Response of Knee Ligaments to Prolotherapy in a Rat Injury Model

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Background: Prolotherapy is an alternative therapy for chronic musculoskeletal injury including joint laxity. The commonly used injectant, D-glucose (dextrose), is hypothesized to improve ligament mechanics and decrease pain through an inflammatory mechanism. No study has investigated the mechanical effects of prolotherapy on stretch-injured ligaments.

Hypotheses: Dextrose injections will enlarge cross-sectional area, decrease laxity, strengthen, and stiffen stretch-injured medial collateral ligaments (MCLs) compared with controls. Dextrose prolotherapy will increase collagen fibril diameter and density of stretch-injured MCLs.

Study Design: Controlled laboratory study.

Methods: Twenty-four rats were bilaterally MCL stretch-injured, and the induced laxity was measured. After 2 weeks, 32 MCLs were injected twice, 1 week apart, with either dextrose or saline control; 16 MCLs received no injection. Seven uninjured rats (14 MCLs) were additional controls. Two weeks after the second injection, ligament laxity, mechanical properties (n = 8), and collagen fibril diameter and density (n = 3) were assessed.

Results: The injury model created consistent ligament laxity ($P < .05$) that was not altered by dextrose injections. Cross-sectional area of dextrose-injected MCLs was increased 30% and 90% compared with saline and uninjured controls, respectively ($P < .05$). Collagen fibril diameter and density were decreased in injured ligaments compared with uninjured controls ($P < .05$), but collagen fibril characteristics were not different between injured groups.

Conclusion: Dextrose injections increased the cross-sectional area of MCLs compared with saline-injected and uninjured controls. Dextrose injections did not alter other measured properties in this model.

Clinical Relevance: Our results suggest that clinical improvement from prolotherapy may not result from direct effects on ligament biomechanics.

Keywords: dextrose; saline; healing; subfailure damage; medial collateral ligament

Incomplete healing from ligamentous rupture or severe stretch injury can lead to chronic pain,[27] joint instability and laxity,[37] and possibly osteoarthritis.[9] Subfailure stretch injuries account for more than 85% of sprains in humans.[3] Clinically, these sprain and strain injuries account for more than 5 million visits to United States emergency departments every year.[26] Many of these patients are refractory to usual care therapies.[40]

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While little is known about subfailure healing, animal models of complete ligament rupture show that changes in ligament structure and function can be chronic. In a rabbit model, healing from complete ligament rupture and gap injury leads to decreased mechanical and structural properties such as failure force and elastic modulus that persist beyond 1 year.[13,14,41,42] Laxity is also present beyond 1 year,[13] and decreased collagen fibril diameters remain for at least 2 years after injury.[12]

Stretch injury creates a persistent mechanical deficit in the injured tissue. Healing from such injury may result in alterations similar to that of fully ruptured ligaments. Limited animal studies have investigated healing after stretch injury.[22,29,30] These studies found that healing occurs by an intrinsic response thought to be mediated by fibroblasts and minimal recruitment of inflammatory cells (neutrophils, macrophages, or lymphocytes). In contrast,

complete rupture creates inflammation postinjury.[13] In an ovine medial collateral ligament (MCL) subfailure injury model, Laws and Walton[22] found decreased ultimate strength and laxity up to 3 weeks after injury but found no differences from controls at 6 weeks. An increase in the number of fibroblasts was found up to 6 weeks after injury, but no change in inflammatory cell number was found. In rat MCL and lateral collateral ligament (LCL) subfailure injury models, Provenzano et al[30] reported that ultimate stress was decreased immediately after injury and was only partially recovered at 14 days, and injured LCLs had an increased length (laxity) 0, 7, and 14 days after injury compared with controls. Gene expression related to inflammation was not increased 0, 3, or 7 days after injury compared with controls.[29]

Prolotherapy is an increasingly popular alternative therapy used to treat chronic musculoskeletal and arthritic pain. It has been advocated as a treatment for chronic ligament and joint laxity.[17] Treatment involves injection of an irritant solution at painful ligament or tendon insertions and in adjacent joint spaces.[17,23] Prolotherapy is hypothesized to create a host inflammatory response,[5] which results in stronger connective tissue, improved biomechanics and joint function, and decreased pain.[17,23] If true, prolotherapy could become an important therapy for chronic musculoskeletal pain and ligament laxity. Beneficial effects using prolotherapy for a variety of musculoskeletal conditions have been documented in 34 clinical case series and case reports and 5 randomized controlled trials; 2 randomized controlled trials have reported equivocal results.[32,36] The potential mechanism of action has not been well studied in either clinical or animal models.

Four studies using animal models suggest biological effects of prolotherapy; however, each has methodological limitations. Liu et al[24] injected 5% sodium morrhuate into healthy rabbit MCLs on days 1, 5, 9, 19, and 26. Compared with saline control, sodium morrhuate injections increased ligament mass, thickness, and failure strength 16 days after the last injection. The testing technique, however, may have permitted grip slippage, and tissue displacement was not rigorously quantified. To investigate the cause of the increased failure strength in the Liu study, Maynard et al[25] examined microscale differences in the patellar tendon and Achilles tendon in rabbits after injection of sodium morrhuate. They reported general trends toward increased tendon circumference, cell number, water content, proteoglycans, and decreased collagen. However, 3 different numbers of injections (1, 3, and 5 injections) and 3 time points for evaluation (1, 4, and 9 weeks after the last injection) were used, with only 1 animal per group, precluding statistical analysis. Sodium morrhuate injected into healthy rat patellar tendons was recently reported to decrease tendon length and increase tendon strength compared to the contralateral control (no treatment) 4 weeks after injection.[4] A rat model study that used a combination of dextrose and sodium morrhuate, in addition to mepivicaine and cyanocobalamine (also known as Pomeroy's solution), injected into crush-injured Achilles tendons found no change in strength or elastic modulus 3 weeks after 7 weekly injections.[18]

To our knowledge, no study has investigated the effects of injections with dextrose, the most commonly used prolotherapy solution, on injured ligaments in an animal model. Our goal was to characterize the biological, morphological, and mechanical healing responses of stretch-injured MCLs treated with prolotherapy. Our hypotheses were (1) dextrose prolotherapy will enlarge the cross-sectional area, strengthen, and stiffen stretch-injured MCLs compared to controls; (2) dextrose prolotherapy will reduce ligament laxity of stretch-injured MCLs compared to controls; and (3) dextrose prolotherapy will increase collagen fibril diameter and density of stretch-injured MCLs compared with controls.

