Investigating the histological and structural properties of tendon gel as an artificial biomaterial using the film model method in rabbits

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Abstract

Tendon gel, a substance formed during intrinsic tendon regeneration using the film model method, could be a promising artificial biomaterial for revolutionizing tendon healing treatments. Using a rabbit model, this study aimed to detect the properties of tendon gel by investigating the histological and structural differences among tendon gels under different preservation periods. Forty mature female rabbits were divided into four groups of ten rabbits. Achilles tendon rupture models were established using the film model method to obtain tendon gels. A tensile stress was applied to the tendon gel to promote maturation. Although the day-3 and -5 tendon gels were histologically more immature than the day-10 and -15 ones prior to the application of tensile stress, type I collagen fibers equivalent to those of normal tendons were observed in all groups after the tensile process. According to surface and molecular structural evaluations, day-3 tendon gels following tensile process were molecularly cross-linked, and thick collagen fibers similar to those present in normal tendons were observed. Structural maturation observed in day-3 tendon gels caused by traction was hardly observed in day-5, -10, and -15 tendon gels. Hence, the day-3 tendon gel was considered as a novel biomaterial with the best regenerative potential.

Introduction

Tendon regeneration following injury involves intrinsic and extrinsic regenerative processes *in vivo* [1]; more specifically, during the extrinsic regenerative process, neovessels and inflammatory cells invade the surrounding tissues, and fibroblasts are ultimately recruited to continue this regenerative mechanism [2]. Even in case of surgical repair, the strength of the tendon decreases as it regenerates from scar tissue—which is composed of fibroblasts—through an extrinsic regenerative process [3,4]. Thus, to solve this problem, animal-based and clinical studies [5,6] utilizing artificial ligaments and/or tendons as augmented materials have been performed, yielding encouraging results [7–9]. On the other hand, complications such as noninfectious effusions, synovitis, and foreign body reactions—often resulting in surgical failure—have been reported during treatment with such materials [9,10]. In addition to these complications, healing by artificial tendons and/or ligaments is different from normal healing; moreover, it is still not widely used in clinical practice. Therefore, new materials are needed for further development in this field.

Torigoe et al. [11] observed the intrinsic tendon regenerative process by placing the ends of transected murine Achilles tendons between two thin films; this technique, known as the *film model method*, was originally developed for evaluating the regeneration of peripheral nerves [12] as well as for observing tendon intrinsic regeneration. They also reported that a translucent gel-like substance—called *tendon gel*—was secreted from the cut tendon stump and it was a key material for tendon regeneration [11]; in addition, maturation of tendon gel induced by the application of mechanical stress was observed via electron microscopy [11]. Similarly, Ohashi et al. [13] reported that tendon gel matures physiologically and histologically under traction; moreover, an appropriate *in vivo* preservation period is needed for optimal traction-induced tendon gel maturation in murine models. These results support other previous reports showing the importance of applying mechanical force for regeneration of injured tendons [14,15].

Therefore, these findings open possibilities for the creation of new artificial tendons of biological origin and/or for promoting mature tendon regeneration using new artificial biomaterials *in vivo*. Clinically, this artificial biomaterial could be utilized to reinforce tendon components in treating several types of injuries, such as Achilles tendon ruptures and rotator cuff tears, per chance with potentially revolutionary outcomes.

However, to the extent of our knowledge, there are no studies concerning the histological and structural aspects of intrinsic tendon regeneration *in vivo* using animal models other than murine models. Therefore, to develop a novel intrinsic-regeneration-based technology, we focused on studying the properties of tendon gel; hence, we performed experiments using rabbits, which are medium-sized animals, to obtain more gel samples and examine their structures in detail. The core purpose of this study was to determine suitable *in vivo* preservation periods for the treatment of tendon injuries using the film model method in rabbits; in essence, the potential of tendon gel as an artificial biomaterial could be evaluated by identifying the best traction timing when the ability of the tendon gel is utilized to the maximum.

