Adipose-Derived Stem Cells Improve Collagenase-Induced Tendinopathy in a Rat Model

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Abstract

Background: Tendinopathy is a common and highly prevalent musculoskeletal disorder characterized by repetitive activity-related pain and focal tendon tenderness. Histopathologically, tendinopathic tissue mainly shows degenerative changes ~~with little inflammation~~. Therefore, tendinopathy is not affected by anti-inflammatory therapies, and a novel approach, including a stem cell-based therapy, may be beneficial for its treatment. The purpose of this study was to evaluate the effects of adipose-derived stem cells (ASCs) on tendon healing in a rat tendinopathy model.

Hypothesis: ASC transplantation would improve degeneration in collagenase-induced tendinopathy.

Study Design: Controlled laboratory study.

Materials and Method: Sixteen F344/NSlc rats underwent collagenase injection into the Achilles tendon to induce tendinopathy. At 1 week after collagenase injection, eight animals received ASCs (ASC group) and eight animals received phosphate-buffered saline alone (PBS group). Animals were sacrificed at 4 or 12 weeks after ASC administration and the degree of degeneration in each tendon was histologically evaluated according to the Bonar scale. The microstructure of healing tendons was observed by scanning electron microscopy. Reverse transcription (RT)-PCR was performed to measure the ratio of type III collagen mRNA to type I collagen mRNA in tendons.

Results: The median Bonar scale score in the ASC and PBS groups was 2.5 and 5.33, respectively, at 4 weeks after treatment, and 1.0 and 4.0, respectively, at 12 weeks after

treatment. Histologically, the ASC group showed a significantly lower degree of tendon degeneration than the PBS group at both time points. In RT-PCR analysis, the ratio of type III collagen to type I collagen was significantly lower in the ASC group than in the PBS group at 12 weeks after treatment. Moreover, this ratio decreased over time in the ASC group, whereas it increased over time in the PBS group.

Conclusions: These findings demonstrate that the application of ASCs results in significant improvement in the pathological findings associated with tendinopathy and the normalization of collagen ratios within the affected tendon.

Clinical Relevance: Subcutaneous adipose tissue can be harvested easily and ASC administration might have the potential to rapidly treat tendinopathy.

Key Terms: tendinopathy; degeneration; ASCs; collagen ratio.

What is known about the subject: Bone marrow-derived mesenchymal stem cell implantation might accelerate wound healing in tendon injury.

What this study adds to existing knowledge: ASC administration can improve degeneration in tendinopathy.

INTRODUCTION

Tendinopathy is a common musculoskeletal disorder characterized by repetitive activity-related pain and focal tendon tenderness. It can be disabling and frequently results in lost productivity, reduced physical activity, and early retirement from sports or labor [6].

Many studies have shown that inflammatory change is not a major cause of this disorder. Inflammation occurs in the acute stage of tendinopathy. Although the inflammatory process might affect the development of tendinopathy, histopathologically, tendinopathic tissue eventually shows degenerative changes characterized by disrupted collagen fibers, an increase in cellularity, hypervascularity, and ground substance deposition [4, 24, 27, 29, 35]. Moreover, Lui et al. showed that the degree of tendon degeneration is related to tendinopathic pain in animal models [16]. Denaturation of the collagen matrix is also observed. Tendons consist of large amounts of type I collagen and small amounts of other collagens, such as type III collagen [23, 32, 35]. In tendinopathic disorders, the ratio of type III to type I collagen increases and this is sustained over the long-term [17, 35].

Despite its prevalence, tendinopathy is poorly understood and consequently there is little evidence supporting the efficacy of many described remedies. Nonsteroidal anti-inflammatory drugs and corticosteroids have been a mainstay in the clinical treatment of tendinopathy; however, their usefulness remains controversial. As stated above, inflammation is not a main cause of tendinopathy. Thus, these drugs have little effect on degeneration in tendinopathy. Moreover, corticosteroids reportedly

inhibit collagen synthesis and decrease the load to failure of tendons [1]. Relative rest, strengthening exercises, and surgeries have been advocated as a treatment for decades, but this is not an established treatment owing to a lack of evidence [1, 12]. Therefore, a ~~radical~~ new approach is needed to treat tendinopathy. Some authors have suggested that a decreased healing response leads to structural deterioration in tendons and that this results in chronic tendinopathy [21, 35].

Mesenchymal stem cells (MSCs) are non-hematopoietic stromal cells that can differentiate into bone, cartilage, muscle, ligament, tendon, and adipose tissue, and contribute to the regeneration of mesenchymal tissues [21]. The ability of MSCs to secrete growth factors was recently identified and these play an essential role in regenerating tissues [10, 25, 33]. It is that unique characteristic that has led to the exploration of their use and application in the setting of tendon injuries where they may accelerate healing [1]. Transplanting MSCs into tendons has shown good results in the treatment of tendinopathy in animal models [20, 21, 30]. Although MSCs can be isolated from various tissue sources, their procurement from bone marrow is extremely painful for donors and general anesthesia is often required. Moreover, the yield of harvested cells is low.

Adipose tissue is an attractive cell source for stem cell therapies. Adipose-derived stem cells (ASCs) are multipotent, and adipose tissue is ubiquitous and easily obtained in large quantities with little donor site morbidity and discomfort. Therefore, the use of autologous ASCs as therapies is feasible and has been shown to be safe and efficacious in preclinical and clinical studies [18, 36]. Several studies have shown the efficacy of ASC transplantation for tendon disorders [30]. However, there are few reports

that specifically evaluated the effects of ASC transplantation on tendinopathy. The purpose of this study was to evaluate the effects of ASC transplantation on tendon healing in a rat tendinopathy model.

MATERIALS AND METHODS

The following procedures were approved by the animal research ethics committee of Juntendo University School of Medicine (240181).

