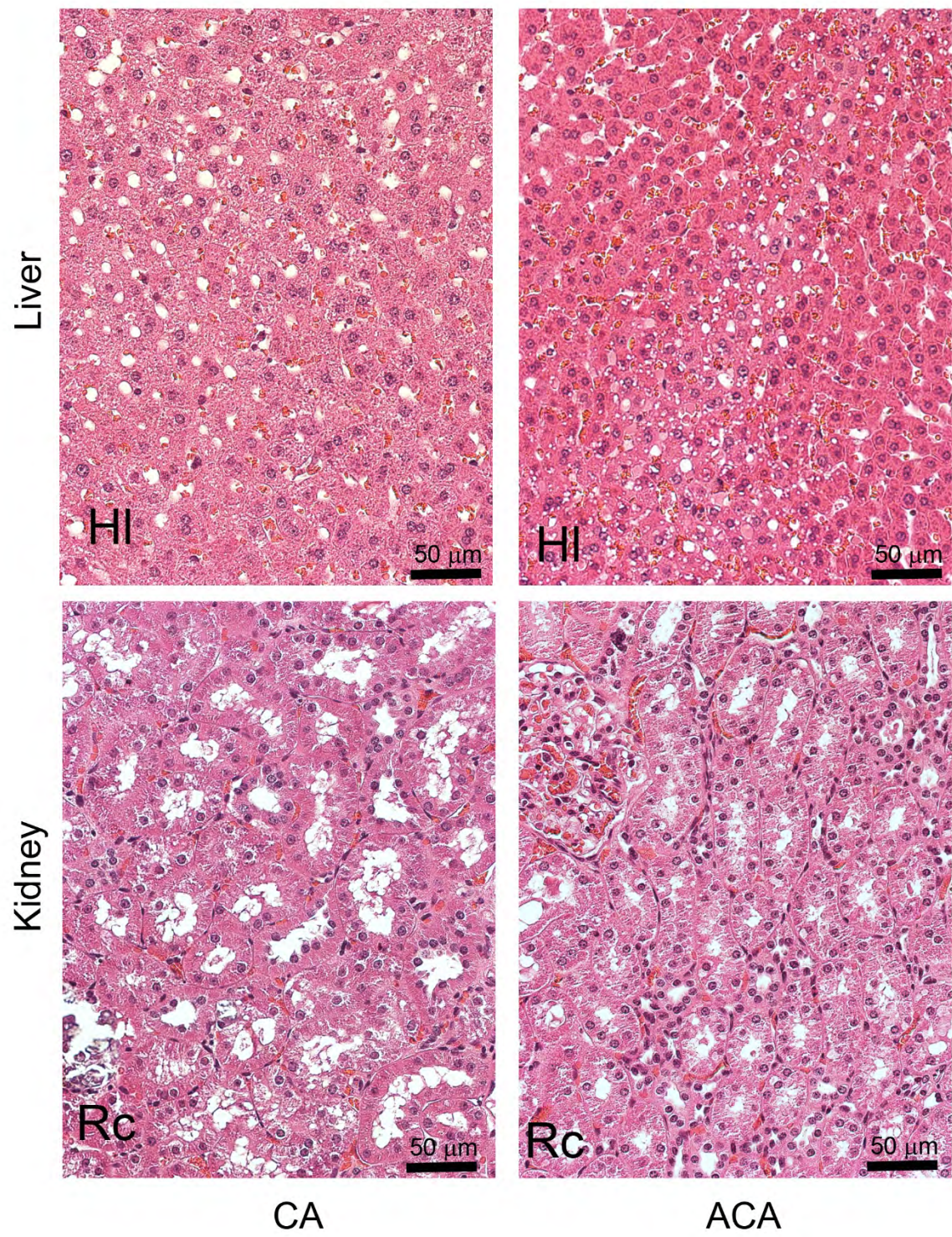


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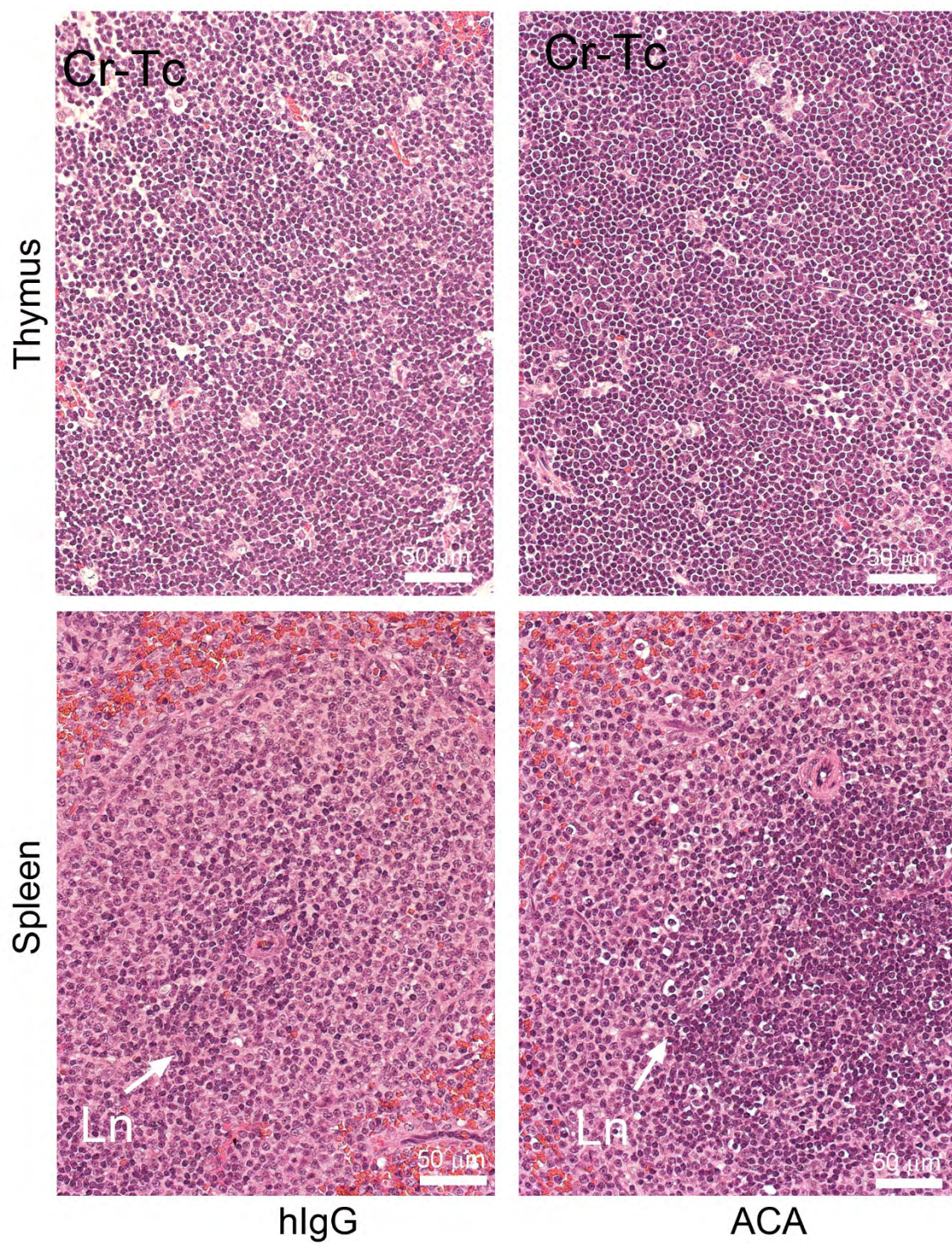


