Evaluation of Porcine Small Intestinal Submucosa in Achilles Tendon Repair

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KEY WORDS: Achilles tendon, small intestinal submucosa, tensiometry, collagen densitometry

ABSTRACT

Background: Tendon defects remain a major concern in orthopedic surgery because of the limited availability of tendon autografts. Since the extracellular collagen-based matrix derived from porcine small intestinal submucosa (SIS) has been successfully used in repair of many tissues, such as abdominal wall, vessels and bladder, we hypothesized that SIS can be used to repair an experimentally induced Achilles tendon defect.

Material and Methods: Seventeen New Zealand rabbits were submitted to Achilles tendon resection (1.5 cm length) in the right posterior limb and this defect was repaired with SIS. The left Achilles tendon (sham operated) was used as a control. Daily examination was performed for 30 days. On the day of sacrifice, Achilles tendon from both limbs was excised and signs of infection, dehiscence, incorporation and degree of adhesion were evaluated. Tensiometry, and histological analysis, including inflammatory and foreign body reactions, fibroblastic proliferation and collagen densitometry were performed. Data were analyzed using *t* test.

Results: In SIS versus control, presence of infection, dehiscence, incorporation and adhesions were not different ($P < 0.05$). The mean rupture strength in SIS versus control was not different (68.5 N vs 61.3 N). In SIS there was a predominantly chronic inflammatory reaction with presence of fibroblasts and foreign body reaction was evident in 41%. In SIS versus control, densitometry showed a similar amount of mature (88.9 vs 93.3%) and immature (11.1 vs 6.7%) collagen.

Conclusion: These data indicate that SIS when assessed using clinical, histological and tensiometric indices, can be used to repair Achilles tendon defect.

INTRODUCTION

The Achilles tendon is essential for normal ankle joint movement and particularly critical for activities like running

or stair climbing. The best method of treatment for acute closed ruptures of the Achilles tendon is controversial, with advocates of operative treatment recommending early surgical repair and advocates of non-operative treatment preferring cast immobilization. Currently, ruptured Achilles tendons are most often operatively treated. Operative treatment is associated with shorter period of disability and low frequency of re-ruptures, although it is associated with potential wound complications, which occur in approximately 7.5% of cases.[1,2] Because of differences in rehabilitation protocols, it is still unclear whether differences between surgical and non-surgical treatment have a better outcome. Modern concepts of non-operative treatment have been shown to produce good results with acceptable complication rates.[3,4] The rupture rate of non-surgical treatment varies from 0% to 17%,[4,5] with average of 12.1%, whereas the re-rupture rate from surgical repair is only 2.2%.[6]

However, loss of Achilles tendon poses a difficult reconstructive problem. A successful reconstruction must have the appropriate strength, durability, and tension to meet the dynamic workload. The lack of suitable local tissue at this site makes the task even more difficult. Various innovative procedures have been used, including fascia lata autografts,[7] antero-lateral thigh free flap,[8] latissimus dorsi muscle,[9] fasciocutaneous infragluteal flap,[10] and marlex mesh.[11] Although various local flaps and free flaps have been described for reconstruction of Achilles tendon, it is not a procedure free of complication. There is a need for a biomaterial capable of Achilles tendon reconstruction and possibly, allowing complete tendon regeneration.

Porcine small intestinal submucosa (SIS) is an acellular resorbable biomaterial that can serve as scaffolding for new tissue growth. It has been used experimentally in animals as vascular grafts,[12] abdominal wall repairs,[13,14] and for bladder[15] and esophagus reconstruction.[16] In orthopedic surgery, SIS has been successfully used to regenerate fascial defects,[17] ligaments,[18] and tendons.[19]

The aim of this study was to determine the efficacy of SIS in Achilles tendon repair. We hypothesis that porcine SIS can be used in repair experimentally induced Achilles tendon defect. SIS induces a gross and histological appearance and mechanical strength similar to that of the contralateral sham-operated tendon over a period of 30 days.

