

# An in vivo rabbit joint injury model to measure trauma-induced coagulopathy and the effect of timing of administration of ketotifen fumarate on posttraumatic joint contracture

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## Abstract

**Objectives:** Using a rabbit in vivo joint injury model, the primary objective of the study was to determine if a relationship exists between earlier time to initiation of ketotifen fumarate (KF) treatment and posttraumatic joint contracture (PTJC) reduction. The secondary objective was to determine if a coagulation response could be detected with serial thrombelastography (TEG) analysis following acute trauma in this model.

**Methods:** PTJC of the knee were created in 25 skeletally mature, New Zealand White rabbits. Five groups of 5 animals were studied: a control group that received twice daily subcutaneous injections of normal saline and 4 treatment groups that received twice daily subcutaneous injections of KF (0.5 mg/kg) starting immediately, 1-, 2-, and 4-weeks post-injury. After 8 weeks of immobilization, flexion contractures were measured biomechanically. Serial TEG analysis was performed on the control group animals pre-injury and weekly post-injury.

**Results:** The average joint contracture in the Control Group ( $43.1^\circ \pm 16.2^\circ$ ) was higher than all KF treatment groups; however, the differences were not statistically significant. The average joint contracture was lowest in the 2-week post-injury treatment group ( $29.4^\circ \pm 12.1^\circ$ ), although not statistically significant compared to the other treatment groups. Serial TEG analysis demonstrated significantly higher mean maximal amplitude (maximal amplitude =  $68.9 \pm 1.7$  mm;  $P < .001$ ), alpha-angle ( $81.9^\circ \pm 0.9^\circ$ ;  $P < .001$ ), and coagulation index ( $4.5 \pm 0.3$ ;  $P < .001$ ) 1-week post-injury, which normalized to pre-injury values by 5-weeks post-injury.

**Conclusions:** The use of the mast cell stabilizer KF within 2 weeks of injury demonstrated a nonsignificant trend towards reducing joint contracture in a rabbit in vivo model of PTJC. TEG and the in vivo rabbit joint injury model may be valuable in future preclinical studies of venous thromboembolism prevention and furthering our understanding of the pathophysiology of posttraumatic hypercoagulability.

**Keywords:** joint contracture, ketotifen fumarate, mast cell stabilizer, posttraumatic, range of motion

## 1. Introduction

Posttraumatic joint contractures (PTJC) limit function and the ability to perform activities of daily living secondary to the loss of

motion.<sup>[1]</sup> The elbow joint is particularly prone to PTJC secondary to its highly congruent bony morphology and confined joint space provided by the capsular, ligamentous, and muscular stabilizers.<sup>[2]</sup> Up to 15% of patients with PTJC of the elbow require contracture release or excision of the joint capsule due to functional deficits.<sup>[3,4]</sup> Despite surgical contracture release, 10% of patients undergoing secondary operations to improve motion experience recurrence or contracture progression that continues to limit function.<sup>[5,6]</sup>

Our research group has hypothesized that joint capsule fibrosis is caused in part by activation of a mast cell-mediated pathway.<sup>[7]</sup> Both human and animal studies have shown that elbow range of motion following injury is inversely proportional to the myofibroblast and mast cell number within the joint capsule acutely, and the amount of fibrous collagen tissue present chronically.<sup>[8–10]</sup> Ketotifen fumarate (KF) is a mast cell stabilizer currently approved by the US Food and Drug Administration and Health Canada for the treatment of asthma. Using a rabbit in vivo PTJC model, our prior research has demonstrated that KF lessens contracture severity as well as myofibroblast and mast cell numbers.<sup>[8,11,12]</sup> Furthermore, increasing doses of KF were associated with decreased PTJC.<sup>[11]</sup> However, it is currently unknown how soon after injury treatment with KF should begin,

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especially given that some patients have a delay in presentation to an orthopedic surgeon after a periarticular elbow injury.

In addition, patients are at increased risk for venous thromboembolic events (VTE) following musculoskeletal trauma due to the insults of fracture, surgery, and reduced mobility.<sup>[13,14]</sup> Thromboelastography (TEG) is a whole blood assay capable of providing real-time assessment of individual coagulation states.<sup>[15]</sup> An elevated maximal amplitude (MA), a measure of clot strength, has been shown to be predictive of VTE.<sup>[16–18]</sup> Furthermore, TEG can detect anticoagulant type and dose-dependent changes in coagulation.<sup>[19,20]</sup> We hypothesized that our previously established rabbit in vivo PTJC model may be ideal to assess the coagulation response following acute trauma.

