

Influence of Manual Therapy on Functional Mobility After Joint Injury in a Rat Model

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Context: Animal models can be used to investigate manual therapy mechanisms, but testing manipulation in animal models is problematic because animals cannot directly report their pain.

Objective: To develop a rat model of inflammatory joint injury to test the efficacy of manual therapy in reducing nociception and restoring function.

Methods: The authors induced acute inflammatory joint injury in rats by injecting carrageenan into the ankle and then measured voluntary running wheel activity in treated and untreated rats. Treatments included manual therapy applied to the ankle and knee of the injured limb and several analgesic medications (eg, morphine, ketorolac, prednisone).

Results: Intra-articular injection of carrageenan to the ankle produced significant swelling (diameter of the ankle increased by 64% after injection; $P=.004$) and a robust reduction in voluntary running wheel activity (running distance reduced by 91% compared with controls; $P<.001$). Injured rats gradually returned to running levels equal to controls over 10 days. Neither manual therapy nor analgesic medications increased running wheel activity relative to untreated rats.

Conclusion: Voluntary running wheel activity appears to be an appropriate functional measure to evaluate the impact of an acute inflammatory joint injury. However, efforts to treat the injury did not restore running relative to untreated rats.

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Evidence from a well-powered clinical trial,^{1,2} as well as a systematic review and a meta-analysis,³ supports the efficacy of osteopathic manual treatment for low back pain.⁴ The mechanisms of action, however, are poorly understood. Animal models are needed to investigate the mechanisms of manual therapies (MTs) such as osteopathic manipulative treatment, but testing manipulation in animal models is problematic because reduction of pain, the endpoint associated with many osteopathic treatments, is a subjective experience that animals cannot describe and that investigators cannot directly measure in animals. Therefore, pain must be assessed indirectly by observing pain-related behavior.

Some researchers have investigated MTs in animal models.⁵⁻⁷ Knott et al⁵ observed that lymphatic pump techniques increase lymphatic flow in dogs. Skyba et al,⁶ Sluka et al,⁷ and Sluka and Wright⁸ found decreased mechanical withdrawal threshold after knee mobilization in a rat model of ankle joint inflammation. Both the dog model⁵ and the rat model⁶⁻⁸ required technical instrumentation, limiting each study's utility.

Angeby-Möller et al⁹ and Buvanendran et al¹⁰ tracked pain-related behavior in rodents by measuring voluntary locomotion and weight bearing. These measures are objective, with easily assessed endpoints that can be measured by means of cages outfitted with photosensors⁹ or pressure-sensitive devices,¹⁰ or by using subjective analyses of video recordings of the rodents.¹¹ Cobos et al¹² used activity wheels to measure locomotion. In the current study, we used activity wheels because they were relatively inexpensive and yielded objective endpoints that were easy to record.

Classes of medications appropriate for treating acute pain include opioids, steroids, and non-steroidal anti-inflammatory drugs (NSAIDs).¹³ In the current study, we examined each of these medications as a potential positive control.

The objective of the current study was to assess the effect of MT on the function of rats with an inflamed joint compared with rats with an inflamed joint that were not treated. We hypothesized that rats with inflammatory joint injury would run less and that MT and analgesic medications would improve running distance compared with that of injured, untreated rats.

Methods

Animals

All experiments were approved by the local Animal Care and Use Committee and were carried out in accordance with guidelines from the National Institutes of Health and from the International Association for the Study of Pain. Male Sprague Dawley rats (250-350 g, Hilltop Lab Animals) were housed on a 12-hour light cycle in polypropylene cages with cedar bedding and were fed 5001 Laboratory Rodent Diet (Purina Mills).

On arriving at the animal facility, rats were allowed to acclimatize 2 days to 2 weeks before being placed in polycarbonate cages that contained activity wheels connected to digital display counters to establish baseline running (Thermo Fisher Scientific). Rats were singly

housed in wheel cages. Opaque barriers were placed between cages to minimize the influence of an adjacent rat's activity on each rat's running behavior. Because virtually all running occurs during the dark cycle,¹⁴ wheel revolutions were recorded daily within 4 hours after the beginning of the light cycle for the convenience of the investigators, as very little running occurred after the end of the dark cycle. Each wheel revolution measured 1 meter.

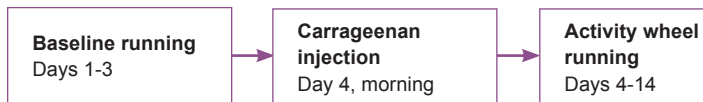
All rats exhibited inherent running behavior. Although all rats increased their daily running over time, rats that ran relatively more continued to outperform other rats, whereas rats that ran relatively less continued to underperform compared with other rats. To control for this inherent behavior, we recorded the initial 3 days of running data and ranked rats by total 3-day baseline running distance to group rats with similar running distances (*Figure 1*). Then we used blocked randomization to assign rats to groups in which blocks were determined by baseline running. The number of rats in each block was equal to the number of treatment groups. For example, to compare the effects of morphine with that of saline, the 2 rats with the lowest baseline running distance were paired, and then the next 2, and so on. One rat in each pair was randomly assigned to receive morphine, and the other rat was assigned to receive saline.

Experimental Design

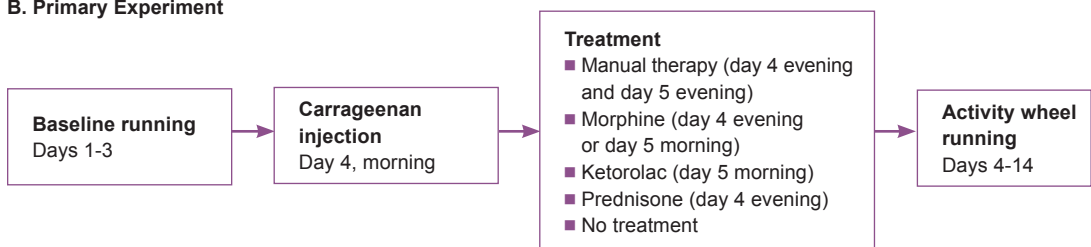
After the 3-day baseline running distance was established for each rat, the ankle joints of some rats were injected with carrageenan (injured) and treated with MT or analgesic medications. Several forms and doses of MT were tested. Of the 3 classes of medications that are appropriate for managing acute pain—opioids, NSAIDs, and steroids—one analgesic medication from each class (morphine, an opioid; ketorolac, an NSAID; and prednisone, a corticosteroid) was selected to test for use as a positive control. After treatment, rat running was monitored for an additional 6 to 10 days.