MATERIAL AND METHODS

The study was approved by the Committee for the Humane Use of Animals at Catholic University of Parana and was in accordance with guidelines established by the ethical principles in use of experimental animals.[20]

Preoperative Procedures

Seventeen New Zealand male rabbits (Tecpar, Curitiba, Brazil) with mean weight of 3296 ± 246 g, were kept in standard rabbit cages (85 x 64 x 68 cm) and acclimated for one week to the constant study environmental conditions: 12 to 12 hour light-dark cycle (lights on at 06:00), 25 ± 4°C room temperature, and 45% humidity. The rabbits were allowed ad libitum diets and municipal tap water. Prior to operation, rabbits were food deprived for 12 hours.

Anesthesia

Rabbits were anesthetized with ketamine and acepromazine mixture (150:5 mg/mL) via intramuscular injection (0.8 mL/kg) to obtain effective anesthesia. Inhalation anesthesia with Isoflurane (Baxter Caribe Inc, Guayama, Puerto Rico) in oxygen, was used to maintain anesthesia during the surgical proce-

dures. Buprenorphine hydrochloride (Medley Pharmaceutics, Campinas, Brazil) 0.05 mg/kg was given twice a day to all postoperative rabbits for three days as analgesic therapy.

Preparation of Small Intestinal Submucosa (SIS)

A segment of proximal jejunum was obtained following a midline incision in a porcine cadaver, within 2 hours of donor pig euthanasia, as previously described.[21] The proximal jejunum was wrapped in surgical sponges that were soaked in physiologic saline solution. All mesenteric tissues were removed and the segment of jejunum was everted. The tunica mucosa was removed using a longitudinal wiping motion with a scalpel handle and moistened gauze. The segment was returned to original orientation and the tunica serosa and tunica muscularis were removed from the intestine by similar method. The remaining thin, whitish, translucent tube consisted of the tunica submucosa with attached stratum compactum and muscularis mucosa of the tunica mucosa.[22] The SIS was rinsed in sterile normal saline and refrigerated and stored for 1 to 10 days in neomycin sulfate 10% (Medley Pharmaceutics, Campinas, Brazil). Single ply SIS is an anisotropic material exhibiting grater hoop strength than longitudinal strength. Therefore, to produce and isotropic configuration of sufficient mechanical strength, each SIS implant consisted of 4 individual sheets of SIS.

Surgical Procedure

Both back limbs were prepared for aseptic surgery. Their limbs were shaved and prepared with Betadine solution (Rimed, Sao Paulo, Brazil), a 5 cm incision was performed in both limbs and the Achilles tendon complex was dissected free from the surrounding tissues. The peri-tendon was split longitudinally and the Achilles tendon was exposed, and a segment of 1.5 cm was excised between its musculotendinous junction and the bone insertion. A 1.5 cm SIS implant was used to replace the resected tendon. Nonabsorbable sutures of 5-0 polypropylene (Ethicon, Cincinnati, Ohio) were passed through both SIS implant and remaining tendon. The peritendon was closed with 6-0 polypropylene running sutures and the skin was closed with 3-0 nylon (Ethicon, Cincinnati, Ohio). For the sham operation, the contralateral back limb was incised, the Achilles tendon was exposed and manipulated for the same duration required in the repair procedure. Thereafter the surgery site was closed in layers.

Postoperative Procedures

After recovery, all animals were individually housed and allowed free movement. No post-operative bandages were used. During the post-operative time, clinical parameters including appetite, activity, infection, bleeding and wound dehiscence were devaluated daily. All of the animals were euthanized 30 days after the surgical procedures using an overdose of pentobarbital (Medley Pharmaceutics, Campinas, Brazil). On the day of sacrifice, Achilles tendon from both limbs was excised and signs of infection, dehiscence, incorporation, and degree of adhesion were evaluated. Tendons were harvested en bloc from just proximal to the adjacent musculotendinous junction to the bone insertion, and immediately submitted to tensiometric evaluation.

