The Dose-Response Effect of the Mast Cell Stabilizer Ketotifen Fumarate on Posttraumatic Joint Contracture

An in Vivo Study in a Rabbit Model

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Background: Posttraumatic joint contracture is a debilitating complication following an acute fracture or intra-articular injury that can lead to loss of motion and an inability to complete activities of daily living. In prior studies using an established in vivo model, we found that ketotifen fumarate (KF), a mast cell stabilizer, was associated with a significant reduction in the severity of posttraumatic joint contracture. Our primary research question in the current study was to determine whether a dose-response relationship exists between KF and posttraumatic joint contracture reduction.

Methods: A standardized operative method to create posttraumatic joint contracture in a knee was performed on skeletally mature New Zealand White rabbits. The animals were randomly assigned to 1 of 5 groups (n = 10 per group): a nonoperative control group, an operative control group, or 1 of 3 experimental KF groups (0.01 mg/kg [the KF 0.01 group], 0.1 mg/kg [KF 0.1], or 5.0 mg/kg [KF 5.0]). Flexion contractures were measured following 8 weeks of knee immobilization using a hydraulic material-testing machine. The posterior knee joint capsules were then harvested for quantification of myofibroblast and mast cell numbers with immunohistochemistry analysis.

Results: Forty-five rabbits were used in the final analysis. Contracture severity was significantly reduced in the KF 0.1 group (p = 0.016) and the KF 5.0 group (p = 0.001) compared with the operative control group. When converted to a percent response, posttraumatic joint contracture reduction was 13%, 45%, and 63% for the KF 0.01, KF 0.1, and KF 5.0 groups, respectively. A half-maximal effective concentration (EC_{50}) for KF of 0.22 mg/kg was established. There was also a decrease in myofibroblasts, mast cells, and substance P-containing nerve fiber counts with increasing doses of KF.

Conclusions: Using a preclinical, rabbit in vivo model of posttraumatic joint contracture, increasing doses of KF were associated with decreasing biomechanical estimates of knee posttraumatic joint contracture as well as decreasing numbers of myofibroblasts, mast cells, and substance P-containing nerve fibers.

Clinical Relevance: KF has been used safely in humans for more than 40 years and, to our knowledge, is the first and only agent ready to be potentially translated into an effective treatment for posttraumatic joint contracture.

Posttraumatic joint contracture is a debilitating complication following an acute fracture or intra-articular injury that can lead to loss of motion at the affected joint and an inability to complete activities of daily living[1]. Despite treatment with physical therapy, static progressive splinting, dynamic splinting, or the application of hinged external fixators[2-4], 10% to 15% of patients who develop posttraumatic joint contracture of the elbow require additional surgical release or excision of the joint capsule[5-7]. Patients with posttraumatic joint contracture may continue to experience a

TABLE I Demographic Comparison of Treatment Arms*					
	Control Groups		Ketotifen Fumarate Groups		
	Nonoperative (N = 10)	Operative (N = 8)	0.01 mg/kg (N = 7)	0.10 mg/kg (N = 10)	5.00 mg/kg (N = 10)
Mean animal weight (kg)					
Preop.	5.29	5.15	5.29	5.18	5.38
Postop.	5.48	4.85	5.32	4.89	5.10
Change	+0.19	−0.30	+0.03	−0.29	−0.28
Right/left (no.)	5/5	4/4	3/4	5/5	5/5

*There were no significant differences between groups pre- or postoperatively.

decline in motion and function despite surgical intervention or nonoperative treatment[6,8-10].