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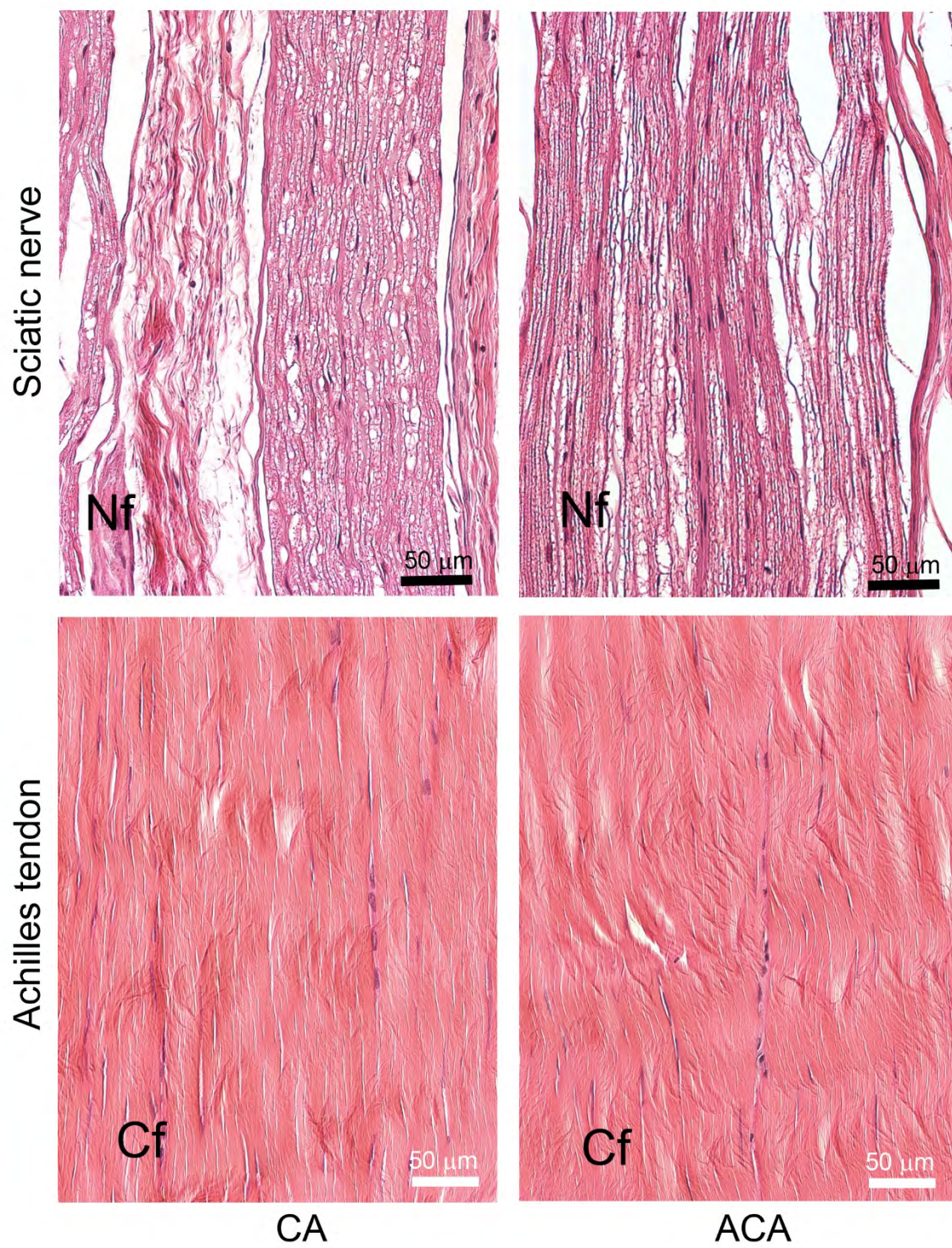