Tensiometric Evaluation

Mechanical evaluation was performed with a tensiometric testing machine (EMIC, Sao Paulo, Brazil). The proximal end was scraped with a blunt scalpel to remove all muscle tissue. The tendon fibers were spread out in a fan-like

shape, sandwiched between two pieces of sandpaper (1.0 x 1.0 cm) and tightly fixed in a metal clamp. The tendon was pulled with a constant speed of 1 mm/sec until failure. Peak tendon load at failure was automatically calculated.

Histologic Evaluation

Tendons were fixed in 10% buffered formaldehyde and prepared for histologic evaluation. The specimens were embedded in paraffin and 5-µm thick longitudinal sections were taken every 0.3 mm through the specimens and stained with hematoxylin and eosin and sirius-red techniques.

Collagen Densitometry

Sirius red is a simple, sensitive, and quantitative procedure for the measurement of collagen and protein content in tissue sections.[23] Briefly, Sirius red staining is based on the selective binding of Sirius red F3BA and Fast green FCF to collagen components, when the sections are stained with both dyes dissolved in aqueous saturated picric acid. The picrosirius staining permits the quantification of mature and immature collagen.[24,25] Mature collagen type I fibers presents in yellow, orange, and red, while immature collagen type III appears in green[26]. The microscopic fields were taken from the midportions of the tendon, using polarized light. The sampling fields were digitized to a computer database via a polarization microscope (Leitz-Ortholux, Wetzlar, Germany), through a 200x objective lens, coupled to a high-resolution color camera (Sony CCD, Tokyo, Japan). Images were captured using the digital camera that interfaced with an IBM-compatible Pentium computer (Everex, Freemont, Calif). The digitized sample fields were analyzed using Optimas 5.2 image analysis software (Optimas Inc., Edmonds, Wash), as previously described.[27]

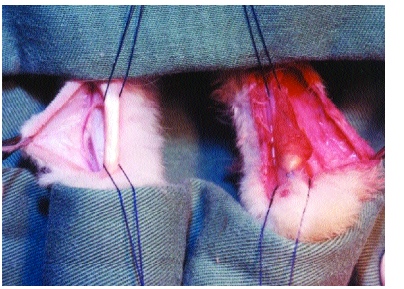

Figure 1. Sham-operated tendon and SIS, 30 days after surgical procedure.

RESULTS

Complications

One rabbit showed a minor wound infection that was locally treated and one rabbit had dehiscence of the graft at day 4, which was evidenced by palpation of the surgical site. This animal was not used in tensiometric evaluation. Local abscess was present in one rabbit. No complications were noted in the sham-operated limb. The remaining rabbits showed normal activity and appetite, and there was no evidence of clinical complications such as local infection or wound dehiscence. In all rabbits, the skin was freely movable across the implant site.

Pathologic Evaluation

Gross Evaluation: A thick scar of the graft and a thin portion of the tendon proximally and distally to the graft was easily palpable on clinical examination. By visual inspection, the graft was slightly thicker compared with the sham-operated tendon (Figure 1); however, the limits between the graft and the native tendon were difficult to determine, with good continuity and solid integration.

Histologic Evaluation: Histologically, the inflammatory response was semiquantitatively evaluated (Table). There was an acute inflammatory response in 5 of the 12 grafts (29%). Foreign body reaction

Table. Semiquantitative Analyses of Inflammatory Response*

Rabbits	Acute Inflammatory Response	Chronic Inflammatory Response
1	-	+
2	-	+
3	++	+++
4	-	++
5	-	++
6	-	++
7	++	+++
8	-	++
9	-	++
10	+++	+++
11	-	+
12	-	+++
13	-	+
14	-	+
15	-	+
16	++	+++
17	+	+++

* - indicates absence; +, mild; ++, moderate; and +++, severe.

represented by mononuclear macrophages was present next to the suture lines. The graft was essentially composed of an abundant, laminated, and organized collagenous extracellular matrix (Figure 2). Parallel collagen fibers were oriented along the longitudinal axes of the tendon. Fibroblasts were distributed between the collagen fibers. The collagen fiber disposition was similar to that of normal tendons. A moderately dense, regular collagenous connective tissue was noted across the entire thickness of the graft. The SIS was also well integrated to the native tendon and no evidence of separation was noted. Histologic examination showed no signs of cartilage or bone formation within the tendon. Remnants of the original SIS was identified, but not quantified. Collagen densitometry showed no statistical significance between SIS and sham operated tendon, regarding the amount of mature type I (88.90% vs 93.34%, respectively), and immature type III collagen (11.10% vs 6.87%, respectively), as shown in Figure 3.