MATERIALS AND METHODS

This study was approved by the University of Wisconsin Institutional Animal Care and Use Committee. A total of 44 Sprague-Dawley rats were used in this study. To characterize the stretch injury model without injection, 12 of these rats were analyzed immediately after injury and after 2 weeks and 4 weeks of healing (n = 4). These MCLs were mechanically tested using the contralateral uninjured leg for control. To investigate the effects of prolotherapy on injured ligaments, 24 rats underwent bilateral MCL subfailure stretch injury on day 0 (Figure 1). Ligaments were allowed to heal for 14 days. This time period was chosen because the stretch-injury characterization part of our study showed that failure strength was fully recovered 2 weeks after stretch injury (Table 1) and was stable throughout this study. Clinically, prolotherapy is often performed at 3- to 6-week intervals.[32] Because rats heal quickly from ligament injury, the shorter interval of 1 week between injections was used. On day 14 and day 21, both MCLs of 16 injured rats were injected bilaterally at the tibial and femoral insertions with 15% dextrose or saline. Rats were euthanized 14 days after the second injection. The remaining 8 injured rats were not injected and were euthanized 5 weeks after injury. An additional group of 7 rats was used as noninjured and noninjected controls. In each rat, 1 MCL was used for pull-to-failure mechanical testing, and the contralateral MCL was used for transmission electron microscopy (TEM) analysis (n = 3) or histological and immunohistochemical (IHC) analysis (n = 5).

Surgical Technique

Anesthesia was induced and maintained using an induction chamber and facemask with 1% to 3% isoflurane in 100% oxygen. After induction, both stifles were shaved and aseptically prepared. An incision approximately 2 cm long was made on the medial side of the stifle. Fascia and muscle above the MCL were incised to expose the MCL. A specialized device was used to stretch the MCL and create a consistent subfailure damage injury (see Subfailure Stretch Injury, below). Immediately before and after the stretch injury, the inherent and induced laxities, respectively, were measured with a specialized measuring device (see Laxity Measurement, below). The muscle and fascia were closed with 3-0 Dexon suture, and the skin was apposed with skin staples. A dose of buprenorphine (0.01 mg/kg subcutaneous) was administered as an analgesic before the animal regained consciousness from anesthesia. At sacrifice, final laxity was measured using the specialized measuring device. Animals were

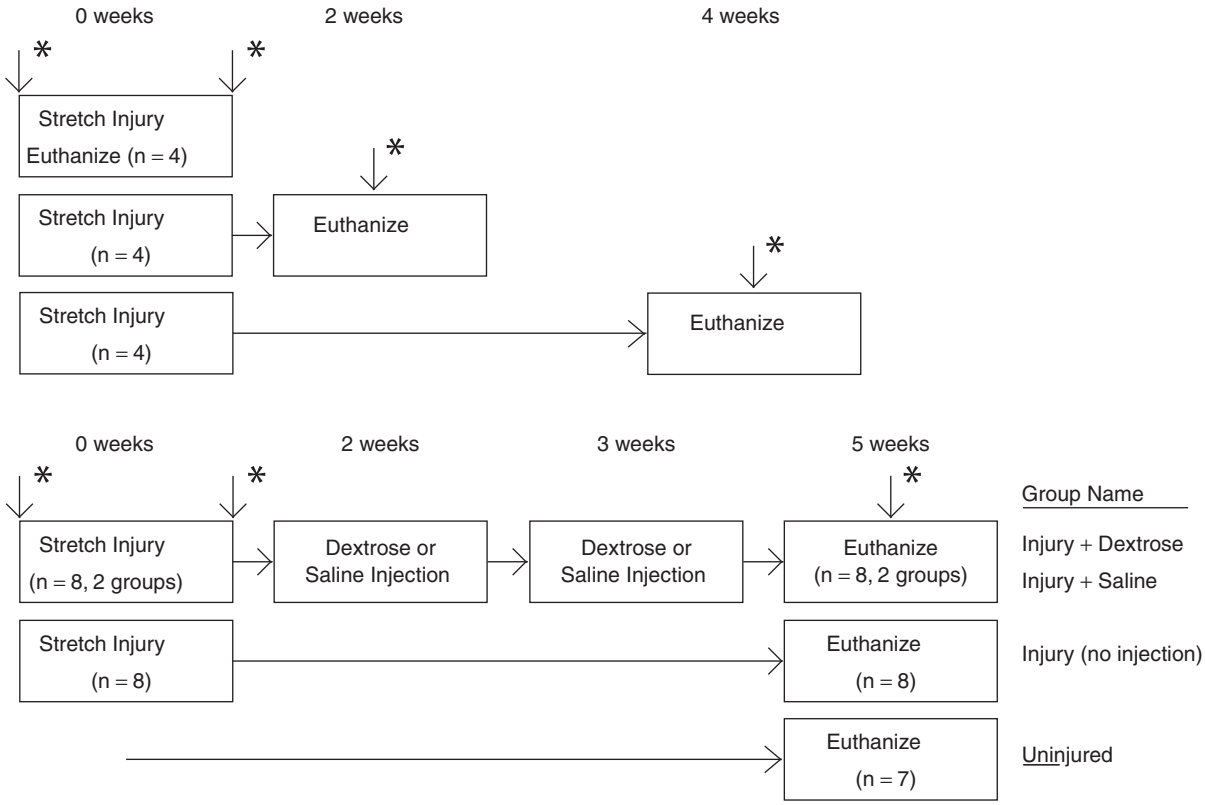

Figure 1. Study design: characterize stretch injury and investigate response from prolotherapy injection. Top 3 rows: To characterize the stretch-injury model, 12 rats underwent unilateral medial collateral ligament (MCL) stretch injury at time 0. Rats were euthanized immediately after injury and after 2 weeks and 4 weeks of healing (n = 4). The contralateral (uninjured) leg was used as control. Bottom 3 rows: To investigate the response to prolotherapy injections, 32 additional rats were used. Sixteen rats underwent bilateral MCL stretch injury at time 0. At 2 and 3 weeks after injury, rats were injected with the most commonly used solution (dextrose) or saline (control) and were euthanized 2 weeks after the second injection. Eight rats underwent bilateral MCL stretch injury with no injections and were euthanized 5 weeks after stretch injury. Seven rats did not undergo stretch injury.

sacrificed with an overdose of pentobarbital (150-200 mg/kg intraperitoneal to effect).

