Published in final edited form as:

J Orthop Res. 2020 November ; 38(11): 2474–2483. doi:10.1002/jor.24647.

Reduction of arthrofibrosis utilizing a collagen membrane drug-eluting scaffold with celecoxib and subcutaneous injections with ketotifen

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Abstract

The dense formation of abnormal scar tissue after total knee arthroplasty results in arthrofibrosis, an unfortunate sequela of inflammation. The purpose of this study was to use a validated rabbit model to assess the effects on surgically-induced knee joint contractures of two combined pharmacological interventions: celecoxib (CXB) loaded on an implanted collagen membrane, and subcutaneously (SQ) injected ketotifen. Thirty rabbits were randomly divided into five groups. The first group received no intervention after the index surgery. The remaining four groups underwent intra-articular implantation of collagen membranes loaded with or without CXB at the time of the index surgery; two of which were also treated with SQ ketotifen. Biomechanical joint contracture data were collected at 8, 10, 16, and 24 weeks. At the time of necropsy (24 weeks), posterior capsule tissue was collected for mRNA and histopathologic analyses. At 24 weeks, there was a statistically significant increase in passive extension among rabbits in all groups treated with CXB and/or ketotifen compared to those in the contracture control group. There was a statistically significant decrease in *COL3A1*, *COL6A1*, and *ACTA2* gene expression in the treatment groups compared to the contracture control group ($p < 0.001$). Histopathologic data also demonstrated a trend towards decreased fibrous tissue density in the CXB membrane group compared to the vehicle membrane group. The present data suggest that intra-articular placement of a treated collagen membrane blunts the severity of contracture development in a rabbit model of arthrofibrosis, and that ketotifen and CXB may independently contribute to the prevention of arthrofibrosis.

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AUTHOR CONTRIBUTIONS

AKL, MET, CGS, ARM, TWT, CEB, AGJ, BB, MEM, JSS, AVW, MPA made substantial contributions to the research design, or acquisition, analysis or interpretation of data; AKL, MET, BB, AVW, MPA, MEM drafted the paper and revised it critically; AKL, MET, JMC, BB, DJB, MEM, JSS, AVW, MPA approved the submitted and final manuscript versions. All authors have read and approved the final submitted manuscript.

*denotes equal contribution

Keywords

Acquired Idiopathic Stiffness; Arthrofibrosis; Celecoxib; Ketotifen; Total Knee Arthroplasty

INTRODUCTION

Acquired idiopathic stiffness is a complication which can result from a multitude of etiologies, including injury or surgery to a joint¹. With a prevalence of 4–30% after primary total knee arthroplasty (TKA), acquired idiopathic stiffness affects thousands of people every year in the United States alone¹. A subset of these patients exhibit true arthrofibrosis, which is characterized histopathologically by extensive scar tissue which painfully restricts motion, develops most commonly in the elbow or knee, and cannot be attributed to component malposition, infection or other reasons^{1, 2}. Current treatment modalities are limited to physical therapy, manipulation under anesthesia, or revision surgery. Furthermore, current understanding of the molecular pathogenesis of arthrofibrosis is limited, adding difficulty to finding an effective pharmacological treatment.

To examine the biomechanical, molecular, and structural effects of contracture induction, we utilized our previously validated rabbit model^{3–7}. This model has been shown to closely replicate the clinical presentation of post-traumatic contracture formation^{3, 7}. Other animal models often result in contractures that reverse after a remobilization period, which does not recapitulate the clinical scenario of a permanent contracture. This makes interpretation of pharmacologic therapies targeted at this condition challenging for their translational significance^{3, 8}. Utilizing a clinically relevant rabbit model, we have demonstrated the development of a durable contracture, early increases in myofibroblasts, increased extracellular matrix (ECM) deposition, and changes in the molecular landscape^{3, 5, 6, 9, 10}.

Using this animal model, several pharmacologic agents have been trialed as possible therapies for this condition. These have included ketotifen fumarate, decorin, rosiglitazone, and a substance P inhibitor^{4, 11–15}. More recent investigations in our laboratory have shown that celecoxib (CXB), a selective cyclooxygenase-2 (COX-2) inhibitor, leads to increased motion, decreased contracture stiffness, and down-regulation of collagen gene markers compared to untreated controls¹⁶. CXB is hypothesized to blunt contracture formation via its inhibition of prostaglandin E₂, thereby reducing the inflammatory process and allowing more physiologic collagen remodeling to occur¹⁷.

In previous studies, local administration of CXB was performed via repeated intra-articular injections¹⁰. However, multiple intra-articular injections over a short period are cumbersome and may result in repetitive microtrauma that could, in and of itself, be pro-fibrotic. This has made the isolation of a potential effect from CXB somewhat more challenging. In an effort to avoid the use of repetitive injections, we identified a U.S. Food and Drug Administration (FDA)-approved, biodegradable collagen scaffold that previously has been demonstrated to be safe when implanted intra-articularly, without induction of synovitis, cartilage damage, or fibrosis in non-contracted joints¹⁸. The ultimate goal of this research is to identify a novel and effective therapeutic modality to treat and/or prevent arthrofibrosis in human patients.

