Carbodiimide-Derivatized Hyaluronic Acid Surface Modification of Lyophilized Flexor Tendon

A Biomechanical Study in a Canine in Vitro Model

By Jun Ikeda, MD, PhD, Chunfeng Zhao, MD, Yu-Long Sun, PhD, Kai-Nan An, PhD, and Peter C. Amadio, MD

Investigation performed at the Orthopedic Biomechanics Laboratory, Division of Orthopedic Research, Mayo Clinic, Rochester, Minnesota

Background: Intrasynovial grafts are the ideal solution to replace defects in intrasynovial flexor tendons, but autologous graft sources are rarely available. The purpose of the present study was to test the hypotheses that an intrasynovial tendon prepared with repetitive freeze-thaw cycles and lyophilization (as a means of reducing immunogenicity) has increased frictional force (gliding resistance) in comparison with fresh intrasynovial tendons and that a lyophilized intrasynovial flexor tendon that is modified with carbodiimide-derivatized hyaluronic acid and gelatin has decreased frictional force in comparison with untreated lyophilized tendons.

Methods: Thirty-six flexor digitorum profundus tendons from the second and fifth digits of canine hind paws were randomly assigned to three groups. Twelve tendons were immediately assessed both mechanically and morphologically and served as the normal tendon group. The other twenty-four tendons were prepared with repetitive freeze-thaw cycles and lyophilization and were randomly assigned to two groups, including one group in which the tendons were treated with carbodiimide-derivatized hyaluronic acid and gelatin and one group in which the tendons were not treated. The frictional force was measured during 1000 cycles of simulated flexion-extension motion in all tendons, and the mean frictional forces were compared. The tendons were then observed with use of transmitted light microscopy for residual hyaluronic acid on the tendon surface, and the smoothness of the surface was evaluated with use of scanning electron microscopy.

Results: The frictional force after lyophilization was significantly increased by 104.9% after the first cycle and by 99.5% after 1000 cycles in comparison with the normal tendon ($p < 0.05$). The frictional force of the lyophilized tendons after treatment with carbodiimide-derivatized hyaluronic acid and gelatin was not significantly different from that of normal tendons. The untreated lyophilized tendon surfaces were observed on scanning electron microscopy to be rough in appearance, whereas the normal surface and the surface treated with carbodiimide-derivatized hyaluronic acid and gelatin were smooth, with residual hyaluronic acid present on the gliding surface.

Conclusions: Lyophilization alters tendon surface morphology and increases tendon frictional force. Surface modification with carbodiimide-derivatized hyaluronic acid and gelatin can mitigate this adverse effect.

Clinical Relevance: Tendon surface modification with carbodiimide-derivatized hyaluronic acid and gelatin can improve the gliding ability of lyophilized flexor tendons and therefore may improve the utility of lyophilized tendon allografts as a tendon graft substitute.

Injuries to the flexor tendons in the digits are common, especially in the young and working-age populations. Although immediate primary repair after injury has been generally accepted and outcomes have been improved in association with the use of new regimens of primary repair and postoperative controlled mobilization[1-5], poor functional out-

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comes are still common, especially in zone II[6-10] (i.e., the portion of the flexor tendon sheath extending from the middle part of the palm to the middle one-third of the finger). This zone contains two tendons in a fibrous sheath in which the blood supply to the tendon is at its most tenuous, being limited to one or two small vessels that are easily injured. Consequently, this is the zone in which tendon repair is most difficult technically and in which the worst results of tendon repair are recorded. In such cases, tendon autografts play an important role in reconstruction to restore hand function. However, clinical outcomes after treatment with tendon autograft are generally poor as well because of complications such as adhesion formation. Although experimental studies have shown that intrasynovial tendons have better outcomes than extrasynovial grafts do[11-13], extrasynovial tendons usually are used clinically[14-17] because autologous intrasynovial tendons are rarely available for use as tendon grafts. Intrasynovial tendon allografts are another possibility[18-20], but fresh allografts may induce an immune reaction. To reduce immunogenicity and to facilitate storage, allografts may be subjected to repetitive freeze-thaw cycles and lyophilization[18,21-25], but the effect of lyophilization on allograft function is unclear.