Subfailure Stretch Injury

This subfailure stretch injury has been developed and characterized.[29,30] In brief, the leg is placed in a reproducible position, and a device is placed on the MCL. This device constrains the MCL insertions and stretches the MCL by lifting the midsubstance of the MCL medially a specified amount (2.4 mm) in a "bowstring" fashion. This action stretches the ligament a specific distance while keeping the insertions intact and creates a consistent subfailure stretch injury of the ligament. Previous studies show that mechanical and cellular damage to the ligament is not substantially different than with pure axial stretch of an excised ligament.[30,31]

Laxity Measurement

Inherent, induced, and final MCL laxity parameters were determined using a specialized measuring device (Figure 2). This device functions similarly to the device used for stretch injury except that it is smaller, does not constrain the ligament insertions, and stops moving when a specific load is reached (instead of a specific displacement). The leg was placed in a reproducible position, and the device was placed under the midsubstance of the MCL. The midsubstance was lifted medially in a bowstring fashion. The distance that the device moves medially until it applies 0.74 N of force is defined as the laxity parameter. The inherent (before stretch) laxity parameter value was subtracted from the induced (immediately after stretch) and final (at sacrifice) laxity parameters to calculate the change immediately after stretch and at sacrifice, respectively.

Injection Technique

Solutions were injected at the tibial and femoral insertion sites of the MCL. Bony landmarks were used to determine the location of the tibial and femoral insertion sites. A total of 0.1 mL of solution was injected at the tibial and femoral insertion sites of the MCL.

TABLE 1
Mechanical and Structural Properties of Uninjured and Injured Medial Collateral Ligaments (MCLs) Immediately, 2, 4, and 5 Weeks After Injury and Injured MCLs 5 Weeks After Dextrose, Saline, and No Injection[a]

		Failure Force (N)		Failure Stress (MPa)		Failure Strain (%)		Failure Displacement (mm)	
Time Point	Injection Solution	Uninjured	Injured	Uninjured	Injured	Uninjured	Injured	Uninjured	Injured
0 weeks	No injection	24.9	17.3[b]	30.4	26.5	5.3	7.3[b]	0.58	0.82[b]
	(n = 4)	(2.0)	(1.9)	(3.2)	(1.3)	(0.7)	(1.1)	(0.08)	(0.10)
2 weeks	No injection	35.3	32.2	41.0	15.0[b]	6.7	7.9	0.75	0.89
	(n = 4)	(1.3)	(1.1)	(2.3)	(2.2)	(0.2)	(0.8)	(0.04)	(0.08)
4 weeks	No injection	45.2	40.3	44.1	25.6[b]	6.7	6.2	0.83	0.78
	(n = 4)	(3.5)	(4.4)	(6.0)	(5.3)	(1.1)	(0.6)	(0.13)	(0.07)
5 weeks	No injection	30.1	32.3	39.3	26.0[b]	8.7	6.5	0.97	0.76
	(n = 7, 8)	(2.6)	(3.6)	(3.2)	(2.4)	(0.9)	(0.4)	(0.11)	(0.05)
5 weeks	Saline		30.6		27.9[b]		6.1		0.73
	(n = 8)		(3.4)		(3.7)		(0.7)		(0.08)
5 weeks	Dextrose		34.7		24.5[b]		6.9		0.82
	(n = 8)		(2.0)		(2.1)		(0.7)		(0.09)

[a]Mean ± standard error (SE).
[b]$P < .05$ versus uninjured at the same time point.

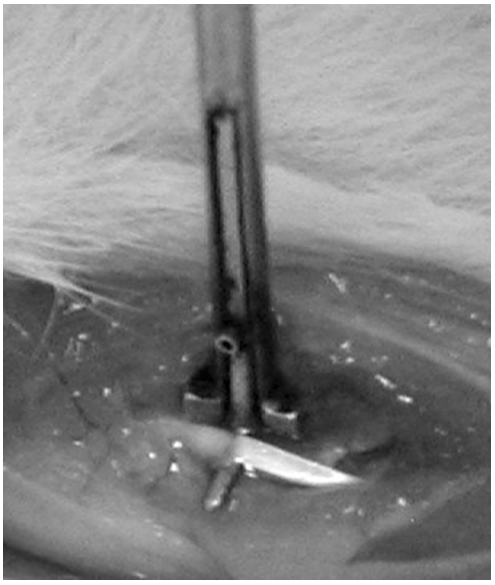

Figure 2. Stretch-injury measurement device. The leg was placed in a reproducible position, and the device was placed under the midsubstance of the medial collateral ligament. The midsubstance was lifted medially in a "bowstring" fashion. The distance that the device moves medially until it applies 0.74 N of force is defined as the laxity parameter.

Immunohistochemistry and Histology

At sacrifice, MCLs were carefully dissected, placed in optimal cutting temperature compound, flash-frozen, and stored at –80°C. Specimens were cryosectioned onto microscope slides and labeled with an anti-ED1 antibody (ED1 labels monocytes and most macrophage subpopulations) and an anti-ED2 antibody (ED2 labels most tissue macrophages) (AbD Serotec, Raleigh, NC). Additional slides were stained with hematoxylin and eosin to examine cell number and collagen fibril alignment. Labeled and stained sections were visually compared to determine if the number of cells or alignment of fibrils was different between treatment groups.