The aforementioned membrane is well suited for this translational application as it has been shown to be safe, and resorb completely by 8 weeks.

Additionally, we sought to investigate the use of ketotifen fumarate in combination with CXB. Ketotifen has been shown to reduce the formation of joint contracture, as well as decrease the numbers of myofibroblast and mast cells^{11, 12}. It is a mast cell stabilizer and a histamine H₁- antagonist¹⁹. Mast cells have been previously implicated in a number fibrotic diseases including both cardiac and pulmonary fibrosis^{20, 21}. It is hypothesized that mast cells contribute to fibrosis by modulating myofibroblasts' interaction with neuropeptides²². Our group has previously demonstrated the significance of myofibroblasts in the development and treatment of arthrofibrosis⁵. Furthermore, many pro-fibrotic cytokines and growth factors are present in mast cells and their granules^{23, 24}. Therefore, the aim of the present study was to assess the biomechanical, gene expression, and histopathologic effects of CXB, delivered via a collagen membrane, with and without SQ ketotifen injections, in a rabbit model of arthrofibrosis. There is a paucity of data in the literature combining ketotifen and intra-articular CXB for the prevention or treatment of arthrofibrosis. Consequently, a secondary aim of the study was to determine the relative effects of the collagen membrane in the same rabbit model. Our hypotheses were that CXB alone would help to reduce arthrofibrosis, and that the combination of CXB and ketotifen would provide an additive anti-fibrotic effect.

MATERIALS AND METHODS

Animals and Husbandry

This study was approved in advance by the Institutional Animal Care and Use Committee (IACUC) and our institution's Department of Comparative Medicine. All study rabbits were acquired from Charles River Laboratories, Inc. (Wilmington, MA) and were quarantined for one week after arrival at our institution before being transferred to the experimental room. Rabbits were socially housed (n = 2/cage) when applicable, in polycarbonate cages (1 m³), supplied with commercial pelleted chow (Purina Laboratory Rabbit Diet HF 5326), and filter-purified tap water *ad libitum*, and were housed in a room with constant temperature (21 ± 2°C) and humidity (45 ± 10%). Each animal was exposed to 12 hours of light daily and provided with enrichment toys.

Study Design

Thirty skeletally mature, intact New Zealand White (NZW) female rabbits (*Oryctolagus cuniculus*) weighing 2.5 – 3.5 kg were randomly divided into five different groups, each with six rabbits. Group 1 ("contracture control") acted as the control group, receiving no additional intervention after the contracture-induction operation. Groups 2–5 underwent implantation of collagen membranes into the stifle (knee) joint at the time of their index surgery for contracture induction (Figures 1A–C). Membranes implanted in Groups 2 and 4 were treated with a vehicle solution (described below), while membranes implanted in Groups 3 and 5 were imbued with CXB dissolved in the same vehicle solution via an established and published protocol²⁵. Of note, the calculated initial dose of CXB was 1 mg/

membrane. In addition to the collagen membrane, the rabbits in Groups 4 and 5 also received SQ ketotifen injections (1 mg/kg) twice daily for 14 days postoperatively.

Identical index surgical procedures were performed on the right knee of each rabbit as previously described^{3, 6, 7}. Briefly a lateral parapatellar incision was made to provide access to disrupt the ACL, PCL, hyperextend the knee to disrupt the posterior capsule, and drill holes in the medial and lateral femoral condyles. A distal incision was made over the tibia where a Kirschner wire (K-wire) was introduced through the tibia, and through a separate proximal incision the K-wire was retrieved and wrapped around the femur to immobilize the knee in flexion. A collagen membrane, for Groups 2–5, was placed immediately posterior to the patellar tendon through the exposure (Figures 1A–C). Following surgery, rabbits were permitted free cage activity (cage volume, 1 m³). Injections of ketotifen were administered in the immediate postoperative period and subsequently administered twice daily for 14 days total following surgery. No additional analgesic or anti-inflammatory drugs were given after the initial dose of Buprenorphine SR (an opioid analgesic; 0.18 mg/kg SQ) at the time of surgery, since such agents have the potential to impact inflammation and fibrous tissue production. All rabbits returned to their initial body weight by 8 weeks from surgery. The K-wire was removed under anesthesia 8 weeks after the index procedure in all animals. Extra-articular heterotopic ossification (HO) and excessive callus were removed at the time of K-wire removal from all animals where the K-wire hooked over the femur and at the insertion point of the K-wire on the tibia. For an additional 16 weeks after K-wire removal, all rabbits were allowed free cage activity to allow for joint remobilization.