Previous studies also have shown that tendon frictional force (gliding resistance) is an important factor influencing the outcome of tendon repair. Higher gliding resistance results in greater adhesion formation[26], and extrasynovial tendon has a higher gliding resistance than intrasynovial tendon does[27]. Treating the extrasynovial tendon surface with a carbodiimide-derivatized hyaluronic acid (cd-HA) and gelatin mixture has been shown to improve tendon gliding ability in vitro[28-30] and to improve digital function in vivo[31]. Such treatment can also improve the gliding of repaired intrasynovial tendons[32].

The overall goal of our research program is to improve outcomes after tendon repair and reconstruction through engineering of the tendon-tendon sheath interface in order to reduce friction and, consequently, adhesion formation. The purpose of the present study was to test the hypotheses that (1) an intrasynovial tendon prepared with repetitive freeze-thaw cycles and lyophilization has increased frictional force compared with fresh intrasynovial tendon and (2) the resulting lyophilized intrasynovial flexor tendon allograft modified with cd-HA-gelatin has decreased frictional force compared with untreated lyophilized tendon.

Materials and Methods

A total of thirty-six flexor digitorum profundus tendons from the second and fifth digits were harvested from the hind paws of nine adult mongrel dogs that were killed for other studies approved by our Institutional Animal Care and Use Committee. The tendons were randomly divided into three experimental groups. Twelve tendons were immediately assessed for both mechanical and morphological evaluation after harvesting; these tendons served as the normal tendon group (Group 1). The other twenty-four tendons were treated with five freeze-thaw cycles followed by lyophilization to render them acellular[21,24,33,34] and were stored at –80°C until testing. These twenty-four tendons were then randomly divided into two groups; in one group (Group 2) the tendons were treated with cd-HA-gelatin, and in the other group (Group 3) the tendons were not treated and served as the lyophilized tendon control group. The tendons were then evaluated mechanically and morphologically.

Tendon Preparation

After the dogs were killed, the tendons in Group 1 were tested as soon as possible after harvest, without any special preparation. The tendons in Groups 2 and 3 were immediately immersed into liquid nitrogen for one minute and then were thawed for five minutes in warmed saline solution at 37°C. This procedure was repeated five times, after which the tendons were lyophilized. Previously, this protocol has been shown to produce necrosis of 97% to 100% of all viable tenocytes[24]. After lyophilization, the tendons were stored at −80°C until testing.

Prior to mechanical testing, the tendons in Groups 2 and 3 were rehydrated in a saline solution bath for twenty-four hours at 4°C. Although our pilot data showed that the weight of the lyophilized tendons was restored to that of the pre-lyophilization level between five and twenty-four hours, a rehydration time of twenty-four hours was chosen on the basis of a published report in which the investigators noted that rehydration for twenty-four hours was preferable from the perspective of the restoration of mechanical properties[20].

Treatment of Group-2 Tendons with cd-HA-Gelatin

For the cd-HA-gelatin treatment group, the tendons were immersed in a combined solution of 1% hyaluronic acid and 1% 1-ethyl 1-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Sigma Chemical, St. Louis, Missouri)[35]/1% N-hydroxysuccinimide (NHS; Pierce Biotechnology, Rockford, Illinois)[36] with gelatin mixture for thirty seconds, after which the reaction was allowed to proceed for five minutes with the tendon wrapped in a towel moistened with phosphate-buffered saline solution. Excess reagent was washed off with phosphate-buffered saline solution. Sodium hyaluronate (Acros Organics, Pittsburgh, Pennsylvania) was dissolved in phosphate-buffered saline solution (pH 6) at 20 mg/mL. EDC/NHS activates the CO_2H in the hyaluronic acid molecule and forms the intermediate O-acylisourea, which can chemically bind to exposed amino groups on the tendon surface. This chemical modification of hyaluronic acid not only increases the half-life of the hyaluronic acid but also increases the binding strength between hyaluronic acid and the tendon surface[28,37-39].