Mechanical Testing

Pull-to-failure testing was performed as described previously.[30] After sacrifice, extraneous tissue was dissected away to carefully expose the MCL. Specimens were trimmed to the femur-MCL-tibia (FMT) complex while care was taken not to harm the MCL insertions. During dissection and mounting, the FMT complex was kept hydrated with phosphate-buffered saline. Thickness and width were measured optically, and cross-sectional area was estimated by elliptical geometry. The FMT complex was mounted into a custom bath and test machine. A preload (0.1 N) was applied to the MCL, and the MCL was preconditioned (loaded cyclically to 1% strain for 10 cycles). The ligament initial length was measured, and the cross-sectional area was calculated at the preload. Then the ligament was pulled to failure at 10% per second. Failure force was measured at the highest force before failure. Failure stress was calculated by dividing the failure force by the initial cross-sectional area of the ligament. Failure strain was calculated by subtracting the ligament length at preload (initial length) from the ligament length at failure and dividing by the initial length. Stiffness was determined using a probabilistic microstructural model.[21]

Transmission Electron Microscopy

The MCL was exposed and drip-fixed with Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer). The MCL was carefully dissected and fixed in Karnovsky's fixative for 24 hours at 4°C. The ligament was washed in 0.1 M phosphate buffer to remove the fixative before

postfixing in 2% osmium tetroxide for 3 hours. The samples were block-stained in 5% uranyl acetate in 50% ethanol and dehydrated in graded alcohols over 90 minutes. The samples were serially infiltrated in increasing concentrations of epoxy resin (EMbed-812, DDSA [dodecenyl succinic anhydride], Araldite 502, and DMP-30 [2,4,6-Tri(dimethylaminomethyl) phenol]) over 16 hours with the final step of 100% resin in a vacuum of 15 mm Hg for 12 hours. The samples were imbedded in resin and polymerized for at least 48 hours at 60°C. Thin cross-sections (70 nm) were cut with a diamond blade on an Ultracut E microtome (Reichert, Vienna, Austria). Sections were stained with 7.7% uranyl acetate for 30 minutes at 60°C and lead citrate for 5 minutes. Samples were viewed at 31 000× magnification on a Philips CM120 STEM (Philips Electron Optics BV, FEI Applications Group, Eindhoven, the Netherlands). Digital images were taken of the collagen fibrils while avoiding pericellular regions, which may contain higher proportions of smaller collagen fibrils.[10] Five images were taken per ligament. Image analysis was performed on MetaMorph Imaging System Version 6.1r0 (University Imaging Corp, Downingtown, Pa). The minimum diameter of the collagen fibrils was used to minimize the effect of oblique fibrils.[10] The distribution of collagen fibril diameters and collagen fibril density was determined. A minimum of 950 fibrils were analyzed per ligament (1380 ± 358 fibrils, mean ± standard deviation [SD]).

Statistical Methods

Analysis of variance, followed by Fisher protected least significant difference tests, were used to determine if differences existed between treatment groups. Outcomes compared were failure force, failure stress, failure strain, displacement at failure, stiffness, cross-sectional area, collagen fibril density, mean and median collagen fibril diameters, and percentage of collagen fibrils contained within 40-nm classes of fibril diameters. For the laxity data, temporal differences (induced laxity and final laxity after 2, 4, or 5 weeks of healing without treatment) were examined using paired *t* tests. In data with a contralateral control (mechanical testing at 0, 2, and 4 weeks), the injured minus uninjured difference was considered as the measure of interest, and paired Student *t* tests were performed. For the collagen fibril diameter, injured animals were also combined (injured dextrose-injected, saline-injected, and no-injection animals) and compared to the uninjured animals using Student *t* tests to determine the difference between injured and uninjured ligaments. Analysis using ranked data produced results similar to those using numerical data. For comparisons, significance was set to $\alpha = .05$. With 8 rats per group, the power for finding a significant change in laxity and ultimate stress was 92% and 99.6%, respectively, based on the magnitude of change found in a previous study[30] (9% and 20%, respectively). All analyses were performed using SAS statistical software version 9.1 (SAS Institute, Cary, NC).

RESULTS

Within minutes of surgical intervention and injection treatments, all rats recovered and resumed normal movement and behavior (ie, grooming, feeding, walking without limping, and standing on hind limbs). Our results immediately, 2, and 4 weeks after injury were based on 4 animals per group. Our results 5 weeks after injury were based on 8 rats in each of the injured groups (dextrose injection, saline injection, or no injection) and 7 rats in the uninjured control group. Rats were 11.5 to 13 weeks old at surgery, with a weight of 341 ± 31 g (mean ± SD).

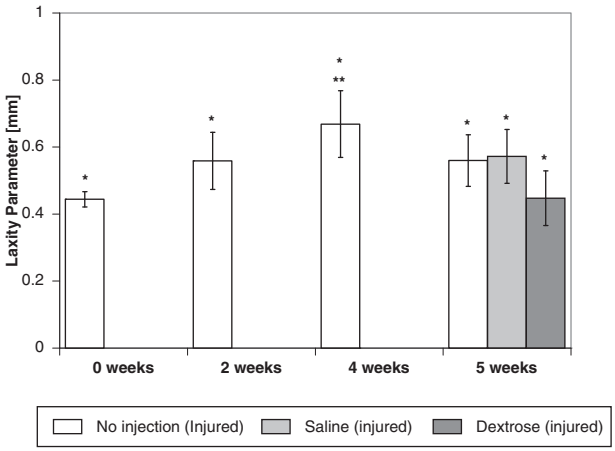

Figure 3. Change in laxity parameter immediately, 2, 4, and 5 weeks after injury. Inherent laxity, as quantified by our bowstring laxity parameter, was subtracted from this same parameter at each time point to obtain the data shown in this figure. Ligaments were consistently injured. Immediately after injury, change in laxity was 0.44 ± 0.14 mm of medial displacement (*$P < .0001$), which was estimated to be approximately 1.6% change in total ligament length. Ligaments remained lax 2, 4, and 5 weeks after injury (*$P < .05$). In addition, there was more laxity 4 weeks after injury than immediately after injury (**$P < .05$). Laxity was not altered by injection treatment of dextrose or saline. Mean ± standard error.