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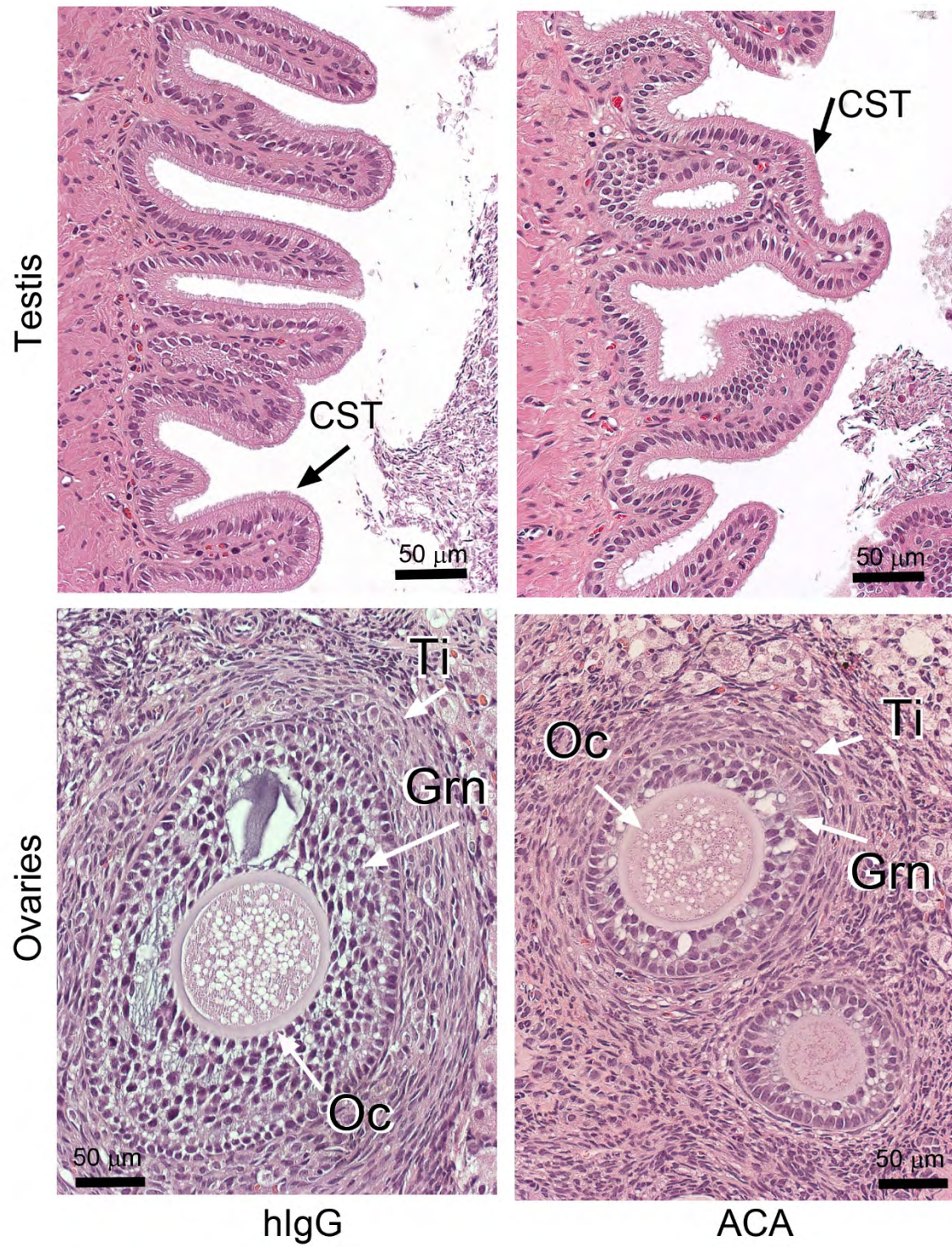