Measurement of Frictional Force

The frictional force between the flexor digitorum profundus tendon and the proximal pulley of the digit was evaluated with use of a testing device that was developed previously[40-42]. The experimental setup consisted of one mechanical actuator, a linear potentiometer, and a tensile load transducer, which were connected to the proximal tendon end. Another tensile load transducer, a pulley unit, and a 4.9-N weight were connected to

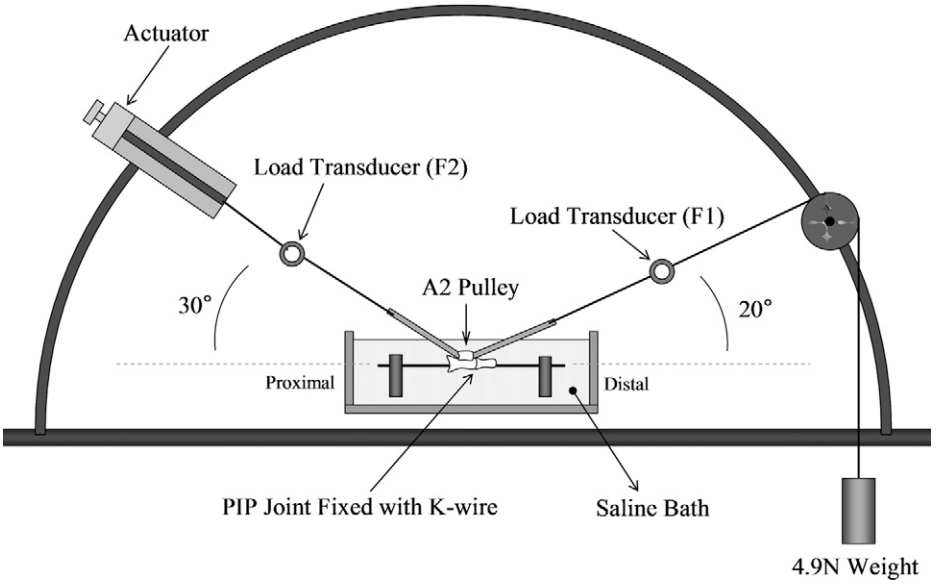

Fig. 1

Illustration depicting the testing device used to measure frictional force (gliding resistance). The actuator was positioned at an angle of 30° and the distal load-transducer was positioned at an angle of 20°. F1 = force 1, F2 = force 2, PIP = proximal interphalangeal, and K-wire = Kirschner wire.

the distal tendon end (Fig. 1). The resolution of the recorded output of the force transducers in this setup is <1 g. A normal canine proximal phalanx with an intact A2 pulley was secured on the custom-made device with the volar side upward (Figs. 2-A and 2-B) in a saline solution bath (Isotemp 202; Fisher Scientific, Houston, Texas) at 37°C. Each tendon was tested