Preparation of ASCs

Two F344/NSlc rats (12-week-old; SLC Co., Ltd., Shizuoka, Japan) were used as donors. F344/NSlc rats were chosen because they are inbred to the point that they are considered syngeneic. Therefore, transplantation of cells from one rat to another is analogous and limits the risk of graft rejection. ASCs were harvested from the inguinal fat pads of rats as described previously [22]. In brief, fat pads were excised, finely minced, and enzymatically digested using 0.15% type I collagenase (Wako, Osaka, Japan) at 37°C with vigorous agitation. An equal volume of Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) was added to neutralize the collagenase. The cells were centrifuged at 1500 rpm for 5 minutes (CF16RXII; Hitachi, Tokyo, Japan). After cell counting using trypan blue, the cells were seeded at a density of 10^5 cells per 100 mm^2 tissue culture plate and maintained in control medium (DMEM containing 10% FBS and 1% antibiotic-antimycotic solution) at 37°C and 5% CO_2. The medium was changed every 3–4 days

and cells were passaged using 0.25% trypsin when they reached 80–90% confluency. Cells from passage #3 were used for experiments.

Generation of a collagenase-induced tendinopathy rat model

Sixteen male F344/NSlc rats (12-week-old; weight, 230–250 g) were used in this study. Under general anesthesia with 2% isoflurane (Forane®; Abbott Japan, Tokyo, Japan), a small incision was made over each Achilles tendon at 7 mm from the calcaneal insertion after the lower limbs were depilated bilaterally. Under direct vision, 250 units of type I collagenase (Wako, Osaka, Japan) prepared in 25 µL of phosphate-buffered saline (PBS) was injected into the Achilles tendon of bilateral limbs with a 29-gauge needle [16]. The skin was sutured with 5-0 monofilament nylon. Each rat was kept separately and free cage activity was allowed during the study period. This study was performed in line with the ARRIVE animal care guidelines. No obvious side effects appeared during the study period.

Administration of ASCs

The rats were randomly divided into two groups: the ASC group (ASC-treated tendons, n = 16) and the PBS group (PBS-treated tendons, n = 16). Specifically, ASCs were injected into bilateral tendons of eight rats (16 tendons) and PBS was injected into bilateral tendons of eight rats (16 tendons). One week after collagenase injection, a small skin incision was made at the same point in each lower limb under the same anesthesia. Under direct vision, either 50 µL of PBS containing 5×10^5 ASCs or 50 µL of

PBS alone was injected into the collagenase-induced lesion with a 29-gauge needle. The concentration of ASCs was decided referring to the past study [5, 30]. Both studies used $1x10^7$ /ml concentration of cells to treat tendon injury, and showed the significant recovery in the cell treated group. The skin was sutured as previously described. At 4 or 12 weeks after treatment, rats were sacrificed with an overdose of CO_2 and Achilles tendons were harvested for evaluation (n = 8 tendons per time point per group). Each tendon was cut to two samples for histological analysis, and RNA isolation.

Hematoxylin and eosin (H&E) and Alcian blue staining

Slides of rat Achilles tendons were prepared as described previously [11]. In brief, the Achilles tendon was washed in PBS, frozen in cooled hexane, and freeze-embedded with 4–5% carboxymethyl cellulose in the coolant. After a specially prepared adhesive film was fastened to the cut surface, the sample was cut longitudinally into 5 µm thick sections. The sections were stained with H&E and Alcian blue, and then mounted onto 3-amino-coated slides. The specimens were examined by standard light microscopy (BIOREVO BZ-9000; KEYENCE, Osaka, Japan) and photomicrographs were obtained at 200× magnification.

Semi-quantitative Bonar histopathological scale analysis

Longitudinal sections of Achilles tendons were histopathologically analyzed using the semi-quantitative Bonar histopathological scale as previously described [7]. In brief, this scale consists of four features, each of which is graded as 0, 1, 2, or 3: (1)

tenocyte (spindle cell) morphology and proliferation, (2) the presence or absence of ground substance, (3) collagen bundle characteristics, and (4) vascularity [5]. The histological slides from each sample were randomly selected and evaluated in a blinded fashion by two independent pathologists. The average score was used for the analysis.

Scanning electron microscopy (SEM)

Excised tissue was fixed in 2.0% glutaraldehyde for 24 hours and post-fixed in 2% osmium tetroxide for 2 hours. Subsequently, samples were dehydrated through a graded ethanol series. Samples were cut into longitudinal sections for observation by SEM and then further fixed in 2.0% glutaraldehyde. Finally, specimens were dehydrated through a graded ethanol series and underwent critical point drying with liquid CO_2. The dried specimens were mounted onto metal stubs and observed under a scanning electron microscope (S-4800; Hitachi, Tokyo, Japan). The diameter, density and condition of collagen fibers were observed at 5000× magnification (not quantitated).

Immunohistochemistry

Immunohistochemical analysis was performed to assess the formation of type I and type III collagen proteins in tissue sections. In brief, sections were blocked in 3% H_2O_2 prepared in 95% ethanol for 10 minutes and in 10% goat serum for 30 minutes at room temperature. After rinsing in PBS, sections were incubated with primary polyclonal rabbit antibodies against rat type I or type III collagen (AbD Serotec, Kidlington, UK; P02454 and P13941, respectively; and 1:10 and 1:250, respectively)

for 30 minutes at 4°C and then with secondary antibodies (1:100) for 3 minutes at room temperature. After rinsing in PBS three times for 5 minutes, samples were developed in diaminobenzidine solution for 10 minutes. After rinsing in distilled water, samples were counterstained with Harris hematoxylin solution for 5 minutes, washed under running tap water for 5 minutes, and mounted onto 3-amino-coated slides. The glass slides were examined by standard light microscopy (BIOREVO BZ-9000; KEYENCE, Osaka, Japan) and photomicrographs were obtained at 200× magnification.

Reverse transcription (RT)-PCR

The ratio of type III collagen mRNA to type I collagen mRNA was determined. Total RNA was isolated from tendon tissues using a High Pure RNA Tissue Kit (Roche, Mannheim, Germany). cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol. Real-time RT-PCR was performed using a 7500 HT Fast Real-Time PCR system (Applied Biosystems, Warrington, UK).

Statistical analysis

Significant differences among groups were evaluated at each time point using the Mann-Whitney U-test for Bonar scale data and Student's t-test for relative mRNA expression data. Data are expressed as mean ± standard deviation. A value of $p < 0.05$ was regarded as statistically significant.

RESULTS

H&E and Alcian blue staining

In H&E-stained samples, the ASC group showed a lower level of degenerative changes in tendons than the PBS group at 4 and 12 weeks after treatment. The ASC group exhibited decreased levels of disrupted collagen fibers, cellularity, and hypervascularity (Figure 1A–D). In Alcian blue-stained samples, the PBS group exhibited more ground substance deposition between collagen fibers than the ASC group at 4 and 12 weeks after treatment (Figure 1E–H).