Figure S22

APPENDIX #3

Request for Supplemental Funds Due to the Impacts of the COVID-19 Pandemic

AWARD NUMBER: W81XWH-18-1-0554

TITLE: "Prevention of the Post-Traumatic Fibrotic Response in Joints: a Critical Preclinical Evaluation of an Antifibrotic Antibody"

PRINCIPAL INVESTIGATOR: Andrzej Fertala, Ph.D.

CONTRACTING ORGANIZATION: Thomas Jefferson University, 1020 Walnut Str, Philadelphia, PA 19107-5543

Although we managed to follow the plan described in the original SOW, we could not avoid significant roadblocks caused by the ongoing pandemic. The attached copy of the SOW indicates the current status of each task.

After careful review of the current status of the project entitled: "Prevention of the Post-Traumatic Fibrotic Response in Joints: a Critical Preclinical Evaluation of an Antifibrotic Antibody" (award number W81XWH-18-1-0554), we request extra funds to mitigate some roadblocks caused exclusively by challenges resulting from the COVID-19 pandemic:

Challenges with supply-chain

• Problem 1

Inability to purchase bioreactor vessels. We experienced a problem with purchasing disposable bioreactor vessels needed to produce a crucial antibody utilized in this study. In brief, the bioreactor producer (Eppendorf) has informed us that all vessels for growing surface-attached cells are shipped only to companies working on COVID-19 and cannot be sold to academia (see attached email).

Impact on the project:

Delay in antibody production. Since the beginning of our study, the system we developed to produce the anti-collagen antibody (ACA) depended on growing CHO cells anchored to the bioreactor's porous discs. Because the bioreactor vessels were not available for our purchase, we faced a delay in antibody production.

Countermeasures:

Changing cell culture conditions. To circumvent the bioreactor problem, we had to change the anchorage-dependent cell culture conditions to growing the cells in suspension. Growing cells in suspension is an intrinsically slow process, which requires an investment to purchase specific cell culture media and pieces of hardware necessary to connect a cell-suspension vessel to a controller. Although we successfully changed the cell-culture conditions, this change cost us time and money.

Current status: We successfully produce the ACA in CHO cells grown in suspension.

• Problem 2

Delays with supply of animals. Covance Inc., the original supplier of the rabbits we use in the study, has stopped selling them. Buying the rabbits from other potential suppliers was challenging due to the high demand of the biotech sector working to combat COVID-19.

Impact on the project:

Delays in some aspects of the study: Because of this disruption in the supply of rabbits, we faced unexpected delays.

Countermeasures:

We secured the standing order to purchase the rabbits from Charles River Laboratories so we now have a reliable source of experimental animals (see attached order).

Consequences of the need to pay salaries during the COVID-19 ramp down.

• Problem 1

Ultimate reduction of funds for experimental research. Due to the need to maintain salaries during the COVID-19 pandemic ramp down, when "wet laboratory" research and animal studies could not be conducted as planned, the actual dollars available for experiments have decreased.

