

The use of porcine small intestinal submucosa to enhance the healing of the medial collateral ligament—a functional tissue engineering study in rabbits

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Abstract

Introduction: Small intestinal submucosa (SIS) from porcine has been successfully used as a collagen scaffold for the repair of various tissues, including those of the human vascular, urogenital, and musculoskeletal systems. The objective of this study was to evaluate whether SIS can be used to enhance the healing process of a medial collateral ligament (MCL) with a gap injury in a rabbit model.

Methods: A 6 mm wide gap was surgically created in the right MCL of 20 skeletally mature, female New Zealand White rabbits. In 10 rabbits, a strip of SIS was sutured onto the two ends of the MCL, while for the other 10 animals their injured MCL remained untreated and served as a non-treated group. The left MCL of all animals was exposed and undermined serving as the sham-operated side. At 12 weeks post-healing, eight hind limbs from each group were used for mechanical testing. The cross-sectional areas (CSA) of the MCLs were measured. The femur–MCL–tibia complex (FMTC) was tensile tested to failure. The load–elongation curves representing the structural properties of the FMTC and the stress–strain curves representing the mechanical properties of the healing MCL were obtained. The remaining two animals from each group were prepared for histological evaluation.

Results: The CSA between the SIS-treated and non-treated groups were not significantly different ($p > 0.05$). Both treatment groups appeared to increase by nearly 40% compared to the sham-operated side, although statistical significance was not found for the non-treated group ($p > 0.05$). The stiffness of the FMTC from the SIS-treated group was 56% higher than the non-treated group (45.7 ± 13.3 N/mm vs. 29.2 ± 9.2 N/mm, respectively, $p < 0.05$) and the ultimate load also nearly doubled ($117.434.5$ N vs. 66.4 ± 31.4 N, respectively, $p < 0.05$). These values were lower compared to the sham-operated side (89.7 ± 15.3 N/mm and 332.0 ± 50.8 N, respectively). The tangent modulus of the healing MCL (279.7 ± 132.1 MPa vs. 149.0 ± 76.5 MPa, respectively) and stress at failure (15.7 ± 4.1 MPa vs. 10.2 ± 3.9 MPa, respectively) both increased by more than 50% with SIS treatment ($p < 0.05$). Yet, each remained lower compared to the sham-operated side (936.3 ± 283.6 MPa and 75.6 ± 14.2 MPa, respectively). Blinded histological comparisons between the SIS-treated MCL and the non-treated control demonstrated qualitatively that the SIS treated group had increased cellularity, greater collagen density, and improved collagen fiber alignment.

Conclusion: Healing of a gap MCL injury was significantly enhanced with SIS. The improved mechanical properties and histological appearance of the MCL suggest that SIS treatment improves the quality of tissue and renders the possibility for future studies investigating functional tissue engineering of healing ligaments.

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Introduction

Medial collateral ligament (MCL) injuries are common in sport and work-related activities [16,20]. Isolated

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MCL injuries have been found to heal with conservative treatment, yet the mechanical properties, histological appearance, and biochemical composition are not restored to pre-injury levels even after one to two years [17,22,26,28,31]. Several approaches, including growth factor treatment, gene transfer, cell therapy, and the use of scaffolds are being investigated in hopes to improve the quality of healing ligaments [2,10,14,18,21]. The use of polymeric or naturally occurring biodegradable scaffolds has recently been demonstrated to be feasible to help with the healing process, while further enhancement by processing or conditioning of these scaffolds has been demonstrated *in vitro* [11,12,15,18,21,30].