Semi-quantitative Bonar histopathological scale analysis

The median Bonar scale score in the ASC and PBS groups was 2.5 and 5.33, respectively, at 4 weeks after treatment, and 1 and 4, respectively, at 12 weeks after treatment. The ASC group exhibited a significantly lower degree of tendon degeneration than the PBS group at both time points.

SEM

In both collagenase-treated groups, fibers had a shorter diameter than intact tendon fibers. The density of collagen fibers was higher in the ASC group than in the PBS group at both time points. Collagen fibers in the PBS group were randomly orientated and wavelike at both time points (Figure 2A–E).

Immunohistochemistry

In immunohistochemical analyses, the area positively stained for type I collagen increased over time in the ASC group. In the PBS group, there were no apparent changes in this staining between 4 and 12 weeks after treatment (Figure 3A–D). In immunohistochemical analyses, the area positively stained for type III collagen decreased over time in the ASC group. In the PBS group, there were no apparent changes in this staining between 4 and 12 weeks after treatment (Figure 3E–H).

RT-PCR

The ratio of type III collagen to type I collagen decreased over time in the ASC group (from 0.169 to 0.116), whereas it increased over time in the PBS group (from 0.099 to 0.224). At 12 weeks after treatment, the ratio of type III collagen to type I collagen was significantly lower in the ASC group than in the PBS group ($p < 0.05$) (Figure 4).

DISCUSSION

This study showed that the ASC administration improved pathological characteristics of collagenase-induced tendinopathy in both the acute (4 weeks) and chronic (12 weeks) stages compared to continued degeneration throughout both stages in a control model.

Several basic research studies reported that MSC administration accelerates the healing of tendon injuries and improves the degeneration of tendons [20, 23, 30, 34]. In this study, tendons in the ASC group had a structure that was histologically analogous to that

of normal tendons. ASC administration decreased the level of degeneration at 4 and 12 weeks after treatment. Specifically, ASC administration inhibited the disruption of collagen fibers and the increase in cellularity from the acute stage. Moreover, chondrocyte-like cells, calcific deposits, and ground substances were not observed in ASC-treated tendons up to 12 weeks after treatment. These findings suggest that ASC administration induced processes to heal degeneration in tendinopathy during the acute stage.

A collagenase-induced tendinopathy model shows inflammation and micro-injury in tendons during the acute stage, and the ratio of type I collagen in tendons decreases in this stage [4, 5, 29]. It is generally accepted that the level of type III collagen increases and scar tissue forms during the acute stage of wound healing. Thereafter, type III collagen is replaced by type I collagen over time [3, 4, 9, 26]. The same process occurs in injured tendons. An increased level of type III collagen is necessary during the acute stage of wound healing. However, type III collagen is mechanically weaker than type I collagen. Therefore, rapid ~~conversion~~ replacement of type III collagen into type I collagen is necessary for tendon repair [15, 32, 35].

In this study, ASC administration rapidly increased the ratio of type III collagen to type I collagen, after which it decreased. By contrast, this ratio increased slowly over the entire observation period in the PBS group. These findings indicate that ASC administration accelerated wound healing from the acute stage and normalized the tendon structure in tendinopathy at the chronic stage. Meanwhile, in the PBS group, the

ratio of type III collagen to type I collagen increased and the process of wound healing continued throughout the entire observation period.

Several studies have suggested the “negative cycle” concept in tendon wound healing, which may cause prolonged symptoms of tendinopathy [2, 17, 35]. In other words, the abnormal collagen ratio (i.e., a high ratio of type III collagen to type I collagen) is maintained in tendons upon repetitive or over injury, when tenocytes cannot rapidly repair the injury. A sustained abnormal collagen ratio decreases tendon strength, which can cause mechanical micro-injuries and degenerative changes in tendons. This inhibits replacement of type III collagen by type I collagen and maintains the abnormal collagen ratio. Kraus et al. showed that tenocytes and ASCs have an increased proliferation rate and gene expression when they are co-cultured in vitro [14]. Therefore, ASC administration might stimulate tenocytes and reinforce their functions, leading to the accelerated formation of scar tissue and conversion of this tissue into normal tissue at the acute stage. ASC administration accelerated extracellular matrix turnover, in particular normalization of the collagen ratio and tendon structures from the acute stage, which might increase tendon strength. Early recovery of tendon strength would inhibit mechanical micro-damage and further degenerative changes in tendons, leading to reduced scar tissue. This positive cycle ~~would~~ could rapidly heal degeneration in tendinopathy.

Valencia et al. and Chen et al. reported that MSC transplantation alone did not repair tendon injury [5, 31]. These conclusions are in contrast to our results. However, the cell transplantation conditions differ between the studies, especially with regard to the

timing of cell administration. In our study, ASCs were administered 1 week after collagenase injection, in contrast to 4 weeks in these previous studies. The difference in the timing of transplantation might result in different conclusions. Therefore, additional studies are needed to determine whether there are different responses if the treatment is delivered at different time points.

A limitation of this study is the lack of information on the fate of the transplanted cells. Whether transplanted stem cells differentiate into tenocytes is not clearly understood [8]. Although one study showed the long-term survival of stem cells transplanted into tendons [30], several studies reported that transplanted labeled cells could not be detected a few weeks after transplantation [13, 19, 28]. Several studies have shown that growth factors, such as hepatocyte growth factor, insulin-like growth factor-1, transforming growth factor-ß1, and vascular endothelial growth factor, play an important role in tendon healing [32, 34]. Recent studies reported that several growth factors secreted by MSCs are required for effective wound healing [13, 25, 28]. In this respect, the paracrine effects of ASCs might be important for the improvement of tendinopathy in this study, although further studies are needed to trace the fate of transplanted cells and to understand the mechanism by which ASC treatment improves tendinopathy.

In conclusion, ASC administration improved degeneration in tendinopathy and rapidly normalized the collagen ratio in tendon tissue.