Following injury, the etiology of posttraumatic joint contracture is multifactorial. Our research group hypothesized that a mast cell-mediated pathway is likely activated, resulting in joint-capsule fibrosis[11]. In a human study of patients with chronic elbow posttraumatic joint contracture, range of motion was inversely proportional to the number of myofibroblasts and mast cells in the joint capsule[12]. Similarly, in a prior rabbit in vivo model of posttraumatic joint contracture eval-uated by our group, the amount of alpha-smooth muscle actin (α-SMA)-positive myofibroblasts, tryptase-positive mast cells, and neuropeptide-containing nerve fibers was significantly greater in the contracture capsules when compared with the control capsules[12,13]. Ketotifen fumarate (KF) is a mast cell stabilizer that has been approved by the U.S. Food and Drug Administration and Health Canada for topical ophthalmic treatment and is also approved by the latter as an oral medi-cation for asthma. Prior research by our group showed that, in a rabbit model, treatment with KF significantly reduced the
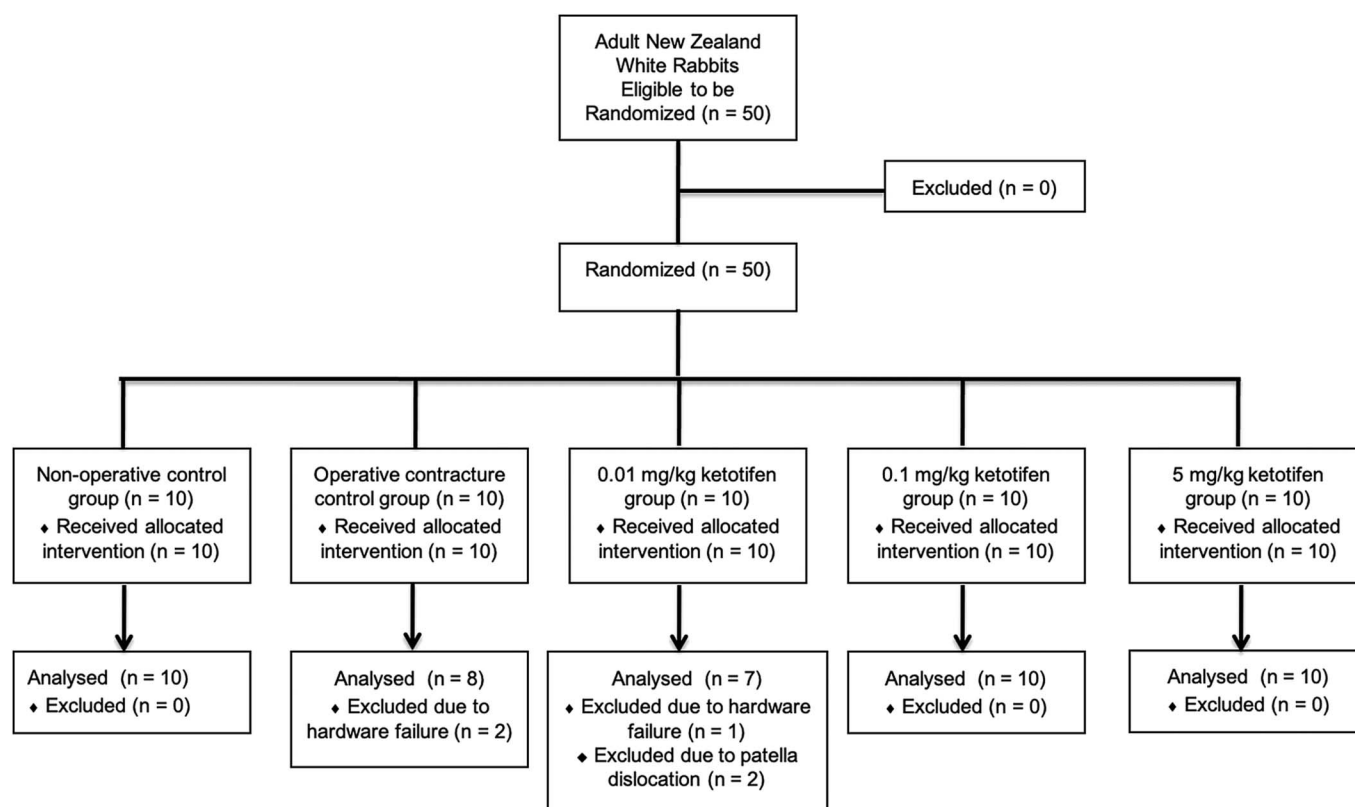

Fig. 1

CONSORT (Consolidated Standards of Reporting Trials) diagram demonstrating the number of animals randomized to each treatment arm and the final number of animals analyzed per treatment arm.
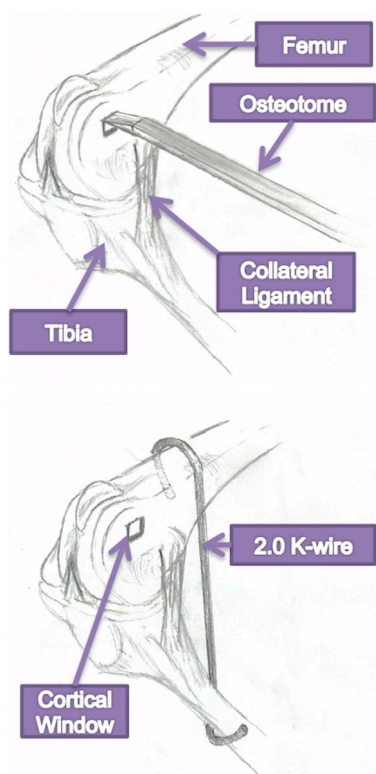
Fig. 2

Surgical posttraumatic joint contracture model demonstrating intra-articular, yet extra-capsular, corticotomy of 5-mm × 5-mm and 2.0-mm Kirschner wire (K-wire) placement for joint immobilization.

severity of joint contracture; however, the doses used previously were 0.5 or 1.0 mg/kg twice daily, and there was no dose-response relationship shown over this narrow range[14,15]. Quantifying the dose-response relationship will be valuable, and an optimal dose range needs to be identified whereby contracture reduction is maximized while side effects are avoided.

