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 (EC50) for KF of 0.22 mg/kg was established. There was also a decrease in myofibroblasts, mast cells, and substance P-containing nerve fibers 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.

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Decline in motion and function despite surgical intervention or nonoperative treatment.

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. 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. Similarly, in a prior rabbit in vivo model of posttraumatic joint contracture evaluated 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. 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 medication for asthma. Prior research by our group showed that, in a rabbit model, treatment with KF significantly reduced the 

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

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

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.
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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 \times 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...
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, 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. A double-labeling IHC methodology that has previously been described was used to quantify myofibroblast and mast cell numbers in joint capsule preparations. 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. 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 (EC₅₀) 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 α = 0.05 (SPSS version 23; IBM).

Fig. 4 Ketotifen fumarate dose-response curve for flexion contracture, demonstrating a relationship with a threshold response, with a half-maximal effective concentration (EC₅₀) of 0.22 mg/kg.

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).
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 (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).
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 ketotifen fumarate (KF) and decreasing levels of α-SMA and Col I. RT-PCR data are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**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 EC50 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.

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. 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.

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. 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 been 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 EC₅₀ 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 post-traumatic 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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