In the preliminary experiments, rats ran for 3 days to

A. Preliminary Experiment



B. Primary Experiment



C. Primary Experiment With Sedentary Period

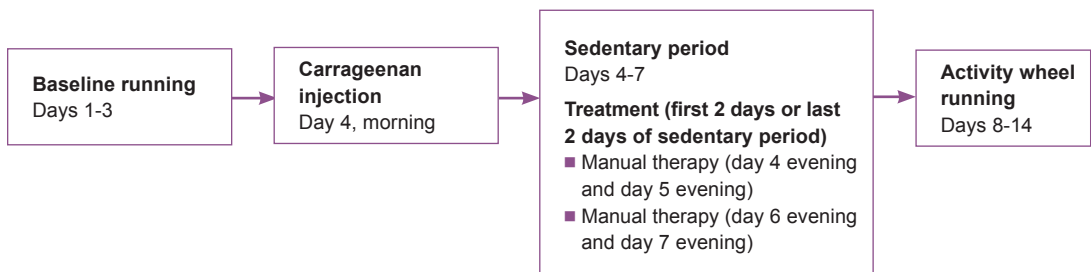


Figure 1.

Overview of experimental designs for (A) preliminary experiments, (B) primary experiments, and (C) primary experiments with an enforced 4-day sedentary period.

collect baseline running data, were injected with carrageenan, and then were monitored for daily running for up to 10 days (*Figure 1A*). Uninjured control rats did not receive carrageenan injection. In the primary experiments, rats ran for 3 days to collect baseline running data, were injected with carrageenan in the morning, were treated with either MT or with an analgesic medication, and were monitored for daily running for up to 10 days (*Figure 1B* and *Figure 1C*). Injured controls received no touch or vehicle. Two sessions of MT were performed, the first on the evening of the injection day and the second on the following evening. Rats were followed for

up to 10 days. Pharmaceutical agents were administered as a single dose. Morphine was administered either the evening of the injection day (ie, early morphine) or the following morning (ie, late morphine). Ketorolac was administered the morning after carrageenan injection. Prednisone was administered the evening of injection day. Rats that were treated with morphine or ketorolac, which do not persist in the body, were followed for up to 24 hours after treatment. Rats treated with prednisone, which is long acting, were followed for 10 days. To control for the potential therapeutic effect of exercise, we conducted 2 experiments with an enforced sedentary

period immediately after injury by immobilizing the wheels. Manual therapy occurred the evenings of either the first or the last 2 days of the 4-day sedentary period, which began on the day of injection. Wheel mobility was restored at the end of the sedentary period so rats could resume running (*Figure 1C*).

Inflammatory Joint Injury

To induce inflammatory injury, rats were anesthetized with 2% to 5% isoflurane mixed with 95% O₂/5% CO₂ and placed in the left lateral recumbent position. With the right foot in neutral position, the junction of the tibia and fibula just superior to the talus was identified. At this location, a 0.5-inch, 27-gauge needle was advanced in a posterior medial direction through the skin and subcutaneous tissue and into the joint capsule. If resistance was encountered, the ankle was dorsiflexed while the needle was advanced into the joint space. After the needle entered the joint space, 0.05 mL of 0.75%, 1.5%, or 3% carrageenan (non-gelling, mixture of κ and λ carrageenans, catalog #C1867, Sigma Aldrich) in 0.9% saline (pH 7.4) or saline alone was injected. In one of the ex-

periments, to create a bilateral injury, the procedure was repeated for the left foot. To standardize intra-articular injections, each investigator was trained to find the articular capsule and perform the injection in less than 30 seconds. The injection site was monitored for bleeding and regurgitation of injection fluid. Intra-articular injections were performed between 6 AM and 8 AM.

Manual Therapy

To determine if MT improved voluntary running behavior after injury to the joint, we applied MT to the knee or the ankle on anesthetized rats under 2% to 5% isoflurane for 2 or 3 repetitions of 1, 2, or 3 minutes, with or without 1 minute of effleurage, with a 1- to 2-minute rest interval between repetitions (*Table 1*). Knee mobilization/translation (*Table 1*, protocols 1-4 and 6) was applied by flexing and extending the ipsilateral knee joint to its end range of extension while the tibia was simultaneously translated (joint surfaces moved parallel to one another) in an anterior-to-posterior direction using grade III and IV mobilization forces, a technique previously shown to influence pain-related behavior in rats with in-

Table 1.
Manual Therapy (MT) Protocols Used to Assess Functional Mobility After Joint Injury in Rats

Protocol	MT	Repetitions, No.	Duration, min	Rest Between Repetitions, min	Total Time, min
1	Knee mobilization/translation	3	3	1	11
2	Knee mobilization/translation	2	3	1	7
3	Knee mobilization/translation	2	2	1	5
4	Knee mobilization/translation; ankle effleurage	2	1; 1	1	5
5	Ankle oscillation; ankle effleurage	2	1; 1	1	5
6	Knee mobilization/translation	2	2	2	6

flamed ankles.⁶ Flexion and extension were performed at a frequency of 20 times per minute. Ankle oscillation (*Table 1*, protocol 5) was applied by anterior to posterior oscillation at a rate of 100 to 120 oscillations per minute with the ankle in a neutral position. Effleurage to the ankle (*Table 1*, protocols 4 and 5) was applied by gentle compression of the tissue in a distal-to-proximal direction above the ankle, across the ankle, and across the foot. Injured control rats were anesthetized for the same period of time and no touch was applied to the hindlimb. An osteopathic physician (E.J.S.) who is board certified in neuromusculoskeletal and osteopathic manipulative medicine trained osteopathic medical students (including B.D.W.) in the manual techniques. Training consisted of 1 session of instruction and practice, followed by at least 1 supervised session. Technique was further observed periodically to minimize inconsistency. Manual therapy was performed the evening after carrageenan injection and again the following evening.

