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■ **ARTHROPLASTY**

Anti-fibrotic effects of the antihistamine ketotifen in a rabbit model of arthrofibrosis

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Aims

Arthrofibrosis is a relatively common complication after joint injuries and surgery, particularly in the knee. The present study used a previously described and validated rabbit model to assess the biomechanical, histopathological, and molecular effects of the mast cell stabilizer ketotifen on surgically induced knee joint contractures in female rabbits.

Methods

A group of 12 skeletally mature rabbits were randomly divided into two groups. One group received subcutaneous (SQ) saline, and a second group received SQ ketotifen injections. Biomechanical data were collected at eight, ten, 16, and 24 weeks. At the time of necropsy, posterior capsule tissue was collected for histopathological and gene expression analyses (messenger RNA (mRNA) and protein).

Results

At the 24-week timepoint, there was a statistically significant increase in passive extension among rabbits treated with ketotifen compared to those treated with saline (p = 0.03). However, no difference in capsular stiffness was detected. Histopathological data failed to demonstrate a decrease in the density of fibrous tissue or a decrease in α-smooth muscle actin (α-SMA) staining with ketotifen treatment. In contrast, tryptase and α-SMA protein expression in the ketotifen group were decreased when compared to saline controls (p = 0.007 and p = 0.01, respectively). Furthermore, there was a significant decrease in α-SMA (*ACTA2*) gene expression in the ketotifen group compared to the control group (p < 0.001).

Conclusion

Collectively, these data suggest that ketotifen mitigates the severity of contracture formation in a rabbit model of arthrofibrosis.

Article focus

■ Given that arthrofibrosis can occur in up to 5% of patients following primary total knee arthroplasty (TKA), pharmacological prophylaxis and effective treatment methods are required to improve the clinical care of the large number of patients with this debilitating condition.

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Key messages

■ Subcutaneous (SQ) injection of ketotifen improved passive extension compared to the control group in this rabbit model for arthrofibrosis. However, no change was noted in the capsular stiffness.

■ Ketotifen reduced α-smooth muscle actin (α-SMA; encoded by the *ACTA2* gene), a myofibroblast marker, at both the protein and gene expression levels, although a difference was not seen histopathologically.

Strengths and limitations

■ The present study demonstrates promising results with the use of ketotifen to treat arthrofibrosis. This is consistent with prior reports; furthermore, our model

has proven to result in a more severe and permanent contracture when compared with other animal models. Additionally, our model included monitoring the effects of ketotifen over the course of 16 weeks of remobilization.

- Given that some forms of ketotifen are already Food and Drug Administration (FDA)-approved and being utilized in clinical trials to treat joint fibrosis, this would ease the process of translating this treatment to patients with arthrofibrosis.

- Due to the lengthy time interval from the initial trauma to study analyses, crucial markers in the inflammatory cascade found at earlier timepoints, both at the genetic and protein expression levels, may have been missed.

Introduction

Joint contracture secondary to arthrofibrosis, also recently referred to as acquired idiopathic stiffness of the joint,[1] is a relatively common complication after injuries and surgical procedures, affecting up to 10% of all arthroplasty procedures.[2] Arthrofibrosis is characterized histopathologically by diffuse proliferation of scar tissue in periarticular soft tissues, and causes substantial pain and restricted movement for patients.[3] It is particularly common in the elbow and knee joints. Prevalence rates of arthrofibrosis after total knee arthroplasty (TKA) in the literature vary from 2% to 10%, thus affecting thousands of patients in the USA alone.[1,4-9] Arthrofibrosis continues to be a difficult clinical problem to treat due to a limited understanding of the underlying molecular pathogenesis. Current treatments for these patients are limited to physical therapy, manipulation under anaesthesia (MUA), or arthroscopic scar debridement.[10-12]

To address this clinical problem by systematic experimentation, we have developed a rabbit model to study the biomechanical, histopathological, and molecular changes that occur after surgical induction of contracture. The joint lesions in this model closely resemble changes occurring in humans during post-traumatic contracture formation.[13] The aforementioned changes include decreased range of movement (contracture), early increases in the myofibroblast population,[14] and increased extracellular matrix (ECM) production, as well as alterations in the messenger RNA (mRNA) transcript and protein composition of fibrotic tissue.[14,15] Our rabbit model has been shown to create a permanent contracture that does not resolve on its own. This outcome is accomplished through a combination of trauma, rupture of the posterior capsule, an immobilization period, and a remobilization period.[16]

Several pharmacological agents have been evaluated with regard to arthrofibrosis prevention and treatment in experimental models of arthrofibrosis, including subcutaneous (SQ) ketotifen, intra-articular decorin, and intramuscular or oral rosiglitazone.[17-21] It has been demonstrated that ketotifen, which inhibits mast cell degranulation, decreases the number of myofibroblasts, serum mast cell tryptase, α-smooth muscle actin (α-SMA, myofibroblast marker; encoded by the *ACTA2* gene), protein and mRNA levels, transforming growth factor-β1 (TGFB1), and collagen I (COL1A1) compared to untreated contracture groups.[19,21,22] Previous studies have examined the short-term effects of ketotifen in a rabbit contracture model, with all animals being killed after eight weeks of immobilization.[19,20] However, there is a paucity of data in the literature examining the long-term effects of ketotifen for the prevention or treatment of arthrofibrosis.