Tensiometric Evaluation

The mean failure loads of SIS was 68.53 N and in the sham operated tendons was 61.34 N, which was not statistically different. All of the sham operated Achilles tendons ruptured in the proximal[10] and distal part of the tendon.[6] Thirteen of the 16 SIS grafts failed in the proximal part of the tendon and the remaining three in the middle part. The tendon callus was the weakest point in three SIS grafts.

DISCUSSION

Loss of Achilles tendon is a difficult reconstructive problem because of the limited availability of tendon autografts. A successful reconstruction must have the appropriate strength, durability and tension to meet the dynamic workload. Various procedures have been used; however, local flaps and free flaps are

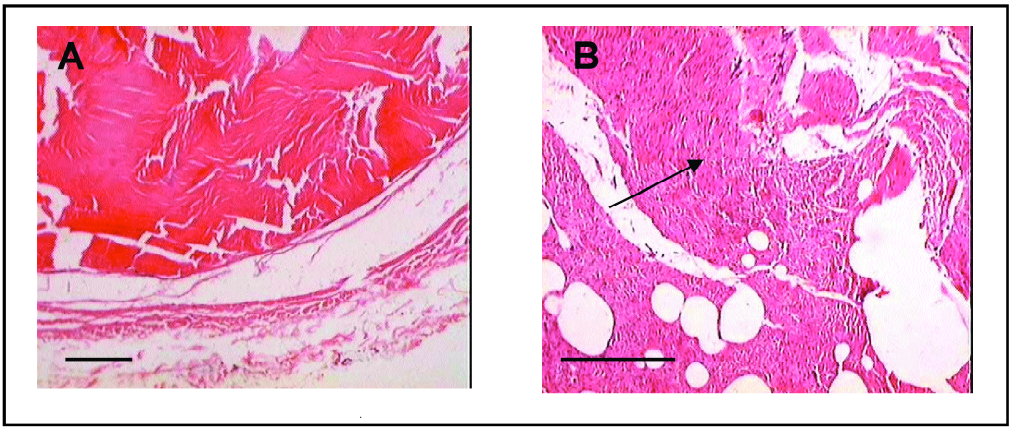

Figure 2. Histologic evaluation. Figure 2A shows Sham operated tendon. Figure 2B shows SIS. In Figure 2A, the Achilles tendon is covered by a thin peri-tendon later in sham operated tendon (50x). In Figure 2B, a moderately dense, regular collagenous and connective tissue next to the native tendon (arrow) was noted across the entire thickness of the graft (100x).

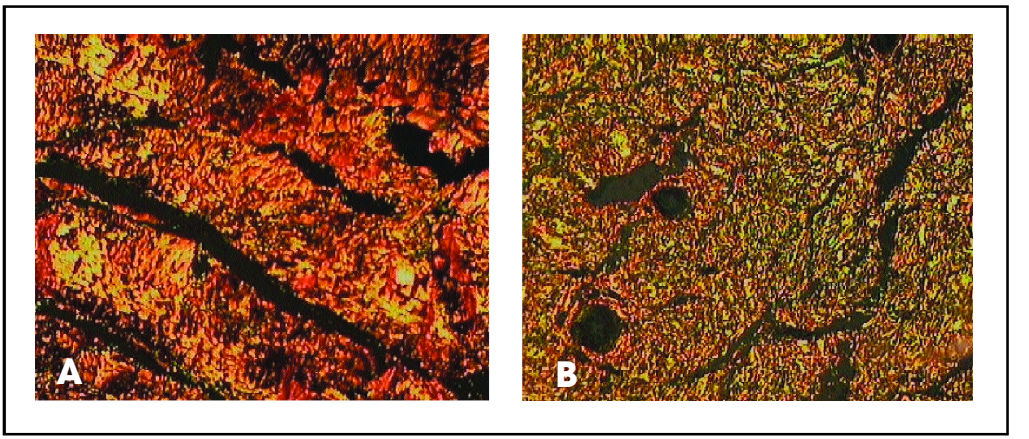

Figure 3. Collagen densitometry in sham operated tendon and SIS graft. Figure 3A shows a Sham operated tendon. Figure 3B shows SIS. Mature collagen fibers type I presents in yellow, orange, and red, while immature collagen type III appears in green (200x).