Pharmaceutical Treatments

For treatment using pharmaceutical agents, a single dose of morphine (catalog #M8777, Sigma Aldrich) at 1 mg/kg body weight in approximately 0.25 mL in 0.9% saline was injected into rats subcutaneously the evening after carrageenan injection (early morphine) or the next morning (late morphine). The experiment with evening treatment was included because morphine has a half-life of 6 hours and would possibly be rendered ineffective by night, when rats that were injected in the morning do the majority of their running. Injured controls for the morphine treatment received an injection of 0.25 mL of 0.9% saline.

We injected ketorolac (catalog #K1136, Sigma Aldrich) at 20 mg/kg body weight in 0.3 mL distilled water intraperitoneally the evening after carrageenan injection. Injured control rats for the ketorolac treatment received an intraperitoneal injection of 0.3 mL water alone at the same time.

Prednisone (catalog #1559006, Fisher Scientific), 0.05 mL of 30 mg/mL in ethanol, was injected subcuta-

neously the morning after carrageenan injection. Uninjured controls received no injection because injection alone has no effect on running behavior.

Preliminary Experiments

The 7 preliminary experiments are described in *Table 2* and illustrated in *Figure 1A*. The first preliminary experiment was conducted to assess inherent running variability. Both 3-day baseline running and injection-day running were recorded and correlated to 10-day running after injection. To test the influence of the barriers, data from our pilot studies, which were performed without barriers, were compared with the current experiments, which used barriers. To determine whether injury was caused by the needle (negative control), in 1 experiment rats received either an intra-articular injection of 0.9% saline (pH 7.4) or no injection. To determine the optimal dose required to induce inflammation, rats were injected with 0.05 mL of 0.75%, 1.5%, or 3% carrageenan as described previously.

To assess whether swelling correlated with running, the basic experimental design was used but with no MT or analgesic treatment (*Table 2*). Hindlimb swelling was measured immediately before and 24 hours after carrageenan injection. For consistent measurements, a line was drawn at the edge of the fur on the hindlimb of the lightly anesthetized rat using a nontoxic permanent marker. The limb was then dipped into a plethysmometer (IITC Life Science Inc) until the water level reached the line drawn on the limb, and the volume displacement was recorded. In addition, the anteroposterior diameter at the same location on that hindlimb was measured with a digital caliper (Thermo Fisher Scientific Inc) and recorded.

Primary Experiments

The 5 primary experiments (*Table 3*) were designed to control for numerous variables that could influence the outcomes in the rat model of the current study. The first experiment was to determine the optimal duration

Table 2.
List of Preliminary Experiments Performed on Rats

Experiment	Description
Inherent running variability	Baseline running and injection day running were recorded to determine if data were predictive of cumulative running.
Barriers	Opaque barriers were compared with no barriers to assess effect on running behavior.
Saline or no injection as a negative control	Running behavior was compared after either saline injection or no injection to determine if injury was caused by the needle during injection. Running behavior after carrageenan injection was also compared with running after saline injection or no injection.
Carrageenan dose	The effects of 3 doses of carrageenan on running behavior were tested to determine the optimal carrageenan dose.
Carrageenan and swelling (1 day)	The effect of carrageenan on swelling was recorded the day after injection to determine whether initial swelling correlated with running behavior.
Carrageenan and swelling (1 week)	The effect of carrageenan on swelling was recorded daily after injection for 7 days to determine whether a decrease in swelling correlates with an increase in running behavior.
Analgesic medications	Morphine, ketorolac, and prednisone were evaluated for use as positive controls.

Table 3.
List of Primary Experiments Performed on Rats

Experiment	Description
Duration of manual therapy (MT)	To determine the optimal dose of MT, 2 or 3 repetitions of a 3-minute MT protocol with 1-minute rest periods were compared.
Sedentary Period	An enforced 4-day sedentary period with MT during the first 2 days or the last 2 days of the sedentary period was included to control for the potential therapeutic effect of exercise.
MT with ankle effleurage	Ankle effleurage was performed with knee MT to increase lymph drainage.
Ankle MT	Ankle MT with ankle effleurage was performed to treat the injured joint directly.
Bilateral injury	Both ankles were injected with carrageenan, and MT was applied to both knees to control for the ability of rats to compensate for injury.

(number of repetitions) of MT. Experiments were conducted in which MT was repeated for 2 or 3 cycles on 2 days (*Table 1*, protocols 1 and 2). Next, we conducted 2 experiments with an enforced 4-day sedentary period to control for the potential therapeutic effect of exercise, as there is evidence that exercise improves ankle function

after sprain.¹⁵ Running wheels were immobilized immediately after injury to create a 4-day sedentary period. Wheel mobility was restored at the end of the sedentary period so rats could resume running. We performed MT for 2 minutes with 1 minute of rest for 2 repetitions (*Table 1*, protocol 3) either during the first 2 days or the

last 2 days of the 4-day sedentary period.

Two additional experiments assessed the effect of different MTs. One experiment evaluated the combined use of knee and ankle MTs (*Table 1*, protocol 4), whereas another evaluated ankle MT to address primary hyperalgesia (*Table 1*, protocol 5).

We also studied a bilateral injury model. To treat both hindlimbs efficiently in this model, we modified the MT protocol slightly, extending the rest period from 1 minute to 2 minutes (*Table 1*, protocol 6). Thus, while one hindlimb was being treated for 2 minutes, the other hindlimb was resting.

Statistical Analyses

Spearman correlation coefficients were used to determine whether baseline running or injection day running was more strongly related to 10-day running and whether daily hindlimb swelling was associated with the change in daily running. Analysis of covariance was used to compare the effect of varying doses of carrageenan. The Mann-Whitney test was used to compare baseline running between rats in cages with and without barriers. Wilcoxon signed ranks tests were used for testing evidence of postinjection swelling, and a Friedman test was used to test for change in swelling over time. We used mixed-effects regression models to perform statistical analysis for the repeated measures data. Because the outcome was a non-normally distributed count variable, Box-Cox transformations were implemented prior to model fitting. Model fitting involved evaluating the covariance structure of the data using the small-sample-size corrected version of Akaike information criterion and log likelihood values to assess the model fit. In addition, the transformed baseline running was used as a covariate to control for variability in the daily revolutions due to the variability in baseline running. Final models were used to determine whether carrageenan injection reduced running compared with saline injection or no injection and to compare treatment with uninjured control or with no-touch injured control. Analysis of covari-

ance was also used to test for differences in swelling between treatment and vehicle. SAS 9.3 (SAS Institute Inc) was used for the statistical analyses. We set statistical significance at $P \leq .05$.