The aim of the present study was to address the hypothesis that ketotifen may alleviate the biomechanical and histopathological changes of arthrofibrosis in a long-term rabbit model for arthrofibrosis, which includes eight weeks of immobilization and 16 weeks of remobilization. We also sought to gain insights into the molecular mechanism of action of ketotifen by examining expression of select mRNA transcripts and proteins which may be involved in arthrofibrosis and mast cell function.

Methods

Ethical treatment of animals. All animal work was conducted at our institution. This study was reviewed and approved in advance by the Institutional Animal Care and Use Committee (IACUC) and our institution's Department of Comparative Medicine.

Study design. A total of 12 skeletally mature, intact New Zealand White (NZW) female rabbits (*Oryctolagus cuniculus*) weighing 2.5 kg to 3.5 kg were randomly divided into two different groups, each with six rabbits. Group 1 received SQ saline injections (control) and Group 2 received SQ ketotifen injections (1 mg/kg). Injections were performed in both groups twice daily for 14 days postoperatively.

All rabbits underwent identical index surgical procedures performed on the right knee as previously described.[16,23,24] Briefly, the joint cavity was exposed, and intra-articular haematoma was created by drilling the nonarticulating portions of the femur, capsular disruption was created by transecting the cruciate ligaments and hyperextending the knee, and the knee was then immobilized in maximum flexion with a Kirschner wire (K-wire) threaded through a hole drilled in the tibia and hooked over the femur. Following surgery, rabbits were permitted free cage activity (cage volume, 1 m³). SQ injections were administered in the immediate postoperative period and subsequently administered twice daily for a total of 14 days following surgery. No other analgesic or anti-inflammatory drugs were given, since such agents may impact fibrous tissue production. All rabbits regained their initial weight by eight weeks from the index surgical procedure. The K-wire was removed under anaesthesia eight weeks after the index procedure in all rabbits. Extra-articular heterotopic ossification (HO) and excessive callus were removed at the time of K-wire
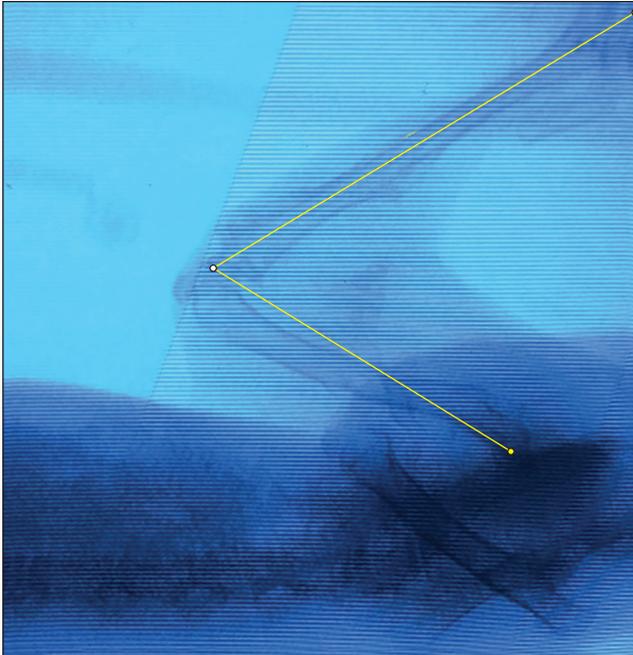
Fig. 1

In live rabbits, the passive extension angle was measured using fluoroscopic imaging. The angle formed at the centre of the knee joint (parallel to the transepicondylar axis) was measured using a line bisecting the tibial shaft and a line along the posterior femoral cortex centred at the hip joint.

Fig. 2

Capsular stiffness calculation as defined by a line tangential to the slope of the torque versus angular displacement curve.

removal. All rabbits were then allowed free cage activity for an additional 16 weeks after K-wire removal to allow for joint remobilization prior to sacrifice.

Preparation of ketotifen injections. Research-grade ketotifen (Tocris; Minneapolis, Minnesota, USA) was dissolved in dimethylsulfoxide (DMSO) in order to create a 25 mg/ml stock solution at room temperature. Injection volumes were then calculated based on each rabbit's weight on 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 two weeks.