Laxity Measurement

To characterize the stretch-injury model, laxity measurements were taken immediately before and after stretch injury and after 2, 4, or 5 weeks of healing (Figure 3). Laxity was consistently created by this subfailure injury model ($P < .0001$). Laxity persisted after 2, 4, and 5 weeks of healing. After 5 weeks of healing, dextrose and saline injections did not alter laxity ($P = .28$).

Mechanical Testing: Stretch-Injury Model

To characterize the stretch-injury model, rats were euthanized immediately after injury and after 2 and 4 weeks of healing. No injections were performed on these animals. The rats euthanized at 5 weeks without prolotherapy injection are also presented in this section.

The injury created by subfailure stretch can be seen in Figure 4, which shows the force displacement curve of injured ligaments compared with the contralateral control ligaments immediately after injury and after 2 weeks of

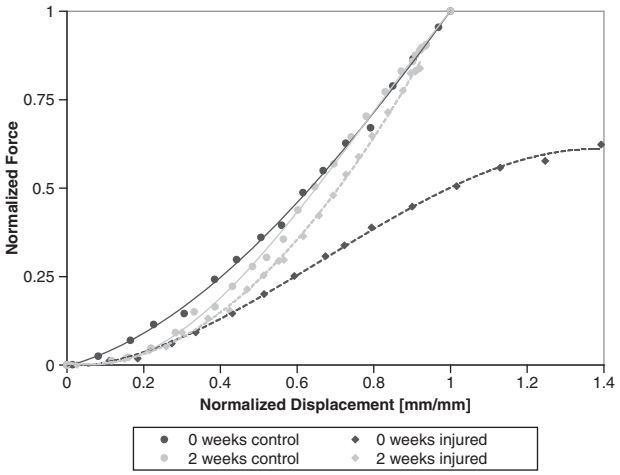

Figure 4. Force displacement curve comparing injured ligaments and the contralateral control immediately after injury and after 2 weeks of healing. Immediately after injury, all ligaments had a lower failure force and lower stiffness than uninjured controls. After 2 weeks of healing from injury, failure forces were similar between injured and uninjured ligaments. Injured ligaments were normalized to the force and displacement of the contralateral control because the rats were growing.

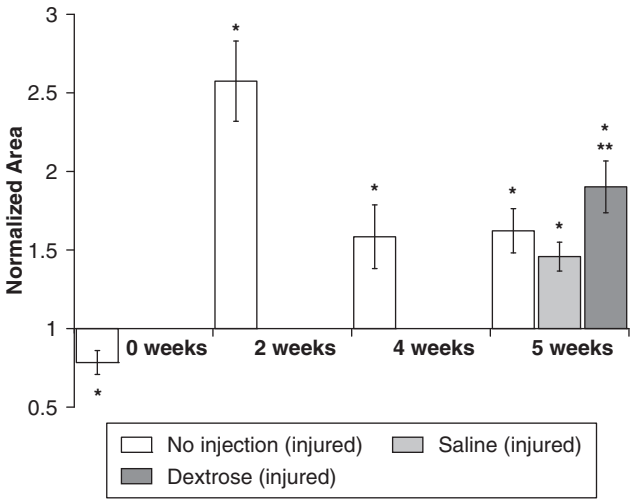

Figure 5. Cross-sectional area of medial collateral ligaments immediately, 2, 4, and 5 weeks after injury normalized to uninjured area. Injured (no injection) ligaments initially had a 22% smaller cross-sectional area than contralateral uninjured controls. After 2 and 4 weeks of healing, injured (no injection) ligaments had 157% and 58% larger cross-sectional areas, respectively, than contralateral uninjured controls (*P < .05 vs uninjured at the same time point). After 5 weeks of healing, dextrose-injected, saline-injected, and no injection ligaments had 90%, 46%, and 62% larger cross-sectional areas, respectively, than uninjured controls (*P < .05). In addition, injured ligaments with dextrose injection had a 30% larger area compared with injured ligaments with saline injection (**P < .05). Mean ± standard error.

healing. Failure force was decreased by 31% of uninjured controls immediately after injury (P < .05) (Table 1). Similar failure forces were found at 2, 4, and 5 weeks after injury when comparing injured and uninjured ligaments within each time point (P = not significant). All ligaments failed in the tibial third of the MCL. Stiffness was decreased in every injured ligament compared with every uninjured control ligament immediately after injury. Stiffness was not different after 2, 4, or 5 weeks of healing. Compared with uninjured ligaments, the cross-sectional area of injured ligaments was 22% smaller immediately after injury and was 157%, 58%, and 62% larger after 2, 4, and 5 weeks of healing, respectively (P < .05) (Figure 5). Failure stress, which takes into account the cross-sectional area, in injured ligaments was decreased by 63% at 2 weeks, 42% at 4 weeks, and 34% at 5 weeks after injury compared with uninjured controls (P < .05) (Figure 6). Displacement and strain at failure were 41% and 38% larger, respectively, immediately after injury compared with uninjured controls (P < .05) (Table 1). Displacement and strain at failure were not altered 2, 4, or 5 weeks after injury compared with uninjured controls at each time point (P = not significant).

Mechanical Testing: 5 Weeks After Stretch Injury With and Without Injection

Injured ligaments with dextrose injection, saline injection, and no injection had 90%, 46%, and 62% larger cross-sectional area than uninjured ligaments, respectively (P < .05) (Figure 5). In addition, injured ligaments with dextrose injections had a 30% larger cross-sectional area than injured ligaments with saline injections (P < .05).