Results

Preliminary Experiments

For inherent running variability, 3-day baseline running distance was more strongly related to subsequent running in injured rats ($\rho=0.68, P<.001$) than running distance for the initial postinjection, 24-hour period ($\rho=0.37, P=.006$) (*eFigure 1*). In addition, 24-hour and 48-hour total baseline running distances were strongly related to subsequent running in injured rats (24-hour: $\rho=0.62, P<.001$; 48-hour: $\rho=0.68, P<.001$) (data not shown).

The use of opaque barriers affected running behavior. Rats in cages with opaque barriers ran slightly more (21%) during the 3-day baseline running period than rats that could see their neighbors (mean [standard deviation (SD)] revolutions 3358 [1736] vs 2776 [2719], $P<.05$).

Saline injection and no injection as controls gave the same results; saline did not affect running distance ($P>.30$; *Figure 2A*). Carrageenan injection reduced running (*Figure 2B*). The day after injection, rats injected with carrageenan ran 9% of the distance that uninjured controls (which received saline injection or no injection) ran ($P<.001$). The running of injured rats increased each day relative to uninjured controls. By 4 days after injection, rats reached their pre-injection running distance, and by 5 days after injection they caught up to the running distances of uninjured controls. There was no dose-dependent effect of carrageenan for the 3 doses from 0.75% to 3% carrageenan ($P=.26$; *Figure 2B*). The running behavior of all injected but untreated rats from all of the experiments is shown in *Figure 2C*.

Carrageenan induced visible hindlimb swelling as seen in a 55% increase in volume ($P=.002$) and a 64% increase in diameter ($P=.004$) after 24 hours (*Figure 3*). Hindlimb volume and diameter did not change in no-in-

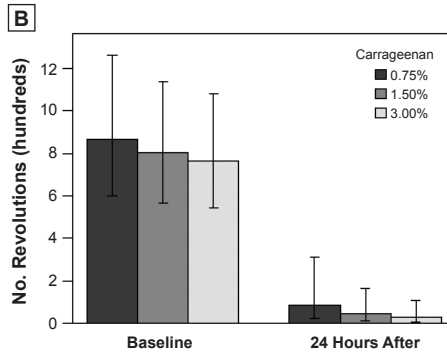
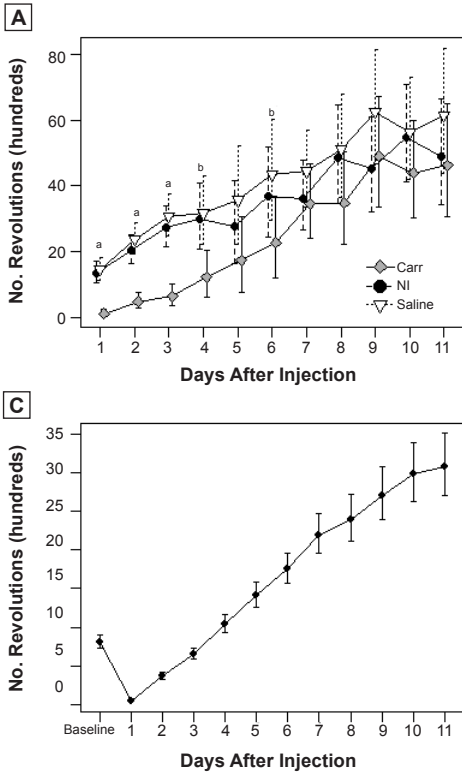


Figure 2. Effect of saline injection, no injection (NI), and carrageenan injection (Carr) on rat running wheel activity over time. All data shown as back-transformed means (95% confidence limits). (A) Activity wheel daily running of rats injected with 3% carrageenan (n=10) and rats injected with saline or not injected (n=11) (^a*P*<.05, ^b*P*<.01; injured vs uninjured, mixed model). (B) Mean running 24 hours after injection with 0.75% (n=11), 1.5% (n=11), or 3% (n=10) carrageenan compared with mean daily running during 72 hours prior to injection (*P*=.26, analysis of covariance). (C) Activity wheel daily running of rats injected with 3% carrageenan (n=196).

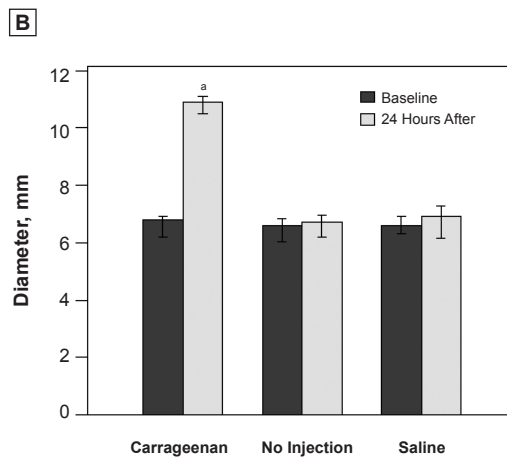
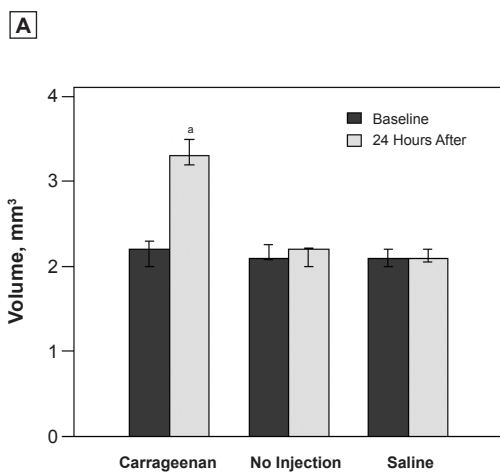


Figure 3. Effect of saline injection, no injection, and carrageenan injection on hindlimb swelling as measured by (A) plethysmometry and (B) calipers. Rats were injected with saline (n=11), not injected (n=11), or injected with 3% carrageenan (n=10). Data are shown as median with 25th and 75th percentiles. ^a*P*<.01, Wilcoxon signed rank test.

jection or saline-injected rats after 24 hours ($P > .31$).