The objective of the current study was to evaluate the efficacy of KF administration over a wide range of doses in the reduction of posttraumatic joint contracture. We hypothesized that there would be a linear dose-response effect of KF on posttraumatic contractures and joint-capsule properties across doses of 0.01 to 5.0 mg/kg, using a rabbit in vivo model of posttraumatic joint contracture.

Materials and Methods

Local research ethics board approval was obtained prior to study initiation. All animals underwent a 2-week acclimatization period prior to experiments. An in vivo model of posttraumatic joint contracture of the knee was created using a combination of intra-articular injury and internal immobilization in skeletally mature New Zealand White rabbits (mean preoperative weight of 5.26 kg) (Table I). The animals were randomly assigned to 1 of 5 groups (n = 10 per group). The first group (the nonoperative control group) consisted of rabbits

that did not receive operative or pharmacological interventions. The second group (the operative control group) comprised rabbits that underwent surgical intra-articular joint injury and 8 weeks of immobilization, in order to create a stable posttraumatic joint contracture[11,13-15]. No pharmacological interventions or placebo injections were given to rabbits in this study arm. The animals in the remaining 3 study arms received the identical surgical interventions as the operative control group, but they were also treated with a subcutaneous dose of the mast cell stabilizer KF, twice daily for 8 weeks; 1 of 3 doses was administered: 0.01 mg/kg (the KF 0.01 group), 0.1 mg/kg (the KF 0.1 group), or 5.0 mg/kg (the KF 5.0 group) starting immediately after the surgical intervention (Fig. 1). All injections were given subcutaneously, alternating between bilateral upper-back areas, as this method has been previously reported in the literature and used in our prior research[14-16]. Each animal was inspected twice daily to ensure there was no local skin irritation due to the injections.

Posttraumatic Joint Contracture Model
Surgical joint interventions were performed using a previously established model[12,14]. The right or left knee was randomly selected in advance. Following the administration of general anesthesia, a skin incision was made on the lateral aspect of the randomly selected right or left thigh. The intermuscular interval that was utilized was between the lateral aspect of the quadriceps and the hamstring musculature. After exposure of the lateral aspect of the femur, both medial and lateral knee joint arthrotomies were made by removing 5-mm × 5-mm cortical windows from the portions of the medial and lateral femoral condyles that did not contain articular cartilage. Care was taken not to injure the
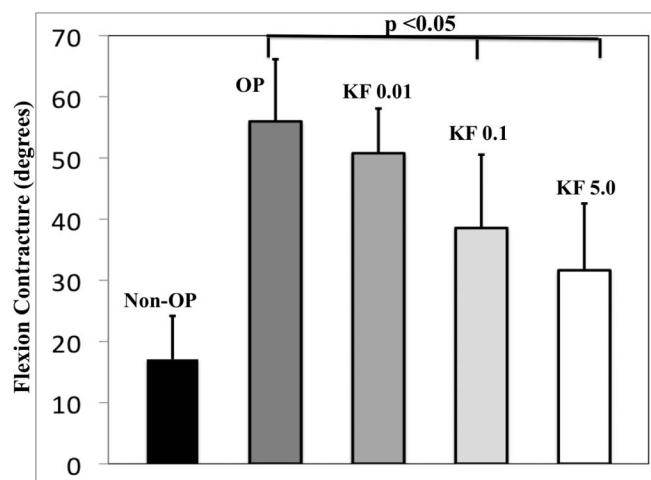