not procedures free of complication. We tested the use of SIS in the repair of experimentally induced Achilles tendon defect. Results of this study suggest that, in a rabbit model, SIS can be used as a tissue-engineered replacement of a resected Achilles tendon. The SIS graft maintained sufficient strength, while serving as a temporary scaffold for host tissue ingrowth and remodeling. Furthermore, SIS graft was not associated with significant presence of infection, wound and graft dehiscence and showed a good incorporation with the adjacent tendon. Collagen densitometry showed a similar amount of mature and immature collagen, when compared to sham operated tendons.

Porcine small intestinal submucosa (SIS) is a 0.1-mm thick laminar material composed of more than 90% protein (essentially collagen), less than 10% lipids and traces of carbohydrates. Components of the SIS are collagen (I, III, IV, V, and VI), fibronectin, laminin, glycoprotein and growth factors.[28] This collagenous resorbable material has been successfully used as an autograft,

allograft, and xenograft in many organs and tissues in animal models. Studies have demonstrated that after implantation, this biomaterial induces a site-specific remodeling of various connective tissues, supporting host tissue ingrowth, and appears to promote cellular differentiation. The regenerative process leads to a tissue structurally and functionally similar to the original tissue and the collagenous connective tissue is analogous to scar tissue that occurs in most parts of the body.[17,18,19] Other significant features of SIS have been identified: capillary growth into the tissue maintaining graft viability and permitting the diffusion of oxygen and other nutrients to the vascular tissue, conferring resistance to infection,[21] and no signs of rejection have been described.[22,29]

Two other authors have described the use of porcine SIS as a graft material in Achilles tendon repair. Badylak et al[19] created a 1.5 cm segmental defect of the Achilles tendon in twenty dogs, and replaced it with SIS. The neotendons were evaluated at 1, 2, 4, 8, 12, 16 and 48 weeks and four dogs were euthanized in each time point. Four additional dogs were submitted to 1.5 cm segmental Achilles tendon defect and were surgically repaired with the same tendon that was resected. They showed that the SIS remodeled neotendons were stronger than the sham operated tendons and the histological evaluation consisted of organized collagen similar to the normal tendons. This is in accordance to our findings. Of the four dogs in which the SIS was not implanted, inferior strength was observed compared to SIS implants. Gu et al[30] removed part of the Achilles tendon in 20 rabbits and substituted porcine SIS, using the contralateral limb as control. As in Badylak et al, four rabbits were evaluated at each euthanasia point, 1, 4, 8, 12 and 16 weeks after surgery. The differences of maximum load at 4 weeks after surgery was inferior in SIS regenerated tendons compared to sham operated limbs, and after 8 weeks no differences were observed. Changes in physical characteristics of the SIS over time is an important consideration, however the number of animals tested 30 days after the implants in both studies was small, motivating us to use a large number of animals. In our study we evaluated the specimens 30 days after surgery and this period was sufficient to maintain strength compared to sham operated Achilles tendon.

Histologically, there was an acute inflammatory response in 5 of the 12 grafts because the analyses were performed a short period of time after the surgical procedure. However, signs of chronic inflammation were observed in all of the SIS implants and abundant, laminated and organized collagenous extracellular matrix was noted. The parallel collagen fibers were oriented along the longitudinal axes of the tendon and this is in accordance with previous studies.