The hindlimb swelling observed 24 hours after carrageenan injection did not correlate with the distance run on that day ($\rho = -0.07$, $P = .68$) (eFigure 2). Also, daily percentage change from baseline in hindlimb swelling (diameter) did not correlate with daily percentage change from baseline in running ($|\rho| < .17$, $P > .37$) (eFigure 3A). Swelling peaked on day 3 when the mean (SD) hindlimb diameter was 59% (1.59 [0.22] mm) greater than baseline measurements (eFigure 3B). On day 6, carrageenan-induced swelling was still discernible, with hindlimb diameter measuring 34% (1.34 [0.21] mm) greater than baseline measurement ($P = .002$).

There were no statistically significant differences for any of the pharmaceutical agents compared with no treatment for running (Table 4 and Table 5) or hindlimb swelling (data not shown).

Primary Experiments

Protocols 1 through 5 did not significantly affect the running behavior of injured rats compared with injured no-touch controls (Figure 4, Figure 5, and Table 1). However, some investigators noted that joint stiffness in rats was reduced after treatment.

Rats with bilateral injuries ran fewer revolutions than rats injured in 1 ankle only (ie, unilaterally), running 2.5% of the distance unilateral injury rats ran the day after injection and gaining up to 48% of the distance run by unilateral injury rats 7 days after injection ($P < .01$). Protocol 6—MT administered to both injured knees of the rats (Table 1)—did not significantly affect the running behavior of injured rats compared with injured no-touch control rats ($P = .47$) (Figure 5C).

Table 4.
Effect of Analgesic Treatments on Activity Wheel Running in Rats^a

Analgesic Medication	Revolutions, mean (CI)		P Value
	Treatment	No Treatment	
Early Morphine (n=7)			
Day 0	21 (6-72)	38 (11-128)	.48
Day 1	426 (264-686)	584 (362-941)	.32
Late Morphine (n=8)			
Day 0	130 (65-262)	136 (68-275)	.92
Day 1	599 (430-836)	672 (482-938)	.61
Day 2	817 (520-1285)	1147 (730-1804)	.27
Ketorolac (n=16)			
Day 0	63 (32-124)	23 (11-45)	.04
Day 1	391 (278-548)	412 (294-578)	.82

^a Carrageenan (3%) was injected at the beginning of Day 0. Treatments occurred on the evening of the day of injection (early morphine, ketorolac), or the morning of the following day (late morphine). Mixed model comparisons are between treatment and control.

Abbreviation: CI, confidence interval.

Comment

In the current study, we intended to identify a successful MT model in rats and to lay the groundwork for subsequent studies that could be performed to delineate underlying therapeutic mechanisms for MT. We established that carrageenan injected into the ankle induced an inflammatory injury in the joint, which reliably compromised activity wheel use. A meta-analysis of clinical trials¹⁶ observed that a variety of MT techniques yielded better results than mobilization alone, so the current study used a variety of MT techniques. In our subjective assessment, we found that MT and ankle effleurage were sufficient to loosen the treated joint whether MT was applied to the proximal joint (the knee) or the injured joint (the ankle); greater flexibility in either joint indicated a physiologic response to the treatment. Clinically, mobilization and range-of-motion exercises reduce pain and recovery time

after ankle sprain.^{17,18} In the current study, however, the effect of MT, no matter the location or duration of treatment, and the effects of analgesic medications on running wheel activity were not apparent.

Our research design and MT protocols directed to the knee were based on studies by Skyba et al,⁶ Sluka et al,⁷ and Sluka and Wright,⁸ which showed that MT to the knee modulated pain behavior secondary to ankle inflammation from injection. Sluka and Wright⁸ observed that knee mobilization 2 hours after capsaicin injection into the ankle increased mechanical withdrawal threshold in the von Frey filament test. This effect was abolished by monoamine blockers but not gamma-aminobutyric acid or opioid blockers, suggesting that mobilization affects central descending serotonergic pathways.⁶ In the current study, we initially used capsaicin at the same and greater concentration levels as

Table 5.
Cumulative Effect of Steroid Treatment
on Activity Wheel Running in Rats^a

Day	Revolutions, mean (CI)		P Value
	Prednisone (n=24)	No Treatment (n=23)	
0	64 (30-136)	39 (18-85)	.36
1	457(282-741)	300 (183-492)	.23
2	687 (517-913)	569 (425-762)	.36
3	1067 (847-1344)	1055 (832-1336)	.94
4	1387 (1127-1708)	1310 (1058-1622)	.70
5	1900 (1505-2400)	1767 (1392-2243)	.66
6	2142 (1739-2638)	2189 (1769-2708)	.88
7	2552 (2058-3166)	2603 (2089-3245)	.90
8	2741 (2163-3472)	2818 (2213-3589)	.87
9	3022 (2426-3763)	3085 (2465-3861)	.90
10	3069 (2379-3960)	3332 (2569-4323)	.65

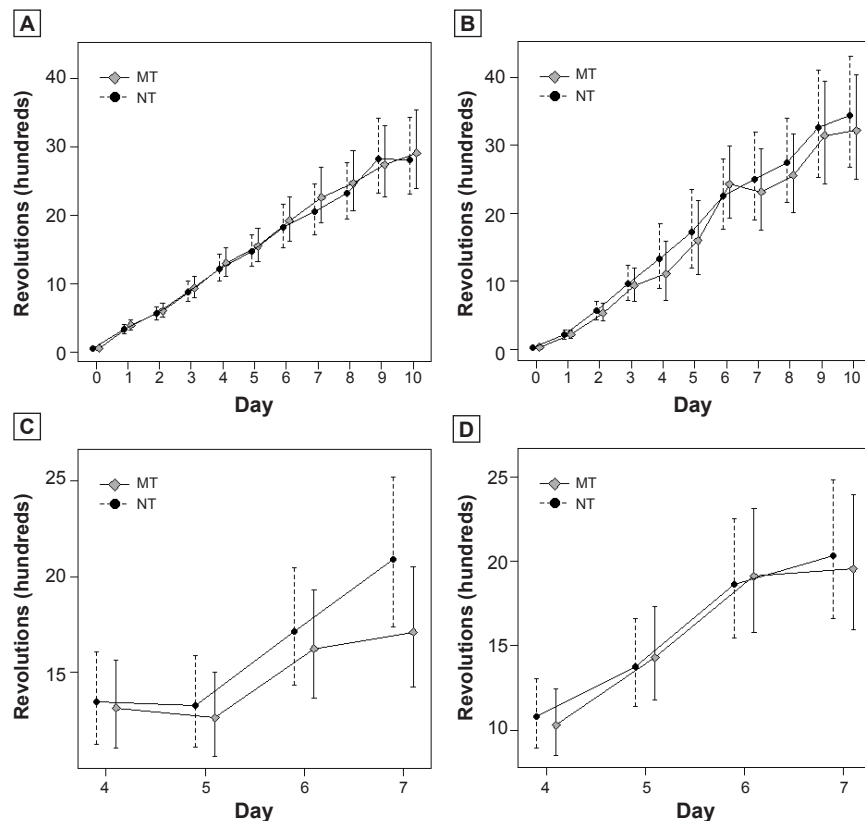
^a Carrageenan (3%) was injected at the beginning of Day 0. Treatment occurred on the morning of the following day. Data shown as back-transformed mean (95% confidence interval [CI]) and P values. Mixed model comparisons are between treatment and control.