After 5 weeks of healing, failure force was not altered by injection of dextrose or saline compared to no injection (P = not significant). However, failure stress, which takes the cross-sectional area into account, was altered after injury. Injured ligaments with dextrose, saline, or no injection had 38%, 29%, and 34% smaller failure stress, respectively, compared with uninjured ligaments (P < .05) (Figure 6). Injured ligaments with saline injection and with no injection had a trend toward 30% and 25% smaller strain at failure, respectively, compared with uninjured controls ($F_{3,27} = 2.75$, $P = .06$; [Pr > $|t_{27}|$] < .05). Displacement at failure was not altered by dextrose or saline injection (P = not significant). No further differences were found between injured groups 5 weeks after injury.

Immunohistochemistry: 5 Weeks After Stretch Injury With and Without Injection

Sections labeled for ED1-positive and ED2-positive macrophages were compared visually. Few macrophages were found in injured and injected ligaments 5 weeks after injury. No differences could be found between groups in hematoxylin and eosin stained slides. Therefore, slides were not analyzed further.

Transmission Electron Microscopy: 5 Weeks After Stretch Injury With and Without Injection

Representative TEM images are shown in Figure 7. Injured ligaments, regardless of injection group, had decreased collagen fibril density and diameter. The collagen fibril density

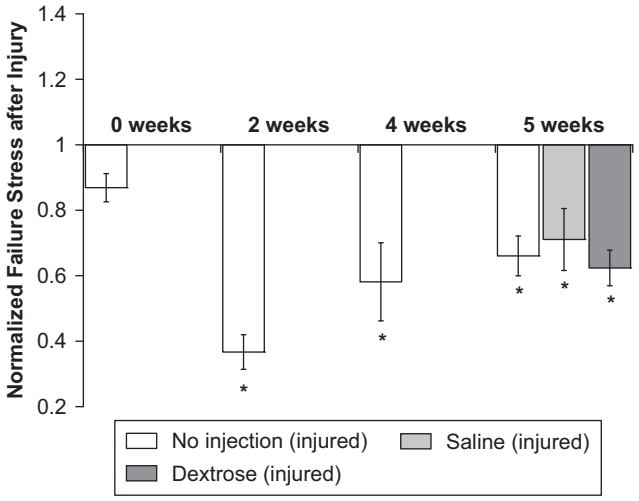

Figure 6. Decreases in failure stress of medial collateral ligaments immediately, 2, 4, and 5 weeks after stretch injury. Immediately after stretch injury, the failure stress was similar in injured and uninjured ligaments. At 2 and 4 weeks after injury, injured ligaments failed at 63% and 42% lower stress, respectively, than uninjured controls at the same time point (*$P < .05$). After 5 weeks of healing, injured ligaments with dextrose injection, with saline injection, and without injection had 38%, 29%, and 34% smaller failure stresses, respectively, than uninjured controls (*$P < .05$). No additional differences were found between injured groups at 5 weeks. Mean ± standard error.

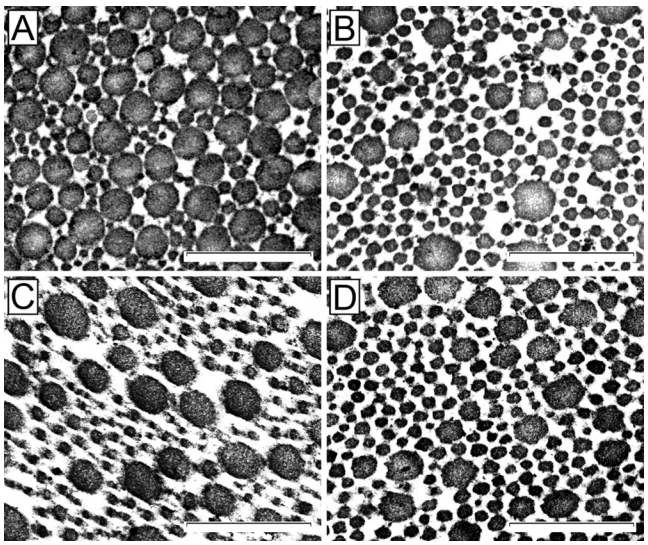

Figure 7. Transmission electron microscopy images of the cross-section of ligaments. Five weeks after injury, collagen fibril diameter and density were decreased compared with uninjured ligaments. This effect did not change with injection treatment. A, uninjured with no injection; B, injured with no injection; C, injured with saline injection; and D, injured with dextrose injection. Scale bar = 500 nm.

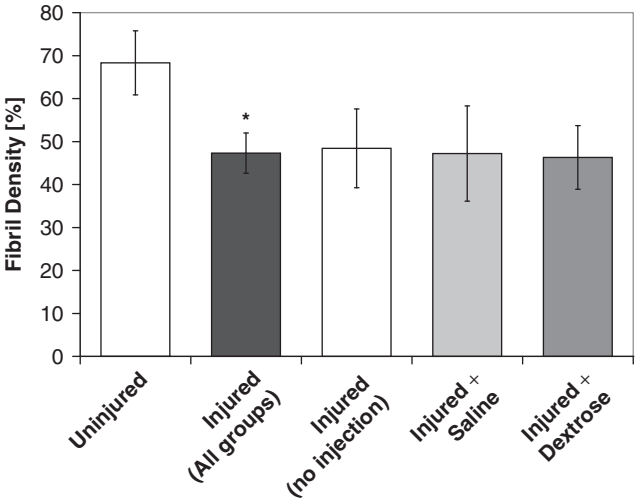

Figure 8. Collagen fibril density in ligaments 5 weeks after injury with and without injection. When grouped together, all injured ligaments (dextrose-injected, saline-injected, and no injection) had a 31% smaller collagen fibril density compared with uninjured controls (*$P < .05$). No differences were found between injection treatments. Mean ± standard error.