Fig. 3

Mean flexion contracture (and standard deviation) across all treatment arms. A mean flexion contracture of 56° developed in the operative control group (OP), and this did not significantly improve at the lowest ketotifen fumarate (KF) dose (KF 0.01). Both the KF 0.1 and KF 5.0 doses were associated with a significant reduction in contracture severity, with a mean flexion contracture of 39° and 32°, respectively.
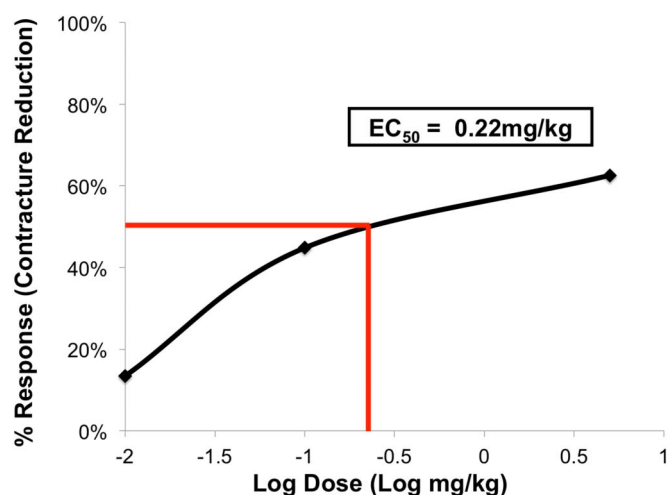
Fig. 4

Ketotifen fumarate dose-response curve for flexion contracture, demonstrating a relationship with a threshold response, with a half-maximal effective concentration (EC_{50}) of 0.22 mg/kg.

collateral ligaments, in order to mimic a traumatic intra-articular injury while still maintaining the articular congruity of the joint (Fig. 2).

Next, an incision was made approximately 3 cm distal to the tibial tubercle along the easily palpable subcutaneous border of the tibia. A transosseous 2.0-mm-diameter Kirschner wire (Zimmer Orthopaedic Solutions) was placed through the tibia and then bent posterior to the knee. The knee was flexed to 150°, and the Kirschner wire was contoured around both the tibia and the lateral aspect of the femur. The tibial end of the Kirschner wire was cut flush with the bone to ensure stable immobilization, while protecting the skin from irritation. Postoperatively, all animals were housed in individual cages and permitted to move freely (0.1-m³ cages), without restrictions postoperatively. Twice-daily animal assessments included evaluation of any substantially reduced eating, drinking, or movement. Each animal was killed 8 weeks following the day of surgery, in order to ensure a stable contracture.

Posttraumatic Joint Contracture Testing

The lower limbs were harvested and flexion contractures were measured using a custom rabbit-knee-gripping device[15,17], with the axis of rotation centered on the femoral insertion of the medial collateral ligament. This device was attached to a hydraulic material-testing machine (MTS). A standardized torque of 0.2 Nm was applied to each limb to reach terminal extension for 5 cycles, and the contracture angle was calculated with reference to full extension, averaging the 5 cycles.

Immunohistochemistry (IHC) Analysis

The posterior knee joint capsules were harvested and used for quantification of myofibroblast and mast cell numbers with IHC, and estimation of α-SMA, type-I collagen (Col I), and tryptase levels using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot gel electrophoresis, as has been previously described[13-15]. A double-labeling IHC methodology that has previously been described was used to quantify myofibroblast and mast cell numbers in joint capsule preparations[13]. The RT-PCR levels and western blot intensities were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Triple-label IHC combined with DAPI (4′,6-diamidino-2-phenylindole) nuclear labeling was also completed, and cell counts for myofibroblasts, mast cells, and substance P (SP)-containing cells were calculated as a percentage relative to the total cell count.

Statistical Analysis

Data are presented as the mean and standard deviation. A random-effects regression model was used to analyze data from the biomechanical experiments in order to account for the repeated measures involved in the contracture measurement for each limb, in keeping with our prior research[14,15]. Comparisons of contracture severity between individual groups were analyzed by testing linear combinations (of model estimates) of the coefficients. For calculating the dose-response relationship, the percent response of the joint contracture was calculated relative to the nonoperative controls across the 3 experimental KF groups. This relationship was plotted against the log dose to assess the relationship and to obtain the half-maximal effective concentration (EC_{50}) value. Statistical analysis for IHC, western blotting, and RT-PCR consisted of a 1-way analysis of variance (ANOVA) with Tukey post-hoc analysis. Significance was $\alpha = 0.05$ (SPSS version 23; IBM).
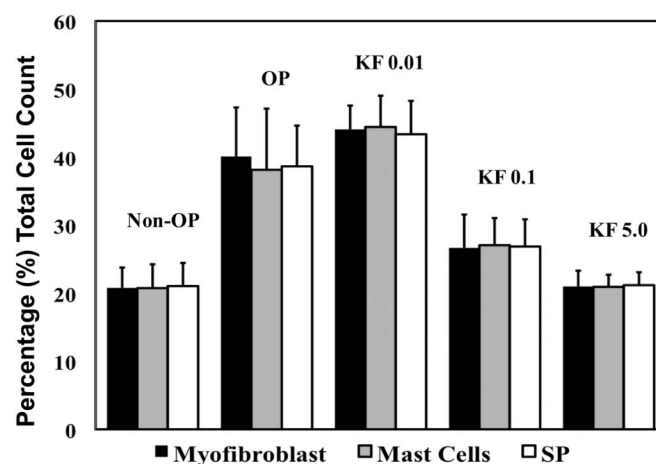