REFERENCES

1. Andres BM, Murrell GA. Treatment of tendinopathy: what works, what does not, and what is on the horizon. *Clin Orhop Relat Res.* 2008;466(7):1539-1554. PMID: 18446422

2. Arnoczky SP, Lavagnino M, Egerbacher M, Caballero O, Gardner K. Matrix metalloproteinase inhibitors prevent a decrease in the mechanical properties of stress-deprived tendons: an in vitro experimental study. *Am.J Sports Med* 2007;35(5):763-769. PMID: 17293464

3. Bailey AJ, Bazin S, Sims TJ, Le Lous M, Nicoletis C, Delaunay A. Characterization of the collagen of human hypertrophic and normal scars. *Biochim Biophys Acta.* 1975;405(2):412-421. PMID: 1180964

4. Casalechi HL, Leal-Junior EC, Xavier M, et al. Lower-level laser therapy in experimental model of collagenase-induced tendinitis in rats: effects in acute and chronic inflammatory phase. *Lasers Med Sci.* 2013;28(3):989-995. PMID: 22926534

5. Chen L, Liu JP, Tang KL, et al. Tendon derived stem cells promote platelet-rich plasma healing in collagenase-induced rat achilles tendinopathy. *Cell Physical Biochem.* 2014;34(6):2153-2168. PMID: 25562162

6. Connell D, Datir A, Alyas F, Curtis M. Treatment of lateral epicondylitis using skin-derived tenocyte-like cells. *Br J Sports Med.* 2009;43(4):293-298. PMID: 19224912

346 **7.** Cook JL, Feller JA, Bonar SF, Khan KM. Abnormal tenocyte morphology is
347 more prevalent than collagen disruption in asymptomatic athletes' patellar
348 tendons. *J Orthop Res.* 2004;22(2):334-338. PMID: 15013093
349 **8.** Guest DJ, Smith MR, Allen WR. Monitoring the fate of autologous and
350 allogeneic mesenchymal progenitor cells injected into the superficial digital
351 flexor tendon of horses: preliminary study. *Equine vet J.* 2008;40(2):178-181.
352 PMID: 18267891
353 **9.** Hayakawa T, Hashimoto Y, Myokei Y, Aoyama H, Izawa Y. Changes in type of
354 collagen during the development of human post-burn hypertrophic scars. *Clin*
355 *Chim Acta.* 1979;93(1):119-125. PMID: 436291
356 **10.** Jung H, Kim HH, Lee DH, Hwang YS, Yang HC, Park JC. Transforming growth
357 factor-beta 1 in adipose derived stem cells conditioned medium is a dominant
358 paracrine mediator determines hyaluronic acid and collagen expression profile.
359 *Cytotechnology.* 2011;63(1):57-66. PMID: 21203839
360 **11.** Kawamoto T. Use of a new adhesive film for the preparation of multi-purpose
361 fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch*
362 *Histol Cytol.* 2003;66(2):123-143. PMID: 12846553
363 **12.** Khan KM, Cook JL, Bonar F, Harcourt P, Astrom M. Histopathology of
364 common tendinopathies. Update and implications for clinical management.
365 *Sports Med.* 1999;27(6):393-408. PMID: 10418074

13. Kinnaird T. Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion Through Paracrine Mechanisms. *Circulation.* 2004;109(12):1543-1549. PMID: 15023891

14. Kraus A, Woon C, Raghavan S, Megerle K, Pham H, Chang J. Co-culture of human adipose-derived stem cells with tenocytes increases proliferation and induces differentiation into a tenogenic lineage. *Plast Reconstr Surg.* 2013;132(5):754e-766e. PMID: 24165627

15. Lin TW, Cardenas L, Soslowsky LJ. Biomechanics of tendon injury and repair. *J Biomech.* 2004;37(6):865-877. PMID: 15111074

16. Lui PP, Chan LS, Fu SC, Chan KM. Expression of sensory neuropeptides in tendon is associated with failed healing and activity-related tendon pain in collagenase-induced tendon injury. *Am J Sports Med.* 2010;38(4):757-764. PMID: 20139325

17. Maffulli N, Ewen SW, Waterston SW, Reaper J, Barrass V. Tenocytes from ruptured and tendinopathic achilles tendons produce greater quantities of type III collagen than tenocytes from normal achilles tendons. An in vitro model of human tendon healing. *Am.J Sports Med.* 2000;28(4):499-505. PMID: 10921640

18. Mizuno H, Tobita M, Uysal AC. Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem cells.* 2012;30(5):804-810. PMID: 22415904

386 **19.** Ni M, Lui PP, Rui YF, et al. Tendon-derived stem cells (TDSCs) promote tendon
387 repair in a rat patellar tendon window defect model. *J Orthop Res.*
388 2012;30(4):613-619. PMID: 21928428
389 **20.** Nourissat G, Diop A, Maurel N, et al. Mesenchymal stem cell therapy
390 regenerates the native bone-tendon junction after surgical repair in a
391 degenerative rat model. *PloS one.* 2010;5(8):e12248. PMID: 20805884
392 **21.** Obaid H, Connell D. Cell therapy in tendon disorders: what is the current
393 evidence? *Am.J Sports Med.* 2010;38(10):2123-2132. PMID: 20699425
394 **22.** Ogawa R, Mizuno H, Watanabe A, Migita M, Shimada T, Hyakusoku H.
395 Osteogenic and chondrogenic differentiation by adipose-derived stem cells
396 harvested from GFP transgenic mice. *Biochem Biophys Res Commun.*
397 2004;313(4):871-877. PMID: 14706623
398 **23.** Okamoto N, Kushida T, Oe K, Umeda M, Ikehara S, Iida H. Treating Achilles
399 tendon rupture in rats with bone-marrow-cell transplantation therapy. *J Bone*
400 *Joint Surg Am.* 2010;92(17):2776-2784. PMID: 21123607
401 **24.** Rees JD, Maffulli N, Cook J. Management of tendinopathy. *Am.J Sports Med.*
402 2009;37(9):1855-1867. PMID: 19188560
403 **25.** Salgado AJ, Reis RL, Sousa NJ, Gimble JM. Adipose tissue derived stem cells
404 secretome: soluble factors and their roles in regenerative medicine. *Curr Stem*
405 *Cell Res Ther.* 2010;5(2):103-110. PMID: 19941460
406 **26.** Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med.*
407 1999;341(10):738-746. PMID: 10471461

