The Dose-Response Effect of Ketotifen Fumarate on Substance P-Containing Nerves, Mast Cells, and Myofibroblasts in Posttraumatic Joint Contractures

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Purpose: Posttraumatic joint contracture (PTJC) is a debilitating complication following intra-articular injury. Prior research has shown that treatment with ketotifen can significantly reduce PTJC severity in a rabbit model. Prior to clinical testing, knowledge of the dose-response relationship is required. We hypothesize that there will be a dose-response effect between ketotifen and PTJC severity and measures of fibrosis.

Methods: After obtaining IRB approval, an in vivo model of PTJC of the knee was created, using a combination of intra-articular injury and internal immobilization in skeletally mature New Zealand White rabbits. Five groups of animals were studied (n = 10 per group): a nonoperative control group (Non-OP), a group with the operatively created PTJC and no pharmacological treatment (operative contracture group - OP), and 3 groups with the operatively created PTJC treated with a mast cell stabilizer, ketotifen fumarate, at doses of 0.01 mg/kg (KF 0.01), 0.1 mg/kg (KF 0.1), and 5.0 mg/kg (KF 5.0) injected subcutaneously twice daily for 8 weeks. After 8 weeks of immobilization, PTJC was measured using a hydraulic materials testing machine. The posterior knee joint capsules were then harvested for immunohistochemistry (IHC), Western blot gel electrophoresis, and reverse transcription-polymerase chain reaction (RT-PCR) quantification of α-smooth muscle actin (SMA), collagen type 1 (Col 1), and mast cell tryptase. The Western blot and RT-PCR levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Triple label IHC combined with DAPI nuclear labeling was also completed and cell counts for myofibroblasts (MFs), mast cells (MCs), and Substance P (SP) were calculated as a percentage relative to the total cell count. Statistical analysis consisted of a one-way analysis of variance (ANOVA) with Tukey's post hoc analysis. Statistical significance was $P < 0.05$.

Results: Five rabbits were excluded due to hardware failure or patellar subluxation. Relative to the Non-OP, the OP group had an average flexion contracture of 39° ± 10°, while contracture severity was reduced to 34° ± 7° ($P = 0.32$), 21° ± 12° ($P = 0.016$) and 15° ± 11° ($P = 0.001$) in the KF 0.01, KF 0.1, and KF 5.0 ketotifen groups, respectively. Using IHC analysis, there was a decrease in MFs, MCs, and SP nerve fiber counts with increasing doses of ketotifen (Fig. 1). Expressed as a percentage of total cells, there were statistically significant differences in MF, MC, and SP values between the OP group and the KF 0.1 and KF 5.0 groups ($P < 0.05$). There were no significant differences between the Non-OP and the KF 5.0 groups; KF 0.1 and KF 5.0 groups; and the OP and KF 0.01 groups, for MFs, MCs, and SP ($P > 0.05$). The Western blot gel showed a dose-response effect of ketotifen on SMA, Col 1, and tryptase levels. The trend was for increasing doses of ketotifen to be associated with

decreasing levels of all three molecules, the differences statistically different between the OP and the KF 5.0 groups. The Non-OP group was statistically different from the OP group while there was no statistically significant difference between the Non-OP and KF 5.0 groups. The RT-PCR analysis for SMA and Col 1 followed a similar pattern as the Western blot. We did not analyze tryptase mRNA levels, as there is no rabbit specific tryptase PCR primer.

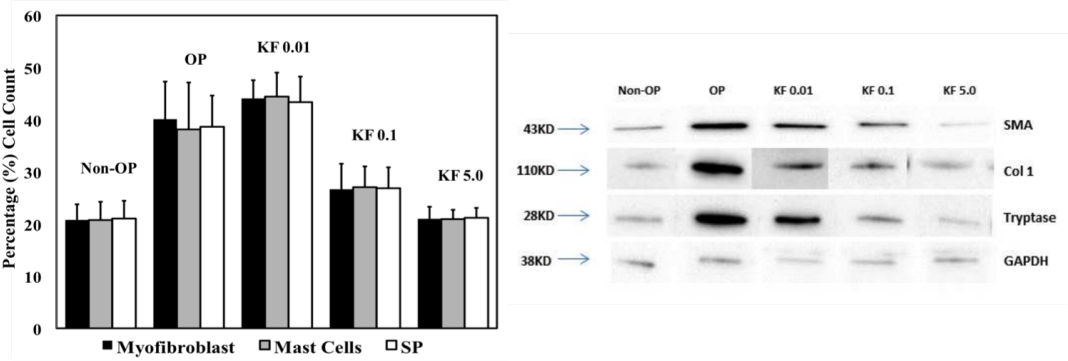

Figure 1: Immunohistochemistry (left) for cell counts for myofibroblasts, mast cells, and Substance P (SP) cell counts, calculated as a percentage relative to the total cell count; and Western blot gel electrophoresis (right) quantification of α-Smooth Muscle Actin (SMA), Collagen type 1 (Col 1), and mast cell tryptase, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Conclusion: Using the rabbit in vivo preclinical model of PTJC, a dose-response of ketotifen treatment was observed. Increasing doses of ketotifen were associated with decreasing biomechanical estimates of PTJC coupled with decreasing numbers of MFs, MCs, and SP containing nerve fibers. Western blot analysis of SMA (myofibroblast marker), tryptase (mast cell marker), and Col 1 protein levels, and RT-PCR analysis of SMA (myofibroblast marker) and Col 1 mRNA levels also decreased with increasing doses of ketotifen. PTJC severity reduced 63% while the IHC, Western blot, and RT-PCR levels were similar to Non-OP controls at the highest dose of ketotifen. A threshold response EC50 ketotifen dose of 0.22 mg/kg was calculated, which has not been previously shown across a narrow range of ketotifen doses.

See pages 49 - 106 for financial disclosure information.