Results

Concerning the histology before the tensile process, a significant difference in the total histological evaluation score (0–8) among the four groups was detected using ANOVA ($p < 0.01$). The *post hoc* multiple comparison test revealed a significant difference between the groups euthanized before D5 and after D10 (D3, 2.0 ± 0.8 vs. D5, 2.7 ± 0.5 , $p = 0.20$; D3 vs. D10, 4.9 ± 0.7 , $p < 0.01$, D3 vs. D15, 6.0 ± 0.8 ; $p < 0.01$; D5 vs. D10, $p < 0.01$; D5 vs. D15, $p < 0.01$; D10 vs. D15: $p = 0.08$) (Supplementary Fig S1). Tendon gel specimens with HE and EVG stains are shown in Figs 1 and 2. The cell nucleus of the tendon gel changed from round to flat, moving inward along with maturation. Additionally, the gels turned red over time with EVG stain. The red color in the EVG stain suggests the presence of collagen fibers, with the amount of red increasing with the amount of collagen fibers. No invasion by new blood vessels was observed in any tendon gel. After the tensile process, collagen fibers arranged similarly to those in a normal tendon were observed in all the groups (Fig 3). In the immunohistological evaluation, type I collagen fibers in the tendon gel were demonstrated to exist in D15 before the tensile process although only the normal tendon was stained in the other groups (Fig 4). In contrast, type I collagen fiber was observed in every group after the tensile process. Type III collagen fibers in the tendon gel were verified to exist in D10 and D15 before the tensile process, but were barely observed in any of the groups after the tensile process. This result indicated that the tendon gels of all the groups had matured into collagen fibers composed mainly of type I collagen as a result of the tensile process. The AFM surface analysis results showed that there were almost no collagen fibers in D3 and D5 before the tensile process. However, in D10 and D15 before the tensile process, collagen fibers were thinner than those in normal tendons (D10: $51.4 \pm 3.6 \mu\text{m} \times 10^{-3}$; D15: $87.2 \pm 3.9 \mu\text{m} \times 10^{-3}$; Normal tendon: $281.0 \pm 3.9 \mu\text{m} \times 10^{-3}$). Moreover, collagen fibers in D3 after the tensile process were arranged as in normal tendons; in contrast, collagen fibers in D5, D10, and D15 were still thinner than those in normal tendons (Fig 5). Furthermore, a

significant difference in the thicknesses of collagen fibers after the tensile process was detected between D3/D5 and D10/D15, with D3 and D5 having thicker fibers. In addition, the collagen fibers in D3 were significantly thicker than those in D5. However, there was no significant difference between the collagen fibers in D3 and those in normal tendons (Supplementary Fig S2). According to molecular structural analysis results, a -CHO peak close to $1,750\text{ cm}^{-1}$ indicating the occurrence of cross-linking of collagen fibers was observed in all groups after the tensile process, which was not observed either before the tensile process or in normal tendons (Fig 6). In addition, this peak tended to be larger in D3 than in D5, D10, and D15.

Discussion

The most significant findings of this study were that the gel in D3 before the tensile process showed better regeneration-promoting ability—through application of a traction force—than the gels in D5, D10, and D15 before the tensile process. Although the tendon gels in all the four groups were histologically mature after the application of a traction force, those in D3 and D5 before the tensile process were significantly immature compared to the gels in D10 and D15 before the tensile process. Based on the histological and immunohistological evaluations, we inferred that maturation of the tendon gel through mechanical stress induced by muscle contraction after the junction between the tendon parenchyma and tendon gel started from the fifth day onwards. On the other hand, surface and molecular analyses indicated that tendon gels in D3 before the tensile process, although histologically immature, were actually mature like in normal tendons owing to the traction force unlike the gels in D5, D10, and D15. Therefore, the tendon gel in D3 showed high potential as a novel biomaterial for applications in regenerative medicine.