408 **27.** Solchaga LA, Bendele A, Shah V, et al. Comparison of the effect of intra-tendon
409 applications of recombinant human platelet-derived growth factor-BB, platelet-
410 rich plasma, steroids in a rat achilles tendon collagenase model. *J Orthop Res.*
411 2014;32(1):145-150. PMID: 24018586
412 **28.** Togel F, Weiss K, Yang Y, Hu ZM, Zhang P, Westenfelder C. Vasculotropic,
413 paracrine actions of infused mesenchymal stem cells are important to the
414 recovery from acute kidney injury. *Am J Physiol-Renal.* 2007;292(5):F1626-
415 F1635. PMID: 17213465
416 **29.** Torres- Silva R, Lopes-Martins RA, Bjordal JM, et al. The low level laser
417 therapy (LLLT) operating in 660 nm reduce gene expression of inflammatory
418 mediators in the experimental model of collagenase-induced rat tendinitis.
419 *Lasers Med Sci.* 2015;30(7):1985-1990. PMID: 25380666
420 **30.** Uysal CA, Tobita M, Hyakusoku H, Mizuno H. Adipose-derived stem cells
421 enhance primary tendon repair: Biomechanical and immunohistochemical
422 evaluation. *J Plast Reconstr Aeshet Surg.* 2012;65(12):1712-9. PMID: 22771087
423 **31.** Valencia Mora M, Antuna Antuna S, Garcia Arranz M, Carrascal MT, Barco R.
424 Application of adipose tissue-derived stem cells in a rat rotator cuff repair model.
425 *Injury.* 2014;45 Suppl 4:S22-27. PMID: 25384471
426 **32.** Wang JH. Mechanobiology of tendon. *J Biomech.* 2006;39(9):1563-1582.
427 PMID: 16000201

33. Wang XJ, Dong Z, Zhong XH, et al. Transforming growth factor-beta1 enhanced vascular endothelial growth factor synthesis in mesenchymal stem cells. *Biochem Biophys Res Commun.* 2008;365(3):548-554. PMID: 18023419

34. Woo SL, Hildebrand K, Watanabe N, Fenwick JA, Papageorgiou CD, Wang JH. Tissue engineering of ligament and tendon healing. *Clin Orthop Relat Res.* 1999(367 Suppl):S312-323. PMID: 10546655

35. Xu Y, Murrell GA. The basic science of tendinopathy. *Clin Orthop Relat Res.* 2008;466(7):1528-1538. PMID: 18478310

36. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* 2001;7(2):211-228. PMID: 11304456

441 FIGURE LEGENDS

442

443 Figure 1.

444 Representative histology images of longitudinal tendon sections. (A) Hematoxylin and
445 eosin (H&E) staining of the adipose-derived stem cell (ASC) group at 4 weeks after
446 treatment. (B) H&E staining of the ASC group at 12 weeks after treatment. (C) H&E
447 staining of the phosphate-buffered saline (PBS) group at 4 weeks after treatment. (D)
448 H&E staining of the PBS group at 12 weeks after treatment. There was a lower level of
449 degenerative changes in tendons in the ASC group than in the PBS group at 4 and 12
450 weeks after treatment. The ASC group exhibited decreased levels of disrupted collagen
451 fibers, cellularity, and hypervascularity. (E) Alcian blue staining of the ASC group at 4
452 weeks after treatment. (F) Alcian blue staining of the ASC group at 12 weeks after
453 treatment. (G) Alcian blue staining of the PBS group at 4 weeks after treatment. (H)
454 Alcian blue staining of the PBS group at 12 weeks after treatment. The PBS group
455 exhibited a large amount of ground substance deposition between collagen fibers at 4
456 and 12 weeks after treatment. H&E staining mainly showed tenocyte (spindle cell)
457 morphology and proliferation, collagen bundle characteristics, and vascularity. Alcian
458 blue staining showed the presence or absence of the ground substance. Magnification
459 200×; bar = 50 μm; arrow, vascular structure; arrowhead, chondrocyte-like cell.

460

461 Figure 2.

Representative scanning electron microscopy images of longitudinal tendon sections. (A) Normal tendon for comparison. (B) Tendon in the adipose-derived stem cell (ASC) group at 4 weeks after treatment. (C) Tendon in the ASC group at 12 weeks after treatment. (D) Tendon in the phosphate-buffered saline (PBS) group at 4 weeks after treatment. (E) Tendon in the PBS group at 12 weeks after treatment. The density of collagen fibers was higher in the ASC group than in the PBS group at both time points. Collagen fibers in the PBS group were randomly orientated and wavelike at both time points. Magnification, 5000×.

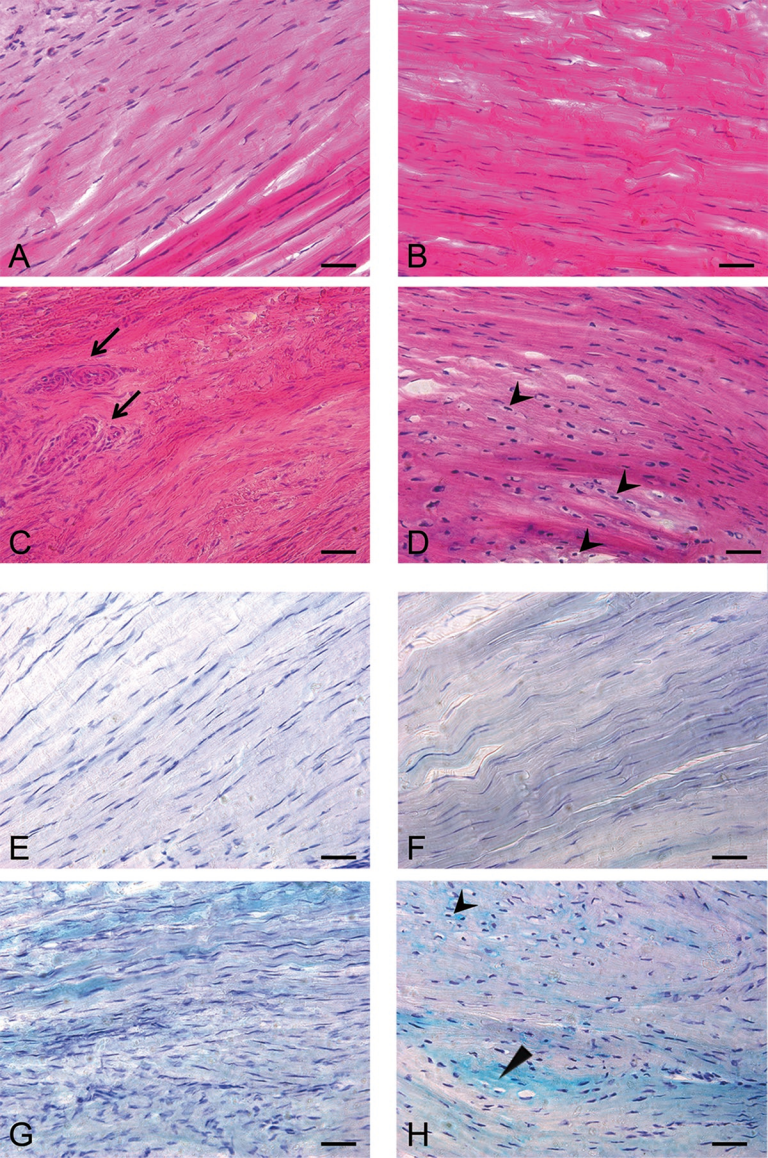
Figure 3.