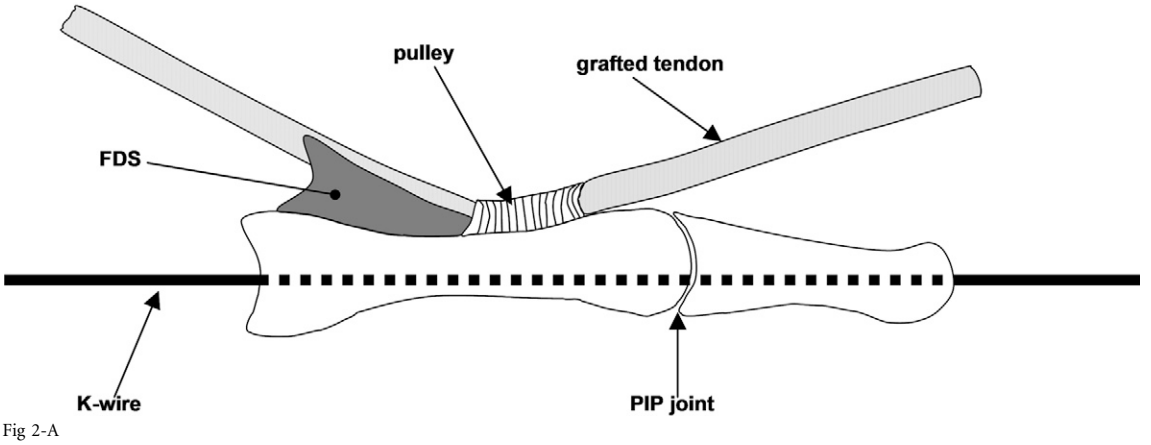

Fig 2-A

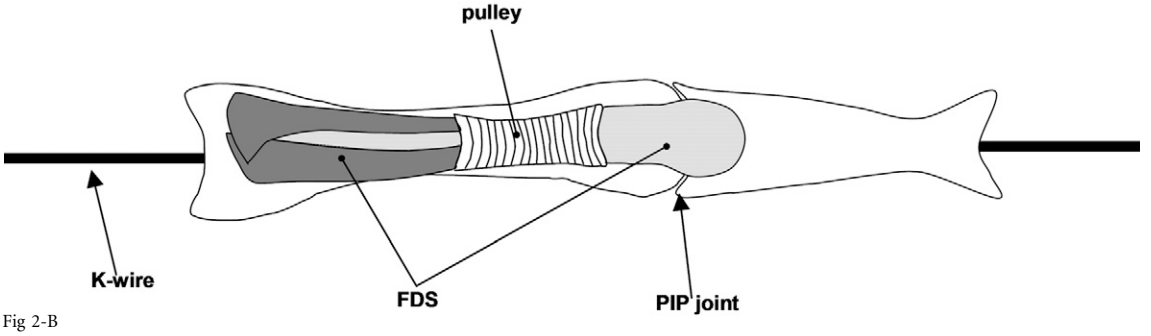

Fig 2-B

Figs. 2-A and 2-B Illustrations depicting the lateral view (Fig. 2-A) and top view (Fig. 2-B) of the pulley unit. The proximal phalanx with an intact A2 pulley was mounted in the device and was fixed in full proximal interphalangeal (PIP) joint extension with a longitudinal Kirschner wire (K-wire). FDS = flexor digitorum superficialis.

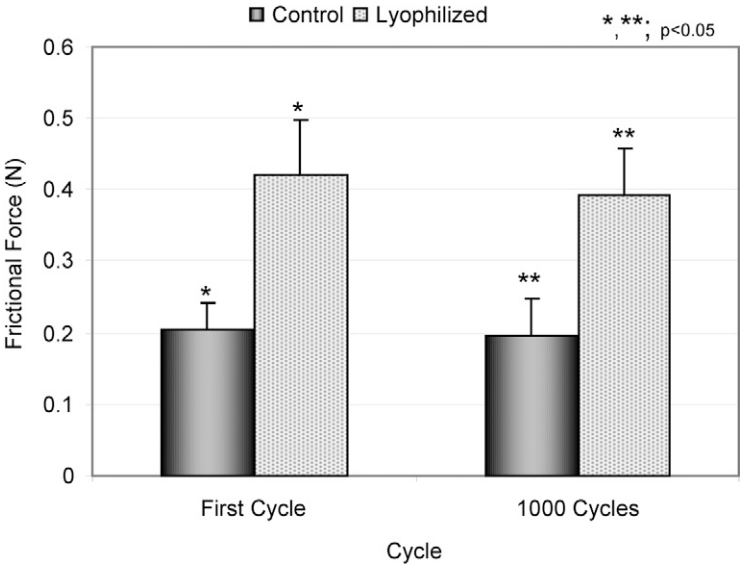

Fig. 3

Bar graph illustrating the frictional force (gliding resistance) for the groups of normal and untreated lyophilized tendons. The values are expressed as the mean and the standard deviation.