The primary objective of the study was to determine if earlier time to initiation of KF treatment following injury would have a greater reduction in joint contracture. The secondary objective was to determine if a coagulation response could be detected with serial TEG analysis following acute trauma using our rabbit in vivo knee joint injury model.

## 2. Materials and methods

Before study initiation, local animal care committee approval was obtained (AC17-0197), and all animals underwent a 2-week acclimatization period. In total, 25 skeletally mature New Zealand White rabbits (mean preoperative weight of 3.33 kg) underwent an in vivo PTJC of the knee using a combination of intra-articular injury and internal immobilization.

### 2.1. PTJC model

Using a previously established PTJC model, surgical joint interventions were performed under inhalational general anesthesia on either the right or left knee, which was randomly selected before surgery.<sup>[8,11,21]</sup> A lateral thigh incision was made and the lateral aspect of the distal femur was exposed utilizing the intermuscular interval between the quadriceps and hamstring musculature. Medial and lateral parapatellar arthrotomies were made to expose the femoral condyles. Care was taken during the arthrotomies to avoid iatrogenic injury to the collateral ligaments and articular cartilage. Through the parapatellar arthrotomies, 5-mm<sup>2</sup> cortical windows from the extra-articular portion of the

medial and lateral femoral condyles were removed to create a traumatic intra-articular hemarthrosis while maintaining joint integrity.

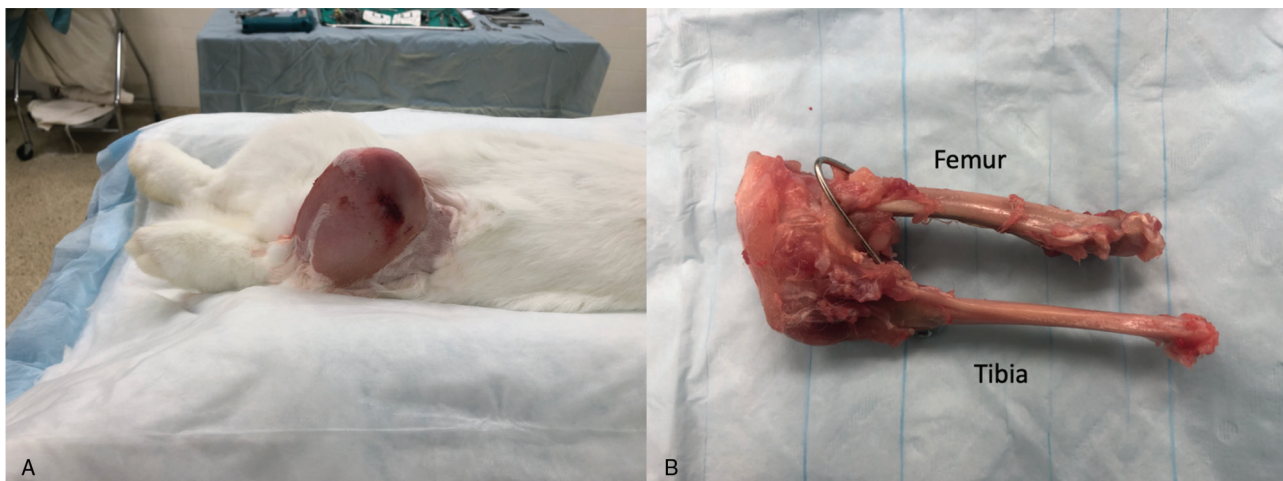
To immobilize the knee, a separate incision was made 3 cm distal to the tibial tubercle and a transosseous 1.6-mm diameter Kirschner wire (Zimmer Biomet, Mississauga, Ontario) was placed through the tibia from anterior to posterior, passed behind the knee and bent around the femur (Fig. 1). All knees were immobilized at 150° of flexion. For 3 days postoperatively, a broad-spectrum antibiotic and morphine derivative were administered. All rabbits were allowed free cage activity within their individual cages (0.1 m<sup>3</sup>).

