Original article

Collagen membrane alleviates peritendinous adhesion in the rat Achilles tendon injury model

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Keywords: *collagen membrane; Achilles tendon rupture; peritendinous adhesion; tendon repair*

Background Tendon adhesion is one of the most common causes of disability following tendon surgery. Therefore, prevention of peritendinous adhesion after surgical repair of tendon is a major challenge. The aim of this study was to explore the possible application of a collagen membrane for the prevention or attenuation of peritendinous adhesions.

Methods Sprague-Dawley (SD) rat Achilles tendon was cut and sutured by a modified Kessler's technique with or without the collagen membrane wrapped. Macroscopic, morphological and biomechanical evaluations were applied to examine the recovery of the injured tendon at 4 and 8 weeks after surgery.

Results The surgery group wrapped by collagen membranes had a better outcome than the group with surgery repair only. In the collagen membrane-treated group, less adhesion appeared, stronger tensile strength was detected, and more tendon fibers and collagen I expression were observed morphologically.

Conclusion Wrapping the tendon with a collagen membrane may be an efficient approach for tendon repair and preventing tendon adhesion after its ruptures.

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Achilles tendon rupture often occurs in 30–50-year old patients. With rapidly growing traumatic damages to tendons and ligaments, it has accounted for about 35% of all tendon injuries.[1] Ligaments and tendons have poor regenerative capacity despite recent improvements in surgical and rehabilitative techniques. The techniques for the repair of tendon injury include surgical treatment, biological treatment, physical therapy, biomaterials of adjuvant therapy and so on.[2] Despite improvements in surgical techniques and postoperative rehabilitation programs,[3] the formation of fibrous adhesions between the healing tendon and the surrounding tissues is still the most common complication after lacerated tendon repair. Currently, the modalities used for preventing adhesions without affecting the healing process include local injection of growth factors or platelet concentrate,[4] local application of extracorporeal shock wave therapy or ultrasound therapy,[5] systemic administration of certain anti-inflammatory drugs[6] or local injection of bone-marrow-derived mesenchymal stem cells.[7] Thus, how to induce regeneration of large diameter collagen fibers and the mechanical properties of the regenerative tendon are currently major challenges in tendon repair.

In particular, the use of a barrier during surgery to protect raw tissue surfaces has been shown to be one of the most effective methods of preventing tendon adhesions. The ideal barrier should be easy to use, absorbable, biocompatible, and not interfere with tendon healing. Besides, maintaining tendon movement and the tendon's mechanical properties are also necessary. Several biomaterials have been reportedly applied both in clinical and basic research. But only collagens have been approved for clinical application by American Food and Drug Administration (FDA). Collagen, characterized by low immunogenicity, excellent biocompatibility and biodegradability, has been widely applied in clinical applications for tissue regeneration, such as neural regeneration,[8,9] chronic skin wound recovery,[10] bone regeneration and root coverage procedures and so on.[11,12]

In this study, we explored the application of collagen membranes in the rat Achilles tendon injury model for the prevention or alleviation of peritendinous adhesion and guidance of Achilles tendon regeneration.

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METHODS

Preparation of collagen membranes

Collagen (Shandong Zhenghai, China) with porous stereoscopic structure was prepared from bovine skin, freeze-dried and sterilized by 12 kGy Co^{60} irradiation and cut into 4 mm×4 mm×0.5 mm membranes, following a well-established method.[13] Scanning electron microscopy (SEM) was used to visualize the surface features of collagen membranes.

Rat model of Achilles tendon rupture

Male Sprague-Dawley rats, (250±18) g, were used in this experiment. All animals were housed in standard cages, following the American NIH Guide for Care and Use of Laboratory Animals. The research protocol was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

All procedures were performed under sterile conditions in an animal operating facility at a room temperature of 21°C and a relative air humidity of approximately 60%–65%. The rats were intraperitoneally anesthetized with ketamine (50 mg/kg) and diazepam (50 mg/kg). The skin on the left hind limb was shaved and washed with Anerdian. A horizontal skin incision was made directly over each Achilles tendon. The tendon was isolated by blunt dissection from the underlying tissue, with a small curved forceps, which was left extended beneath the tendon to prominently expose it. A complete transverse incision was made with a surgical blade on the middle region of each Achilles tendon (1.0 cm from the Achilles tendon bone end). The damage was repaired by a modified Kessler's technique with 5-0 nylon sutures. The suture was not completely closed, but had a 2 mm gap (Figure 1). The animals were randomly divided into three groups, sham group without tendon incision (G1, *n*=40), surgery group (G2, tendon incised and sutured only, *n*=40), collagen membrane-treated group (G3, tendon incised, sutured and wrapped with collagen membrane, *n*=40). After surgery, discomfort due to pain or infection was not encountered in any of the groups.