Preparation of Celecoxib Membranes

Prior investigators in our laboratory have identified the absorption properties, cellular toxicity, fibrotic gene expression, and release kinetics of CXB from the HeliMEND collagen membrane (Integra® Miltenex®, York, PA) via electron microscopy, as well as assessment of eluted concentrations by UV spectroscopy and HPLC mass spectrometry, respectively²⁵. Based on these data, the collagen membrane was loaded with CXB for Groups 3 and 5 in the present study. Briefly, CXB (Tocris; Minneapolis, MN) was dissolved in a mixed solvent solution of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and phosphate-buffered saline (PBS 1X, pH 7.4; ThermoFisher Scientific, Waltham, MA). The mixed solvent solution was 63% DMSO. The collagen membranes were submerged in the drug solution and placed on a rocking platform for 12 hours at room temperature (RT). The membranes were then placed in a covered, sterile petri dish and into a vacuum apparatus at 100 mmHg for 12 hours at RT in order to evaporate the mixed solvent solution and concentrate the CXB. The submersion and vacuum evaporation steps were repeated once utilizing the same initial drug solution, to maximize the amount of CXB bound to the membrane prior to implantation (Figures 1D–F). Membranes intended for Groups 2 and 4 were submerged in the mixed solvent solution alone, without CXB, but the additional processing steps were identical.

Preparation of Ketotifen Injections

Research-grade ketotifen (Tocris; Minneapolis, MN) was dissolved in DMSO to create a 25 mg/mL stock solution. Injection volumes were then calculated based on each rabbit's weight

at the date of initial contracture induction surgery to ensure that a dose of 1 mg/kg total body weight was administered to each experimental animal twice daily for 2 weeks following the index surgery. The ketotifen was injected subcutaneously between the scapulae of all animals.

Radiographic Joint Angle Measurement

Joint angle measurements were performed on the operative limb at 8, 10, 16 and 24 weeks in live, sedated rabbits using a previously validated dynamic load cell (DLC) device according to a published protocol^{3, 26}. Briefly, animals were sedated with Ketamine (35 mg/kg, IM) and Xylazine (5 mg/kg, IM) and placed in lateral recumbency (left side down). The sliding arm of the device was used to apply sequential extension torques of 40, 50, and 60 N·cm to the knee, with the rabbit in the supine position in the animal support device²⁶. Lateral fluoroscopic images were captured at each torque to allow for measurement of passive knee extension. Images were then analyzed by two independent observers (MET and AKL) using ImageJ 1.50i (U.S. National Institutes of Health; Bethesda, MD) and a consensus measurement was established. Total passive extension angles were defined as the angle formed by the intersection of a line drawn along the anatomic axis of the femur, and a line along the anatomic axis of the tibia²⁶. Due to challenges in obtaining a true lateral orientation of the knee for each experimental animal, the error due to out-of-plane rotation was calculated and found to be negligible when rotation was within 20° of the target value.

Biomechanical Analysis of the Limb at Necropsy

Animals were euthanized with Fatal-Plus® (100 mg/kg) at 24 weeks after the initial surgery. All skin, muscle, and tendons on the femur and tibia proximal and distal to the knee joint were removed, taking care to preserve the entire joint capsule from the upper pole of the patella to the tibial tubercle. Both the tibia and femur were transected 7 cm from the knee joint line and subsequently attached to a torque cell via intramedullary rods³. A maximum extension torque of 20 N·Cm was applied to the tibia at a rate of 1°/s. Collected data were plotted, and the passive extension angle at maximum torque was determined³.

Utilizing data acquired from the torque cell, graphs of passive extension angle versus torque on the limb were created using Matlab 2016a (Mathworks Inc., Natick, MA). Capsular stiffness was calculated based on the slope of a line tangential to the exponential curve, drawn at the steepest, linear segment. The mean capsular stiffness for each treatment group was calculated based on individual values for each included rabbit.

Joint Capsule Arthrotomy

At the time of necropsy, posterior capsule tissue was collected from the operative limbs of each rabbit. Additionally, the anterior portion of the joint was also dissected. All collagen membranes were found to be completely dissolved, however all animals treated with a membrane did have a piece of scar tissue underneath the patellar tendon. This scar tissue was observed to be near the anatomical location of the infrapatellar fat pad, which was excised at the time of index surgery.

Gene Expression

At necropsy, once piece of posterior capsule tissue was snap-frozen in liquid nitrogen. Total RNA was extracted, after which cDNA was synthesized and used for quantitative PCR (qPCR)¹⁰. Before assessing expression levels of the genes of interest, each sample was tested for contamination of bone, skeletal muscle, blood, and fat using qPCR. If contamination was detected, the sample was excluded from all further analyses.

Histologic Processing

Harvested posterior capsule tissue samples were immediately immersed in 5 mL of neutral buffered 10% formalin (NBF), stored at RT (22°C) for 48 – 72 hours, and subsequently processed routinely into paraffin. For paraffin embedding, tissue was transferred into a 95% ethanol solution and placed in an Isotemp® Vacuum Oven at 65°C and 12 mm Hg for 1.5 hours; it was then removed and left at standard temperature and pressure (STP) for 1 hour. The previous step was repeated with 100% ethanol after which the samples were left at STP for 3 hours. Next, the samples were suspended in 100% xylene and placed into the oven at 68°C for 1 hour and then left at STP overnight. The samples were then transferred into 50% xylene/50% paraffin solution and placed in the oven at 68°C for 2 hours. Finally, the samples were infiltrated with 100% paraffin, embedded in paraffin, and sectioned serially at 5 microns (µM). One section was stained with hematoxylin and eosin (H&E). The second section was processed by indirect immunohistochemistry (IHC) to localize α -smooth muscle actin (α -SMA) by application of a primary monoclonal mouse anti-rabbit IgG (Abcam, Cat. No. ab7817) at 1:2000 g/ml for 1 hour at RT followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse polyclonal secondary antibody for 30 min at RT and then 3,3'-diaminobenzidine (DAB) for 5 min at RT to demonstrate antigen localization. Following IHC, sections were counterstained with hematoxylin. Staining was performed on all samples at the Mayo Clinic Pathology Research Core.