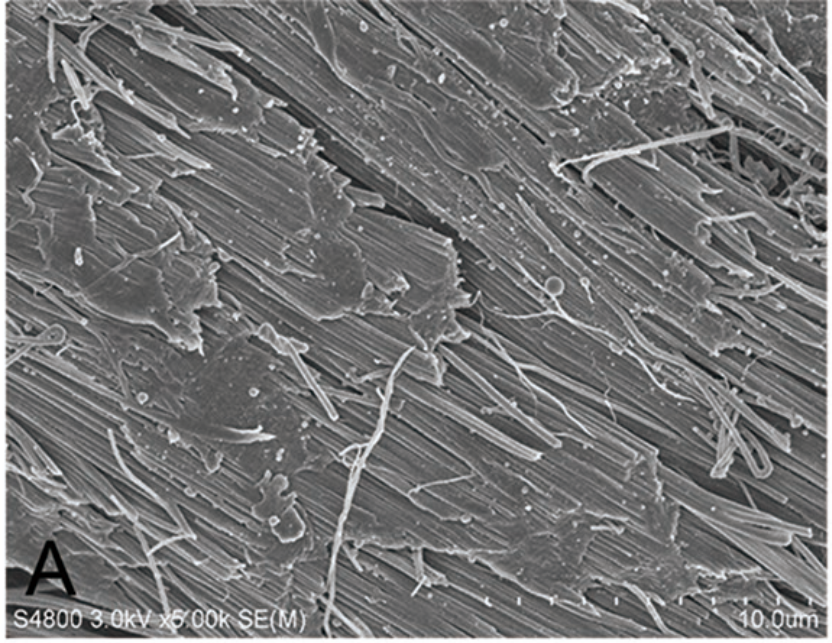
Representative immunohistochemistry images of longitudinal tendon sections. (A) Type I collagen staining of the adipose-derived stem cell (ASC) group at 4 weeks after treatment. (B) Type I collagen staining of the ASC group at 12 weeks after treatment. (C) Type I collagen staining of the phosphate-buffered saline (PBS) group at 4 weeks after treatment. (D) Type I collagen staining of the PBS group at 12 weeks after treatment. The area positively stained for type I collagen increased over time in the ASC group. In the PBS group, there were no apparent changes in this staining between 4 and 12 weeks after treatment. (E) Type III collagen staining of the ASC group at 4 weeks after treatment. (F) Type III collagen staining of the ASC group at 12 weeks after treatment. (G) Type III collagen staining of the PBS group at 4 weeks after treatment. (H) Type III collagen staining of the PBS group at 12 weeks after treatment. In the ASC group, the area positively stained for type III collagen decreased over time. In the PBS

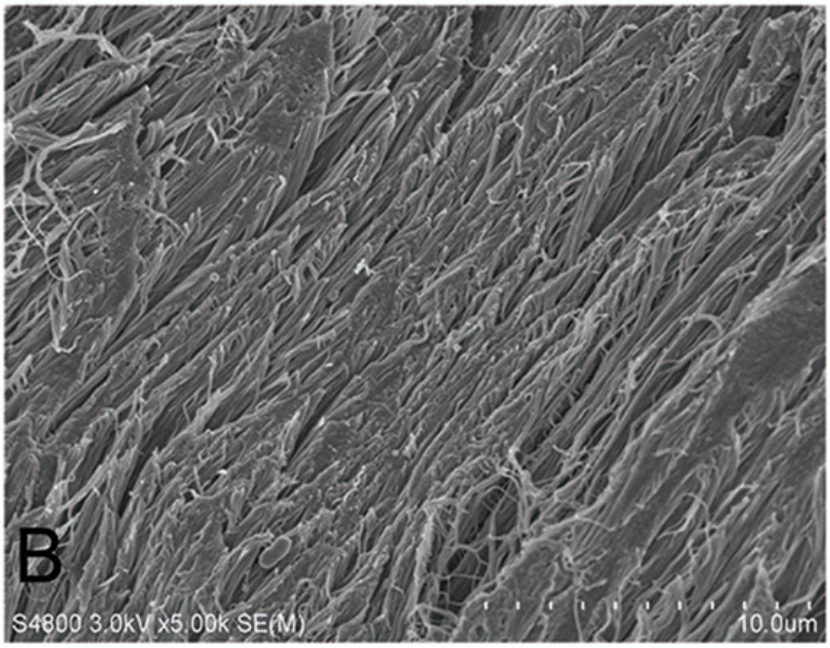
group, there were no apparent changes in this staining between 4 and 12 weeks after treatment. Magnification 200×; bar = 50 μm; CR, calcific deposits.