Radiological joint angle measurement. Joint angle measurements were performed on the operative and non-operative limbs at eight, ten, 16, and 24 weeks in live, sedated rabbits using a previously validated dynamic load cell (DLC) device according to the published protocol.[16,25] The sliding arm of the device was used to apply sequential extension torques of 40 N·cm, 50 N·cm, and 60 N·cm to the knee, with the rabbits in a supine position in the animal support device.[25] Lateral fluoroscopic images were captured at each torque to allow for measurement of passive knee extension (Figure 1). Images were then analyzed by two independent observers (MET and AKL) using ImageJ 1.50i (National Institute of Health, Bethesda, Maryland, USA).

Total passive extension angles were defined as the angle formed by the intersection of a line drawn along the anatomical axis of the femur, and a line along the anatomical axis of the tibia.[25] Due to challenges in obtaining a
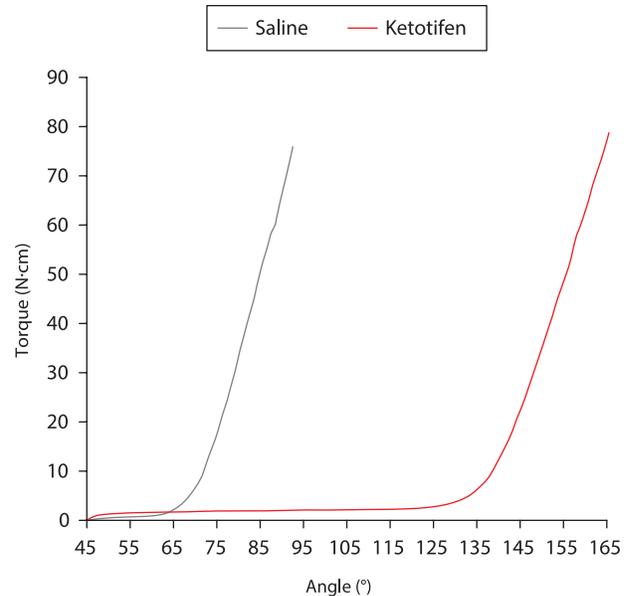
true lateral orientation of the knee for each experimental animal, the error due to out-of-plane rotation was calculated and found to be negligible when rotation is within 20° of the target value (Supplementary Figure a).

Biomechanical analysis of the limb at necropsy. At the end of the experimental period, animals were euthanized with intravenous Fatal-Plus (100 mg/kg; Vortech Pharmaceuticals Ltd, Dearborn, Michigan, USA). Skin, muscle, and tendon were then removed from both the femur and tibia, 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 rods.[16] A maximum extension torque of 20 N·cm was applied to the tibia at a rate of 1° per second. Collected data were plotted, and the passive extension angle at maximum torque was determined.[16]

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, Massachusetts, USA). We defined 'capsular stiffness' as the stiffness of the posterior capsule, once all soft tissues had been removed from the tibia and femur. The capsular stiffness was calculated based on the slope of a line tangential to the exponential curve, drawn at the steepest, linear segment (Figure 2). The mean capsular stiffness for each treatment group was calculated based on individual values for each included rabbit (Table I).

Following all biomechanical testing, posterior capsule tissue was harvested from the operative limb of each rabbit. This tissue was divided into three pieces for histopathology, RNA, and protein analyses. Experimental

Table I. Tabular representation of capsular stiffness in saline versus ketotifen treatment groups.

Treatment group	Mean capsular stiffness (SD)	p-value*
Saline	3.19 (0.01)	0.64
Ketotifen	2.52 (0.30)	0.64

*Mann-Whitney U test.

Table II. Mean passive extension angle measurements taken from rabbits at different timepoints in the saline and ketotifen groups.

Timepoint wks	Mean extension angle, ° (SD)		p-value
	Saline	Ketotifen	
8	46 (13.2)	49 (13.3)	0.904
10	70 (9.6)	74 (9.6)	0.548
16	92 (17.6)	104 (7.9)	0.222
24	98 (11.6)	118 (6.7)	0.032*

*Statistically significant.

Fig. 3

Graphic representation of passive extension angles at 40 N·cm of torque for different timepoints in the presence of saline or ketotifen. Horizontal dotted lines show the mean difference in passive extension among groups at 24 weeks. Data are presented as means and SDs. Statistical significance was assessed using the Mann-Whitney U test. $p < 0.05$.

methods describing histological processing, histopathological evaluation, and RNA and protein expression analysis can be found in the Supplementary Material.