Fig. 5

Results of immunohistochemistry analysis, showing cell count (percentage of total cell count [mean and standard deviation]) for myofibroblasts, mast cells, and substance P (SP)-containing nerve fibers for the nonoperative control group (Non-OP), the operative control group (OP), and the 3 ketotifen fumarate (KF) dosing groups (KF 0.01, KF 0.1, and KF 5.0). There were significant decreases in myofibroblast, mast cell, and SP values between the operative control group and the KF 0.1 group, and the operative control group and the KF 5.0 group ($p < 0.05$).
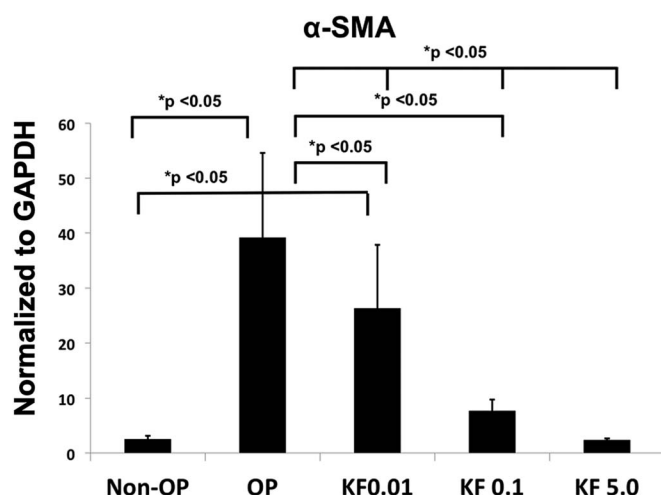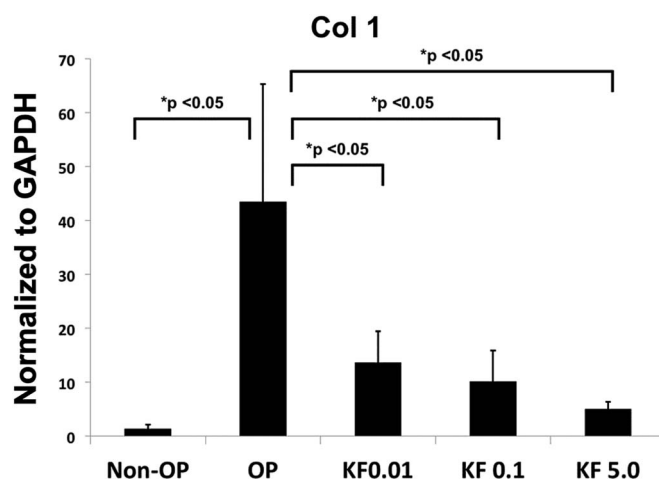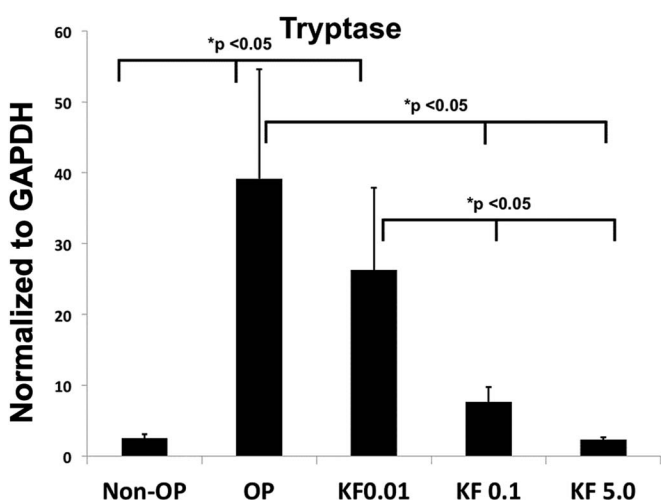
Fig. 6-A

Fig. 6-B

Fig. 6-C

Figs. 6-A, 6-B, and 6-C Western blot analysis, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results, presented as the mean and standard deviation, indicate a dose-response effect of ketotifen fumarate (KF) on α-smooth muscle actin (SMA) (**Fig. 6-A**), type-I collagen (Col 1) (**Fig. 6-B**), and tryptase (**Fig. 6-C**). We observed a trend of an association between increasing doses of KF and decreasing levels of all 3 molecules, with significant differences between the operative control group (OP) and the group that received a KF dose of 5.0 mg/kg (KF 5.0).