noted in the study by Sluka but did not achieve noticeable inflammation. Consequently, we injected carrageenan rather than capsaicin to create a more robust and longer-lasting inflammation. This modification was consistent with the study by Sluka et al,⁷ wherein knee mobilization of the contralateral (but not the ipsilateral) limb increased mechanical withdrawal threshold compared with untreated rats when administered 4 weeks, but not 1 or 2 weeks, after carrageenan was injected into the ankle. Thus, mobilization of either the proximal joint⁸ or the contralateral limb⁷ affected nociception, supporting the role of the central nervous system in mediating the analgesic effect of mobilization.

The von Frey filament test, in which the latency of the rat's reaction to a flexible filament pressed against the hindpaw is observed, is an accepted test of mechanical

allodynia, and its limitations are also widely accepted. The test is sensitive to many variables, including temperature, humidity,¹⁹ and surface material.²⁰ We discontinued initial attempts to use von Frey filaments as an outcome measure because we found the results of the test too subjective. Automated von Frey filament tests address issues with variability and reproducibility,²¹ but such an approach was not available for use in the current studies. While we considered several other outcome measures, we chose to use running wheels that recorded precise measures of daily running distance, which we assumed would produce a more obvious and relevant outcome to body function and would objectively demonstrate the effect of induced injury and analgesic and MT treatments. This decision was supported by the increasing popularity of running wheel activity as an indi-

Figure 4. Effect of different manual therapy (MT) protocols after hindlimb carrageenan injection on rat running wheel activity. (A) Protocol 1: MT of the knee, 3 repetitions of 3 minutes and 1 minute rest (MT, n=38; injured no-treatment control [NT], n=37; $P=.57$). (B) Protocol 2: MT, 2 repetitions of 3 minutes and 1 minute rest (MT, n=16; NT, n=16; $P=.73$). (C) Protocol 3: MT, 2 repetitions of 2 minutes and 1 minute rest applied on the first 2 days (MT, n=24; NT, n=23; $P=.45$) of an enforced sedentary period. (D) Protocol 3: MT, 2 repetitions of 2 minutes and 1 minute rest applied on the final 2 days (lower right, MT, n=23; NT, n=24; $P=.96$) of an enforced sedentary period. Data are shown as back-transformed mean (95% confidence interval). P values reflect mixed-model tests for treatment.



cator of nociception. Voluntary running wheel activity was shown to decrease after carrageenan was injected into the hindpaw¹² and after monosodium iodoacetate was injected into the knee to induce osteoarthritis.²² In the current study, voluntary running wheel activity consistently demonstrated decreased 24-hour running distance after carrageenan was injected into the ankle.

Modeling our methods on those of Sluka and Wright,⁸ we injected carrageenan into the ankle to create inflammatory injury, and we treated the proximal joint. Rats ran very little the first night after carrageenan injection, but on each subsequent night they ran more, catching up to their uninjured counterparts within 5 days. This progression is clinically relevant: after a grade 1 ankle sprain, on average, human patients return to full weight bearing when walking at 6 days after injury, and return to normal pre-injury walking rates after 11 days.²³ In the current study, mobilization to the knee did not affect the course of recovery, and so the MT was modified to include effleurage, a lymphatic drainage technique, which was performed on the injected ankle, an approach more consistent with current osteopathic treatment models. Again, we did not observe a beneficial effect on running behavior. These approaches did not influence the running characteristics of rats with inflamed ankles.

Several factors could explain why there was no improvement in running distances after MT compared with no treatment. Schött et al²⁴ reported that rats shift their weight off the injured limb after carrageenan injection into the ankle. In the current study, voluntary running activity did not increase in injured treated rats compared with injured control rats because rats may have shifted their weight off the injured limb. That is, they compensated for the injured limb by running on their 3 uninjured limbs. We tried to control for this shift in running style by testing MT in a bilateral injury model. However, MT did not improve running in the bilateral injury model.

To confirm that the tested forms of MT were not therapeutically beneficial in this model, injured rats were treated with morphine, ketorolac, and prednisone. None

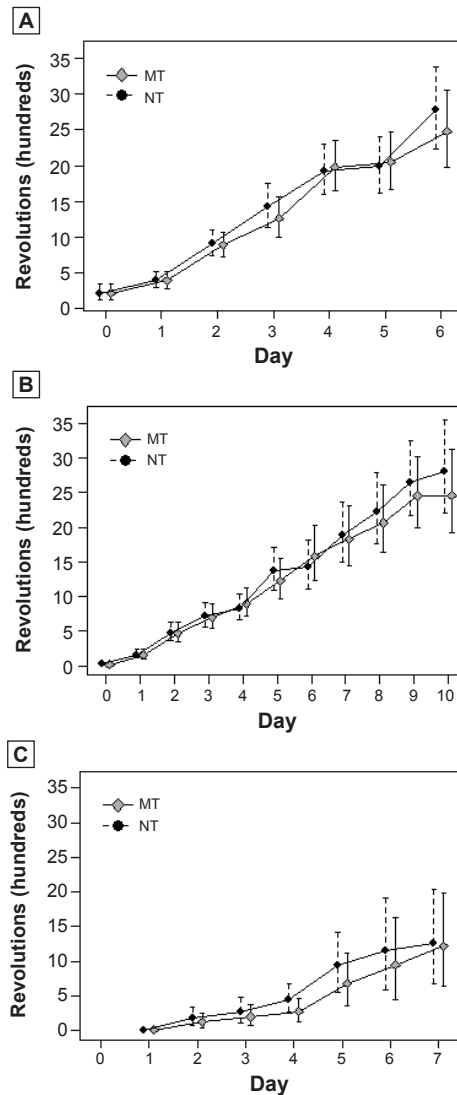


Figure 5.