Statistical analysis. For all data, comparisons between each treatment group and its respective control group were carried out using the Mann-Whitney U test, which assumes non-parametric variance between groups. Statistical significance was set at a probability of $p < 0.05$. Data are reported via means and SDs. Group sample size was determined by assuming a 5% type 1 error and 80% power to detect a mean effect difference of 30° with a SD of 20°.
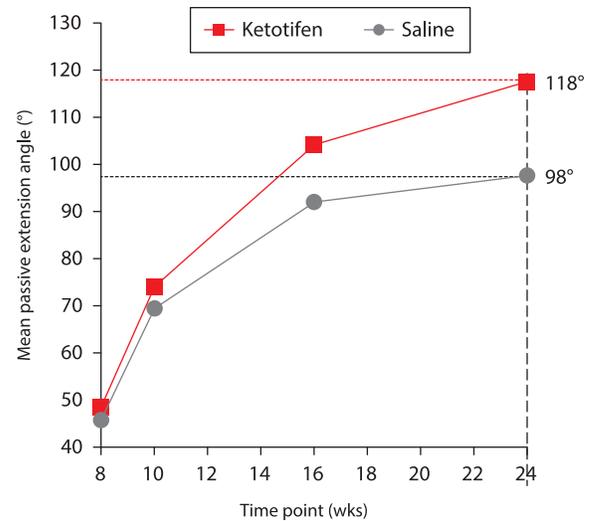
Results

Radiological measurements. Passive extension angle measurements obtained in live, sedated rabbits at eight, ten, 16, and 24 weeks after the index surgical procedure demonstrated more rapid and superior progressive improvement in the ketotifen treatment group compared to the saline group (Table II and Figure 3, Supplementary Figure b, Supplementary Table i).

At the 24-week timepoint, 16 weeks after K-wire removal, there was a statistically significant increase in passive extension of approximately 20° among rabbits in the ketotifen group (Group 2) compared to those in the saline group (Group 1) (117.7° vs 97.8°; $p = 0.03$, Mann-Whitney U test; Supplementary Figure ca). In addition, animals treated with ketotifen reached 90° of passive extension earlier than animals treated with saline (15.3 weeks vs 18.2 weeks, respectively; Supplementary Figure cb).

Capsular stiffness measurements. We examined capsular stiffness by monitoring torque on the limbs relative to the passive extension angle. The ketotifen treatment led to a decrease in capsular stiffness compared to the saline control group, but this did not achieve statistical significance within our experimental cohort ($p = 0.64$, Mann-Whitney U test; Figure 2 and Table I).

Histopathological data. Scoring criteria for the various changes are given for haematoxylin and eosin (H&E)

staining of posterior fossa tissue sections utilizing the same criteria as previously published[26] and α-SMA immunoreactivity signals (Table III). These criteria reflect the degree of fibrosis (reliably defined in H&E-stained sections)[27,28] and neovascularization (in α-SMA-labelled sections) within affected periarticular soft tissues.

In H&E-stained sections, periarticular soft tissue from the posterior fossa in naïve (nonoperated) control rabbits consists almost entirely of white adipose tissue (fat), penetrated by very few narrow, pale eosinophilic, poorly cellular columns of fibrous connective tissue (unpublished data). Mast cells - readily identifiable in H&E-stained sections by their numerous large, dark basophilic, cytoplasmic granules - are essentially absent throughout this region of non-reactive tissue in normal animals, occurring rarely as isolated cells located at the interface between two adjacent fat cells (unpublished data).[29,30] In contrast, rabbits given a joint contracture operation exhibited an increase in the quantity of fibrous connective tissues in the posterior fossa, which appears prominently in H&E-stained sections as extensive arthroplasty of normal adipose tissue by coalescing, variably cellular columns and fields of dense fibrous connective tissue. No granule-laden mast cells were seen in these fibrosis-ridden sections, even in zones where other inflammatory cells (mainly lymphocytes) were observed, which is consistent with the general tissue response in animal models of chronic joint disease.

The microscopic appearance of tissue samples in both groups covered a relatively wide spectrum, which thus prevented discrimination of treated versus control rabbits. By coded evaluation of tissue sections, rabbits that received ketotifen appeared to have no significant reduction in fibrosis (H&E, α-SMA immunohistochemistry

Table III. Tabular representation of the criteria used for obtaining histopathological scores.