Results

There were no significant differences in animal weight between the groups at the time of randomization or at the completion of the experiment (Table I). All rabbits treated with KF tolerated the medication. Throughout the study period, a total of 5 rabbits were excluded for failure of hardware (2 in the operative control group and 1 in the KF 0.01 group) or patellar dislocation (2 in the KF 0.01 group) (Fig. 1).

The average flexion contracture was $56° ± 10°$ in the operative control group (n = 8) compared with $51° ± 7°$ in the KF 0.01 group (n = 7), $39° ± 12°$ in the KF 0.1 group (n = 10), and $32° ± 11°$ in the KF 5.0 group (n = 10) (Fig. 3). A baseline flexion contracture averaging $17° ± 7°$ was present in the nonoperative control group (n = 10). Contracture severity was significantly reduced in the KF 0.1 group (p = 0.016) and the KF 5.0 group (p = 0.001) compared with the operative control group. When converted to a percent response for the overall effect on contracture, the reduction in contracture was 13%, 45%, and 63% for the KF 0.01, KF 0.1, and KF 5.0 groups, respectively. This relationship was plotted against the log dose,

and an EC_{50} for KF of 0.22 mg/kg was established (Fig. 4). Animal weight and operative side did not significantly influence contracture values.

Using IHC analysis, we noted a decrease in myofibroblasts, mast cells, and SP nerve fiber counts with increasing doses of KF. Expressed as a percentage of total cells, there were significant differences in myofibroblast, mast cell, and SP values between the operative control group and the KF 0.1 group and between the operative control group and the KF 5.0 group (p < 0.05) (Fig. 5). There were no significant differences between the nonoperative control group and the KF 5.0 group, the KF 0.1 group and the KF 5.0 group, and the operative control group and the KF 0.01 group in myofibroblast, mast cell, and SP values (p > 0.05) (Fig. 5).

The western blot gel electrophoresis showed a dose-response effect of KF on α-SMA (Fig. 6-A), Col I (Fig. 6-B), and tryptase levels (Fig. 6-C). We observed a trend of an association between increasing doses of KF and decreasing levels of all 3 molecules, with significant differences between the operative control group and the KF 5.0 group (p < 0.05).
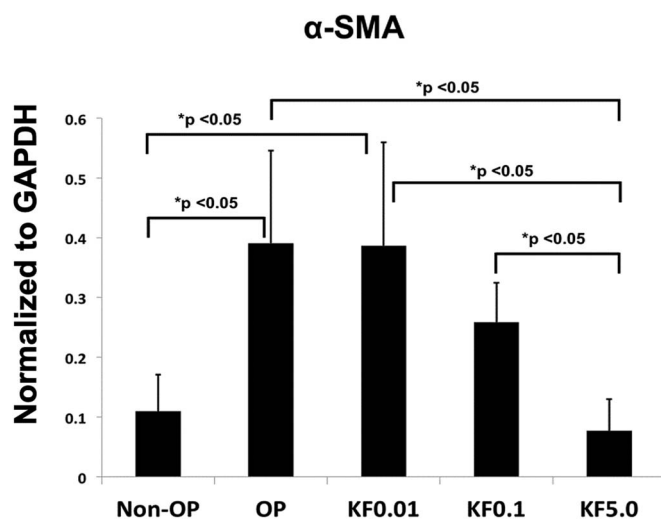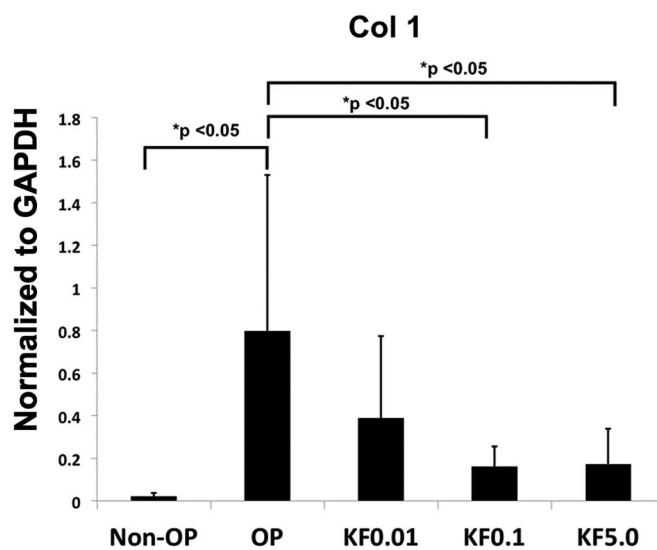
Fig. 7-A