SIS and collagen biomaterials can promote calcification.[31,32] Histological examination showed no signs of cartilage or bone formation within the tendon in all of the specimens evaluated. This can be explained because the histological analyses was performed 30 days after the surgery and the rabbits were allowed free movement after the surgical procedures. Tendon mobilization has been shown to accelerate the healing process whereas immobilization was shown to impair healing. The early mechanical stimulation of the tendon callus would probably minimize the risk of bone formation.[33,34]

Biomechanical and histologic comparisons between ruptured and intact tendons in rabbits have shown a collagen content in transected tendons amounting to 80% of the controls and collagen cross-linking (measured by the hydroxy-pyridinium content) amounting

to 60% of the intact tendon controls.[35] In the present study, collagen densitometry showed no statistical significance between SIS and sham operated tendon, regarding the amount of mature and immature collagen. It was impressive how fast the healing tendon regained strength and stiffness.

CONCLUSION

Small intestinal submucosa may serve as a structural framework for the application of tissue engineering technologies in the development of the ideal Achilles tendon reconstruction material. The present study should be considered a promising preliminary finding in the search for alternatives to Achilles tendon repair.

REFERENCES

1. Moller M, Movin T, Granhed H, et al. Acute rupture of tendon Achilles. A prospective randomized study of comparison between surgical and non-surgical treatment. *J Bone Joint Surg.* 2001;83B:843-848.
2. Cetti R, Christensen SE, Ejsted R, et al. Operative versus nonoperative treatment of Achilles tendon rupture. A prospective randomized study and review of the literature. *Am J Sports Med.* 1993;21:791-799.
3. McComis GP, Nawoczenski DA, DeHaven KE. Functional bracing for rupture of the Achilles tendon. Clinical results and analysis of ground-reaction forces and temporal data. *J Bone Joint Surg.* 1997;79:1799-1808.
4. Weber M, Niemann M, Lanz R, Muller T. Non-operative treatment of acute rupture of the Achilles tendon. Results of a new protocol and comparison with operative treatment. *Am J Sports Med.* 2003;31:685-691.
5. Thermann H, Zwipp H, Tscherne H. Functional treatment concept of acute rupture of the Achilles tendon. 2 years results of a prospective randomized study. *Unfallchirurg.* 1995;98:21-32.
6. Kocher MS, Bishop J, Marshall T, et al. Operative versus non-operative management of acute Achilles tendon rupture: Expected-value decision analysis. *Am J Sports Med.* 2002;30:783-790.
7. Haas F, Seibert FJ, Koch H, et al. Reconstruction of combined defects of the Achilles tendon and the overlying soft tissue with a fascia lata graft and a free fasciocutaneous lateral arm flap. *Ann Plast Surg.* 2003;51:376-382.
8. Lee JW, Yu JC, Shieh SJ, et al. Reconstruction of the Achilles tendon and overlying soft tissue using antero-lateral thigh free flap. *Br J Plast Surg.* 2000;53:574-577.
9. Lee HB, Lew DH, Oh SH. Simultaneous reconstruction of the Achilles tendon and soft-tissue defect using only a latissimus dorsi muscle free flap. *Plast Reconstr Surg.* 1999;104:111-119.
10. Papp C, Todoroff BP, Windhofer C, Gruber S. Partial and complete reconstruction of achilles tendon defects with the fasciocutaneous infragluteal free flap. *Plast Reconstr Surg.* 2003;112:777-783.
11. Ozaki J, Fujiki J, Sugimoto K, et al. Reconstruction of neglected Achilles tendon rupture with Marlex mesh. *Clin Orthop.* 1989;238:204-208.
12. Lantz GC, Badylak SF, Hiles MC, et al. Small intestinal submucosa as a vascular graft: a review. *J Invest Surg.* 1993;6297-6310.
13. Badylak SF, Kokini K, Tullius B, et al. Morphological study of small intestinal submucosa as a body wall repair device. *J Surg Res.* 2002;103:190-202.
14. Greca FH, Biondo-Simoes MLP, Santos EA, et al. Comparison between a polypropylene mesh and small intestine submucosa used for abdominal wall defects repair: experimental study in dogs. *Acta Cir Bras.* 2001;16:44-50.
15. Pope JC, Davis MM, Smith ER, et al. The ontogeny of canine small intestinal submucosa regenerated bladder. *J Urol.* 1997;158:1105-1110.
16. Badylak SF, Meurling S, Chen M, et al. Reabsorble bioscaffold for esophageal repair in a dog model. *J Pediatr Surg.* 2000;35:1097-1103.
17. Dejardin LM, Arnoczky SP, Clarke RB. Use of small intestinal submucosa implants for regeneration of large fascial defects: An experimental study in dogs. *J Biomed Mater Res.* 1999;46:203-211.
18. Aiken SW, Badylak SF, Toombs JP. Small intestinal submucosa as an intra-articular ligamentous graft material: A pilot study in dogs. *Vet Comp Orthop Trauma.* 1994;7:124-128.
19. Badylak SF, Tullius R, Kokini K, et al. The use of xenogeneic small intestinal submucosa as a biomaterial for Achilles tendon repair in a dog model. *J Biomed Mater Res*. 1995;29:977-985.
20. Silva OC, Zucoloto S, Beer A. *Experimental models in surgical research.* Sao Paulo (Brazil), Robe; 1998.