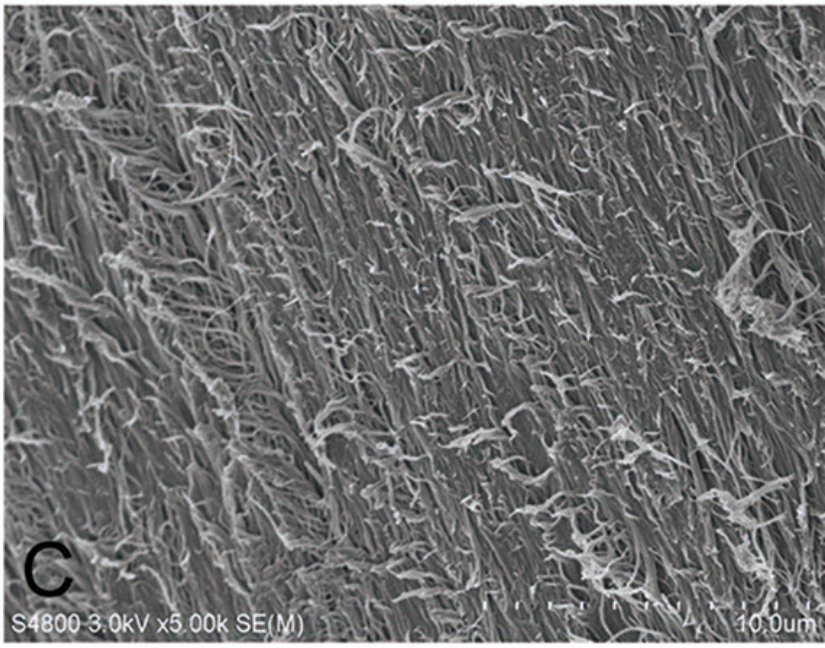
Figure 4.

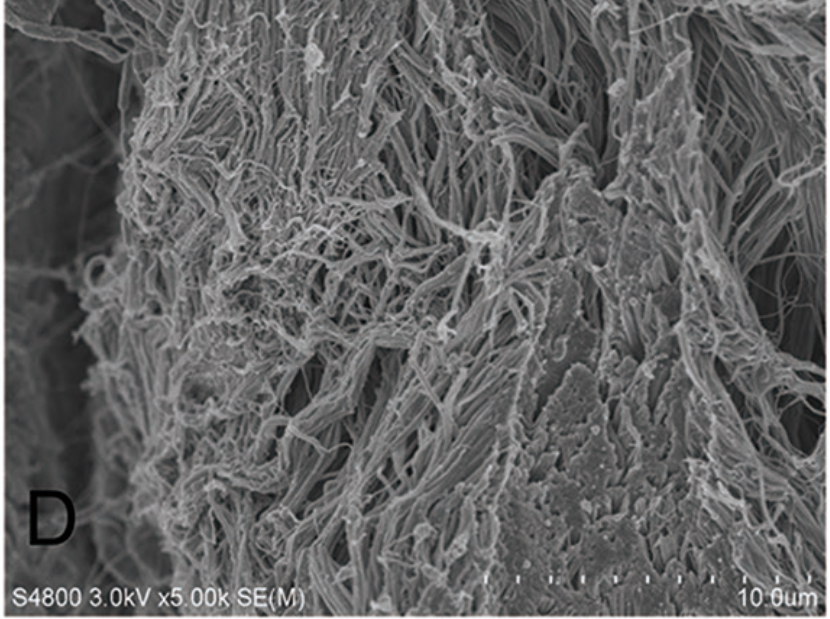
Real-time RT-PCR analysis of the ratio of type III collagen mRNA to type I collagen mRNA. Dots represent the mean for each group at 4 and 12 weeks after treatment. Error bars represent ± one standard deviation. * indicates $p < 0.05$ according to the Student's t-test.