Histopathologic Evaluation

Sections were evaluated using a Nikon E600 bright-field light microscope by an ACVP board-certified veterinary pathologist (BB). Features were scored using tiered, semi-quantitative scales using the following grades: within normal limits (score = 0), or minimal (1), mild (2), moderate (3), marked (4), or severe (5) changes. Scoring criteria for the various changes are given for the H&E stains in Table 1 and the α -SMA IHC in Table 2. These criteria were established by an initial informed (“non-blinded”) histopathologic evaluation, after which the definitive data set was produced by a coded (“blinded”) histopathologic assessment. Additionally this is the same scoring criteria that has been described previously (Tibbo et al., *BJR*, in submission)¹⁰.

Statistical Analysis

For all experimental data, comparisons between each treatment group and its respective control group were carried out using the Wilcoxon-rank sum test, which assumes nonparametric variance between groups. Statistical significance was set at a probability of < 0.05. Data are reported via group means and standard deviations (SD). Group sample size

was determined by assuming a 5% type 1 error and 80% power to detect an effect difference of 30° with a SD of 20°.

RESULTS

Radiographic Measurements

Passive extension angle measurements obtained in live, sedated rabbits at 8, 10, 16, and 24 weeks after the index surgical procedure demonstrated progressive improvement in passive extension in all treatment groups compared to the contracture control group (Figure 2A, Table 3, Supplemental Figure 1). This difference was found to be significant at 24 weeks in all treatment groups except the vehicle membrane group (Group 2; Figure 2B), when compared with the contracture control group. However, no difference was identified among the various treatment groups, when compared to each other. Additionally, it took less time for each of the treatment groups to reach 90° of passive extension when compared to the control group (Figure 2C).

Capsular Stiffness Measurements

Capsular stiffness measurements were similar among all treatment arms at 24 weeks. Groups 2 and 3 (vehicle and CXB membrane groups, respectively) demonstrated significant reduction in capsular stiffness when compared to the control ($p=0.018$ and $p=0.002$, respectively). The combination treatment in Group 5 also showed a significant decrease in capsular stiffness ($p=0.005$; Figures 3A–B; Supplemental Table 1).

Genetic Expression

Significant upregulation of *ACTA2* was identified in the control group (Group 1) when compared to the other four treatment groups ($p<0.001$). This result is consistent with previous findings⁵. In addition, significant differences were detected in the mRNA expression of collagen markers *COL1A1*, *COL3A1*, and *COL6A1* (Figure 4) within periarticular soft tissues near the surgically manipulated joint, as has been noted previously¹⁰. However, *COL1A1* was not significantly decreased in the CXB membrane group (Group 3) when compared to the control.

Histopathology Data

The range of tissue findings on both H&E and α -SMA staining among the treatment groups (Groups 2–5) covered a relatively wide spectrum, which thus prevented discrimination of statistically significant differences for treated rabbits compared to the contracture control group (Group 1; Figure 5). By coded evaluation of tissue sections stained with H&E, rabbits that received CXB-treated membranes (Group 3) appeared to have a modest reduction in periarticular fibrosis and inflammation relative to control animals given vehicle-treated membranes (Group 2) or treated animals given a vehicle-treated membrane and repeated SQ ketotifen injections (Group 4), as indicated by slightly decreased fibrous tissue scores (Supplemental Figure 2).

DISCUSSION

The present study provides data supporting the concept that intra-articular implantation of a treated collagen membrane coated with the anti-inflammatory drug celecoxib (CXB, Group 3) blunts contracture development in a rabbit model of arthrofibrosis. At the 24-week time point, a significant increase in passive extension was observed with the CXB membrane (Group 3) with or without ketotifen (Groups 4 and 5) when compared to the control group. Furthermore, CXB (Groups 3 and 5) decreased the capsular stiffness of the joint at 24 weeks. Downregulation of mRNA encoding *COL3A1*, *COL6A1*, and *ACTA2*, was observed in all rabbits treated with CXB, or a combination of ketotifen and CXB. Additionally, histopathologic data from posterior capsule tissue of rabbits treated with a CXB membrane showed appreciable reduction in the density of fibrous tissue. Therefore, the commercially available, biodegradable, FDA-approved collagen membrane investigated in this study appears to be an efficient carrier to locally deliver pharmacologic therapy which has a considerable clinical advantage compared to multiple intraarticular injections.