Histopathological score	Meaning	Fibrous tissue (H&E and Masson's Trichrome)	Small vessel numbers (α-SMA)
0	WNL	Section consists of white fat and small amounts of dense, sparsely cellular connective tissue	Labelled profiles are widely scattered in entire section
1	Minimal	Section contains up to 10% of more cell-dense connective tissue	Labelled profiles have higher density in < 5% of section
2	Mild	Section contains about 15% to 30% of more cell-dense connective tissue	Labelled profiles exhibit greater density in 5% to 20% of section
3	Moderate	Section contains about 35% to 50% of more cell-dense connective tissue	Labelled profiles exhibit greater density in 25% to 45% of section
4	Marked	Section contains about 55% to 80% of more cell-dense connective tissue	Labelled profiles exhibit greater density in 50% to 75% of section
5	Severe	Section contains more than 85% cell-dense connective tissue	Labelled profiles exhibit greater density in > 80% of section

*SMA, alpha-smooth muscle actin; H&E, haematoxylin and eosin; WNL, within normal limits.

(IHC), and Masson's trichrome) or inflammation (H&E) relative to control saline animals, as indicated by similar microscopic lesion scores (Figures 4a and 4b, Supplementary Figure d). Similarly, coded evaluation of tissue sections (H&E, toluidine blue) confirmed the absence of mast cells within the fibrotic and variably inflamed posterior tissue of both control and ketotifen-treated rabbits.

Gene expression. Significant upregulation in the mRNA for α-SMA was observed in the saline group (Group 1) when compared to the ketotifen group (p < 0.001, Mann-Whitney U test). This result is consistent with previous findings.[14] However, no significant differences were detected in the mRNA expression of collagen markers *COL1A1*, *COL3A1*, and *COL6A1* (Figure 5), as noted previously.[26]

Protein expression data. Protein expression was normalized based on the Ponseau S total protein stain.[31] Data from western blot analysis revealed significantly elevated levels of the mast cell biomarker tryptase (encoded by the *TPSAB1* gene) during progression of arthrofibrosis in the saline group (Group 1) compared to the ketotifen group (p = 0.007, Mann-Whitney U test; Figure 6a). Consistent with the mRNA expression data, α-SMA protein levels were also decreased in the ketotifen group (p = 0.01, Mann-Whitney U test; Figure 6b).

Discussion

The present study provides data to suggest that SQ administration of the antiallergenic drug ketotifen, which stabilizes mast cells from degranulation during the initial stages of inflammation, alleviates the severity of contracture development in a rabbit model of arthrofibrosis when given for two weeks following the initial surgery to immobilize the joint. At the 24-week timepoint examined in this study, a significant increase in passive extension was observed when comparing the ketotifen treatment group with the saline treatment group. Histopathological data from posterior capsule tissue of rabbits treated with ketotifen did not show a reduction in the density of fibrous tissue or myofibroblasts, although sampling error may have precluded a homogenous analysis. However, downregulation of mRNA encoding α-SMA (*ACTA2*) was observed in the rabbits treated with ketotifen. Additionally, systemic administration of ketotifen also diminished tryptase and α-SMA protein expression in the posterior capsule. Taken together, these data indicate that treatment to reduce mast cell-driven components of acute inflammation has a positive effect on functional outcome (joint mobility) and might increase to some degree the profibrotic molecular pathways responsible for joint fibrosis and persistent contracture.

Prior studies have demonstrated a link between COX-2 inhibition and a decreased density of fibrotic lesions, thereby limiting fibrotic tissue formation.[26] Outcomes of the present study corroborate previous results from Kopka et al[21] and Monument et al,[20] demonstrating decreased contracture severity after treatment with systemic ketotifen. Importantly, the data from those prior studies investigate a single acute timepoint: immediately after eight weeks of immobilization. However, in our study the animals were allowed 16 weeks of remobilization. This difference in timing is a key consideration in comparing the data from these studies. All three studies show that acute ketotifen treatment systemically can decrease joint contracture, but our longer-term study shows that this functional benefit persists for a substantial period. In the current study, we identified decreased α-SMA (*ACTA2*) mRNA expression among rabbits in the ketotifen treatment arm when compared to the saline group (Group 1), even after 16 weeks of remobilization. This difference is likely attributable to inhibition of the inflammatory cascade in the immediate postoperative period. The critical period in which to inhibit this pathway has been identified as the first 24 to 72 hours after the inflammatory insult.[32] It is for this reason that we selected a preventive pharmacological treatment schedule (starting on postoperative day zero). Furthermore, extensive upregulation in myofibroblast expression of α-SMA has been demonstrated to plateau by two weeks after insult and decrease continuously thereafter.[14] This, combined with the heterogeneity of the posterior capsule reaction, is likely to explain the limited differences between the histopathological appearance of the fibrotic reactions (assessed using H&E, Masson's trichrome, and α-SMA immunohistochemistry (IHC))