Concerning histological properties, tendons comprise 55–70% of water and 30% of extracellular matrix, which in turn is primarily composed of aligned type I collagen fibers as well as other components, such as elastin, decorin, biglycan, and fibromodulin [21]. Furthermore, tendons generally contain only a few flat tenocytes [22,23], but in the gels of D3 and D5 observed in this study, many round cells—also considered tenocytes—were actually observed. According to the research by Ohashi et al. [13] with murine models, tenocytes were generated at the edges of the tendon in the early stage; subsequently, they were spread throughout the gel and their shape was changed from round to flat, indicating maturation. These findings are similar to ours. In this study, we observed a histologically significant difference between tendon gels in D3/D5 and D10/D15 before the tensile process. We attributed this result to the completion of the connecting junctions between the tendon parenchyma and tendon gel after the fifth day in many tendon gels. Consequently, the contraction of the gastrocnemius exerted traction stress on the tendon gel, which was thought to promote morphological changes in the tenocytes and collagen maturation, as revealed by Torigoe et al. [11] and Ohashi et al. [13] using murine models.

With regards to the immunohistological evaluation, interestingly, collagen fibers are organized in hierarchical levels in tendon tissue; more specifically, tropocollagen—a triple-helix peptide chain—gathers in fibrils, fascicles, tertiary bundles, and ultimately in the whole tendon [24]. When the extrinsic and

intrinsic regeneration processes are mixed—like in clinical scenarios—tenocytes and collagen fibers become aligned in the direction of mechanical stress with concurrent decreases in type III collagen and water content in the forming scar tissue [4]. Furthermore, the increased activity of collagenases eventually supports the resorption of type III collagen, as well as its subsequent replacement with type I collagen over an extended period [2,25]. In this study, no extrinsic regeneration occurred owing to the utilization of the film model method, and we added forced traction to the tendon gel in the tensile process which is not a clinical situation. Therefore, the results of our immunohistological evaluation are not considered similar to those of the natural regeneration occurring [4] in clinical scenarios. However, this result shows that the tissue composed of type I collagen, as in the case of a normal tendon, is regenerated in a short period of time by applying a traction force to the tendon gel produced by the intrinsic regeneration. Similar to the results of the EVG staining, the results of the immunohistological evaluation showed type I collagen only in D15 and type III collagen in D10 and D15 before the tensile process, and no collagen fiber at all in D3 and D5. This fact is supported our hypothesis that the maturity of the tendon gel was gradually achieved after the junctions between the tendon parenchyma and tendon gel were formed.

Concerning the molecular structural evaluation performed in this study, the presence of peaks close to $1,750\text{ cm}^{-1}$ was confirmed in all the four groups after the tensile process. The peak was higher in D3 than in D5, D10, and D15. According to the cross-linking models [26–28], the FT-IR spectrum is analyzed as follows: the first cross-linking reaction is known as *aldol condensation* where a saturated -CHO group is formed [26], resulting in a peak close to $1,750\text{ cm}^{-1}$. Furthermore, the height of this peak relates to the extent cross-linking. Therefore, no peak was found in normal mature tendons [20], as shown by our results. As highlighted by a previous study [29], while the structural stability of collagen fibers is affected mostly by intermolecular cross-links rather than intramolecular cross-links, collagen fibers are more strongly bound together when more fibers are cross-linked in type I collagen. In addition, mechanical stress is an essential element for maturation of collagen fibers [20,30]. Kwansa et al. [31] also indicated that application of a traction force promotes cross-linking between the amino- and carboxy-terminal extremities of the collagen fibers, which structurally strengthen the whole collagen tissue. Therefore, the result of this study showed that intermolecular cross-links occurred more frequently in D3 than in the other groups.

According to our surface analysis using AFM, thick collagen fibers similar to those in normal tendons were identified in D3 after the tensile process. We considered that the intermolecular cross-links formed in collagen on applying traction force actually led to collagen maturation; moreover, the presence of thick collagen fibers, equivalent to those in normal tendons, was confirmed even on the surface of the tendon gel. On the other hand, in D5, D10, and D15, as the -CHO peak was small, the number of intermolecular cross-links was also considered small as thick collagen fibers were not detected during surface analysis. According to molecular and surface analyses, the D3 tendon gels before the tensile process can form collagen intermolecular cross-links and thick collagen fibers through traction force and subsequently undergo maturation.