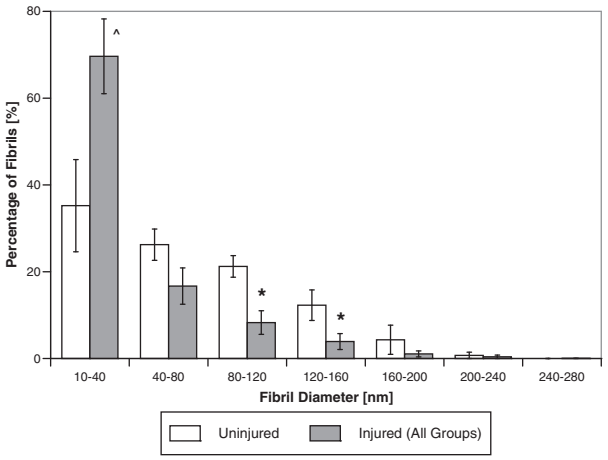

Figure 9. Collagen fibril diameter distributions in injured and uninjured ligaments. Five weeks after injury, there were 61% and 68% fewer larger collagen fibrils (80-120 nm and 120-160 nm) in injured ligaments (including dextrose-injected, saline-injected, and no injection) compared with uninjured ligaments (*$P < .05$). In addition, there was a trend toward 98% more fibrils with smaller diameters of 10 to 40 nm in injured ligaments compared with uninjured (^$P = .06$). Mean ± standard error.

was decreased by 31% when all injured ligaments (dextrose-injected, saline-injected, and no injection) were compared with uninjured controls ($P < .05$) (Figure 8). When individual injection groups were compared, no differences were found between injection groups. The distribution of collagen fibril diameters was also altered by stretch injury. The mean and median collagen fibril diameters had a trend toward significance with injured ligaments having a 39% smaller mean and median fibril diameter compared with uninjured controls ($P = .053$ and .058, respectively) (Table 2). In addition, there were fewer larger collagen fibrils in all injured groups compared with the uninjured group (Figure 9). Specifically, there were 61% and 68% fewer collagen fibrils

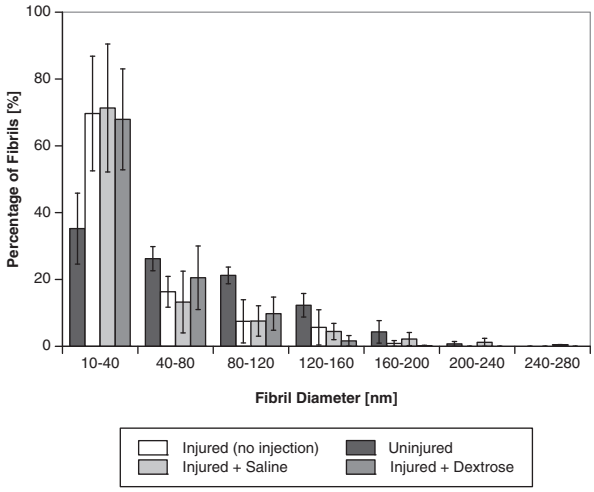

Figure 10. Collagen fibril diameter distribution by treatment group 5 weeks after injury. There were no differences in collagen fibril diameter distribution between injection groups. Mean ± standard error.

with a diameter of 80 to 120 nm and 120 to 160 nm, respectively, in the injured groups combined compared with the uninjured control ($P < .05$). There was also a trend toward 98% more fibrils with smaller diameters of 10 to 40 nm ($P = .06$). No differences were found between injection groups (Figure 10).

DISCUSSION

To our knowledge, this is the first study to assess whether injections with dextrose, the most commonly used prolotherapy solution, change the mechanical, morphological, or biological healing characteristics of MCLs in a stretch-injured animal model controlling for volume effects (saline control) and procedural effects (noninjected control). Injured ligaments injected with dextrose had a significantly larger cross-sectional area compared to saline-injected ligaments and uninjured controls. The clinical significance of this finding is unclear. Dextrose injections did not have any other effects on laxity, mechanical properties, or collagen fibril density or diameter compared to control injections.

This study also investigated the response of the MCL to this stretch injury for up to 5 weeks after injury. This stretch-injury model created and sustained laxity within the MCL for at least 5 weeks after injury and, therefore, provides a suitable model to study the effects of prolotherapy and other treatments on ligament laxity. Immediately after injury, cross-sectional area, failure force, and stiffness decreased, and strain and stretch at failure increased compared with uninjured controls. The unaided healing response of these stretch-injured ligaments led to an increase in the cross-sectional area and a decrease in failure stress at 2, 4, and 5 weeks after injury. However, the failure force was not altered, and hence the decrease in failure stress was directly related to the increase in area and may not indicate weaker ligaments. Investigating this change in cross-sectional area more closely, the collagen density and the diameters of these fibrils were decreased in injured ligaments 5 weeks after injury compared to uninjured controls. The persistent ligament laxity created in this model is consistent with what is found clinically after stretch injury.[6]

The model used in our study is useful for creating consistent stretch injury and assessing changes in laxity without extremely invasive surgery. The testing of methods to decrease laxity within ligaments and tendons is usually conducted on normal, healthy tissue and not loose or lax tissue.[19,38] Few animal models create laxity within a joint or ligament. Most models that create laxity are either limited by inconsistent stretch injury[22] or the use of surgical techniques to move the ligament insertion by osteotomy.[39]

Previous animal studies investigating prolotherapy have examined the effects of injecting sodium morrhuate on healthy ligaments or tendons compared with saline injections[24,25] or with no injection[4] or have examined the effects of injecting solutions that are not widely used on crush-injured tendons.[18] The current study assessed dextrose because of its wide use in clinical practice[8] and its use in recent randomized, controlled human studies.[33,34,43] We report an increase in the size of the ligament (cross-sectional area) with dextrose injection compared with saline control. This finding is consistent with studies by Liu et al[24] and Maynard et al,[25] who reported an increase in the ligament thickness and tendon circumference,

TABLE 2
Collagen Fibril Diameter Distribution in Injured and Uninjured Ligaments 5 Weeks After Injury[a]

		Fibril Diameter	
Injured or Uninjured	Injection Type	Mean (SE) nm	Median (SE) nm
Uninjured	No injection	71.07 (11.99)	60.09 (15.49)
Injured	No injection	45.15 (12.70)	40.90 (9.11)
Injured	Saline	44.14 (14.97)	34.42 (10.05)
Injured	Dextrose	41.47 (7.67)	34.26 (4.13)
All injured groups		43.59 (6.11)[b]	36.53 (4.24)[b]

[a]Injured ligaments, regardless of injection treatment, had a trend toward a 39% decrease in the mean and median fibril diameter compared with healthy uninjured ligaments. There were no additional changes with injection treatment. Mean ± standard error (SE).
[b]$P = .054$ and $P = .058$, respectively, compared with uninjured ligaments.

respectively, after prolotherapy injection compared to saline. Liu et al[24] and Aneja et al[4] reported an increase in failure force with prolotherapy injections compared with saline injections and no injection, respectively. This is in contrast to the results in the current study and the study by Harrison[18]; neither work found a change in failure force. However, the current study used stretch-injured ligaments with dextrose injections, and the study by Harrison used crush-injured ligaments with Pomeroy injections, each of which may have a different effect than healthy ligaments injected with sodium morrhuate.