Effect of carrageenan injection on running wheel activity of rats that received manual therapy (MT). (A) Protocol 4: Two repetitions of 1-minute MT on the knee, 1 minute of ankle effleurage, and 1 minute rest (MT, $n=22$; injured no-treatment control [NT], $n=22$) ($P=.74$). (B) Protocol 5: Ankle MT with 2 repetitions of 1-minute oscillation, 1 minute of ankle effleurage, and 1 minute of rest (MT, $n=23$; NT, $n=24$) ($P=.82$). (C) Protocol 6: Both ankles were injured (bilateral injury model); both knees were treated with 2 repetitions of 2 minutes MT and with 2 minutes of rest between repetitions (MT, $n=8$; NT, $n=8$) ($P=.47$). Data are shown as back-transformed mean (95% confidence interval). P values reflect mixed-model tests for treatment.

of the medications influenced running behavior to a statistically significant level. We did not anticipate this outcome, because previous reports indicate that morphine and ketorolac at similar doses (1 mg/kg and 20 mg/kg body weight, respectively) restored reduced activity in photosensor cages after knee surgery¹⁰ and morphine restored activity wheel running after carrageenan injection into the hindpaw.¹²

A 2012 study of mice¹² showed that several analgesic medications, some of which we used in the current study, were effective in restoring running behavior after an intraplantar injection of complete Freund's adjuvant.¹² This result suggests that running behavior in rodents may be a useful endpoint when the injury is on the surface of the paw. Some studies^{9,10,25} show an effect of analgesics in restoring locomotion after joint injury. Pharmaceutical agents, including morphine, partially reversed the reduction in grip force caused by osteoarthritis induced by sodium monoiodoacetate (ie, monosodium iodoacetate).²⁵ Buvanendran et al¹⁰ reported that morphine or ketorolac fully restored ambulation in rats housed in cages fitted with photosensors to monitor activity after knee surgery. Angeby-Möller⁹ found that morphine partially restored the reduction in weight bearing caused by intra-articular injection of carrageenan into the rat ankle, as measured by the CatWalk method. The results of the current study, on the other hand, showed that, despite an initial decrease in running behavior of rats with joint inflammation, morphine and ketorolac failed to restore running behavior more quickly than no treatment. A more prolonged use of morphine and ketorolac may be necessary to show an effect in this model. Yet, the ineffectiveness of the long-acting steroid remains an unexpected and unexplained outcome.

Throughout this series of preliminary experiments, we were able to show that running wheel activity is an objective functional endpoint that is not affected by inter-investigator variability. Rats grouped by baseline running consistently exhibited reduced running distance after carrageenan injection that correlated inversely with

the amount of swelling observed. The reduction in running was due to the inflammatory agent and not from damage caused by the needle or from the volume injected, because rats injected with saline did not experience a decrease in running. Voluntary running wheel activity required minimal training for both the researchers and the rats and was implemented inexpensively and easily. Treadmill running, an alternative approach, might have reduced variability but would have been more labor intensive.

A source of variability in voluntary running wheel activity is the change in running behavior over time. Rats become acclimated to running wheels, increasing their running over time, and reaching a plateau after about 4 weeks.²⁶ Although rats tend to run more with time, we sought to address the potential variability with the baseline matching protocol because the preliminary data indicated that rats that run less tend to continue to run less compared with others with equal experience over a 2-week period. Our baseline matching protocol could easily be applied to other behavioral animal models, in which baseline performance can be established prior to the experiment. For instance, baseline performance of locomotor activity (treadmill, activity cages), mechanical sensitivity (von Frey filaments, mechanical pressure), and thermosensitivity assays (tail withdrawal, heat withdrawal) could be considered for randomization if such outcomes display similar individual variability as running behavior does. Further, matching individuals on pretreatment criteria is not unprecedented: for example, Hallas et al²⁷ paired animals by initial ankle circumference after inducing inflammation.

We assumed that the opaque barriers between cages would minimize the possible influence of more active rats on their less active neighbors. Unexpectedly, rats with barriers were more active. This finding may have been attributable to other factors, such as a rat's ability to detect activity of neighboring rats through different senses, such as smell, sound, and touch (eg, vibration). Ideally, rats would be fully separated to eliminate the ef-

fect of social interactions on behavior, but that was not logistically possible for this study. Rats are extremely social animals, so the sight of neighbors may have had a calming effect, resulting in less running, or may have distracted them from running. Social interaction influences circadian rhythms,²⁸ but, to our knowledge, we are the first investigators to report that opaque barriers were associated with increased activity.

The MT techniques we developed for the rat knee and ankle were easily taught to second-year osteopathic medical students. Involving students increased the number of experiments we could perform and had the benefit of exposing students to osteopathic medical research. One disadvantage of multiple investigators, however, was the possibility of variability, but we tried to control for this by observing students' techniques throughout the experiment to minimize inconsistency. Investigator bias was not observed to impact running distance.

A limitation of our study is that joint stiffness, the feedback that we used to modify MT technique, was not quantifiable. Every investigator independently noted that the joint began to loosen after 2 minutes of treatment and that the treated joints were less stiff immediately after treatment and 24 hours after the first treatment, compared with injured joints that did not receive treatment. For this reason, it was helpful to have multiple investigators participate in performing MT. Unfortunately, we were unable to find a reliable, repeatable method to measure joint stiffness. We initially tried to quantify joint stiffness by means of a force transducer or with weights that straighten the limb,²⁹ but we were unsuccessful. Because the quantifiable endpoint we used—running wheel activity—was not influenced by any of the treatments we tested, a valid, meaningful, and quantifiable endpoint must be found before further studies can be undertaken using this animal model.