Macroscopic evaluation of peritendinous adhesion formation

At 8 weeks after operation, peritendinous adhesion was evaluated macroscopically by the criteria described by Tang et al[14] summarized in Table 1.

Table 1. Criteria for histological evaluation of peritendinous adhesions

Points	Features of adhesion
Quantity	
0	No apparent adhesions
1	A number of scattered filaments
2	A large number of filaments
3	Countless filaments
Quality	
0	No apparent adhesions
1	Regular, elongated, fine, filamentous
2	Irregular, mixed, shortened, filamentous
3	Dense, not filamentous
Grading of adhesions	
0	None
1–2	Slight
3–4	Moderate
5–6	Severe

Biomechanical evaluation

At 4 and 8 weeks after surgery, the rats were killed and the Achilles tendons were harvested. All soft and hard tissues were carefully dissected from the calcaneus-Achilles tendon complex. The lateral portion of the Achilles tendon was removed at the musculo-tendinous junction, leaving the medial portion of intramuscular tendinous fibers, the medial Achilles tendon, and the calcaneus complex. The biomechanical experiments were conducted on a mechanical testing system (E1000, Instron, USA), with specially designed grips for connecting and testing the soft tissues. Each tendon was securely held by the clamps and positioned with the healing side located in the center between the clamps. The tendon was pulled at 10 mm/min until terminal rupture occurred, and the tensile failure load force was recorded as the maximum tension force.

Histological evaluation and immunohistochemistry (IHC)

The rats were dislocated and the Achilles tendons were collected. The specimens were fastened by their ends to a firm base in order to preserve tendon anatomy and then fixed in 10% formaldehyde. After embedded in paraffin, 5 μm-thick sagittal sections were obtained and stained

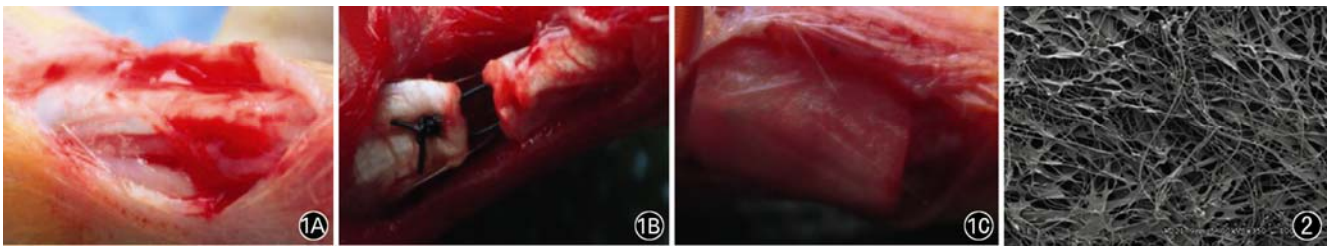

Figure 1. A complete transverse incision was made at the middle region of each Achilles tendon in SD rats. The injury was repaired by a modified Kessler's technique with or without wrapped collagen membrane. **1A:** G1 group, sham group without tendon incision. **1B:** G2 group, surgery group, tendon incised and sutured. **1C:** G3 group, collagen membrane-treated group, tendon incised, sutured and wrapped with collagen membrane.

Figure 2. Morphological image of the collagen membrane obtained by scanning electron microscopy (Model S-2500 Hitachi, Japan. Original magnification ×5000).

with hematoxylin and eosin (HE) for histological evaluation. Images were taken by a light microscope (Zeiss, AxioImager M1, Germany).

Immunohistochemical staining with rabbit anti-rat collagen type I (Abcam, USA) was performed. The sections were visualized and images were collected by a light microscope. Brown staining was regarded as a positive reaction. The relative expressions of collagen type I were quantified by Image-Pro Plus Version 6.0 for Windows software. The darker the brown staining was, the less optical density (OD) value it had, following the instruction of the software.