### 2.2. Study arms

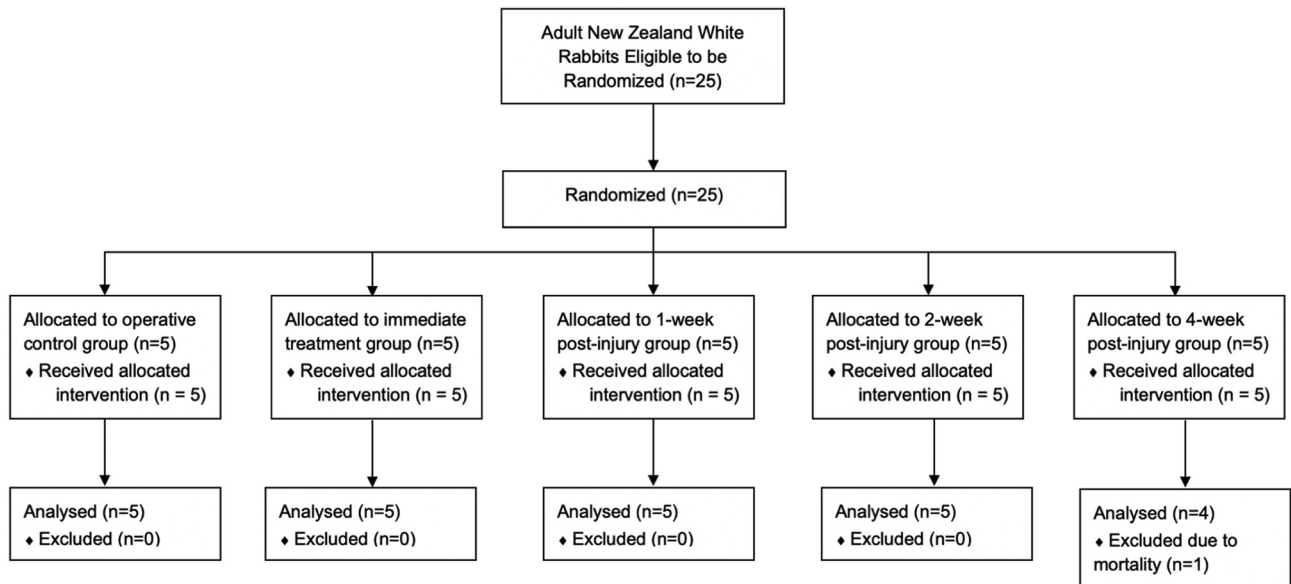
The animals were randomly assigned to 1 of 5 study arms (n=5 per group) (Fig. 2). The first arm (Control Group) consisted of animals that received the intra-articular injury and 8 weeks of internal immobilization with twice daily placebo injections of normal saline. The animals in the 4 study arms underwent identical surgical interventions as the control group followed by the 8-week immobilization period. Additionally, the 4 study arm animals received twice daily subcutaneous injections of KF (0.5 mg/kg) starting at different timepoints postoperatively. The “Immediate Treatment Group” started receiving KF treatment on post-operative day (POD) 1 for a total of 8 weeks. The “1-week Post-injury Group” started receiving KF treatment on POD 7 for a total of 7 weeks. The “2-week Post-injury Group” started receiving KF treatment on POD 14 for a total of 6 weeks. The “4-week Post-injury Group” started receiving KF treatment on POD 28 for a total of 4 weeks. In study arms with delayed initiation of KF treatment, the animals received twice daily injections of normal saline similar to the Control Group until KF treatment initiation. All subcutaneous injections were administered between the upper back areas as per our prior research.<sup>[8,11,12]</sup> Each animal was inspected twice daily by veterinary technicians to ensure no local adverse reactions from the injections.

### 2.3. PTJC testing

Following 8 weeks of immobilization, animals were sacrificed using a barbiturate overdose. Joint range of motion testing was



**Figure 1.** (A) Postoperative image demonstrating lateral thigh incision and knee immobilized at 150° of knee flexion. (B) Postharvest image of rabbit leg with intact knee joint capsule. The 1.6-mm diameter Kirschner wire was placed through the tibia and bent around the femur at time of surgery.