Impact on the project:

A reduction of the funds available for experimental research.

Countermeasures:

We managed to save some funds by running some assays in house instead of outsourcing, as initially planned. Also, we reduced the number of replicates in some ELISA assays, thereby saving some funds.

Requirements for completing the project conclusively

To conclude the project meaningfully, we do not have to make any changes to the original SOW. To achieve crucial goals, we have to perform planned experiments that we did not have a chance to initiate due to the COVID-associated challenges. The following points briefly describe the planned experiments.

1. The original plan was to test the safety of the short-term and the long-term treatment of the rabbits with the ACA. First, we performed dose-escalation studies with three ACA concentrations. These studies were completed successfully.

2. The next step was to test the long-term efficacy of the highest safe ACA dose and evaluate its impact on the healing of joint tissues, including tendons, subchondral bone, and the posterior capsule. Although we managed to determine the impact of the highest dose (7.5 mg/knee/week) on the healing process, we could not determine its long-term efficacy to reduce posttraumatic joint stiffness due to challenges with the bioreactor's supplier. Therefore, it is necessary to produce a new batch of the ACA in cell suspension cultures and create an experimental group of rabbits to perform the required tests.

Possible impact of the award if the request for supplementary funds is granted

Testing the dose-dependent safety and efficacy of a novel therapeutic compound is necessary for its clinical implementation. Supplemental funds will define the efficacy of the highest ACA dose selected for the study, thereby rendering the project complete.

In the long run, supplemental funds will enable designing the next step toward implementing the ACA into the clinic. As the next step, we plan to develop clinically relevant material for the ACA's intraarticular delivery.

Possible impact of the award if the request for supplementary funds is denied

Should the supplemental funds be denied entirely, the study will not be complete, and the missing part will have to be performed one way or another to move the ACA-based therapy to reduce posttraumatic joint stiffness from the bench to the clinic.

Should the additional funds be granted at an amount lower than requested (please see below), the project would still be able to be completed, albeit not at the optimal level.

Tasks that could be reasonably modified should the request be denied such that the final project outcome would still be meaningful

We see no alternatives to performing the studies planned in the original proposal with the highest ACA concentration, and we already took some steps to mitigate the impacts of the COVID-19-related challenges listed above. In brief, we tested the ACA's efficacy at a significantly lower concentration (0.5 mg/knee/week) than the one deemed the highest safe concentration (7.5 mg/knee/week). The highest safe concentration must be tested to ensure the ACA-based technology's full benefits.

A detailed budget for the supplement funds

We present two budget options. Both options require extending the project for six months to produce an additional ACA batch and analyze groups of rabbits treated with the highest safe dose of the ACA. Please note that the rabbits have to be operated on twice and maintained for three months before performing the required tests. Therefore, extending the project for six is unavoidable but reasonable.

Option 1. An optimal supplemental budget that would allow complete execution of the project. In this option, we would create two groups of rabbits: (i) treated with the highest safe concentration of the ACA, and (ii) non-treated control. Based on our results with the lowest ACA concentration, we predict that a sample size of 10 in each group has a 90% power to detect a difference between means of 1.84 with a significance level (alpha) of 0.05 (two-tailed).

The total cost for the optimal option, including indirect costs, is

The following tables present a detailed budget:

Table 1. Personnel.

Personnel (% effort)	6-month salary	FB^a (@21.1%)	IC^b (@56%)	Total 6-month salary+FB+IC
PI (20%)				
Research Associate 1 (25%)				
Research Associate 2 (25%)				
Total				

^aFB; fringe benefits, ^bIC; indirect cost.

In addition to personnel, the following categories of funds are required to perform additional studies:

1. Purchasing the rabbits. We need 20 rabbits @\$160/rabbit (20 x \$160 = **\$3,200**).

2. Maintaining the rabbits. We will maintain the rabbits for 90 days. With per diem of \$3.3, we will spend \$3.3 x 90 days x 20 rabbits = **\$5,940**

3. Cost of veterinary staff and operating room. We operate 5 rabbits/surgery session and each rabbit is operated on twice. Thus, we will perform 8 surgeries to process 20 rabbits twice. The total cost of these surgeries will be **\$2,400** (see Table 2).

Table 2. Estimation of surgery-associated costs.

Procedures	Cost per surgery	Cost per all surgeries
Veterinary technician time	\$150 per surgery @\$50/hr	\$1,200/8 surgeries/5 rabbits per surgery
Pharmaceutical supplies	\$100 per surgery	\$800/8 surgeries/5 rabbits per surgery
Post-surgery care	\$50 per surgery	\$400/8 surgeries/5 rabbits per surgery

Total for purchasing, maintaining and operating the rabbits: \$3,200 + \$5,940 + \$2,400 = \$11,540

Total + indirect cost @56%: \$11,540 + 6462.4 =

Table 3. Breakdown of major supplies needed for this study.