Fig. 7-B

Figs. 7-A, and 7-B Reverse transcription-polymerase chain reaction (RT-PCR) quantification of α-smooth muscle actin (α-SMA)-positive myofibroblasts (**Fig. 7-A**) and type-I collagen (Col 1) (**Fig. 7-B**), presented as the mean and standard deviation. We observed a trend of an association between increasing doses of ketotifen fumarate (KF) and decreasing levels of α-SMA and Col I. RT-PCR data are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The nonoperative control group differed significantly from the operative control group, while there was no significant difference between the nonoperative control group and the KF 0.1 and KF 5.0 groups.

The RT-PCR analysis for Col I and α-SMA followed a similar dose-response pattern as the western blot. We observed a trend of an association between increasing doses of KF and decreasing levels of α-SMA and Col I (Figs. 7-A and 7-B). There was significantly lower Col I expression in the KF 0.1 and KF 5.0 groups compared with the operative control group (p < 0.05). There was significantly greater Col I expression in the operative control group compared with the nonoperative control group. For α-SMA, there was significantly lower α-SMA expression in the KF 5.0 group compared with the operative control group, the KF 0.01 group, and the KF 0.1 group (p < 0.05). There was significantly lower α-SMA expression in the nonoperative control group compared with both the operative control and KF 0.01 groups. There is no rabbit-specific tryptase PCR primer, to our knowledge.

Discussion

Using the preclinical, rabbit in vivo model of posttraumatic joint contracture, a nonlinear dose-response was observed with treatment with the mast cell stabilizer KF for the first time, to our knowledge. Increasing doses of KF were associated with decreasing biomechanical estimates of knee joint flexion contractures as well as decreasing numbers of myofibroblasts, mast cells, and SP-containing nerve fibers in the joint capsule; however, these relationships were not linear, as was previously hypothesized. Over the 500-fold range of doses studied, a threshold dose-response was observed for the effect of KF on posttraumatic joint contracture, with a reduction in biomechanical contracture properties beginning at the KF 0.1 dose, which is lower than previously studied doses. We report the novel finding of an EC_{50} of 0.22 mg/kg in our rabbit model of posttraumatic joint contracture. This result also explains why a dose-response effect was not previously noted in the earlier investigation of KF performed in our laboratory because, although both previous doses studied (0.5 and 1.0 mg/kg) were effective in reducing joint contracture, they fall toward the threshold of the sigmoid curve, and thus minimal difference between the doses was observed[14,15].

Western blot results for α-SMA (a myofibroblast marker), tryptase (a mast cell marker), and Col I protein levels, and RT-PCR analysis of α-SMA and Col I messenger RNA (mRNA) levels also decreased in the joint capsule in association with increasing doses of KF. However, at the highest KF dose, the inhibition of activated mast cell degranulation reached only 63%, indicating that KF alone will not be able to completely eliminate joint contracture. This is in keeping with research on the anti-anaphylactic effect of KF studied in a guinea pig model, where a maximum of 70% mortality reduction was observed at the highest dose. The dose-response in the range tested did not produce a plateau effect; however, higher doses in the rabbit model were not considered, as the recommended human dose is approximately 0.03 mg/kg[18].

The results of this study add further evidence that mast cell activation is an important event in the inflammatory pathway and the genesis of joint capsule fibrosis after traumatic injury. This is the second time that we have observed an association between KF and a decrease in contracture severity in separate experiments, using the same animal model and

testing methods, supporting reproducibility[14]. Notably, at the highest KF dose, the inhibition of activated mast cell degranulation is incomplete, indicating that KF alone will not be able to reduce the incidence of joint contracture entirely. These results provide additional support for the myofibroblast-mast cell-neuropeptide axis of fibrosis, validating the need for further research involving this axis of fibrosis to develop other alternative treatments for posttraumatic joint contracture.