a

b

Fig. 4
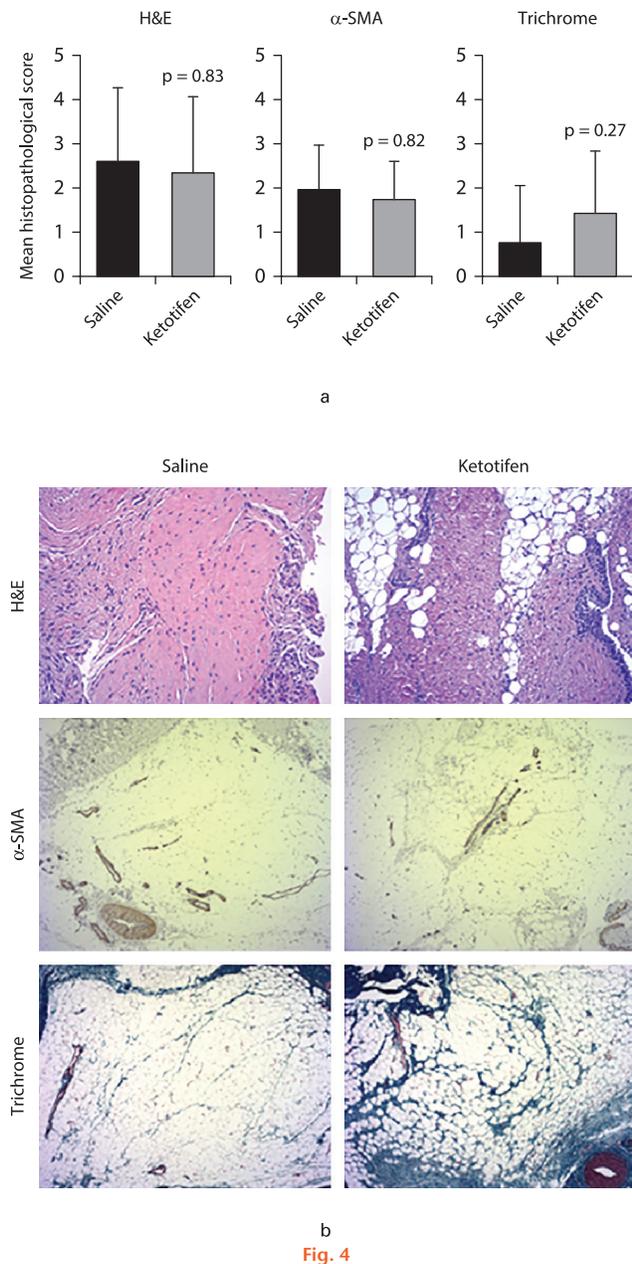
Histopathological data showing no difference in fibrous lesions in periarticular soft tissue as assessed in haematoxylin and eosin (H&E)-stained, α-smooth muscle actin (α-SMA; myofibroblast marker)-labelled, and Masson's trichrome-stained sections, in the rabbits that received ketotifen compared to the rabbits that received saline. This is represented by a) bar graphs depicting the mean histopathological score and b) representative images of the H&E-stained, α-SMA-labelled, and Masson's trichrome-stained sections. Statistical significance was assessed using the Mann-Whitney U test.

observed between the ketotifen-treated animals and the controls. Importantly, the second remobilization surgery removed two irritants (e.g. the K-wire and extra-articular nodules of ossification) that might have been capable of sustaining a chronic active inflammatory process (i.e. one with continued recruitment of innate immune cells such as neutrophils, macrophages, and mast cells in addition to the regional aggregation of acquired immune cells

such as lymphocytes and plasma cells). The elimination of these irritants would have reduced the concentration of signalling molecules capable of driving the acute inflammatory process. This altered microenvironmental milieu coupled with the near-total lack of mast cells in periarticular white adipose tissue or normal rabbits likely explain the demonstrated absence of mast cells within fibrotic joint tissue of animals from our study.

We identified α-SMA protein expression differences between treatment groups. The difference in mRNA and protein expression can be explained by examining the timing of the transcription and translation processes, as well as their association with the inflammatory cascade. At 24 weeks, it is very likely that the initial inflammatory insult has resolved with minimal ongoing cytokine-induced myofibroblastogenesis. Conversely, it has been demonstrated that the levels of mast cells and neuropeptides remain elevated up to 32 weeks after contracture induction in both rabbit knee contractures and human elbow contractures.[33] Western blotting measures the accumulation of proteins that were deposited upon past translation of the corresponding mRNA, whereas mRNA levels are typically associated with ongoing protein synthesis. Our measurement timepoint (24 weeks) may have been too late to identify changes in mRNA levels, since by that timepoint new protein synthesis may have already subsided across treatment groups. We hypothesize that this explanation is plausible with respect to our mRNA expression results for collagens 1, 3, and 6 based on prior work on the temporal expression of such genes.[32] The best modality through which to identify essential differences in early genesis and later progression of profibrotic molecular signatures in specific cell types and tissue regions of the operative joint would be histopathological analyses at early timepoints with optimization of the sampling sites. We plan to pursue this avenue of study in future investigations.