against its own phalanx and pulley. The tendon was then pulled at a rate of 2 mm/sec by the actuator against the weight with a fixed excursion of 16 mm, an estimate of normal canine tendon excursion based on previous studies[43]. The first two cycles of frictional testing were used to precondition the tendon and to remove any loose superficial reagent, and then data were collected during the third cycle (the first data-collection cycle). The force difference between the proximal and distal tendon ends represents the gliding resistance of the flexor digitorum profundus tendon against the A2 pulley. The gliding resistance was obtained with the equation $(F2_{flexion} - F2_{extension})/2$, as described previously[44]. The motion between tendon and pulley was repeated for 1000 cycles. The number of simulated repetitive cycles (1000) was determined on the basis of the estimated total number of postoperative passive motions in a six-week in vivo canine tendon rehabilitation model[31,43,45]. The

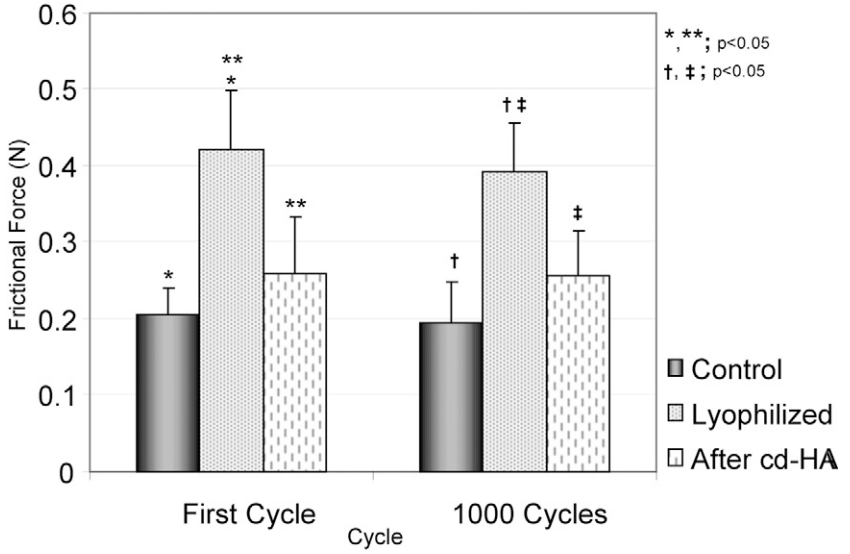

Fig. 4

Bar graph illustrating the frictional force (gliding resistance) for the groups of normal tendons, untreated lyophilized tendons, and lyophilized tendons treated with cd-HA-gelatin. The values are expressed as the mean and the standard deviation.