**Figure 2.** CONSORT (Consolidated Standards of Reporting Trials) diagram demonstrating the number of animals randomized to each study arm and the final number of animals analyzed in each arm.

performed on the same day as the sacrifice using a custom rabbit-knee-gripping device.<sup>[8,11,21]</sup> As per prior research, the axis of rotation for testing was centered on the femoral insertion of the medial collateral ligament. The custom device with the rabbit leg was secured to a hydraulic material-testing machine (MTS, Eden Prairie, Minnesota). Beginning with the knee joint at 90° of flexion, a standardized extension torque of 0.2 Nm was applied for 5 cycles, with the values of each cycle averaged (Fig. 3). Extension was recorded as a number between 0° and 90°, with 0° representing full extension and negative numbers representing hyperextension.

**2.4. Trauma-induced coagulopathy testing**

Whole blood specimens were collected from the 5 control animals which did not receive ketotifen treatment for TEG analysis. Venous blood was collected by direct venipuncture from the marginal ear vein into a 2.7 mL vacutainer blood collection tube containing 0.109M, 3.2% buffered sodium citrate (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Serial TEG analysis (TEG6s Hemostasis Analyzer, Haemonetics, Braintree, Massachusetts) was completed after the acclimatization period and before surgery (baseline), then weekly until normalization of TEG values to baseline. The TEG parameters alpha-angle and MA are measures of fibrin cross-linking as the clot strengthens and maximal clot strength respectively. Coagulation index (CI) is a formula combining all TEG parameters for an overall assessment of coagulation. A MA > 65 mm was used to define hypercoagulability, as per prior human studies.<sup>[16,17]</sup>

**2.5. Statistical analysis**

Results were summarized using means with standard deviation. Comparison of contracture severity between the control group and study groups were analyzed using Mann–Whitney *U* test. Serial TEG results were compared to baseline using paired *t* tests and MA was compared to the >65 mm threshold using a 1

sample *t* test. Significance for all tests was defined as *P* < .05. The statistical software R version 3.5.1 (R Foundations for Statistical Computing) was used for analysis.



**Figure 3.** Joint contracture measurement setup demonstrating the custom rabbit-knee-gripping device placed within the MTS. The femoral insertion of the medial collateral ligament representing the axis of rotation is placed under the plumb bob.

### 3. Results

Over the course of the 8-week experiment, rabbits in all 4 study groups tolerated the ketotifen treatment without any local or systemic adverse reactions. 2 animals were excluded from the measurement of PTJC. One rabbit (4-week Post-injury Group) was excluded from the study due to intraoperative death. A second rabbit (Control Group) was excluded due to inadvertent disruption of the contracture during dissection and the resulting contracture angle was greater than 1 standard deviation from the control group mean. The rabbit remained in the study of trauma-induced coagulopathy, as the contracture was maintained during the immobilization period. There were no failures of hardware during the study period.

#### 3.1. PTJC testing

The average flexion contracture in the control group was  $43.1^\circ \pm 16.2^\circ$ . Relative to the control group, the average flexion contracture angles in the treatment groups were reduced; however, the differences observed were not statistically significant (Fig. 4). Based on numerical differences, the average flexion contracture was lowest in the 2-week Post-injury Group ( $29.4^\circ \pm 12.1^\circ$ ), followed by the 1-week Post-injury Group ( $33.9^\circ \pm 9.3^\circ$ ). The average flexion contracture angles were higher in the Immediate Treatment Group ( $37.9^\circ \pm 7.6^\circ$ ) and 4-week Post-injury Group ( $37.8^\circ \pm 5.3^\circ$ ); however, remained below the average flexion contracture of the Control Group, although not statistically significant.

#### 3.2. Trauma-induced coagulopathy testing

The 5 control animals were included in the assessment of trauma-induced coagulopathy following knee joint injury. All animals demonstrated normal baseline MA (mean MA =  $59.6 \pm 2.4$  mm). One-week post-injury, the mean MA of the group was significantly higher than the MA > 65 mm hypercoagulable threshold (mean MA =  $68.9 \pm 1.7$  mm;  $P = .007$ ). Additionally, the mean MA of the

group 1-week post-injury was significantly higher than the baseline value (mean MA =  $59.6 \pm 2.4$  mm;  $P < .001$ ). By 5-weeks post-injury, the mean MA of the group had returned to baseline values (mean MA =  $60.3 \pm 7.6$  mm) (Fig. 5).