The porcine small intestinal submucosa (SIS) is a naturally occurring scaffold that is commercially available. It consists of an organized collagen matrix, mainly composed of Type I collagen [5] with preferred alignment in the longitudinal direction [24]. SIS has been used to repair musculoskeletal tissues and has been shown to promote cell migration into the healing site to enhance revascularization and repair [3,5,8,9]. Furthermore, animal studies have revealed that the SIS can resist infection [6,4,25] and has an immune response that is consistent with remodeling rather than rejection [1]. When used to treat canine with a surgically created defect of the Achilles tendon, SIS enhanced the ultimate load at failure [5]. Similarly, the tensile strength and histomorphological appearance of the infraspinatus tendon following SIS replacement were found to be similar to sham-operated controls [9]. When SIS was used to repair meniscal defects, the histomorphological appearance of the tissue more closely resembled uninjured tissue than non-treated menisci [8].

Thus, the research question was whether SIS can be used as a scaffold to enhance the mechanical properties of healing ligaments. Using the rabbit knee with an MCL gap injury model, our objective was to examine whether bridging the gap with an organized collagen scaffold, such as the porcine SIS, could improve the mechanical properties of the healing MCL at 12 weeks of healing. The 12-week time period was chosen because preliminary studies using SIS have shown incomplete incorporation of the SIS scaffold at earlier time points.

Since treatment with SIS bridges the surgically created gap with a biologically active Type I collagen scaffold, the hypothesis under study was that the mechanical properties of the MCL midsubstance will be improved over those for an untreated control. Uniaxial tensile tests of femur–MCL–tibia complexes (FMTC) were performed and the mechanical properties of the healing MCL were determined from resulting stress–strain curves. Histological evaluation was used to provide support for these findings.

Methods

A total of twenty skeletally mature female New Zealand White rabbits were used in this study. The average body mass of the animals was 5.7 kg (range 4.9–6.2 kg). The University of Pittsburgh Institutional Animal Care and Use Committee approved the study protocol.

After a three-day acclimation period, the animals were taken to the operating room. Under general anesthesia, the MCL was exposed through a medial incision. In all 20 rabbits, a 6 mm wide gap, centered about the joint line, was created via surgical transection of the MCL of the right hind limb [19] (Fig. 1A). In this model, a piece of the MCL midsubstance, 6 mm in length, was transected and removed using a scalpel with a no. 15 blade. This model provided a reproducible and consistent injury to the midsubstance without injuring the insertion sites at the time of surgery. Left MCLs were sham-operated, whereby the ligaments were exposed but not injured. A single strip of SIS (Cook® Biotech Inc., Bloomington, IN) (~10 mm × 4 mm × 0.2 mm) was then secured on top of the gap (luminal side faced down) with the use of a non-resorbable suture (6-0 silk) at each of the four corners ($n = 10$) (Fig. 1B). For the remaining animals ($n = 10$), the gap injury was surgically created and remained untreated. For all specimens, the wound was then irrigated and fascia and skin were closed.

Post-operatively, animals were allowed free cage activity. For post-operative analgesia, 1 mg/kg ketoprofen was administered every 12 h with intra-muscular (i.m.) injection for two days. Antibiosis was achieved with 100 mg/kg cephalothin sodium, i.m. also for two days. The condition of the wounds, the activity levels, and nutritional behavior were monitored daily. After 12 weeks of healing, the animals were euthanized by lethal injection of intravenous sodium pentobarbital (0.4 ml/kg bodyweight). This time point was selected based on a preliminary study conducted at 6 weeks of healing which demonstrated similar values for stiffness (42.1 ± 12.6 N/mm vs. 35.4 ± 9.4 N/mm for