Outcomes of the present study corroborate previous results from our group demonstrating decreased contracture severity after intra-articular administration of the COX-2 inhibitor CXB via repeated intra-articular injections¹⁰. In the current study, we identified decreased *COL3A1*, *COL6A1*, and *ACTA2* mRNA expression in all treatment arms when compared to the contracture control group (Group 1). We hypothesize that this likely results from early inhibition of the pro-inflammatory cascade and continues during the collagen membrane absorption phase (which has been reported to take approximately 8 weeks). Prior studies have demonstrated a link between COX-2 inhibition and a decreased density of fibrotic lesions, thereby implicating blockade of prostaglandin signaling as a viable means of limiting fibrotic tissue formation¹⁰. Others have identified the importance of inhibiting this pathway in the first 24–72 hours after injury⁹. This evidence informed our decision to initiate drug therapy on postoperative day 0. By utilizing a slowly dissolving collagen membrane imbued with CXB, we aimed to prolong local administration of the drug throughout the immediate postoperative inflammatory period, in an effort to more effectively blunt inflammatory cytokine-induced myofibroblast activation¹⁸. We hypothesized that the addition of CXB to the collagen membrane would improve its ability to decrease contracture formation when compared to implantation of the membrane alone¹⁸. However, we were unable to identify such a difference. This may be due either to the initial burst release of CXB from the membrane followed by rapid exponential decay in drug levels at the site of myofibroblast activation, or our limited ability to administer large doses of the drug secondary to minimal adherence to the collagen membrane²⁵. Efforts to increase the CXB concentration on the membrane at the time of initial implantation or to slow the release from the membrane over time may improve overall treatment effect. The collagen membrane alone also may act as a mechanical barrier to scar tissue formation, similar to membranes utilized in prevention of tendon adhesions, thereby further limiting our ability to detect a difference between the two treatment arms²⁷. Another consideration is that the placement of the collagen membrane slows the overall production of collagen in the joint. As the membrane degrades it releases the collagen, resulting decreased collagen and ECM protein production by fibroblasts and myofibroblasts.

The present study also evaluated the effect of adding ketotifen to create a multimodal treatment regimen with CXB. Ketotifen has been demonstrated to reduce fibrosis in multiple studies (Tibbo et al., *BJR*, in submission)^{11, 12}. It is postulated that ketotifen promotes normal tissue healing by inhibiting mast cell activation^{19, 28}. We hypothesize that CXB, however, carries out its effect via inhibition of pro-inflammatory cytokines, as well as inhibition of white blood cells and inflammatory cell recruitment early in the post-injury period²⁹. Rabbits treated with CXB-loaded collagen membranes (Group 3) did demonstrate decreased density of fibrous lesions, as well as reduced myofibroblast presence, when compared to the vehicle membrane group (Group 2). These data will require further investigation at earlier time points to demonstrate if this method of pharmacologic inhibition is able to decrease the density of myofibroblasts and fibrous tissue overall. Likewise, additional studies will be needed to examine the synergistic effect of CXB and ketotifen. Our hypothesis was that these two anti-fibrotic agents would have an additive effect, although this was not supported by our data.

The vehicle membrane with and without ketotifen led to variable results; the addition of ketotifen increased the overall passive extension at 24 weeks, although this difference was not significant. Conversely, capsular stiffness was increased with the addition of ketotifen to the vehicle membrane. Similarly mixed results were observed with respect to molecular and histopathologic analyses. These data will require further study to understand the relationship between the collagen membrane and SQ ketotifen.

Our study is not without limitations. With respect to the biomechanical measurements, two rabbits were excluded from the final analysis. One rabbit was euthanized for a femur fracture at the 8-week time point (Group 3, Rabbit #17), and one for a suspected sciatic nerve-mediated palsy that had not resolved at the 16-week time point (Group 3, Rabbit #18). Additionally, it is possible that samples of posterior capsule may have been contaminated with bone, blood, skeletal muscle, or fat; however, utilizing our previously published panel of contamination biomarkers¹⁰, no contamination was found to be significant in any of the analyzed posterior capsule tissue. Contamination in these samples could cause variation in our RT-qPCR data. With respect to the histopathologic evaluation, the microscopic data did not permit a definitive conclusion regarding the utility of using CXB and/or ketotifen as potential therapies to combat post-operative joint contracture. This inability is an inherent feature of “blinded” histopathologic assessments, particularly for domains such as periarticular soft tissues that have a narrowly constrained set of phenotypic reactions. The posterior capsule tissue composition varies significantly with anatomic location; this likely influences our ability to detect differences between groups at the histopathologic level. The key message provided by our combined data set is that biopsy to collect unfixed tissue for evaluation of molecular biomarkers of fibrogenesis (e.g., *COL3A1*, *COL6A1*, *ACTA2*) is likely to provide better diagnostic and prognostic answers with respect to the extent of arthrofibrosis than will histopathologic examination of conventional biopsy specimens from periarticular tissues. A final set of limitations for the current experiment were inherent in the study design. First, due to the fact that tissue for gene expression analyses was harvested at the 24-week time point, some crucial markers in the inflammatory cascade may have been missed by the current study methodology. These markers may include cytokines, such as TGFs, interleukins, and FGFs, as well as matrix metalloproteinases (MMPs) and tissue