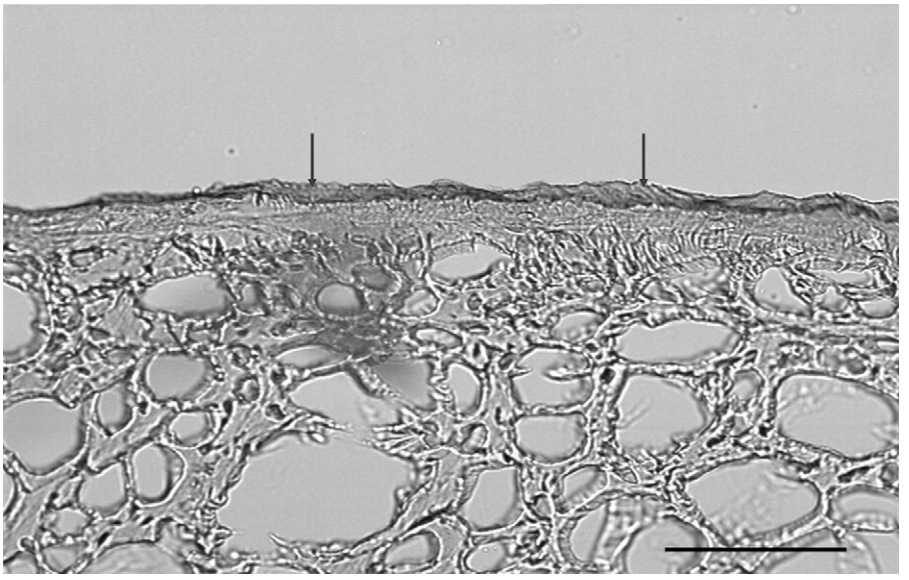

Fig. 5

Transmitted light microscopy image showing the appearance of residual hyaluronic acid binding on the tendon surface. The tendon surface was well covered with a thin layer of cd-HA-gelatin even after 1000 cycles of motion. An area that was well coated with cd-HA-gelatin (arrows) was identified on the surface of the transversely sectioned cd-HA-gelatin-treated tendon (scale bar = 100 μm).

data on the gliding resistance were recorded after every fifty cycles up to 500 cycles and then every 100 cycles up to 1000 cycles.

Evaluation of Residual Hyaluronic Acid Binding on the Tendon Surface

Following mechanical testing, a 5-mm longitudinal segment of the tendon portion that glided against the pulley was excised and was stained with biotinylated hyaluronic acid binding protein (HABP; EMD Biosciences, San Diego, California) to determine residual binding of hyaluronic acid. Tendons were embedded in Tissue-Tek (Sakura Finetek USA, Torrance, California), were sectioned transversely at 8 μm with use of a cryostat (Leica, Heidelberg, Germany), and were collected on supercharged glass slides. After washing in a 0.1-M phosphate-buffered saline solution with 3% Triton X-100 for five minutes, the sections were incubated in a blocking solution consisting of 1% bovine serum albumin in 0.1-M phosphate-buffered saline solution plus 0.5% Triton X-100 for twenty minutes. The sections were then washed with 0.1-M phosphate-buffered saline solution three times and were incubated in 0.2% HABP in 1% bovine serum albumin/0.1-M phosphate-buffered saline solution for two hours at room temperature. Negative control sections were incubated with a 1% bovine serum albumin/0.1-M phosphate-buffered saline solution instead of the biotinylated hyaluronic acid binding protein. The slides were washed with 0.1-M phosphate-buffered saline solution three times for five minutes and were incubated in avidin-biotin peroxidase (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, California) for thirty minutes at room temperature, followed by three washes in 0.1-M phosphate-buffered saline solution for five minutes. The sections were stained with the DAB Peroxidase Substrate Kit (Vector Laboratories) for 3.5 minutes, and then the slides were washed by repeated dipping in double-distilled water. The sections were dehydrated by means of serial washes in 80%, 95%, and 100% alcohol solutions, followed by two xylene washes. Each slide was examined with use of transmitted light microscopy (Nikon Eclipse E400; Nikon, Melville, New York) to evaluate for residual hyaluronic acid binding on the tendon surface.

Scanning Electron Microscopy

Three tendons in each group were prepared for scanning electron microscopy. The selected tendons were washed in phosphate-buffered saline solution and were fixed in a solution of buffered glutaraldehyde and osmium tetroxide. After dehydration in graded acetone, the specimens were coated with gold-palladium alloy and were examined with scanning electron microscopy (FE-SEM S-4700; Hitachi, Hitachi, Japan) in secondary electron mode at 3 kV. The surface of the tendon was qualitatively assessed for the smoothness of the tendon surface.

Statistical Analysis

The mean frictional force (and standard deviation) was determined for each treatment group. One-way analysis of variance was used for the comparison among the three treatment groups (the control group of normal flexor digitorum profundus tendons, the group in which lyophilized tendons were treated with cd-HA-gelatin, and the group in which lyophilized tendons were not treated). A Tukey-Kramer post hoc test for each pairwise comparison was used if there was a significant