We found a change in collagen fibril morphology in injured ligaments compared with uninjured controls regardless of injection (dextrose, saline, and no injection). There was a smaller number of large fibrils in injured ligaments compared with uninjured controls. These results from stretch-injured ligaments are consistent with injury models that have found significantly lower fibril diameters in MCL scars 3 and 6 weeks after transection,[15] in MCL scars up to 2 years after gap injury,[11,12] and in patellar ligament scars after removal of the central third for use as graft tissue[28] compared with healthy ligaments. In addition, we found a smaller fibril density in all injured ligaments compared with uninjured ligaments. No change in fibril density was found when analyzed in previous studies on healing ligaments.[15]

No difference in the collagen fibril diameter or density was found between treatment groups (dextrose, saline, and no injection). Previous studies on the effects of prolotherapy on fibril diameters reported conflicting results. Liu et al[24] found a trend toward larger fibril diameters, and Maynard et al[25] found a trend toward smaller fibril diameters. However, the Liu study analyzed a limited number of fibrils, and the Maynard study analyzed only 1 animal per group. The discrepancy between these 2 studies may be attributable to experimental design (fibril sample size and animal number).

While the current study shows a change in ligament size in response to prolotherapy, we are unable to report a generalized biomechanical response. This study therefore does not provide compelling evidence that dextrose injections cause consistent biomechanical response and does not provide a clear mechanism to explain positive clinical effects[32,36] of decreased pain and disability. Our results suggest that clinical improvement may result from factors not directly assessed in this study, such as an effect on peripheral nerves rather than on ligament biomechanics. Phenol (part of the P2G prolotherapy solution, containing phenol, glycerin, and glucose) is used clinically as a local anesthetic,[35] as an intrathecal neurolytic block for relief of cancer pain,[7] and as a nerve block to reduce spasticity in patients with spinal cord damage.[16] Little is known about the neurological effects of either dextrose or sodium morrhuate, the other 2 common prolotherapy solutions. Both may have an effect on pathologic angiogenesis (neovascularity), which has been hypothesized to be mechanistically related to tendon overuse injury and subsequent pain and disability.[1] When used to destroy neovessels in a procedure very similar to prolotherapy, the sclerosant polidocanol has been reported to reduce pain in lateral epicondyle,[44,45] patellar,[20] and Achilles tendinopathies.[2] Pain reduction is hypothesized to be related to the elimination of nerve fibers associated with neovessels. Relationships between the destruction of pathologic neovascularity and substance P, calcitonin gene-related peptide,[1] and vascular endothelial growth factor have been hypothesized but not clarified. Concentrated dextrose and sodium morrhuate are both vascular sclerosants and may play a similar role when used as injectants in prolotherapy, accounting for reported clinical success with prolotherapy.

The strengths of the current study include the following: (1) a consistent persistent stretch-injury model is used to investigate the effects of prolotherapy injections, (2) the study is well controlled by using control groups for the stretch injury (injured and uninjured groups) and the volume of solution injected (saline), and (3) sufficient animal numbers were used in the 5-week postinjury groups. Limitations include a lack of certainty that this model is a precise surrogate for human chronic ligament damage, given that the healing ability of rats may exceed our ability to detect biomechanical change, if one did occur, and that the degree of similarity between chronic injury in rats and humans is unknown. Type II error is a possibility in the current study, particularly for the stretch-injury characterization section (euthanized immediately, 2 weeks, and 4 weeks after injury). Another potential limitation is our use of younger rats for this study. While it is perhaps true that the use of mature rats would have provided different results, we do not know based on published studies if prolotherapy has any different effects based on subject age. A healing period of 2 weeks before injection and after the second prolotherapy injection is likely adequate to assess healing from a stretch injury in a rat model. The stretch-injury characterization part of our study (euthanize immediately and after 2 and 4 weeks of healing) showed that failure strength was fully recovered 2 weeks after stretch injury. This result demonstrates that the ligament had healed considerably in 2 weeks and was relatively functionally stable. Previous prolotherapy studies using rabbit and rat models had varying protocols. The injections were 4 to 10 days apart, and sacrifices were typically 1 to 4 weeks after the last injection (with 3 rabbits sacrificed at 9 weeks).[4,18,24,25] The time points used in our study are within these parameters.

Future studies are recommended to determine if the mechanism for prolotherapy is an effect on the peripheral nerves or an interaction with vascular endothelial growth factor and related growth factors. Studies are needed to assess whether the effects of sodium morrhuate and P2G on injured ligaments are consistent with the results found in previous animal studies using sodium morrhuate on uninjured ligaments. In addition, studies addressing the effect of injections on more chronically injured ligaments would be beneficial. Researchers should consider larger animal models, as the rat model may not approximate the human case closely enough.

CONCLUSIONS

Rat MCLs injected with dextrose were significantly larger than those injected with saline control solution and uninjured

ligaments, supporting our hypothesis that dextrose prolotherapy will enlarge the cross-sectional area of MCLs compared with controls. However, changes in biomechanical outcomes (laxity and strength) were not seen. The stretch-injury model used in this study created laxity within the MCL that remained for at least 5 weeks and can be measured immediately after injury and at sacrifice, quantitatively demonstrating persistent ligament laxity in an animal model for the first time.

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