A potential limitation of the current study is that the animals in the operative control group did not receive placebo subcutaneous injections. Although the potential confounding effect of stress due to injection has not be quantified, the potential for having introduced a bias between the control and experimental groups may exist. We are also unable to comment on the effect of KF on joint cartilage, as this was not evaluated. Skeletal maturity was not verified in this study, but the animal supplier guarantees skeletal maturity, and the weights of the animals in this study were in accordance with skeletally mature animals.

In conclusion, the use of the mast cell stabilizer KF was associated with a reduction in the biomechanical and molecular manifestations of joint-capsule fibrosis in a rabbit model of posttraumatic joint contracture, and a threshold dose-response relationship was established, with an EC_{50} of 0.22 mg/kg. These results provide additional support for the myofibroblast-mast cell-neuropeptide axis of fibrosis in the development of posttraumatic joint contracture. To our knowledge, KF is the first and only agent ready to be potentially translated into an effective treatment for posttraumatic joint contracture. The use of KF in humans for more than 40 years, such as for chronic asthma, established a wide safety profile and demonstrated its effectiveness as an oral preparation with a relatively low cost. The findings of this study will continue to shape the safe and effective use of KF in the acute peri-articular fracture period as we continue to bridge the gap from basic science investigations to clinical use. ∎

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References

1. Morrey BF. The posttraumatic stiff elbow. Clin Orthop Relat Res. 2005 Feb;431: 26-35.

2. Bonutti PM, Windau JE, Ables BA, Miller BG. Static progressive stretch to reestablish elbow range of motion. Clin Orthop Relat Res. 1994 Jun;303:128-34.

3. Doornberg JN, Ring D, Jupiter JB. Static progressive splinting for posttraumatic elbow stiffness. J Orthop Trauma. 2006 Jul;20(6):400-4.

4. Lindenhovius AL, Doornberg JN, Brouwer KM, Jupiter JB, Mudgal CS, Ring D. A prospective randomized controlled trial of dynamic versus static progressive elbow splinting for posttraumatic elbow stiffness. J Bone Joint Surg Am. 2012 Apr 18; 94(8):694-700.

5. Doornberg JN, van Duijn PJ, Linzel D, Ring DC, Zurakowski D, Marti RK, Kloen P. Surgical treatment of intra-articular fractures of the distal part of the humerus. Functional outcome after twelve to thirty years. J Bone Joint Surg Am. 2007 Jul; 89(7):1524-32.

6. Myden C, Hildebrand K. Elbow joint contracture after traumatic injury. J Shoulder Elbow Surg. 2011 Jan;20(1):39-44. Epub 2010 Nov 3.

7. Forthman C, Henket M, Ring DC. Elbow dislocation with intra-articular fracture: the results of operative treatment without repair of the medial collateral ligament. J Hand Surg Am. 2007 Oct;32(8):1200-9.

8. Jupiter JB, O'Driscoll SW, Cohen MS. The assessment and management of the stiff elbow. Instr Course Lect. 2003;52:93-111.

9. Lindenhovius ALC, Doornberg JN, Ring D, Jupiter JB. Health status after open elbow contracture release. J Bone Joint Surg Am. 2010 Sep 15;92(12):2187-95.

10. Charalambous CP, Morrey BF. Posttraumatic elbow stiffness. J Bone Joint Surg Am. 2012 Aug 1;94(15):1428-37.

11. Monument MJ, Hart DA, Salo PT, Befus AD, Hildebrand KA. Posttraumatic elbow contractures: targeting neuroinflammatory fibrogenic mechanisms. J Orthop Sci. 2013 Nov;18(6):869-77. Epub 2013 Sep 5.

12. Hildebrand KA, Zhang M, van Snellenberg W, King GJW, Hart DA. Myofibroblast numbers are elevated in human elbow capsules after trauma. Clin Orthop Relat Res. 2004 Feb;419:189-97.

13. 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 Oct;26(10):1313-9.

14. 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 Jun;92(6):1468-77.

15. 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 posttraumatic joint contractures. Inflamm Res. 2012 Apr;61(4):285-92. Epub 2011 Dec 16.

16. Chiang CH, Chen JL, Liu YT, Wang DP. Therapeutic effect and pharmacokinetics of ketotifen transdermal delivery system. Drug Dev Ind Pharm. 1998 Mar;24(3):213-7.

17. Hildebrand KA, Holmberg M, Shrive N. A new method to measure post-traumatic joint contractures in the rabbit knee. J Biomech Eng. 2003 Dec;125(6):887-92.

18. Schwarzer G, Bassler D, Mitra A, Ducharme FM, Forster J. Ketotifen alone or as additional medication for long-term control of asthma and wheeze in children. Cochrane Database Syst Rev. 2004;1:CD001384.