The present study assessed ketotifen's ability as a standalone agent to affect passive extension, and the mechanisms through which this may have been accomplished. At the final study timepoint (24 weeks), we noted statistically significant improvements in passive extension in the ketotifen group (Group 2) when compared to the saline controls (Group 1). This effect is thought to be due to inhibition of mast cell degranulation and subsequent decreased inflammatory cascade activation. Ketotifen has previously been demonstrated to reduce mast cell recruitment, tryptase mRNA expression, and the serum mast cell tryptase activity, in addition to reducing contracture formation.[19,21,34] Decreased mast cell recruitment is thought to lead to decreased expression of multiple profibrotic molecules, including basic fibroblast growth factor (bFGF/FGF2), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and TGFB1, thereby decreasing differentiation of fibroblasts into myofibroblasts.[34] Tryptase, a protease released by mast cells, is a potent fibroblast mitogen and is hypothesized to induce
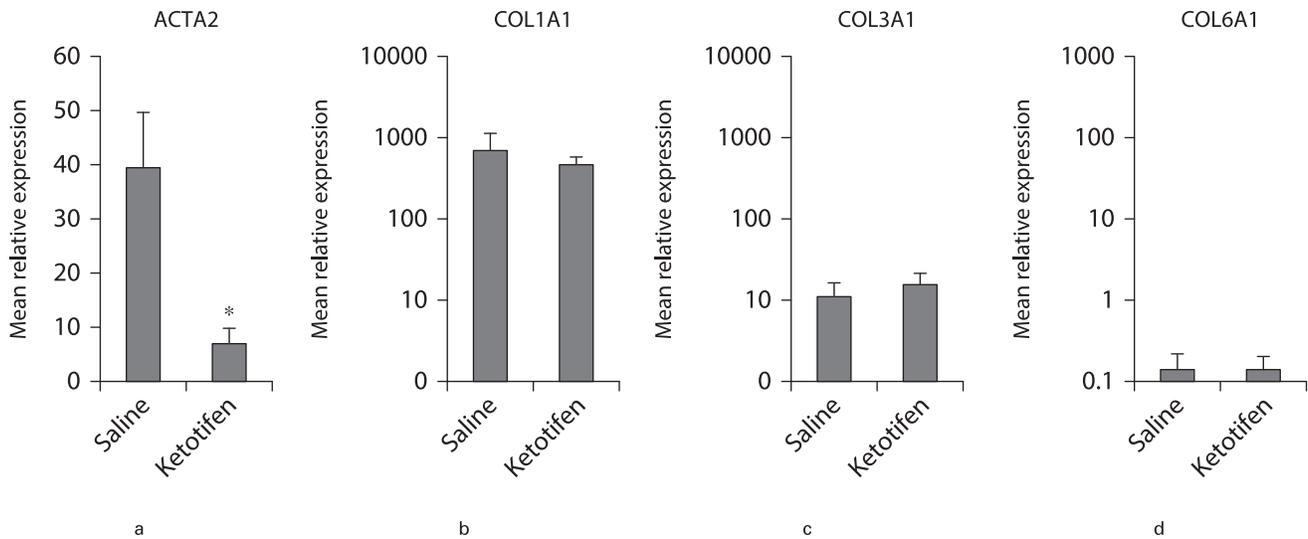
Fig. 5

Mean messenger RNA (mRNA) expression of a) *ACTA2*, b) *COL1A1*, c) *COL3A1*, and d) *COL6A1* relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) across treatment arms at 24 weeks. *p < 0.05, Mann-Whitney U test.
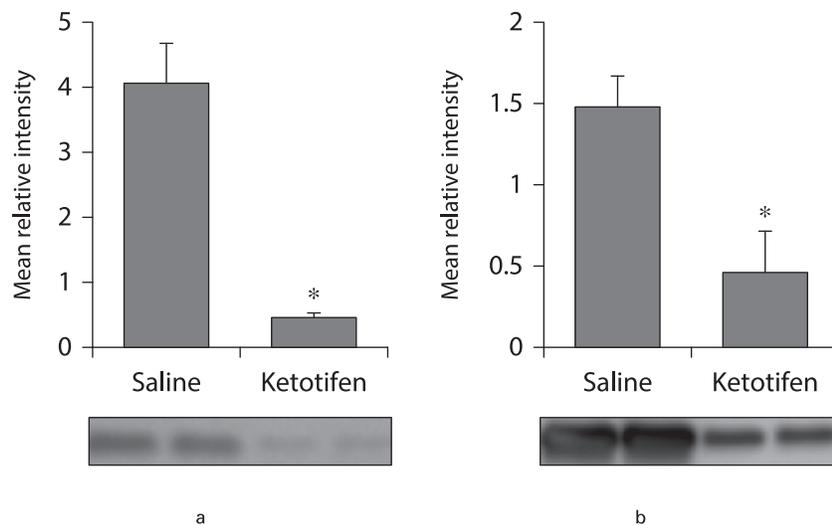