inhibitors of metalloproteinases (TIMPs). Second, our current slow-release, intra-articular drug delivery implants improved upon the previous intermittent delivery via repeated intra-articular injections; however, the membrane approach was not without limitation. The maximum concentration of CXB adsorbed onto the collagen membrane yields lower intra-articular levels than can be achieved by administering bolus doses via intra-articular injection. Therefore, because CXB efficacy is more closely related to total dose (area under the curve [AUC]) rather than the peak quantity (C_{max}), future studies should concentrate on improving this dosing profile²⁵. The benefits associated with using intra-articular collagen membrane implants are many: it is an FDA-approved, safe, non-toxic, easily implantable, slow-release drug carrier that completely dissolves by 8 weeks postoperatively¹⁸. Future studies might also investigate the delivery method of ketotifen, perhaps even administering it using a collagen membrane.

In conclusion, our results suggest that intra-articular administration of CXB via a collagen membrane either without or combined with ketotifen (administered systemically by SQ injection), decreases the severity of contracture development in a rabbit model of arthrofibrosis. At 24 weeks postoperatively, we were able to demonstrate a difference between all treatment modalities and the contracture control group. The collagen membrane loaded with CXB resulted in improved passive extension, effective drug delivery to the joint space, decreased the density of fibrous tissue, and reduced the number of myofibroblasts seen via IHC. Additional studies are necessary to examine the results of these pharmacologic therapies at earlier postoperative time points, with the eventual goal of identifying a translational treatment protocol for reduction of arthrofibrosis in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors would like to acknowledge lab members of the Abdel and van Wijnen laboratories for their critical review of this work and their insightful discussions and/or assistance with reagents and procedures. **Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) of the National Institutes of Health (NIH) under Award Number AR072597-01A1 and the Anna-Maria and Stephen Kellen Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.** We would like to thank the entire Mayo Clinic Department of Comparative Medicine for their expertise in animal care, and Kristin C. Mara, M.S. and Dirk R. Larson, M.S. in the Mayo Clinic Department of Medical Biostatistics and Informatics for their expertise in data analysis throughout the duration of this study. We would also like to thank the Pathology Research Core at the Mayo Clinic for their work on the immunohistochemistry.

REFERENCES

1. Tibbo ME KLA, SC G,Ahmed AT, vWA J, BD J, et al. Acquired Idiopathic Stiffness after Total Knee Arthroplasty: A Systematic Review and Meta-Analysis J Bone Joint Surg Am. 2019;In Press.
2. Kalson NS, Borthwick LA, Mann DA, Deehan DJ, Lewis P, Mann C, et al. International consensus on the definition and classification of fibrosis of the knee joint. The bone & joint journal. 2016 11;98-B(11):1479–88. Epub 2016/11/03. [PubMed: 27803223]
3. Nesterenko S, Morrey ME, Abdel MP, An KN, Steinmann SP, Morrey BF, et al. New rabbit knee model of posttraumatic joint contracture: indirect capsular damage induces a severe contracture.

- Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2009 8;27(8):1028–32. [PubMed: 19165743]
4. Abdel MP, Morrey ME, Barlow JD, Grill DE, Kolbert CP, An KN, et al. Intra-articular decorin influences the fibrosis genetic expression profile in a rabbit model of joint contracture. *Bone Joint Res.* 2014;3(3):82–8. [PubMed: 24671942]
 5. Abdel MP, Morrey ME, Barlow JD, Kreofsky CR, An KN, Steinmann SP, et al. Myofibroblast cells are preferentially expressed early in a rabbit model of joint contracture. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society.* 2012 5;30(5):713–9. [PubMed: 22057979]
 6. Abdel MP, Morrey ME, Grill DE, Kolbert CP, An KN, Steinmann SP, et al. Effects of joint contracture on the contralateral unoperated limb in a rabbit knee contracture model: a biomechanical and genetic study. *J Orthop Res.* 2012 10;30(10):1581–5. [PubMed: 22457083]
 7. Barlow JD, Hartzler RU, Abdel MP, Morrey ME, An KN, Steinmann SP, et al. Surgical capsular release reduces flexion contracture in a rabbit model of arthrofibrosis. *J Orthop Res.* 2013 10;31(10):1529–32. [PubMed: 23703948]
 8. Akesson WH, Woo SL, Amiel D, Doty DH. Rapid recovery from contracture in rabbit hindlimb. A correlative biomechanical and biochemical study. *Clinical orthopaedics and related research.* 1977 (122):359–65. [PubMed: 837619]
 9. Morrey ME, Abdel MP, Riester SM, Dudakovic A, van Wijnen AJ, Morrey BF, et al. Molecular landscape of arthrofibrosis: Microarray and bioinformatic analysis of the temporal expression of 380 genes during contracture genesis. *Gene.* 2017 4 30;610:15–23. [PubMed: 28167142]
 10. Salib CG, Reina N, Trousdale WH, Limberg AK, Tibbo ME, Jay AG, et al. Inhibition of COX-2 pathway as a potential prophylaxis against arthrofibrogenesis in a rabbit model of joint contracture. *J Orthop Res.* 2019;In Press.
 11. Monument MJ, Hart DA, Befus AD, Salo PT, Zhang M, Hildebrand KA. The mast cell stabilizer ketotifen reduces joint capsule fibrosis in a rabbit model of post-traumatic joint contractures. *Inflamm Res.* 2012 4;61(4):285–92. [PubMed: 22173279]
 12. Monument MJ, Hart DA, Befus AD, Salo PT, Zhang M, Hildebrand KA. The mast cell stabilizer ketotifen fumarate lessens contracture severity and myofibroblast hyperplasia: a study of a rabbit model of posttraumatic joint contractures. *J Bone Joint Surg Am.* 2010 6;92(6):1468–77. [PubMed: 20516323]
 13. Barlow JD, Morrey ME, Hartzler RU, Arsoy D, Riester S, van Wijnen AJ, et al. Effectiveness of rosiglitazone in reducing flexion contracture in a rabbit model of arthrofibrosis with surgical capsular release: A biomechanical, histological, and genetic analysis. *Bone Joint Res.* 2016 1;5(1):11–7. [PubMed: 26813567]
 14. Arsoy D, Salib CG, Trousdale WH, Tibbo ME, Limberg AK, Viste A, et al. Joint contracture is reduced by intra-articular implantation of rosiglitazone-loaded hydrogels in a rabbit model of arthrofibrosis. *J Orthop Res.* 2018 6 14 Epub 2018/06/15.
 15. Morrey ME, Sanchez-Sotelo J, Lewallen EA, An KN, Grill DE, Steinmann SP, et al. Intra-articular Injection of a Substance P Inhibitor Affects Gene Expression in a Joint Contracture Model. *J Cell Biochem.* 2017 7 03.
 16. Salib CG, Lewallen EA, Paradise CR, Tibbo ME, Robin JX, Trousdale WH, et al. Molecular pathology of adverse local tissue reaction caused by metal-on-metal implants defined by RNA-seq. *Genomics.* 2018 9 21 Epub 2018/09/25.
 17. Li F, He B, Liu S, Fan C. Celecoxib effectively inhibits the formation of joint adhesions. *Experimental and therapeutic medicine.* 2013 Dec;6(6):1507–11.
 18. Walker JA, Ewald TJ, Lewallen E, Van Wijnen A, Hanssen AD, Morrey BF, et al. Intra-articular implantation of collagen scaffold carriers is safe in both native and arthrofibrotic rabbit knee joints. *Bone & joint research.* 2017 3;6(3):162–71. [PubMed: 28347978]
 19. Grant SM, Goa KL, Fitton A, Sorkin EM. Ketotifen. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in asthma and allergic disorders. *Drugs.* 1990 Sep;40(3):412–48. Epub 1990/09/01.
 20. El-Mohandes EM, Moustafa AM, Khalaf HA, Hassan YF. The role of mast cells and macrophages in amiodarone induced pulmonary fibrosis and the possible attenuating role of atorvastatin.

Biotechnic & histochemistry : official publication of the Biological Stain Commission. 2017;92(7):467–80. Epub 2017/08/25. [PubMed: 28836856]

21. Levick SP, Widiapradja A. Mast Cells: Key Contributors to Cardiac Fibrosis. *Int J Mol Sci.* 2018 12;19(1). Epub 2018/01/13.
22. Hildebrand KA, Zhang M, Salo PT, Hart DA. Joint capsule mast cells and neuropeptides are increased within four weeks of injury and remain elevated in chronic stages of posttraumatic contractures. *J Orthop Res.* 2008 10;26(10):1313–9. [PubMed: 18404724]
23. Gruber BL. Mast cells in the pathogenesis of fibrosis. *Current rheumatology reports.* 2003 4;5(2):147–53. Epub 2003/03/12. [PubMed: 12628046]
24. Mukai K, Tsai M, Saito H, Galli SJ. Mast cells as sources of cytokines, chemokines, and growth factors. *Immunological reviews.* 2018 3;282(1):121–50. Epub 2018/02/13. [PubMed: 29431212]
25. Trousdale WH, Salib CG, Reina N, Lewallen EA, Viste A, Berry DJ, et al. A Drug Eluting Scaffold for the Treatment of Arthrofibrosis. *Tissue Eng Part C Methods.* 2018 8 13 Epub 2018/08/14.
26. Reina N, Trousdale WH, Salib CG, Evertz LQ, Berglund LJ, van Wijnen AJ, et al. Validation of a dynamic joint contracture measuring device in a live rabbit model of arthrofibrosis. *J Orthop Res.* 2018 2 23 Epub 2018/02/24.
27. Legrand A, Kaufman Y, Long C, Fox PM. Molecular Biology of Flexor Tendon Healing in Relation to Reduction of Tendon Adhesions. *J Hand Surg Am.* 2017 9;42(9):722–6. Epub 2017/07/16. [PubMed: 28709791]
28. Torkildsen GL, Abelson MB, Gomes PJ. Bioequivalence of two formulations of ketotifen fumarate ophthalmic solution: a single-center, randomized, double-masked conjunctival allergen challenge investigation in allergic conjunctivitis. *Clin Ther.* 2008 Jul;30(7):1272–82. Epub 2008/08/12.
29. Temp FR, Marafija JR, Milanese LH, Duarte T, Rambo LM, Pillat MM, et al. Cyclooxygenase-2 inhibitors differentially attenuate pentylene-tetrazol-induced seizures and increase of pro- and anti-inflammatory cytokine levels in the cerebral cortex and hippocampus of mice. *Eur J Pharmacol.* 2017 9 5;810:15–25. Epub 2017/06/07. [PubMed: 28583427]