Statistical analysis

Data were expressed as mean±standard deviation (SD). Biomechanical values of groups were compared using analysis of variance statistics. All histological measurements were performed by two independent observers and an average density for positive staining was taken for the subsequent analysis. Statistical Package for Social Science (SPSS 17.0, USA) for data processing was used. Statistically significant values were defined as P <0.05.

RESULTS

Collagen membrane has a porous structure

The biomembranes used here were prepared using a special process and structural improvement method. We observed three dimensional porous structures of the collagen membranes with SEM (Figure 2).

Collagen membrane alleviated peritendinous adhesion formation

In each group, operations went on well and the wounds of all rats healed well without infection after surgery. Four weeks after the operation, the collagen membrane was absorbed completely. At 8 weeks, the severity of tendon adhesion was scored according to the criteria summarized in Table 1. 75% (15/20) of tendons showed no adhesion formation and 25% (5/20) demonstrated slight adhesion formation in the G1 group. In the G2 group, 15% (3/20) of tendons had slight adhesion formation, 55% (11/20) of tendons had moderate adhesion formation, and 30% (6/20) had severe adhesion formation. In the G3 group, 40% (8/20) of tendons showed no adhesion formation, 50% (10/20) had slight adhesion formation and 10% (2/20) of tendons demonstrated moderate adhesion formation. Compared with G2 group, the degree of tendon adhesion in the G3 group was significantly lower (Table 2, P <0.05). These data suggest that collagen membrane has good biocompatibility and alleviates tendon adhesion after injury.

Collagen membrane improved maximum tension force of injured tendons

The average values of maximum tension force at 4 and 8 weeks after operation are summarized in Table 3. As time went on, the injured tendons improved by different degrees in both G2 and G3 groups. Compared to the average tension force of G1 group at 4 weeks after operation, that of G2 or G3 was significantly lower (P <0.05, respectively); while the force of G3 was statistically higher than that of G2 (P <0.05). At 8 weeks after operation, the maximum tension force of the G3 group was significantly higher than that of G2 group (P <0.05). The maximum tension forces showed no significant difference between G1 and G3 groups (P >0.05) at 8 weeks after operation.

Collagen membrane facilitated repair of injured tendons

Normal collagen fiber bundles were observed with a regularly linear structure in the G1 group (Figure 3 A and D). Four weeks after operation, the cut ends of the Achilles tendon in the G2 group were connected by scar tissue enriched with multi-shaped fibroblasts. Although the collagen fiber bundles in the tendons of G3 group were aligned irregularly, they appeared denser and the alignment was better than that in the G2 group (Figure 3 B and C). At 8 weeks post operation, the scar was more mature in both G2 and G3 groups than that at 4 weeks post surgery. They formed a denser collagen matrix, regularly aligned collagen fiber bundles, when compared to their counterparts at 4 weeks. The total collagen matrix area of each group was wider than that of the 4-week groups. The total area of the collagen matrix in the G3 group was more than that of the G2 group at 8 weeks post operation (Figure 3 E and F).

Tendon is mainly composed of collagen type I. For immunohistological staining, the relative expressions of collagen I were quantified by Image-Pro Plus Version 6.0 for Windows software and are summarized in Figure 4. Corresponding to the trend in H&E staining, there was a significantly stronger expression of collagen type I in G3 group than that in G2 group at 4 and 8 weeks postoperatively, respectively (P <0.05). At 8 weeks, the expression of collagen type I was slightly stronger when compared to their counterparts at 4 weeks in both the G2 and G3 groups. These results showed that the injured tendon healed better with collagen membrane wrapped than that without using it.

Table 2. The severity of tendon adhesion score for each group 8 weeks after operation (n=20, n (%))

Groups	None	Slight	Moderate	Severe
G1	15 (75)	5 (25)	0 (0)	0 (0)
G2	0 (0)	3 (15)	11 (55)	6 (30)
G3	8 (40)	10 (50)	2 (10)	0 (0)

Table 3. The average maximum tension force for each group (N, mean±SD, n=10)

Time	G1	G2	G3
4 weeks	83.03±4.68	56.20±4.17†	62.50±2.14*†
8 weeks	87.47±5.47	70.73±4.44†	82.24±5.58*

$^{*}P$ <0.05 compared with G2 group at 4 and 8 weeks; $^{\dagger}P$ <0.05 compared with G1 group at 4 and 8 weeks.