Item/Unit	# of items	cost/item unit [\$]	Cost/all units [\$]	Supplier	Cat#
iPRECIO pumps for antibody delivery/ea	20	275	5,500	Alzet	0006100
Antibody production in a bioreactor; cell suspension culture chamber:/ea	1	939	939	Eppendorf	1386121000
Antibody production in a bioreactor; CD FortiCHO media/1-L bottle	14	137	1,918	Invitrogen	A1148301
Antibody production in a bioreactor; GlutaMax Supplement 100mL bottle	6	40	240	Invitrogen	35050061
Antibody production in a bioreactor; Pen/Strep/100-ml bottle	2	72.06	144.12	FisherSci	MT-30-002-CI
Antibody production in a bioreactor; HT Supplement/50-ml bottle	4	41.47	148	Invitrogen	11067-030
Antibody production in a bioreactor; sterile glucose solution/100-ml bottle	6	40.40	242.40	FisherSci	MT-25-037-CI
Antibody production in a bioreactor; anti-foaming agent/bag	1	256	256	Invitrogen	A1036902
Glucose/lactate assay kit for in-line measurements in the automated TRACE2 system connected to a bioreactor/ea	1	1,191	1,191	PROAnalytics Trace	60.211.080; 50.300.135; 850.305.000; 50.305.004; 130.200.000
Control human IgG	3	216	648	Sigma	14506
Sepharose Protein A for antibody purification/25 ml	1	1019	1,019	Rockland	PA50-00-0025
5 ml pipette/case	1	44	44	FisherSci	13-675-22
10 ml pipette/case	1	45.04	45.04	FisherSci	13-675-20,
25 ml pipette/case	1	142.24	142.24	FisherSci	13-678-11,
Hydroxyproline assay kit/ea	1	532	532	Sigma	MAK008
Cross linked C-Telopeptide of Collagen I ELISA kit/ea	1	660	660	MyBioSource	MBS0736646
ProCollagen I C-terminal peptide ELISA kit/ea	1	660	660	MyBioSource	MBS025817
TOTAL FOR SUPPLES			\$14,346.68		
TOTAL FOR SUPPLES + Indirect cost @56%			\$22,381		

Option 2. A suboptimal supplemental budget that would allow execution of the project. In this option, we would create only one group of rabbits: (i) treated with the highest concentration of the ACA. We would compare results from this group with non-treated control rabbits already processed in earlier experiments.

The total cost for the suboptimal option, including indirect costs, is \$92,626

The following tables present a detailed budget:

Table 1. Personnel.

Personnel (% effort)	6-month salary	FB ^a (@21.1%)	IC ^b (@56%)	Total 6-month salary+FB+IC
PI (20%)	\$19,215.00	\$4,054.37	\$13,030.84	\$36,300.21
Research Associate 1 (25%)	\$8,012.00	\$1,690.53	\$5,433.42	\$15,135.95
Research Associate 2 (25%)	\$8,012.00	\$1,690.53	\$5,433.42	\$15,135.95
Total				\$66,572.11

^aFB; fringe benefits, ^bIC; indirect cost.

In addition to personnel, the following categories of funds are required to perform additional studies:

1. Purchasing the rabbits. We need 10 rabbits @\$160/rabbit (10 x \$160 = **\$1,600**).

2. Maintaining the rabbits. We will maintain the rabbits for 90 days. With per diem of \$3.3, we will spend \$3.3 x 90 days x 10 rabbits = **\$2,970**

3. Cost of veterinary staff and operating room. We operate 5 rabbits/surgery session and each rabbit is operated on twice. Thus, we will perform 4 surgeries to process 10 rabbits twice. The total cost of these surgeries will be **\$1,200** (see Table 2).

Table 2. Estimation of surgery-associated costs.

Procedures	Cost per surgery	Cost per all surgeries
Veterinary technician time	\$150 per surgery@\$50/hr	\$600/4 surgeries/5 rabbits per surgery
Pharmaceutical supplies	\$100 per surgery	\$400/4 surgeries/5 rabbits per surgery
Post-surgery care	\$50 per surgery	\$200/4 surgeries/5 rabbits per surgery

Total for purchasing, maintaining and operating the rabbits: \$1,600 + \$2,970 + \$1,200 = \$5,770

Total + indirect cost @56%: \$5,770 + \$3,231.2 = **\$9,002**

Table 3. Breakdown of major supplies needed for this study.