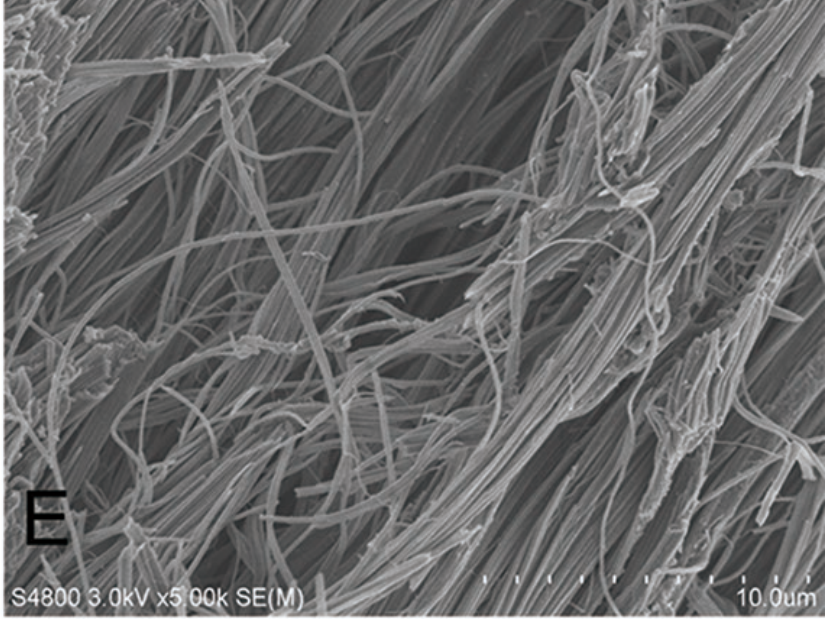

A
B
C
D
E
F
G
H

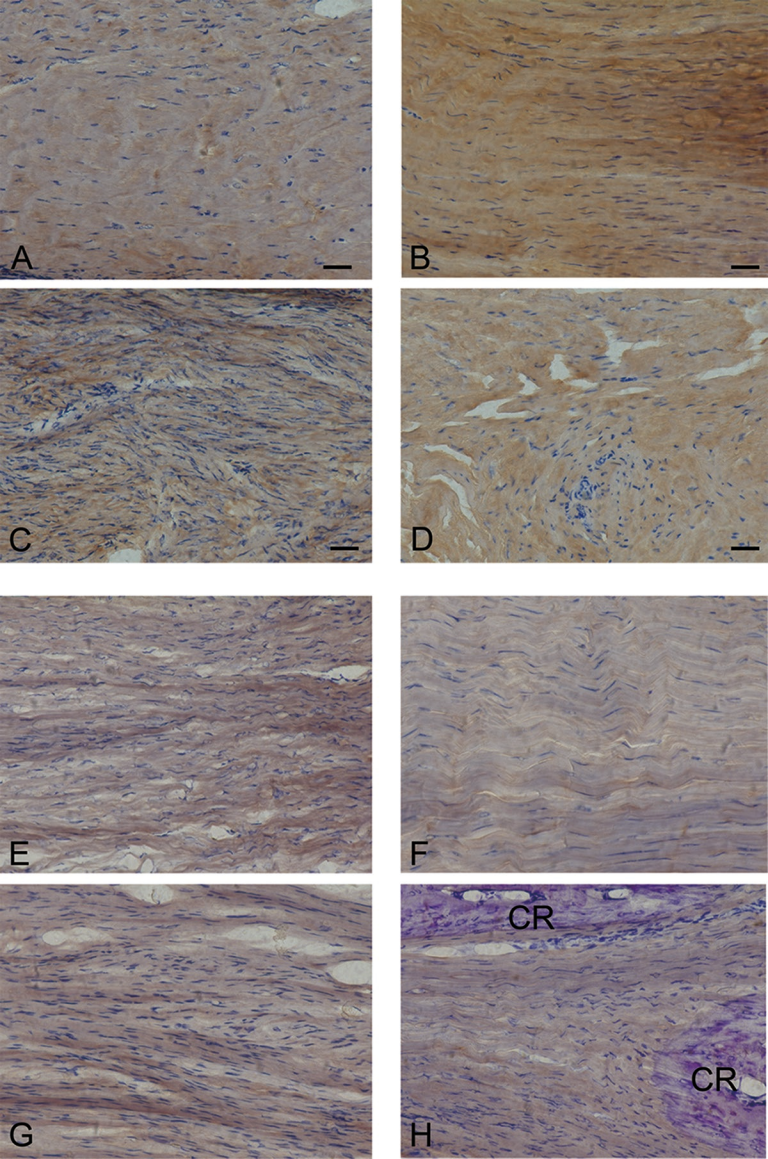

A
S4800 3.0kV x5.00k SE(M)
10.0um

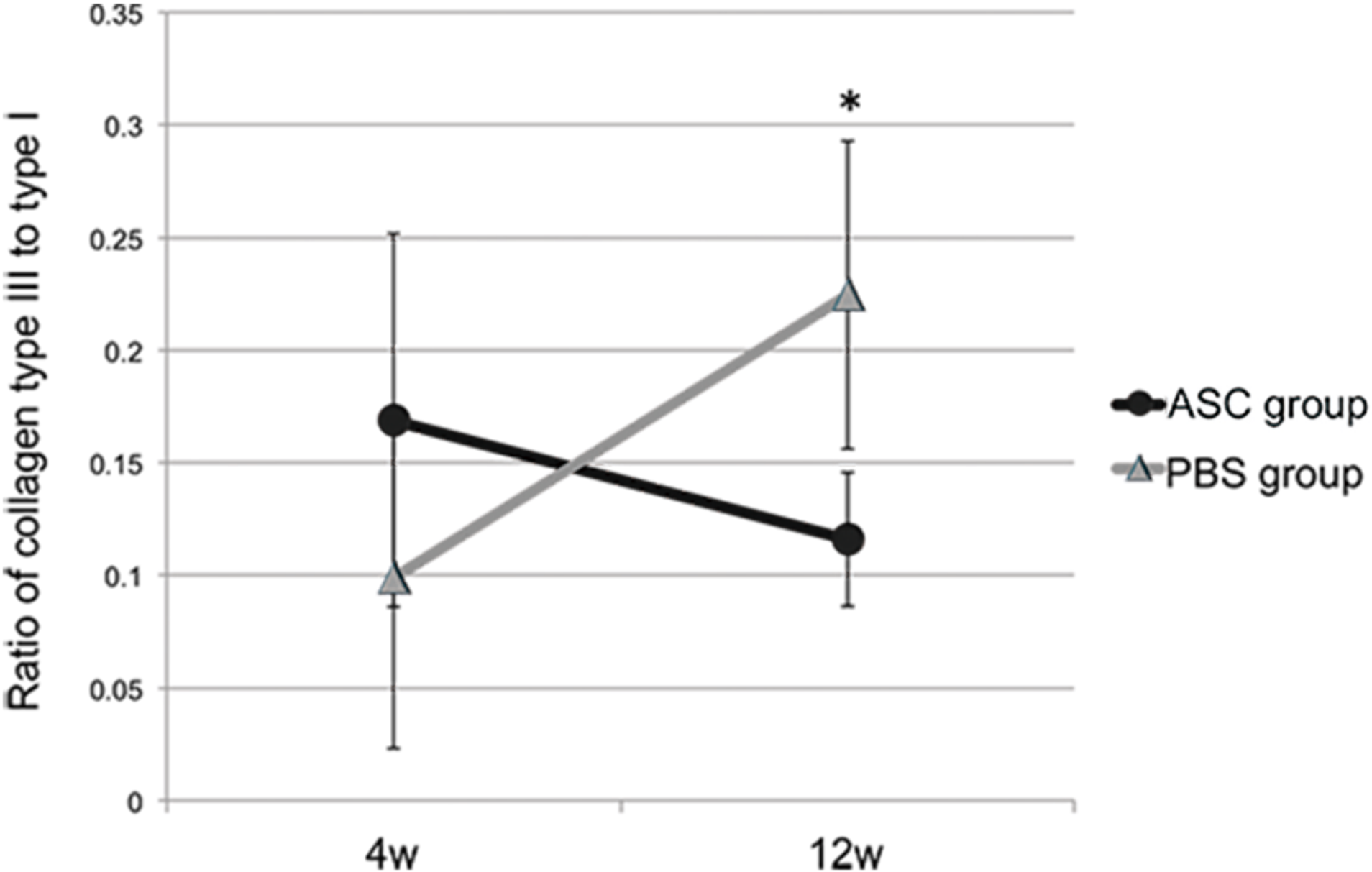

B
S4800 3.0kV x5.00k SE(M)
10.0um


C
S4800 3.0kV x5.00k SE(M)
10.0um


D
S4800 3.0kV x5.00k SE(M)
10.0um


E
S4800 3.0kV x5.00k SE(M)
10.0um


A
B
C
D
E
F
G
CR
CR
H


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Ratio of collagen type III to type I
*
4w
12w
ASC group
PBS group