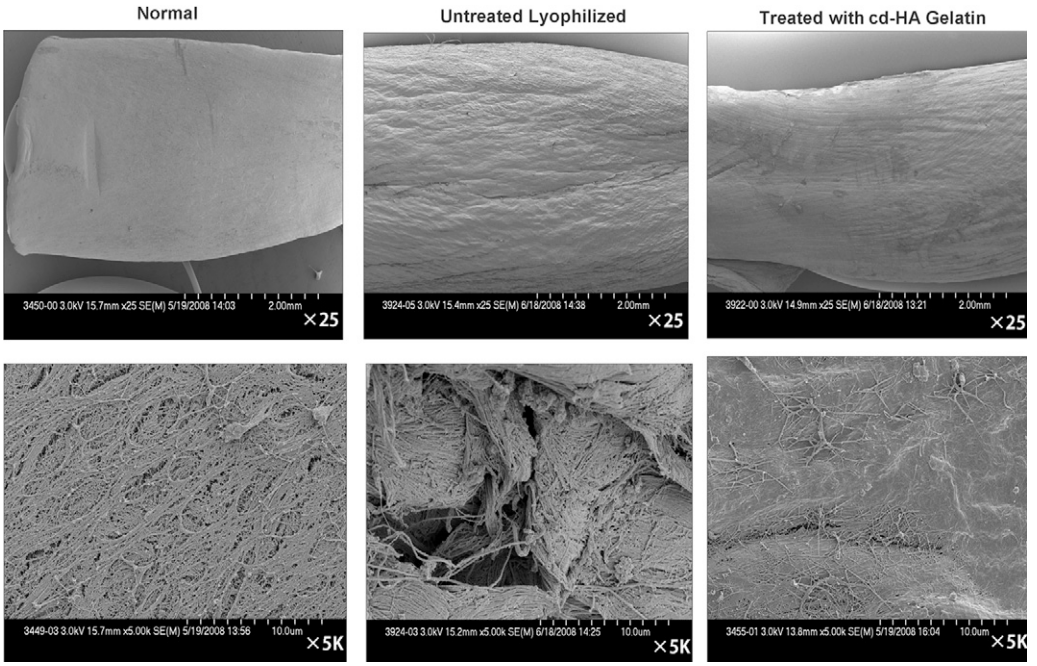

Fig. 6

Selected scanning electron microscopic images, made after 1000 cycles of tendon excursion, showing normal flexor digitorum profundus tendon, untreated lyophilized tendon, and tendon treated with cd-HA-gelatin. The top row of images was made at low magnification (×25), and the bottom row of images was made at high magnification (×5000). The surface of the lyophilized tendons appeared to be rough, whereas the surface of the lyophilized tendon that had been treated with cd-HA-gelatin appeared to be smoother.

difference among the three groups. A p value of <0.05 was considered to indicate significance in all cases.

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Results

The weight of the lyophilized tendon rehydrated in a saline solution bath at 4°C recovered to normal levels within three to four hours. The mean tendon weight (and standard deviation) was 0.87 ± 0.10 g before lyophilization, 0.27 ± 0.04 g after lyophilization, and 0.85 ± 0.10 g after three hours of rehydration. In the normal flexor digitorum profundus tendon group, the frictional force did not change significantly, even after 1000 cycles. The mean frictional force of normal flexor digitorum profundus tendon was 0.206 ± 0.035 N at the first cycle and 0.196 ± 0.052 N at 1000 cycles. The frictional force of the flexor digitorum profundus tendon immediately following lyophilization was significantly increased in comparison with that of the normal flexor digitorum profundus tendon ($p < 0.05$) (0.422 ± 0.076 N at the first cycle and 0.391 ± 0.066 N at 1000 cycles) (Fig. 3). The frictional force of the lyophilized flexor digitorum profundus tendon after surface modification with cd-HA-gelatin was significantly decreased in comparison with the untreated lyophilized tendon ($p < 0.05$) (Fig. 4). The frictional force of the lyophilized flexor digitorum profundus tendon treated with cd-HA-gelatin was 0.259 ± 0.074 N at the first cycle and 0.255 ± 0.061 N at 1000 cycles. There was no significant difference between normal flexor digitorum profundus tendon and lyophilized flexor digitorum profundus tendon treated with cd-HA-gelatin (Fig. 4). After 1000 cycles, the statistical relationship among the three groups remained the same. Transmitted light microscopy showed that the lyophilized tendon surface treated with cd-HA-gelatin was well covered with a thin layer of cd-HA-gelatin even after 1000 cycles of tendon excursion (Fig. 5). Scanning electron microscopy showed that the surface of untreated lyophilized tendons appeared to be rough but that the surface of the normal tendon and the tendon treated with cd-HA-gelatin appeared to be smooth, even after 1000 cycles of tendon excursion (Fig. 6).