Fig. 6

Relative protein expression of a) tryptase and b) α-smooth muscle actin (α-SMA; encoded by *ACTA2*) in all treatment groups at 24 weeks. Protein levels were normalized across lanes by quantifying total protein levels detected by Ponceau S stain. *p < 0.05, Mann-Whitney U test.

fibrosis via protease-activated receptor-2 (PAR-2/F2RL1) and mitogen-activated protein kinase (MAPK) pathway activation, which stimulates transformation from the fibroblast to myofibroblast phenotype.[35] We utilized tryptase as a marker for mast cells, and demonstrated a statistically significant reduction in tryptase expression with ketotifen treatment. A complete understanding of these findings will require additional histopathological and gene expression investigations at earlier timepoints during the initial arthrofibrogenesis process to clarify the biomechanical differences noted in the present study. Such data could provide insight into whether or not pharmacological inhibition of the proinflammatory cascade leads to measurable changes in both promyofibroblastic mRNA and tissue composition. Likewise, the delivery method of ketotifen also warrants further investigation. This will be a key element to facilitate translation of this drug to the clinic.

There are a small number of studies in the literature utilizing ketotifen to reduce joint contracture formation in a rabbit model.[19-21] However, these surgical models do not include the rupture of the posterior capsule, which we believe is vital to maximizing the severity and stability of the contracture, and more closely mimics the functional outcome that occurs in humans following TKA.[16] Additionally, none of the published reports for prior studies examined the effects of ketotifen after the animals have undergone a remobilization period, while our study

defines a model that includes a 16-week remobilization period with multiple knee extension angle measurements during this time. Therefore, our data represent an important extension of the database needed to understand the pathogenesis of postoperative joint fibrosis and assess the efficacy of ketotifen as a treatment providing sustained relief from joint contracture.

Several limitations qualify the interpretation of the data presented in this study. Regarding the biomechanical measurements, two rabbits were excluded from the final analysis. One animal was euthanized following the index procedure for a fractured tibia (Group 1, Rabbit #2) and one animal received non-steroidal anti-inflammatory drugs (NSAIDs) for a self-inflicted foot injury (Group 2, Rabbit #8). It is possible that samples might have been contaminated with bone, blood, muscle, or fat; however, no contamination was found to be substantial in any of the analyzed posterior capsule tissue. All of the gene and protein expression data were obtained from tissue harvested at the 24-week timepoint. Due to the lengthy time interval from the initial trauma, crucial markers in the inflammatory cascade at earlier timepoints, both at the genetic and protein expression levels, may have been missed. We believe that investigations performed at earlier timepoints might allow for more relevant detection of changes in the expression of cytokines, such as TGFs, interleukins, and fibroblast growth factors (FGFs), as well as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Finally, a larger number of animals per group would provide us with more statistical power, and an increased ability to detect smaller differences between the treatment and control groups.

In conclusion, our results suggest that ketotifen administered systemically by SQ injections for two weeks decreases the severity of contracture development in a rabbit model of arthrofibrosis. At 24 weeks postoperatively, ketotifen-treated rabbits exhibited a significantly improved functional outcome (greater passive joint extension) compared to the saline-treated control group. We were unable to demonstrate substantial differences in the degree of fibrous tissue production by histopathological evaluation (by H&E, Masson's trichrome, and α-SMA IHC) of the posterior capsule between the two treatment groups. However, significant ketotifen-induced reductions were found in the expression of three molecules related to fibrosis production, namely gene expression of *ACTA2* as well as protein expression of α-SMA and tryptase. Further studies are necessary to determine whether the histopathological and molecular changes resulting from this pharmacological therapy are better correlated at earlier postoperative timepoints, and whether the divergence at later times can be moderated so that accumulation of fibrous tissue as noted in tissue sections histopathologically can be reduced. The ultimate goal of our programme is to identify a translational treatment protocol that can be prophylactically utilized in human subjects to diminish progression of arthrofibrosis to improve functional outcomes and the quality of life enjoyed by patients following joint arthroplasties.

Supplementary material

ё Text, table, and figures illustrating the experimental methods used for the histological processing, histopathological evaluation, and RNA and protein expression analysis conducted in this study.

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