Clinical Significance:

Current literature has demonstrated that arthrofibrosis may affect up to 5% of primary total knee arthroplasty patients. For that reason, novel pharmacologic prophylaxis and treatment modalities are critical to mitigating reoperations and revisions while improving the quality of life for patients with this debilitating condition.

Figure 1.

Lateral intraoperative image depicting the collagen membrane prior to insertion at the time of the index procedure (A). Intraoperative image of the collagen membrane being slid into the infrapatellar recess at the time of the index procedure (B). Intraoperative image of the completed insertion of the collagen membrane at the time of index procedure (C). Sealing the vacuum apparatus utilized to prepare collagen membranes (D). The vacuum running and drying out the collagen membranes (E). The collagen membranes enclosed in the vacuum (F).

Figure 2.

Mean passive extension angles at 40 N·cm of torque plotted with standard deviation error bars for each treatment group throughout the study time points. A dashed line at 142° indicates mean passive extension of a normal rabbit limb at 40 N·cm (A). Mean difference in passive extension among groups at 24 weeks (B). Treatment groups stratified by the time it took to reach 90° of passive extension (C). * indicates statistical significance, $p < 0.05$. CXB = celecoxib, Keto = ketotifen, SQ = subcutaneous injection.

Figure 3.

Angular displacement vs. torque curves (A) and capsular stiffness calculation as defined by a line tangential to the slope of the angular displacement vs. torque curve (B) stratified by treatment group. * indicates statistical significance, $p < 0.05$. CXB = celecoxib, Keto = ketotifen, SQ = subcutaneous injection.

Figure 4. mRNA expression of ACTA2, COL1A1, COL3A1, and COL6A1 relative to GAPDH across treatment arms at 24 weeks. * indicates statistical significance, $p < 0.05$. CXB = celecoxib, Keto = ketotifen, SQ = subcutaneous injection.

Figure 5. Histopathologic scoring data for all treatment groups for both H&E and α -SMA staining. Scoring criteria are given in Tables 1 and 2, respectively. Scores were assigned during a “blinded” evaluation. CXB = celecoxib, Keto = ketotifen, SQ = subcutaneous injection.

Table 1.

Hematoxylin and eosin (H&E) histopathologic scoring criteria.

Score	Meaning	Amount of Fibrous Tissue
0	WNL	<5%
1	Minimal	5% to 10%
2	Mild	15% to 25%
3	Moderate	30% to 50%
4	Marked	55% to 70%
5	Severe	>75%

Table 2.

α -smooth muscle actin (α -SMA) histopathologic scoring criteria.

Score	Meaning	Fibrous Tissue
0	WNL	Capillaries are widely dispersed, essentially devoid of erythrocytes, and lined by flattened (resting) endothelial cells
1	Minimal	Capillaries are dispersed but slightly increased in density, some contain erythrocytes, and a few are lined by plump (activated) endothelial cells
2	Mild	Capillaries are visibly increased in density, including scattered long linear vessels, and some are lined by plump (activated) endothelial cells
3	Moderate	Capillaries are visibly increased in density, including many rows of long parallel vessels, and are lined by plump endothelial cells
4	Marked	Capillaries are visibly increased in density though up to 50% of the hypercellular connective tissue and are lined by plump endothelial cells
5	Severe	Capillaries are substantially greater in density throughout the hypercellular connective tissue, many are engorged with blood, and they are lined uniformly by plump endothelial cells

Table 3.

Average passive extension angle at 8, 10, 16 and 24 weeks. p-values comparing the various treatment groups to the contracture control group.

	8 weeks (°)	p-value	10 weeks (°)	p-value	16 weeks (°)	p-value	24 weeks (°)	p-value
Contracture Control	47.5	-	76.8	-	87.7	-	80.2	-
Vehicle Membrane	41.5	0.88	72.0	0.99	101.8	0.47	103.2	0.09
CXB Membrane	43.9	0.97	80.1	0.99	110.6	0.02*	111.4	0.002*
Vehicle Membrane + SQ Keto	49.6	0.99	94.4	0.36	103.6	0.18	112.6	0.001*
CXB Membrane + SQ Keto	51.6	0.95	89.0	0.70	109.7	0.03*	110.0	0.003*

* indicates statistical significance, $p < 0.05$