Considering the histological and structural evaluations together, the histologically immature tendon gels, such as those in D3, were considered to be in a pre-mature stage, where histological and structural maturity would ultimately be achieved through traction force, in contrast to D10 and D15. As the collagen in tendons is organized in hierarchical levels [24], we believe that the intermolecular cross-linking occurred along the traction direction in the tensile process because the tendon gels in D3 before the tensile process contained many tropocollagen molecules. Conversely, collagen molecules were cross-linked in multiple directions in D10 and D15 owing to the multidirectional traction forces exerted by muscle contraction *in vivo*; consequently, the number of cross-links after the tensile process *in vivo* was small and the collagen fibers were thin. Although the tendon gels in D5 before the tensile process were also immature histologically and thick collagen fibers—albeit thinner than those in normal tendons—were observed after applying traction force, a significant difference between D3 and D5 after the tensile process was detected in structural analysis. We believe that although immature, D5 was more mature *in vivo* than D3. Therefore, the tendon gels in D3 before the tensile process are more suitable for maturation using traction force and have better regenerative potential as artificial biomaterials. In a preliminary study, day-1 and day-2 tendon gel models (three tendon gel samples for each day) were also prepared; however, no tendon gel of appreciable size could be obtained.

An advantage of this study was that the animal experiments were conducted using the film model method; together with the careful removal of both paratenon and synovial tissues, this method eliminated extrinsic regeneration activity, which inhibits the intrinsic regeneration process. Therefore, elastic fibers or new blood vessels suggesting extrinsic healing [4] were histologically detected in the tendon gel specimens. Furthermore, the possibility of regenerating tendons histologically and structurally similar to those before injury was actually verified through application of traction force to the early-stage tendon gel in D3. We believe that our findings can lead to two possibilities in clinical scenarios: first, if an *in vivo* environment capable of removing the extrinsic regeneration component is created in future—perchance using the inhibiting factors of the vascular endothelial growth factor (VEGF) [32,33] or some inflammatory factors [34]—then tendons similar to those before injury could be regenerated. Second, the early-stage tendon gel could potentially be used as an artificial biomaterial because it could form an artificial tendon through the application of traction force. Furthermore, owing to the characteristics of the thus-matured tendon gel, we believe that new therapeutic options could be ultimately added to other treatments for tendon injuries, such the ones involving stem cells, growth factors, collagen fiber scaffolds, and/or gene transfer among others [35–38]. In future, the performance of the early-stage tendon gel as an artificial biomaterial should be verified through clinical research. Nonetheless, we believe that this study provides useful basic knowledge concerning scar-free intrinsic tendon regenerative processes.

However, there were some limitations in our study. First, immobilization of the lower limbs after surgery was not performed; consequently, differences in the maturation of tendon gels depending on the activity of the rabbit might have occurred. However, as the variation in the histological maturity score of tendon gels in each group was rather small, it is unlikely that the study results were significantly affected by this limitation. Second, the stressing technique was based only on a constant unidirectional traction force; therefore, actual muscle contraction—which consists of cyclic movement patterns—could not be

reproduced, and additional research using cyclic traction should be conducted for achieving more clinically relevant results. Third, as tenocytes— eventually related to intrinsic healing—were not specifically stained, proving that the tendon gel cells were tenocytes was not possible. Nevertheless, as the paratenon was removed and no new blood vessels were observed in the tendon gel, tenocytes were considered present in the tendon gel; however, this aspect must be investigated further in future studies. Fourth, no statistical comparison among the four groups after the tensile process was performed for histologic evaluation, as the tendon gel after the tensile process was separated from the tendon. Therefore, the preparation of tissue specimens oriented in the fiber direction was rather difficult, and it was not possible to judge either the C or J area using the scoring system. However, tendon gels after tensile process uniformly changed according to the histological evaluation, and no significant difference was found. Finally, some problems related to the generation period of tendon gels in human tendons and the methodology for creating an intrinsic-healing-only environment *in vivo* must be solved to facilitate future clinical application of our findings.