Similarly, the mean alpha-angle ( $81.9^\circ \pm 0.9^\circ$ ) and CI ( $4.5 \pm 0.3$ ) of the group were highest 1-week post-injury. These values were also significantly higher than their respective baseline values (mean alpha-angle  $76.0^\circ \pm 1.4^\circ$ ,  $P < .001$ ; mean CI  $3.1 \pm 0.4$ ,  $P < .001$ ). Similar to the mean MA, the mean alpha-angle ( $76.4^\circ \pm 3.5^\circ$ ) and mean CI ( $3.2 \pm 1.2$ ) returned to pre-injury values by 5 weeks postoperatively.

### 4. Discussion

In this preclinical study, we observed a nonsignificant trend towards lower mean flexion joint contracture in animals who started KF treatment within 2-weeks of injury, but not if initiation of KF treatment was delayed until 4-weeks after injury. We were unable to demonstrate a statistically significant improvement in PTJC with earlier initiation of KF treatment.

Using serial TEG analysis, posttraumatic hypercoagulability was demonstrated in the control animals. The animals were most hypercoagulable 1-week following injury with a gradual decline to baseline pre-injury TEG parameters by 5-weeks post-injury.

From the results of our previous preclinical studies, we certainly expected to observe a time-sensitive response to KF treatment. In a study by Hildebrand et al,<sup>[10]</sup> the number of myofibroblasts, mast cells, and neuropeptide containing nerve fibers in joint contracture capsule tissue was found to be maximized at 4 weeks post injury. Later studies demonstrated a significant reduction in PTJC and myofibroblasts, mast cells, tryptase, and neuropeptide containing nerve levels following 8 weeks of immobilization with immediate KF treatment.<sup>[8,11,22]</sup> There are several potential explanations for this discrepancy. First, in previous studies using an in vivo rabbit model, the average flexion contracture of the control group was found to be between  $56^\circ$  and  $58^\circ$ . This is much higher than the average flexion

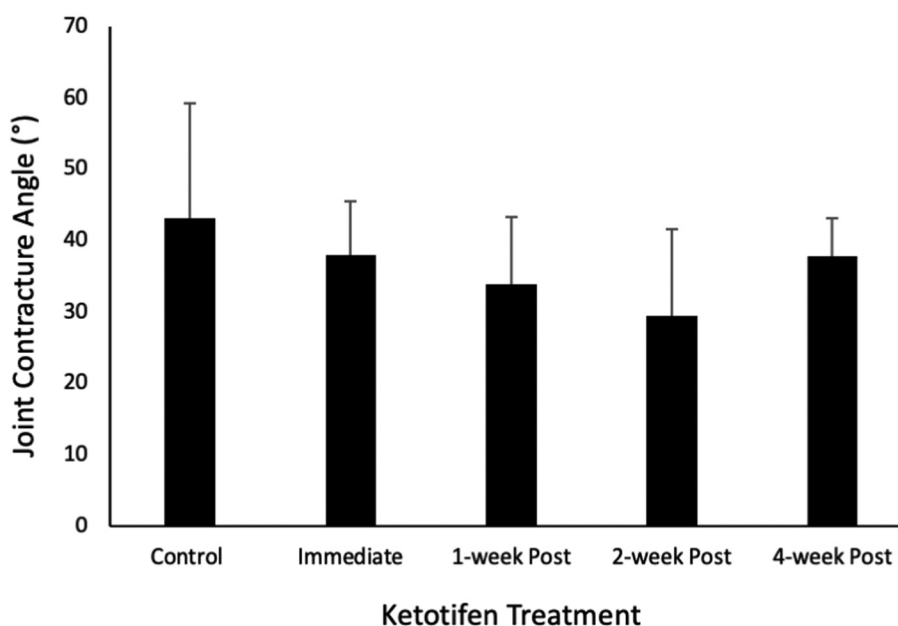
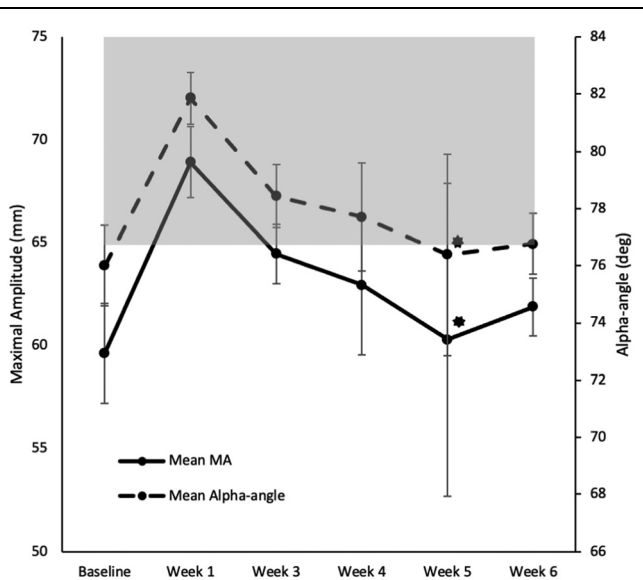