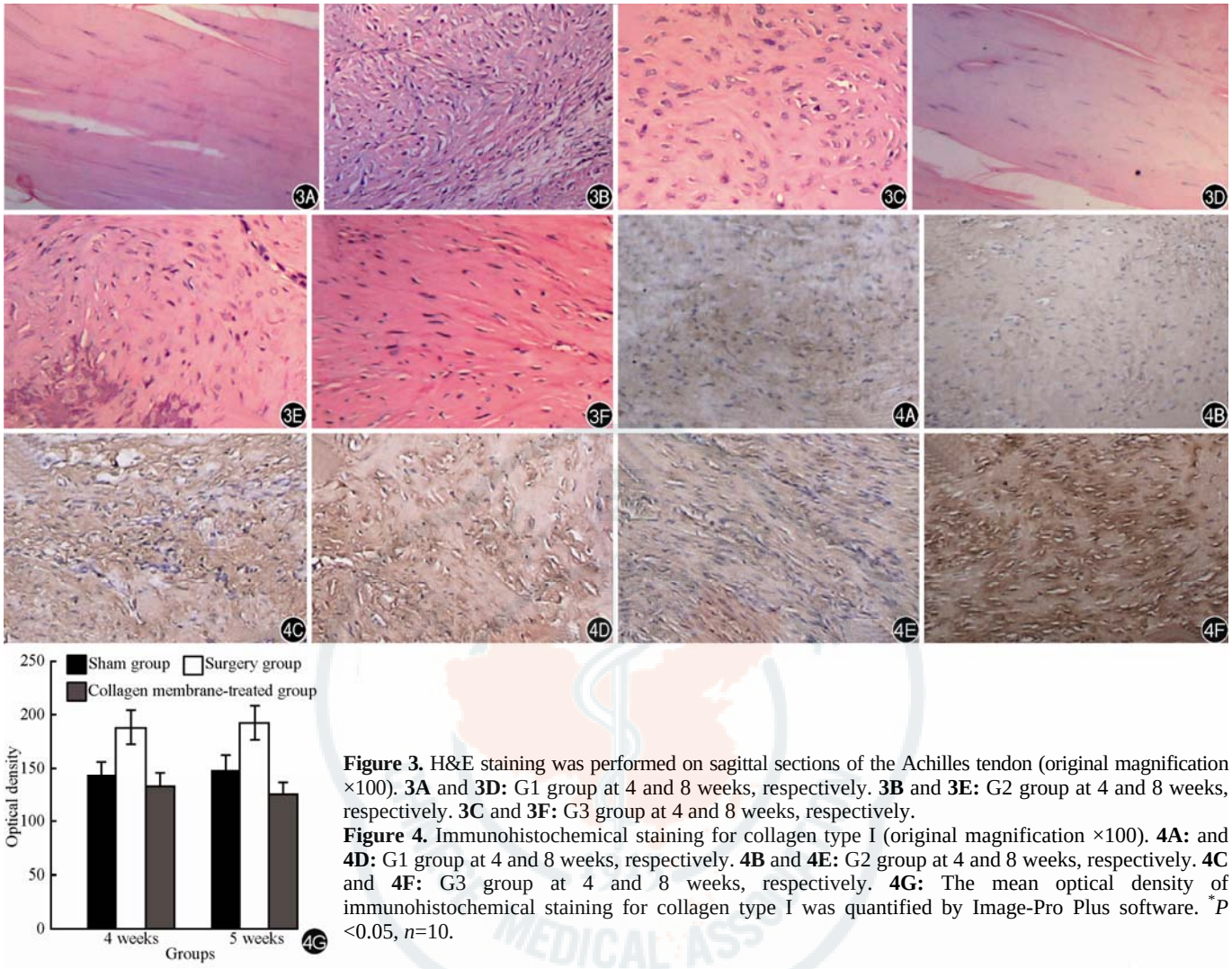

Figure 3. H&E staining was performed on sagittal sections of the Achilles tendon (original magnification ×100). **3A** and **3D:** G1 group at 4 and 8 weeks, respectively. **3B** and **3E:** G2 group at 4 and 8 weeks, respectively. **3C** and **3F:** G3 group at 4 and 8 weeks, respectively.
Figure 4. Immunohistochemical staining for collagen type I (original magnification ×100). **4A:** and **4D:** G1 group at 4 and 8 weeks, respectively. **4B** and **4E:** G2 group at 4 and 8 weeks, respectively. **4C** and **4F:** G3 group at 4 and 8 weeks, respectively. **4G:** The mean optical density of immunohistochemical staining for collagen type I was quantified by Image-Pro Plus software. $^{*}P$ <0.05, n=10.

DISCUSSION

Achilles tendon is the strongest and thickest tendon in the human body and its rupture is one of the most common tendinous lesions due to sports or accidents.[15] Tendon rupture results in excruciating pain and permanent disability. Surgery is the main treatment for injured tendons,[16] but peritendinous adhesion, the most frequent complication following tendon injury surgery, is still a thorny subject for orthopedists.[3]

Tendon healing is a complicated process through extrinsic and intrinsic mechanisms.[17] Biomaterials function as the sheath that block passage of the fibroblasts but allow the passage of cytokines and growth factors.[18] However, some failures were encountered in such usage because of induced severe inflammatory response, or prevention of diffusion of nutrients into the healing tendon that at last leads to tendon necrosis.[19] Meanwhile, some synthetic fibers have various disadvantages such as excessive calcification, reject reaction, and insufficient biocompatibility induced inflammatory response.[20] Thus, ideal mechanical barriers should be suitably flexible, absorbable and biocompatible, such as hydrogel and autogenous vein graft.[21]

In order to prevent peritendinous adhesions while allowing tendon regeneration, we used collagen membrane to explore if it could prevent adhesions between the injured tendon and the surrounding tissues and provide mechanical strength to the incised Achilles tendon in SD rats. Collagen has been widely applied in tissue engineering with evidence of low immunogenicity, proper biocompatibility, biodegradability and excellent hemostasis.[22] We wrapped the sutured tendon with collagen membrane in the Achilles tendon incised SD rats. SEM images showed that collagen membrane had three dimensional porous structures. This structure is beneficial for circulating resolved factors for tendon repair but blocks granules. So it could be a good mechanical barrier between the tendon and the surrounding tissues.