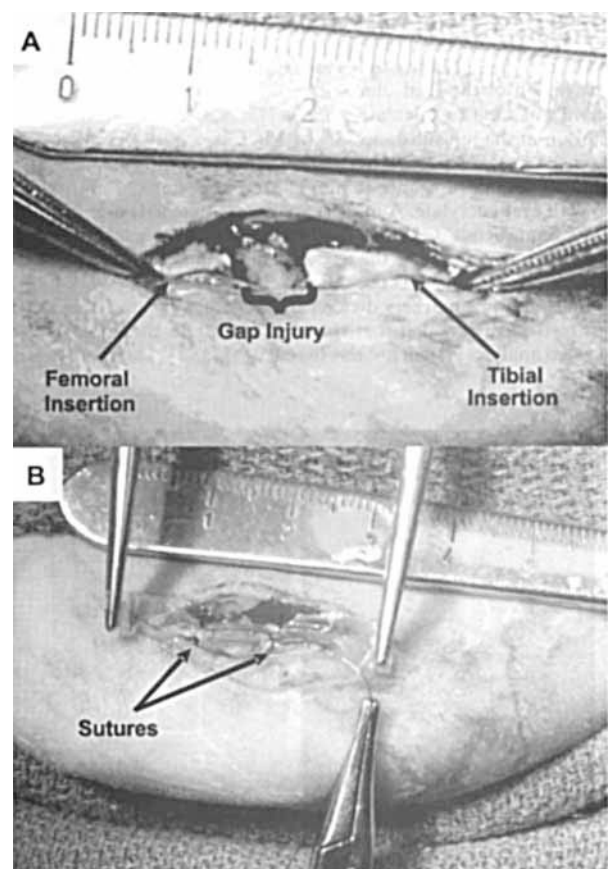


Fig. 1. (A) Rabbit MCL with 6 mm wide gap and (B) a strip of SIS sutured on top of the gap in the MCL.

SIS and non-treated groups, respectively, $p > 0.05$) and ultimate load (107.9 ± 26.9 N vs. 103.9 ± 52.1 N, for SIS and non-treated groups, respectively, $p > 0.05$) between the SIS- and non-treated groups. However, the SIS was incompletely incorporated, and this created difficulties in obtaining mechanical properties for the healing MCL since midsubstance strain measurements represented only strain in the scaffold. These results led to the conclusion that 6 weeks of healing is inadequate for the SIS to be incorporated in the healing ligament. Therefore, this study evaluated a 12-week healing period. It should also be noted that this preliminary study used a mop-end tear model instead of a 6 mm gap injury. Since it is impossible to know the damage that occurs to the insertion sites with this model, the 6 mm gap model was selected for this study to focus on the effect of the SIS treatment on the healing of the midsubstance.

Bilateral hind limbs were disarticulated at the hip joint. All hind limbs were wrapped in saline-soaked gauze, and immediately packed in plastic bags and stored at -20 °C [32]. For biomechanical testing, the frozen, saline-soaked specimens were thawed in plastic bags overnight at room temperature. The specimens were then dissected free of all soft tissue except the MCL, leaving an FMTC. Non-contact measurements of the ligament CSA were performed using a laser micrometer system [29]. CSA measurements were obtained at the three locations: ligament midsubstance, 5 mm proximal, and 5 mm distal to the joint line. These measurements were averaged for stress calculations. Graphical representations of the ligaments CSAs were obtained and documented.

The FMTCs were then rigidly fixed to custom-made clamps at approximately 60° of knee flexion to align the collagen fibers, and placed in a 37 °C normal saline bath. The clamps were fixed to the cross-head and the base of a materials testing machine (Instron, Model 4502, Canton, MA) (Fig. 2), and the FMTC was allowed to adjust to the environment for one hour. After applying a pre-load of 1 N, each FMTC underwent pre-conditioning by cyclic elongation between 0 and 0.75 mm of elongation for 10 cycles at 5 mm/min. This was followed by load to failure testing at an elongation rate of 5 mm/min. The load–elongation behavior of the FMTCs and failure modes were recorded. Structural properties of the FMTC were represented by stiffness (N/mm), ultimate load (N), elongation at failure (mm), and energy absorbed at failure (Nmm). For each FMTC, the greatest slope in the linear region of the load–elongation curve over a 0.5 mm elongation interval was used to calculate stiffness [13,28,33].