21. Badylak SF, Lantz GC, Coffey A, Geddes LA. Small intestinal submucosa as a large diameter vascular graft in the dog. *J Surg Res*. 1989;47:74-80.

22. Clarke KM, Lantz GC, Salisbury SK, et al. Intestine submucosa and polypropylene mesh for abdominal wall repair in dogs. *J Surg Res.* 1996;60:107-114.

23. Lopez-De Leon A, Rojkind M. A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J Histochem Cytochem.* 1985;33:737-743.

24. Constantine VS, Mowry RW. Selective staining of human dermal collagen II. The use of picrosirius red F3BA with polarization microscopy. *J Invest Dermatol.* 1968;50:419-423.

25. Dayan D, Hiss Y, Hirshberg A, et al. Are the polarization colors of picrosirius red-stained collagen determined only by the diameter of the fibers? *Histochemistry.* 1989;93:27-29.

26. Junqueira LC, Cossermelli W, Brentani R. Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy. *Arch Histol Jpn.* 1978;41:267-274.

27. Li XJ, Lei T, Gao JH. Detection of collagens in hypertrophic scars by picrosirius polarization method. *Di Yi Jun Yi Da Xue Xue Bao.* 2002;23:217-219.

28. Dejardin LM, Arnoczky SP, Ewers BJ, et al. Tissue-engineered rotator cuff tendon using porcine small intestine submucosa. Histologic and mechanical evaluation in dogs. *Am J Sports Med.* 2001;29:175-184.

28. Liang HA, Record RD, Hodde JP, Badylack SF. Human microvascular endothelial cell adhesion to small intestinal submucosa. *SIS Symposium* 1998;91.

29. Sandusky GE, Badylak SF, Morff RJ, et at. Histologic findings after in vivo placement of small intestine submucosa vascular grafts and saphenous vein grafts in the carotid artery in dogs. *Am J Pathol*. 1992;140:317-324.

30. Gu Y, Dai K. Substitution of porcine small intestinal submucosa for rabbit Achilles tendon, an experimental study. *Zhonghua Yi Xue Za Zhi*. 2002;82:1279-1282.

31. Owen TJ, Lantz GC, Hiles MC, et al. Calcification potential of small intestinal submucosa in a rat subcutaneous model. *J Surg Res*. 1997;71:179-186.

32. Lee CH, Singla A, Lee Y. Biomedical applications of collagen. *Int J Pharm.* 2001;221:1-22.

33. Pneumaticos SG, McGarvey WC, Mody DR, Trevino SG. The effects of early mobilization in the healing of Achilles tendon repair. *Foot Ankle Int.* 2000;21:551-557.

34. Forslund C, Aspenberg P. Improved healing of transected rabbit Achilles tendon after a single injection of cartilage-derived morphogenetic protein-2. *Am J Sports Med.* 2003;31:555-559.

35. Reddy GK, Stehno-Bittel L, Enwemeka CS. Matrix remodeling in healing rabbit Achilles tendon. *Wound Repair Regen.* 1999;7:518-527.