Figure 4. Mean and standard deviation of posttraumatic flexion contracture angles in the control and four treatment groups.



**Figure 5.** Serial mean and standard deviation of maximal amplitude (MA) and alpha-angle. Shaded area represents MA > 65 (hypercoagulable). \* Represents MA and alpha-angle return to baseline values. Week 2 data removed due to highly variable results.

contracture of 39° measured in our control group. Some of the flexion contracture may have been inadvertently released during dissection of the rabbit leg or during manipulation when placing the limb on the MTS before biomechanical testing. Additionally, the mean animal weight was approximately 2 kg lower compared to the animals used in the study by Schneider et al.<sup>[11]</sup> Although skeletal maturity was guaranteed by the animal supplier, a New Zealand White rabbit weighing 3000g could be between 16 and 30 weeks of age with 28 weeks representing skeletal maturity.<sup>[23]</sup> Skeletal immaturity may have played a role in decreased PTJC development and KF treatment response. Additionally, with a smaller leg, we may not have been able to obtain the same degree of flexion during the initial surgery compared to prior studies. Interestingly, our KF treatment group flexion contracture values were in keeping with previous studies.<sup>[8,11]</sup> We plan to study the joint capsules from this study to quantify myofibroblasts, mast cells, and neuropeptide containing nerve levels in our control and KF treatment groups.

Although animal models have been used to study the effect of antithrombotic agents previously,<sup>[24,25]</sup> to the best of our knowledge, we are not aware of a posttraumatic animal model to study coagulopathy. A posttraumatic animal model may better mimic the human physiologic response to trauma and subsequent hypercoagulable state. Using TEG analysis, posttraumatic hypercoagulability was demonstrated in our in vivo rabbit model. Furthermore, decreased mobility secondary to immobilization of the leg may account for the prolonged hypercoagulable state observed. In both hip fracture and total joint replacement patients, there is a similar TEG MA and CI pattern, to what was observed in this model.<sup>[26–28]</sup> Current thrombosis research in trauma patients does not permit pre-injury TEG analysis. As trauma induced coagulopathy is an individualized response, our in vivo model allows for a pre-injury baseline TEG measurement. Using this animal model, future research involving TEG to study the effects of different antithrombotic agents and early mobility on coagulation, may help in VTE prevention.

A potential limitation of the study was the small sample size. Previous similar experiments used around 10 animals in each treatment arm, double our study size. By increasing the sample size, significant differences in flexion contracture may have been detected. In addition, a KF solution was prepared from tablets dissolved in normal saline, whereas previous experiments used a pre-prepared KF solution, which is no longer available. This may have led to issues with KF dissolution and delivery of KF.

In conclusion, administration of the mast cell stabilizer KF within 2 weeks of injury demonstrated a nonsignificant trend towards decreased PTJC. There is good preclinical support for the use of KF to decrease posttraumatic joint-capsule fibrosis.<sup>[29]</sup> The multicenter randomized clinical trial PrEvention of post-traumatic contractures with Ketotifen 2 study<sup>[30]</sup> is currently in the recruitment phase and we look forward to the clinical results in patients. Finally, TEG and the in vivo rabbit joint injury model may be valuable in future preclinical studies of VTE prevention and furthering our understanding of the pathophysiology of posttraumatic hypercoagulability.

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