Strain in the midsubstance of the MCL was also measured during the tensile test. Two reflective markers (round, 2 mm diameter) were placed on the midsubstance (5 mm apart and centered about the joint line) with cyanoacrylate. A motion analysis system (Motion Analysis™ VP320, Santa Rosa, CA) was used to determine the gauge length (L_0) and to track the motion of the reflective tape markers during the tensile test. Strain was defined as the ratio of the difference in position of the two midsubstance markers divided by the gauge length ($\Delta L/L_0$). Previous tests have revealed that the errors in strain measurement with this video analysis system are less than 0.2% [23,27]. Thus, stress–strain

curves were obtained to represent the mechanical properties of the midsubstance of the MCL, including tangent modulus and stress at failure. For each healing MCL, the greatest slope in the linear region of the stress–strain curve over a 1% strain interval was used to calculate tangent modulus.

For statistical analysis, unpaired *t*-tests were used to compare the structural properties of the FMTC and the mechanical properties of the MCL from the SIS-treated group ($n = 8$) and non-treated group ($n = 8$). Paired *t*-tests were used to compare the treated groups to their respective sham-operated side ($n = 16$). Significance was set as $p < 0.05$. As there were no statistical differences detected between the sham-operated side for the SIS- and non-treated groups, a pooled value is reported for the sham-operated side for all parameters.

Two random rabbits from each treatment group were prepared for histological analysis. The MCLs were prepared with attached femoral and tibial bone blocks, leaving the insertion sites intact. The specimens were immediately fixed in 10% neutral buffered formalin, decalcified in RDO, dehydrated in graded ethanol solutions, and embedded in paraffin. Longitudinal sections 7 μm thick were cut and stained with hematoxylin and eosin and Masson Trichrome. The observer was blinded as to which samples were treated with SIS and which received no treatment. The appearance of each sample was described with regards to cellularity, vascularity, and collagen appearance at the insertion sites and the midsubstance of the ligament. Both light and polarized light microscopies were used to examine the histological sections.

Results

All gap injuries of the MCLs healed with continuity of neoligamentous tissue and appeared to be without signs of inflammation. Overall, the SIS-treated MCLs had a more uniform, solid midsubstance when compared to the non-treated group. One healed MCL in the SIS-treated group and two in the non-treated group revealed a narrowing from the femoral insertion to the midsubstance. Also for the non-treated group, there was a large remodeling area in the distal portion of the tibial insertion where the ligament was more transparent in appearance. For the sham-operated side, gross inspection revealed no significant swelling or inflammation.

The CSA as determined by laser micrometer measurements showed that both treatment groups (SIS-treated group: 7.9 ± 3.4 mm² and non-treated group: 7.2 ± 4.7 mm²) were nearly double the sham-operated side (4.7 ± 1.0 mm²), although the non-treated group was not found to be significantly different ($p > 0.05$). There were also no statistical differences between the SIS-treated group and the non-treated group ($p > 0.05$).

The typical load–elongation curves of healed FMTCs from SIS-treated and non-treated groups are shown in Fig. 3A. There was a toe region up to approximately 1 mm of elongation for both groups. Thereafter, the curves diverged, as the slope of the linear region was noticeably higher for the SIS-treated group. The stiffness of the FMTCs in the SIS-treated group measured 56% greater than that in the non-treated group (45.7 ± 13.3 N/mm vs. 29.2 ± 9.2 N/mm, respectively, $p < 0.05$). These values were 51% and 33%, of that for the sham-operated side (89.7 ± 15.3 N/mm, $p < 0.05$). The ultimate load of the FMTC for the SIS-treated group was 79% higher than the non-treated group (117.4 ± 34.5 N