Conclusions

The histological and structural properties of tendon gels was evaluated for groups with different preservation periods. The tendon gel in D3 was found to histologically and structurally mature in the same way as a normal tendon when subjected to traction force, such that we can state that it offers the best regenerative potential among the new biomaterials addressed in this study.

Methods

Animal experiments

All animal experiments were approved by the Animal Experimental and Use Committee of the Institute for Experimental Animals, Advanced Science Research Center of our institution (approval No. AP-173823), and the all experiments were performed according to the ARRIVE guidelines and the institution's animal experiment regulations.

A total of 40 skeletally mature female Japanese white rabbits (Kitayama Labs, Nagano, Japan, 15–17-week-old) with a mean weight of 3.2 kg (range, 3.0–3.5 kg) were included in the study. The animals were divided into four groups of 10 rabbits each, according to the *in vivo* preservation periods of the film; the preservation periods for the four groups were 3, 5, 10, and 15 days. The sample size was determined by ethical restrictions on the number of experimental animals allowed.

As shown below, intrinsic regeneration models of rabbit Achilles tendon were created using the film model method, and tendon gels were obtained thereafter. Then, a tension force was applied on the tendon gel to promote maturation; histological and structural evaluations of the tendon gel were performed before and after the tensile process to understand both the *in vivo* and tension-driven maturation mechanisms.

Surgical procedure and obtainment of tendon gel

General anesthesia was induced through preoperative intramuscular injection of a mixture containing 10 mg (2.0 mL) of midazolam and 1.0 mg (1.0 mL) of medetomidine. Subsequently, the fur from the hindlimbs, ranging from the gastrocnemius muscle to the Achilles tendon, was shaved; local anesthesia using 30 mg of xylocaine (1.0%, 3.0 mL) per leg was administered, and the surgery was performed using aseptic techniques. Each animal was placed in a prone position on the operating table; in order to create an Achilles tendon rupture model according to the film model method, we bilaterally transected part of the Achilles tendon close to its insertion to the calcaneus on the medial head of the gastrocnemius. After the gastrocnemius muscle belly, the synovium, including the paratenon and the sheath around the tendon [16], was removed furthermore, the proximal end of the transected tendon was inserted between two thin fluorine films (25 μ m, 15 $\times$ 20 mm, Aflex 25 N NT, Asahi Glass, Tokyo, Japan) and fixed using 6-0 nylon suture (Keisei Medical Industrial Co., Ltd., Tokyo, Japan) [11]. Only the proximal end of the collagen bundle was placed on the film after tenotomy; the operated tendon was then moistened with several drops of Ringer's solution and covered with another film. The four corners as well as the ends of the films were also fixed using 6-0 nylon suture with at least eight stitches (Fig 7a). After surgery, no external brace was left on the hindlimbs. The wrapped tendon ends were left in the body for 3 (day-3 group: D3), 5 (day-5 group: D5), 10 (day-10 group: D10), and 15 days (day-15 group: D15) after surgery. All the 10 rabbits per group (20 limbs) were euthanized on day 3, 5, 10, and 15 after tendon surgery to extract the tendon gels (Fig 7b); thus, 20 tendon gel samples were obtained per group.

Tensile process for tendon gel

The tensile process was conducted on 10 tendon gel specimens per group. After film removal and separation from the tendon, only the tendon gels were set on the tensile unit, which consisted of an originally designed specimen holder and traction system (Supplementary Fig S3a). Drops of Ringer's solution were added onto the tendon gel, and the specimens were placed away from the sunlight, under normal temperature and pressure conditions. The tendon gel specimens were pulled with a sustained 1-N load nearly up to breakage (Supplementary Fig S3b). This process was recorded with a digital camera every 30 min. Consequently, we obtained 10 tensed tendon gel specimens per group.