Item/Unit	# of items	cost/item unit [\$]	Cost/all units [\$]	Supplier	Cat#
iPRECIO pumps for antibody delivery/ea	10	275	2,750	Alzet	0006100
Antibody production in a bioreactor; cell suspension culture chamber:/ea	1	939	939	Eppendorf	1386121000
Antibody production in a bioreactor; CD FortiCHO media/1-L bottle	14	137	1,918	Invitrogen	A1148301
Antibody production in a bioreactor; GlutaMax Supplement 100mL bottle	6	40	240	Invitrogen	35050061
Antibody production in a bioreactor; Pen/Strep/100-ml bottle	2	72.06	144.12	FisherSci	MT-30-002-CI
Antibody production in a bioreactor; HT Supplement/50-ml bottle	4	41.47	148	Invitrogen	11067-030
Antibody production in a bioreactor; sterile glucose solution/100-ml bottle	6	40.40	242.40	FisherSci	MT-25-037-CI
Antibody production in a bioreactor; anti-foaming agent/bag	1	256	256	Invitrogen	A1036902
Glucose/lactate assay kit for in-line measurements in the automated TRACE2 system connected to a bioreactor/ea	1	1,191	1,191	PROAnalytics Trace	60.211.080; 50.300.135; 850.305.000; 50.305.004; 130.200.000
Sepharose Protein A for antibody purification/25 ml	1	1019	1,019	Rockland	PA50-00-0025
5 ml pipette/case	1	44	44	FisherSci	13-675-22
10 ml pipette/case	1	45.04	45.04	FisherSci	13-675-20,
25 ml pipette/case	1	142.24	142.24	FisherSci	13-678-11,
Hydroxyproline assay kit/ea	1	532	532	Sigma	MAK008
Cross linked C-Telopeptide of Collagen I ELISA kit/ea	1	660	660	MyBioSource	MBS0736646
ProCollagen I C-terminal peptide ELISA kit/ea	1	660	660	MyBioSource	MBS025817
TOTAL FOR SUPPLES			\$10,931		
TOTAL FOR SUPPLES + Indirect cost @56%			<u>\$17,052</u>		

APPENDIX

1. An updated SOW
2. An information from Eppendorf
3. A quote from Charles River

This generic Statement of Work document is intended to assist applicants with the format preferred by CDMRP. This particular SOW does not contain any specific scientific information and is intended to be easily modifiable for any project. Not all components will be applicable for every project; please consult your Program Announcement for specific award requirements.

STATEMENT OF WORK

CURRENT STATUS & UPDATED TIMELINE

Specific Aim 1	Timeline Months	Site 1	Current Status
To determine the safety of the ACA applied short-term and long-term to reduce post-traumatic joint stiffness			
Major Task 1 Production and purification of therapeutic antibodies (ACA). This task will be performed on a continuous basis for both specific aims.	1-36		Ongoing as planned
Subtask 1 High-density cell culture to produce preparative-scale amounts of therapeutic ACA. In this subtask cell cultures will be carried out in a laboratory-scale bioreactor.	1-36	Dr. Fertala	Ongoing as planned
Subtask 2 Purification of the ACA. The ACA will be purified by affinity chromatography.	1-36	Dr. Fertala	Ongoing as planned
Subtask 3 Quality control of purified ACA. The structural integrity, purity, and affinity of purified ACA will be analyzed.	1-36	Dr. Fertala	Ongoing as planned
Milestone(s) Achieved: Establishing sufficient quantities of functional, highly purified therapeutic antibody for animal-based tests.	1-36	Dr. Fertala	Not fully completed due to supply-chain interruptions

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Local IACUC Approval	1	Dr. Fertala	
Milestone Achieved: IACUC/ACURO Approval	2	Dr. Fertala	Completed
Major Task 2 Concentration-dependent safety assays We plan to employ 20 rabbits for this major task.			Completed
Subtask 1 Dose acceleration. In this subtask, we will determine the safe dose of the ACA in short-term (1 week) tests.	2-6	Dr. Fertala	Completed
Subtask 2 Safety studies. In this subtask, we will determine the effects of the dose of the ACA selected in Subtask 1. This will be done in long-term studies (12 weeks).	6-14	Dr. Fertala	Tests done only with the lowest ACA dose. The highest safe dose not tested for 12 weeks due to problems with supplies of crucial parts for a bioreactor
Subtask 3 Assays of collagen metabolites. In this subtask, we will define markers of collagen metabolism in the context of the ACA treatment.	6-14	Dr. Fertala	Completed
Milestone(s) Achieved: Determining the safe ACA concentration and defining key biomarkers to study the utility of the ACA treatment.	14	Dr. Fertala	Completed
Specific Aim 2 To define the effects of the ACA on the healing of joint tissues.			Partially executed. Done with the lowest ACA dose for 12 weeks. Has to be done with the highest ACA dose.
Major Task 3 To determine the impact of the ACA			Partially executed. Done with the lowest ACA dose for 12 weeks. Has