Discussion

Because of its smooth surface, low friction, and durability, an intrasynovial tendon is the ideal graft tendon for the restoration of finger function[27,45,46]. However, because of limitations in the availability of suitable intrasynovial autograft, an alternative is needed. One possibility is intrasynovial allograft, but, to eliminate allograft immunogenicity, the donor graft cells must be destroyed. We used a freeze-thaw protocol because it is commonly used to prepare allografts when cell viability is not desired as well as to reduce immunogenicity. For the purpose of long-term preservation, lyophilization is often

used[18,21-25] because storage does not require refrigeration and the tissue remains useful for as long as ten years[18].

In the present study, we found that lyophilization changed tendon surface morphology and increased the frictional force, which may hinder tendon gliding when such tendons are used clinically. Although the reason for the tendon surface changes after lyophilization is not clear, it may be related to changes in collagen cross-linking or other surface macromolecules due to water loss. Even after rehydration, the frictional force in the lyophilized tendon remained significantly increased.

The modification of hyaluronic acid with use of the carbodiimide reaction to create new biopolymers by means of cross-linking with other biological molecules such as collagen has been widely investigated for applications such as anti-adhesion membranes[47-51], biodegradable scaffolds for tissue regeneration[52-55], or timed-release drug delivery vehicles[56,57]. The improvement in lyophilized tendon gliding ability with a surface modification with use of cd-HA-gelatin is another such potential application. Our results show that treatment with cd-HA-gelatin restores a smooth surface to the lyophilized tendon and restores the frictional force to control levels. Similar results have been seen with this engineered surface in extrasynovial and intrasynovial tendon that has not been lyophilized, as well as after tendon repair, both in vitro and in vivo[28-32,58,59].

The present study had some limitations. First, it was an in vitro investigation. The effect of this intervention on tendon allograft function in vivo is not known. Second, hyaluronic acid binding protein was used to qualitatively identify residual hyaluronic acid on the tendon surface. No quantitative evaluation was used. Third, although Potenza and Melone reported no difficulty in association with the use of lyophilized tendons that had been stored for as long as ten years[18], we have been unable to identify any other studies that have described the effect of storage on lyophilized tendon. In the present study, we chose two weeks as the storage period, and, therefore, we do not know the effect of longer-term storage. Finally, in theory, the repetitive motion may alter the viscoelasticity of the tendon and may in turn affect the frictional force. However, we only measured frictional force. Other tendon mechanical properties, such as tensile or compressive moduli, were not assessed.

In conclusion, serial freeze-thaw cycles followed by lyophilization alter tendon surface morphology and increase tendon frictional force. Surface modification with cd-HA-gelatin mitigated these adverse effects, restoring the graft to its control frictional force level and making a smoother tendon surface. We believe that the findings from the current study have important clinical implications. They suggest the possibility of improved outcomes after tendon grafting by providing an engineered, lubricated, off-the-shelf alternative to conventional extrasynovial autografts. Additional in vivo study is necessary to confirm that this surface modification can improve the outcome of lyophilized tendon allograft in vivo. ■

Jun Ikeda, MD, PhD
Chunfeng Zhao, MD
Yu-Long Sun, PhD
Kai-Nan An, PhD
Peter C. Amadio, MD
Orthopedic Biomechanics Laboratory,
Division of Orthopedic Research, Mayo Clinic,
200 First Street S.W., Rochester, MN 55905.
E-mail address for C. Zhao: zhao.chunfeng@mayo.edu

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