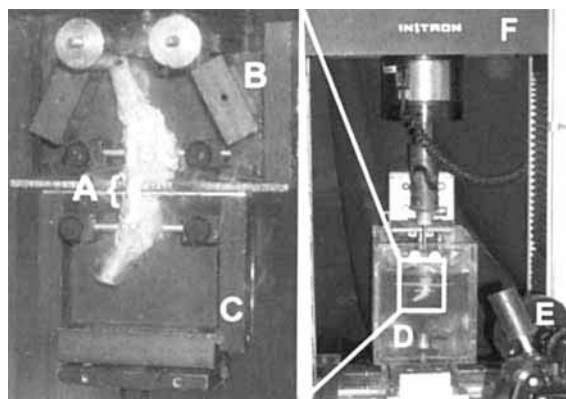


Fig. 2. (A) MCL with strain tracking markers on midsubstance, (B) tibia fixed in customized clamp attached to cross-head, (C) femur fixed in customized clamp attached to the Instron base, (D) saline bath, (E) camera and (F) Instron.

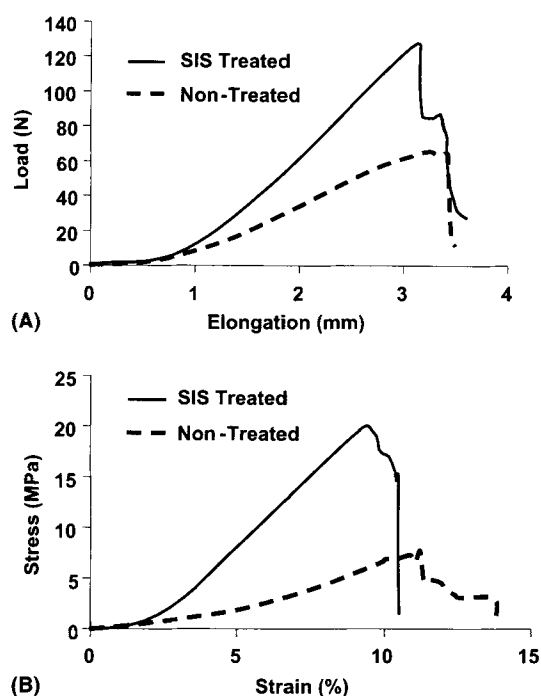


Fig. 3. (A) Typical load–elongation curves and (B) typical stress–strain curves for SIS-treated and non-treated groups.

vs. 66.4 ± 31.4 N, respectively, $p < 0.05$). These values were 36% and 21%, of that for the sham-operated side (332.0 ± 50.8 N, respectively, $p < 0.05$). No significant differences were detected between the SIS-treated group and non-treated group for the elongation at failure (3.7 ± 1.0 mm vs. 3.2 ± 1.0 mm, respectively; $p > 0.05$). The energy absorbed to failure was 198.8 ± 113.3 N mm for the SIS-treated group and 98.0 ± 72.0 N mm for the non-treated group. Both were significantly less than that for the sham-operated side, (1007.9 ± 346.4 N mm, $p < 0.05$).

In terms of the failure mode, three specimens in the SIS-treated group, four in the non-treated group, and all of those in the sham-operated side failed at the midsubstance. Two specimens each in the SIS-treated and non-treated group failed just above the tibial insertion and the remaining specimens ($n = 3$ for SIS-treated group and $n = 2$ for non-treated group) failed by tibial avulsion (Table 1).

The mechanical properties of the ligament midsubstance are detailed in Table 2. Typical stress–strain curves of healed FMTCs from SIS-treated and non-treated groups are shown in Fig. 3B. The tangent modulus of the ligament midsubstance in the SIS-treated group measured 88% greater than that in the non-treated group (280 ± 132 MPa vs. 149 ± 77 MPa, respectively, $p < 0.05$). These values were 30% and 16%, of that for the sham-operated side (936 ± 284 MPa, respectively, $p < 0.05$).