Histological evaluation

The tendon gel specimens were subjected to hematoxylin-eosin (HE) staining, elastica van Gieson (EVG) staining, and immunohistologic staining with type I and III collagen before and after the tensile process. The histological maturation of the tendon gels before the tensile process was assessed using a semi-quantifying original scoring system based on the Bonar scale with HE and EVG stains [17,18], which consisted of four parameters evaluated with a maximum of eight points.

The following parameters of the tendon gels before and after the tensile process were examined using a light microscope (KEYENCE BZ-900, Keyence, Osaka, Japan) at 200 $\times$ magnification: (1) cell count ratio between the tendon parenchyma/tendon gel junction (J) and the central part of the tendon gel (C), (2) ratio between round and flat cells in C, (3) number of connecting junctions between the tendon parenchyma and tendon gel, and (4) amount of collagen fibers. Parameters (1), (2), and (3) were

evaluated using HE stain, and parameter (4) was evaluated using EVG stain. Red regions using the EVG stain suggested the presence of collagen fibers. These regions become more apparent as the amount of collagen fiber increases. These parameters were rated using numerical scores that were used as indices of tendon maturity: (1), 0–1 point; (2), 0–2 points; (3), 0–2 points; and (4), 0–3 points (Table 1). However, as the tendon gels after the tensile process had no connecting junctions between the tendon parenchyma and tendon gel and as it was difficult to confirm the ratio of the positions and shapes of the cells, the numerical scoring system was not suitable for use. For the immunostaining, formalin-fixed, paraffin-embedded tissue sections were stained using a standard protocol as described previously [19]. Goat anti-collagen I (goat IgG, cat. no. 1310-01; Southern Biotech) and mouse monoclonal anti-collagen III (mouse IgG₁ (clone FH-7A), cat. no. ab6310; Abcam) were used as primary antibodies, and the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. The confocal imaging was carried out using an inverted Nikon Eclipse Ti2 confocal microscope (Nikon Instruments/Nikon Corp., Tokyo, Japan) equipped with an Andor Dragonfly spinning-disk unit, Andor EMCCD camera (iXon DU888; Andor Technology Ltd., Oxford Instruments), and a laser unit (Coherent Inc., Santa Clara, CA, USA). Excitation for the DAPI and Alexa 488 chromophores was achieved using a 405- and 488-nm laser, respectively. The presence of type I and III collagen fibers before and after the tensile process was evaluated. In this evaluation of both the type I and III collagen fibers, strong green color, as observed in the normal tendon with collagen 1 staining, was positive for collagen staining, whereas weak green color was considered to be an artifact and evaluated as negative. Blue color indicated the nucleus.

The observation sites were randomly selected from both the J and C areas of the tendon gel specimens, which were independently evaluated by two examiners; the maturity scores were defined as the mean values of scores determined by both examiners.

Table 1. Histological maturity scores of the tendon gels before the tensile process (0–8 points).

	Score 0	Score 1	Score 2	Score 3
Cell count ratio (J/C)	> 1	≤ 1		
Flat-cell-to-round-cell ratio	0	< 1	≥ 1	
Number of connecting junctions between tendon parenchyma and tendon gel	0	1	2	
Amount of collagen fibers	No strain	Mild strain	Moderate strain	Equivalent to normal tendon

Structural evaluation

The surface structures of specimens—particularly collagen fibers—were evaluated using atomic force microscopy (AFM; SPM-9700, SHIMAZDU, Japan). The thicknesses of the collagen fibers in the four groups were evaluated and compared. A cantilever was used for surface observations in the tapping mode. Molecular structural analysis was carried out using microscopic Fourier transform infrared (FT-IR: IRT5000, FT/IR4200A, JASCO, Japan) spectroscopy. This method can detect the presence and amount of cross-linked collagen fibers using the peak of the fatty acid aldehyde group (-CHO), as reported by Kuzumaki et al. [20]. In FT-IR analysis, the aperture diameter was 50 × 50 μm and the integration count was 40. We also evaluated the surface collagen fibers and molecular structures of the tendon gel specimens in each group before and after the tensile process, as well as the normal collagen fibers of rabbit Achilles tendons. The specimens for the AFM observations and FT-IR measurements were placed on a moist glass substrate. We tried not to damage the tendon gels during this placement to avoid affecting the experimental results.