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treatment on the healing of joint tissues.			to be done with the highest ACA dose.
Subtask 1 Performing surgeries for rabbit-based contracture model. All surgeries will be done by participating orthopaedic surgeons. We plan to employ 60 rabbits divided into experimental and control groups.	14-39	Dr. Fertala	Ongoing; on track
Subtask 2 Biomechanical, biochemical, and histological assays of joint tissues. We will study the effects of the ACA applied to reduce post-traumatic joint contracture on the biological, biochemical, and mechanical properties of tendon, cartilage, and subchondral bone.	17-40	Dr. Fertala	Done for the rabbits operated thus far.
Subtask 3 Data analysis and statistical evaluation of results. Data will be collected throughout the duration of the animal-based experiments. Critical analysis of the data will be done with the assistance of Dr. Daskalakis.	20-40		Ongoing; on track
Milestone(s) Achieved: At this stage, we will have established a clinically-relevant method to limit post-traumatic joint stiffness without altering the healing process. All experimental conditions and procedures will position our technology for preclinical tests in large animals, followed by clinical tests. Manuscripts describing key findings will be prepared. All members will participate in data analysis and manuscript preparation.	30-42	Dr. Fertala	Ongoing; on track.

Note: The Government reserves the right to request a revised SOW format and/or additional information.

From: Kenneth Murphy <Murphy.K@eppendorf.com>

Sent: Monday, March 30, 2020 7:51 PM

To: Andrzej Fertala <Andrzej.Fertala@jefferson.edu>

Subject: Eppendorf BioBLU 5p Vessels

WARNING: External Email - This email originated outside of Jefferson.
DO NOT CLICK links or attachments unless you recognize the sender and *are expecting* the email.

Hello Dr. Fertala,

We at Eppendorf hope that you and yours are doing well.

I received notification today from Eppendorf's management board, that all of the BioBLU 5p vessels in their possession are being channeled to Covid-19 research for the time being. I'm still inquiring and trying to pursue them to permit one to be shipped to you to satisfy an open order.

Please advise your need for the BioBLU 5p vessel.

Thank you and be well.

Ken Murphy

Bioprocess Sales, PA, OH, NY, DE

Eppendorf America

Customer Service: 1-800-645-3050

Mobile: 267-559-2524

Murphy.k@eppendorf.com

<image004.png>

<https://www.eppendorf.com/US-en/>

**please note all attachments sent to Eppendorf must be in the following formats:

.xlsx, .docx, .pdf

25 September, 2020



Prof. Andrzej Fertala
Thomas Jefferson University
Orthopaedic Surgery
1020 Locust Street
Philadelphia, PA 19107
United States

Dear Prof. Fertala,

We are pleased to offer you the following pricing discounts. This pricing cannot be combined with any other discounts and will be effective from **02-October-2020** through **31-December-2021**.

These discounts are valid for the following sites only:

CR Sold-to location: 104211

CR Ship-to location(s): 249713

Currency: USD

Animal

<u>Value</u>	<u>Description</u>	<u>Species</u>	<u>Strain</u>	<u>Age Range</u>	<u>Sex</u>
160.00	Flat Research Models Price Based on Age	Rabbit	NZW, ORA NZW	34- 52 Weeks	All

Freight

<u>Value</u>	<u>Description</u>	<u>RM Site</u>	<u>Species</u>	<u>Strain</u>
100.00	% Disc - Long Distance Freight - Specified Strains	All	Rabbit	NZW, ORA NZW

Crate

<u>Value</u>	<u>Description</u>
100.00	% Disc - Rabbit Crates only

Charles River Laboratories, Inc. or **Thomas Jefferson University** has the right to terminate this pricing agreement at any time by giving 30 days notice to the other party. Any new entities acquired by either Charles River Laboratories, Inc. or **Thomas Jefferson University** during the term of this agreement will not be included into the discount structure until approved by both parties.

All products and services are sold subject to and in accordance with the terms and conditions attached hereto and made a part hereof, which can also be found on our website @www.criver.com.

We look forward to working with you. If you agree to the terms and conditions of this discount letter, please counter-sign and return one fully executed copy to Erica Kelly at

Erica.Kelly@crl.com.

Thank you.