As there were different modes of failure, it was not possible to report the ultimate tensile strength for all specimens. Nevertheless, the stress at failure was calculated (ultimate load divided by the cross-sectional area (CSA) of the midsubstance) and compared. It was found that those for the SIS-treated group (15.7 ± 4.1 MPa) were 1.5 times greater than the non-treated group (10.5 ± 3.9 MPa, $p < 0.05$). However, it should be noted that at 12 weeks post-injury, the values for stress at failure were only 20% of that for the sham-operated side (75.6 ± 14.2 MPa, $p < 0.05$).

Histological comparisons confirmed the biomechanical results (Fig. 4). The SIS-treated group was characterized by increased cellularity at the healing site, as well as denser and more highly aligned collagen. The specimens showed accumulation of organized collagenous connective tissue in which the collagen fibers were aligned in parallel with the fibers of the native MCL tissue that existed at the proximal and distal ends of the

Table 1

Structural properties of the FMTC, (a) denotes significance ($p < 0.05$) between the SIS-treated and non-treated group, while (b) denotes significance ($p < 0.05$) between the treatment groups and the sham-operated side

Structural properties of FMTC	SIS-treatment	Non-treatment	Sham-operation
Stiffness (N/mm)	$45.7 \pm 13.3^{a,b}$	29.2 ± 9.2^b	89.7 ± 15.3
Ultimate load (N)	$117.4 \pm 34.5^{a,b}$	66.4 ± 31.4^b	332.0 ± 50.8
Elongation at failure (mm)	3.7 ± 1.0^b	3.2 ± 1.0^b	5.9 ± 1.3
Energy absorbed (N mm)	198.81 ± 113.3^b	98.0 ± 72.0^b	1007.9 ± 346.4

Table 2

Mechanical properties of the healing MCL, (a) denotes significance ($p < 0.05$) between the SIS-treated and non-treated group, while (b) denotes significance ($p < 0.05$) between the treatment groups and the sham-operated side

Mechanical properties of MCL	SIS-treatment	Non-treatment	Sham-operation
Tangent modulus (MPa)	$279.7 \pm 132.1^{a,b}$	149.0 ± 76.5^b	936.3 ± 283.6
Stress at failure (MPa)	$15.7 \pm 4.1^{a,b}$	10.5 ± 3.9^b	75.6 ± 14.2

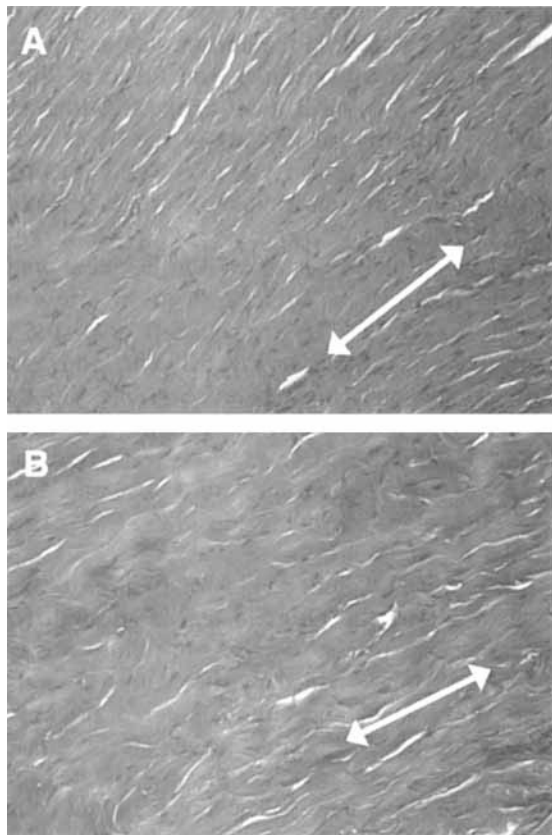


Fig. 4. Histology of the midsubstance of the (A) SIS-treated and (B) non-treated healing MCL. Stained with Masson's Trichrome and magnified 200 \times with light microscopy. Arrow shows longitudinal axis of ligament.