Conclusion

Running wheel activity is a desirable endpoint because it is a functional measurement with obvious human relevance and is not affected by inter-investigator variability. The robust reduction in running elicited by carrageenan injection that we observed is promising for studies of joint inflammation. Although none of the treatments, including established analgesic medications, attenuated the carrageenan-induced reduction in running or accelerated recovery, refining the approach to determine the effect of the interventions on joint inflammation may be possible. Further, the impact of these interventions may only be seen at a molecular level as subtle changes in the levels of endorphins or other biochemical markers. Biochemical changes may provide relief from pain that we could not quantify in the rats' running behavior. Investigations at the level of gene expression may provide some insight about changes in rats with inflammation and rats that also received MT. We are currently investigating the effect of MT at the molecular level.

Acknowledgments

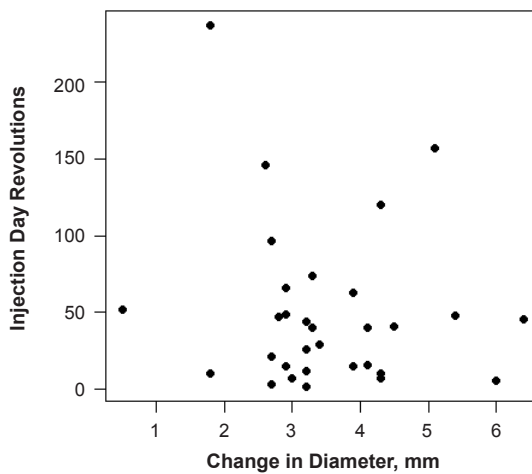
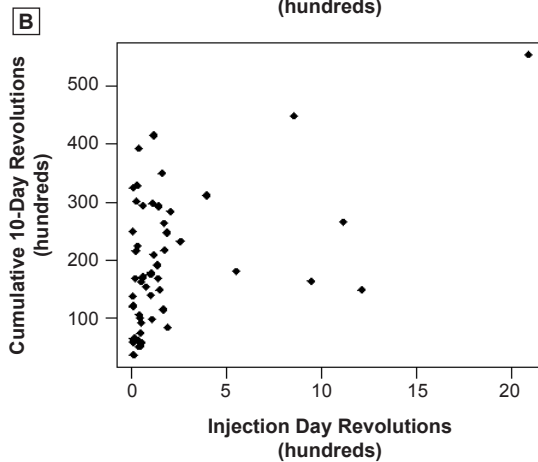
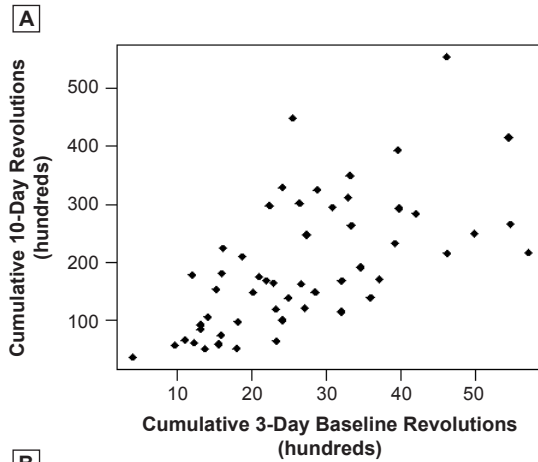
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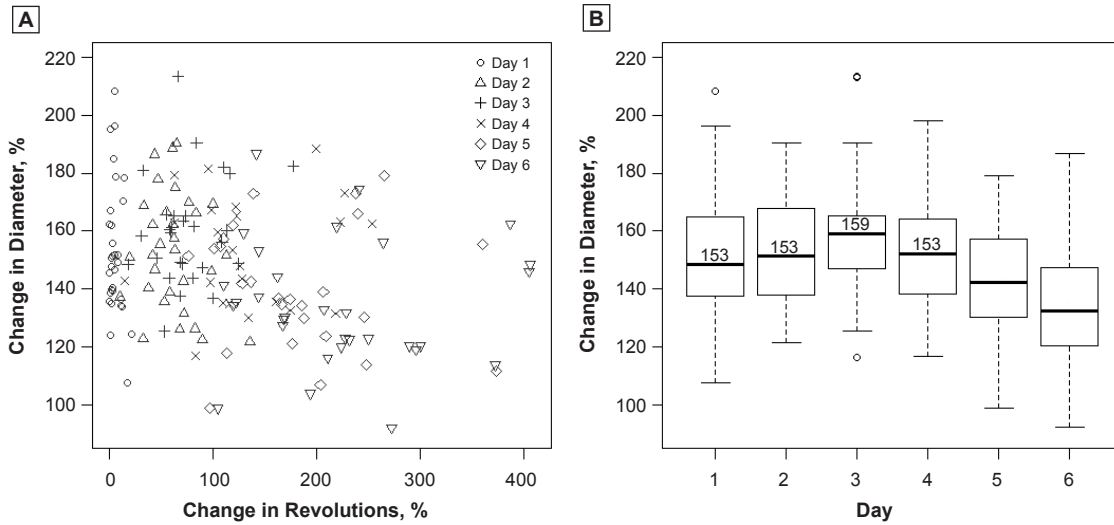


eFigure 1.

Effect of 3% carrageenan injection on inherent running variability. Cumulative 10-day post-injection running correlated with (A) cumulative 3-day baseline running ($\rho=0.68$; $P<.001$) and (B) injection day running ($\rho=0.37$; $P=.006$) on activity wheels in rats ($n=53$) injected with 3% carrageenan. *Abbreviation:* ρ , Spearman rank correlation coefficient.

eFigure 2.

Correlation of activity wheel running and hindlimb swelling in rats ($n=32$) after 3% carrageenan injection as measured by caliper before and after injection. The increase in hindlimb diameter 24 hours after injection was plotted vs the number of revolutions run in the same period ($\rho=-0.07$, $P=.68$). *Abbreviation:* ρ , Spearman rank correlation coefficient.



eFigure 3.

Effect of 3% carrageenan injection on rats ($n=32$) as measured by running wheel activity and by hindlimb swelling over time. (A) Correlation of change in hindlimb diameter (%) and change in activity wheel running (%) ($|\rho|<0.17$, $P>.37$). (B) Change in diameter (%) after carrageenan injection over each day. Points plotted are ≥ 1.5 times the interquartile range above the third quartile or ≤ 1.5 the interquartile range below the first quartile. Change is relative to pre-injection diameter and baseline running. *Abbreviation:* ρ , Spearman rank correlation coefficient.