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences for Windows (SPSS version 23.0; IBM, Armonk, NY, USA). Analysis of variance (ANOVA) was performed on the histological evaluation scores and the thicknesses of the collagen fibers in the four groups, and statistically significant (i.e. $p < 0.05$) items were examined using a *post hoc* multiple comparison test (i.e. Dunnett test). A prior power analysis for the sample size was performed. It revealed that for achieving an effect size of 0.56, a power of 0.8 and a significance level of 0.05 for 40 individuals were required.

Declarations

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Author contributions

All authors made a significant contribution to the research concept, design, data collection, analysis and interpretation of data, critical correction of important intellectual content, and final approval of the submitted version.

The specific contributions were as follows: Conception and design of research: KS, JN, TK, and KT; Data acquisition: KS, and TY; Data analysis and interpretation: KS, TY, and YO; Drafting of the article: KS, JN, TK, KT, and HT; Final approval of submitted version: KS, JN, TK, KT, and HT.

Competing interest

The authors declare no competing interests.

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Data availability

The datasets generated during and/or analyzed during this study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Evaluation of hematoxylin-eosin stain in all groups. (a), (d), (g), and (k): View of tendon gels in low-power field at 40× magnification. (b), (e), (h), and (l): Magnified view of junction between tendon and tendon gel at 200× magnification. (c), (f), (j), and (m): Magnified view of central part of tendon gel at 200× magnification. white arrow: flat cells; black arrow: round cells; T: tendon; G: tendon gel.

Figure 2

Evaluation of elastica van Gieson stain in all groups. D3 (a) and D5 (b) were not stained, D10 was mildly stained (c), and D15 was moderately stained (d).

Figure 3

Histological evaluation after the tensile process. (a) Hematoxylin-eosin (HE) and (b) elastica van Gieson (EVG) stain of D3; (c) HE and (d) EVG stain of D5; (e) HE and (f) EVG stain of D10; (g) HE and (h) EVG

stain of D15. The amount of connective tissue fibers clearly increased following HE staining, and all the tendon gels after the tensile process were EVG-stained equivalently to a normal tendon. Examples of normal tendons following (i) HE staining and (j) EVG staining.

	Type I collagen evaluation		Type III collagen evaluation	
	Before tensile process	After tensile process	Before tensile process	After tensile process
D3
D5
D10
D15

Figure 4

Immunohistological evaluation with type I and III collagen before and after the tensile process. In both staining methods, the presence of each collagen type is indicated by the green area. The presence of type I collagen was validated for all the groups after the tensile process, although it was confirmed only in D15 before the tensile process. Similar to the type I collagen staining, the presence of type III collagen was confirmed in D10 and D15 before the tensile process. There were barely any type 3 collagen fibers in any of the groups after the tensile process. Green color: area where collagen is present; Blue color: nucleus; T: tendon; G: tendon gel.

Figure 5

Surface analysis using atomic force microscopy in each group. There were almost no collagen fibers in (a) D3 and (c) D5 before the tensile process. In (e) D10 and (g) D15 before the tensile process, collagen fibers (white arrows) were identified, albeit thinner than those in (i) normal tendons (black arrow). In contrast, the arrangement of the collagen fibers equivalent to that of normal tendons (white arrowhead) was demonstrated in (b) D3 and (d) D5 after the tensile process. The arrangement of the collagen fibers was also noted in (f) D10 and (h) D15, although fibers were thinner than those in (i) normal tendons.

Figure 6

Results of molecular structural analysis. A -CHO peak close to 1,750 cm^{-1} indicating cross-linking between collagen fibers was observed in all groups after the tensile process (black arrows), which was not observed before the tensile process and in normal tendons. This peak tended to be higher for D3 than for D5, D10, and D15.

Figure 7

Surgical procedure and obtainment of tendon gel. (a) Film model method using the medial head of the gastrocnemius tendon. (b) Tendon gel specimen. MH: medial head; LH: lateral head.

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