MCL. There was no evidence for any remaining SIS scaffold material although no specific immunostaining was used to evaluate the presence of SIS tissue. There was also no active inflammatory reaction present. The vascularity was moderate and slightly greater than the vascularity noted within the non-treated control specimens. No line of attachment could be distinguished between the native tissue and the healing tissue in the specimens examined.

The non-treated healing MCL samples were characterized by an accumulation of mixed connective tissue types in which disorganized collagenous connective tissue was interspersed with adipose connective tissue. The number of blood vessels did not appear different from the adjacent native tissue. There was no clear evidence for inflammation or necrosis in any of the specimens examined based on the absence of inflammatory cell infiltration or associated vascular response, and no clear evidence of tissue edema, cell debris, or tissue degradation products. The proximal and distal ends of the MCL consisted of more organized collagenous connective tissue with normal insertion into the periosteum and underlying bony substance.

Discussion

In this study, treatment with a single layer of SIS collagen scaffold to enhance healing of a 6 mm MCL gap injury in the rabbit knee model was compared to a non-treated group after 12 weeks of healing. As there was no difference in CSA between the SIS-treated and non-treated groups, increases in the mechanical properties of the healing MCL resulted in increases in the structural properties of the FMTC. It was found that the structural properties of the healing FMTC were significantly enhanced with SIS treatment. While the CSA of the MCL, when treated with an SIS scaffold, did not significantly increase compared to non-treated controls, both were significantly larger than the sham-operated side. Such an enlargement of CSA for the healing MCL has been a consistent finding in the literature [7,13,19,28]. The structural properties of the FMTC for the non-treated control and sham-operated side, as reported in this study, are also similar to those found previously [7,13,19,28]. Histological evaluation supported these findings with the SIS-treated healing MCL shown to be continuous between the femoral and tibial insertions and consisting of robust tissue comprised of dense, organized collagen fibers.

The most significant finding of this study was that the mechanical properties of the healing MCL nearly doubled following SIS-treatment compared to a non-treated control, supporting our hypothesis. The results for the control and sham-operated side were similar to those found previously [7,13,19,28]. The tangent modulus and stress at failure of the MCL for the non-treated group were also slightly lower than those reported by Chimich et al., likely due to the length of healing time as well as the severity of injury. Chimich et al. evaluated healing at 14 weeks after the creation of a 4 mm gap-injury, where this study evaluated healing at 12 weeks after the creation of a 6 mm gap-injury. Further, this study reports stress at failure instead of tensile strength due to the low frequency of midsubstance failures for the healing groups. Therefore, the actual tensile strength of the midsubstance may be higher than those values presented for the stress at failure.

Compared to the trends of previous studies that used SIS on other musculoskeletal tissues, similar results can be found. The biomechanical properties for both the healing infraspinatus and Achilles tendons were also significantly enhanced using porcine SIS [3,9]. It should be noted that both of the previous studies used multi-layered structures of SIS to restore function of the ligament immediately after surgery. In this study, a single layer of SIS was used, with very low stiffness and strength as compared to ligaments and tendons [24]. Rather, the SIS was used as an inductive scaffold that was completely degraded, not as a structural element. Therefore, the results of this study are not comparable

to those that have used SIS constructs to restore ligament function after injury, i.e. for ACL reconstruction [3]. Further, differences in environment, i.e. intra-articular vs. extra-articular, may contribute to differences reported between studies.

Although this study is limited to a time period of 12 weeks, functional tissue engineering of healing ligaments utilizing SIS as a scaffold is suggested based on the encouraging results obtained in this study. Ongoing studies focusing on the morphological and biochemical composition of the healed MCL after treatment with SIS as well as long-term effects of SIS treatment are underway. Future studies will apply cell seeding and repetitive mechanical loading, as a means to increase the potential of SIS to enhance the healing process of ligaments.

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