In this study, collagen membrane was placed between the injured tendon and the surrounding tissue. Four weeks after operation, the collagen membrane was degraded. Neither inflammation nor granuloma was found between the tendon and the surrounding tissue. Compared with the surgery group (G2), the degree of tendon adhesion in the collagen membrane-treated group was significantly lower. Data suggested that the collagen membrane we used had good biocompatibility and could attenuate tendon

adhesion after tendon injury.

H&E staining showed that in the collagen membrane-treated group, the arrangements of collagen bundles were better and more regular, and fibroblasts were less than that in the surgery group. The results indicate that collagen membranes can effectively block the extrinsic healing process. Corresponding to the results of H&E staining, the results of IHC also showed more collagen type I expression in the collagen membrane-treated group than those in the surgery group. This suggests that collagen membrane efficiently prevents peritendinous adhesions without impairing healing of the sutured tendon. Biomechanical testing further supported the histological findings. The mechanical and physiological characteristics of the collagen matrix showed its maximum tensile strength. The more the collagen fibers in the tendon, the greater the maximum tension force. The maximum tensile strength was significantly stronger in the collagen membrane-treated group than that in the surgery group at 4 and 8 weeks post operation, respectively (P <0.05). Moreover, there was no significant difference between the sham group and the collagen membrane-treated group. This suggests that the strength of the reparative process is not adversely affected despite slight peritendinous inflammatory response and barrier effect. Diffusion of nutritional materials and growth factors across the microporous membrane may be sufficient to support intrinsic tendon healing and to prevent unnecessary cells from infiltrating in the rupture site. Collagen fibers are considered to be important in the tendon healing process.[23] This study provides a strategy for collagen usage in the treatment of tendon repair. Meanwhile, collagen membrane, an absorbable adhesion barrier, loaded with growth factors or a combination with mesenchymal stem cells needs to be explored in the future.

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