Tenogenic effects of silymarin following experimental Achilles tendon transection in rats

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Abstract

Tendon healing is prolonged due to the small number of cells, poor circulation, and low metabolism. The optimal tendon healing and its complete functional recovery have always been a challenge for researchers. Silymarin possesses anti-inflammatory, anti-oxidant, analgesic, and regenerative properties. The present study aimed to investigate the effects of silymarin on healing the Achilles tendon in rats. Twenty-four male Wistar rats were divided into two groups of control and treatment. After surgical preparation, a complete transverse incision was made in the middle part of the Achilles tendon, and then a modified Kessler suture was placed. The control group received 1.00 mL normal saline for five consecutive days, and the treatment group received 50.00 mg kg⁻¹ of silymarin suspended in 1.00 mL normal saline for five days, orally. During the experimental period, Achilles functional index (AFI) was recorded. Six weeks after surgery, sampling was done. Histopathologically, a significant increase in the density of collagen fibers and reduction in neovascularization and inflammatory cells infiltration were observed in the treatment group. The biomechanical evaluation showed a significant increase in tensile strength of the tendon in the treatment group compared to the control group. The AFI results were concomitant with the results stated above, indicating an improvement in the AFI of rats in the treatment group. The present study results showed that oral administration of silymarin improved tissue healing indices, biomechanical properties, and functional index, leading to optimal healing of experimental Achilles tendon injury in the rat.

Introduction

Tendon injuries are associated with considerable morbidity in sport and the workplace in humans and animals.¹ Noticeable pain and compromised locomotor dysfunction are often known as complications of tendon injury. Among the several approaches, surgical repair is the most common intervention for tendon rupture or laceration. However, adhesion to surrounding connective tissues and inferior mechanical properties of the repairs have been reported frequently after reconstructive tendon surgery adversely affecting the clinical outcome.²

Currently, the clinical therapeutic protocol following surgical repair includes non-steroidal anti-inflammatory drugs (NSAIDs) therapy, mostly for analgesic effects and secondarily for anti-inflammation properties.³ It is believed that reduced edema and consequently decreased production of inflammatory mediators improve wound healing.⁴ Administration of commercially available anti-inflammatory and analgesic agents could result in a relatively improved tendon healing.⁵ Although significant pain relief and reduced inflammation have been reported after NSAIDs,⁶ recent studies have suggested that these agents may hinder the favorable healing response of injured soft tissues like tendons.⁷ Degenerative effects on the healing tendon per se have been observed following NSAIDs therapy,⁸ but several studies have questioned their value due to the serious adverse effects such as hepatotoxicity, nephrotoxicity, coagulopathy, cardiomyopathy, and gastrointestinal mucosal damage.⁹⁻¹³

Alternatively, because of the potential efficacy and reduced risk of side effects, natural medicines have become increasingly noted in recent studies. Silymarin, an extract from Silybum marianum, has demonstrated

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analgesic, anti-oxidant and anti-inflammatory effects and has been clinically studied and proven to be an effective natural treatment for improving wound repair. Topical silymarin could improve dermal wound healing through elevated fibroblast proliferation, collagen organization, and finally improved wound contraction in rats. Enhanced maturity of the wound and increased biomechanical properties were also observed in an experimental study of the rat cutaneous wound healing model. Moreover, the osteogenic effect of orally administered silymarin has been reported in the murine model of tibial fracture. Boosted collagen secretion, improved morphology, and bone strength were observed following silymarin treatment.

In the present study, the effects of orally administered silymarin on the healing of Achilles tendon in rats were investigated using histopathological, biomechanical, and functional evaluations.

Materials and Methods

Animals. Twenty-four adult male Wistar rats (200 ± 20 g) were housed individually in plastic cages on wood chip-type bedding, fed with chow pellet, and had free access to water. Randomly, the rats were divided into two groups of treatment and control (n = 12). The animal experiments were performed in accordance with the authors’ Institutional Animal Care Instructions (93/1445).

Surgery. The rats were anesthetized by an intraperitoneal injection of ketamine (80.00 mg kg⁻¹; Alfasan, Woerden, The Netherlands) and xylazine (5.00 mg kg⁻¹; Alfasan) combination. Following aseptic preparations of the left hind limb, the Achilles tendon was exposed through a longitudinal posterior skin incision. Then, the tendon was completely transected using a 10 blade and sutured with a modified Kessler pattern by 4-0 nylon (Fig. 1). The skin incision was sutured in a simple interrupted pattern by 3-0 nylon. Following recovery, the rats were returned to their cages and roam freely until the end of the study. In the treatment group, 50.00 mg kg⁻¹ silymarin (S0292; Sigma-Aldrich Co., St. Louis, USA) suspended in 1 mL normal saline was orally administered using a gavage needle for five days after surgery. Control rats received only normal saline for a comparable period.

Achilles functional index (AFI). The hind paw prints of rats (n = 6) were used for functional performance of the Achilles tendon at the end of each week after surgery for six consecutive weeks. In this regard, the hind paws of the rats were dipped in ink. The rats were then allowed to walk in a 1.00 m length corridor with white paper on its floor. The AFI was calculated according to Murrell et al. as follows:

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AFI = 74(PLF) + 161(TSF) + 48(ITF) - 5
\]

where, PLF is print length factor, TSF is toe spread factor, and ITF is an intermediate toe factor representing the difference between the experimental and contralateral print measurements.

At the end of the 6th week, rats were sacrificed with an intraperitoneal overdose of ketamine (150.00 mg kg⁻¹; Alfasan) and xylazine (10.00 mg kg⁻¹; Alfasan).

Macroscopic evaluation. After euthanasia at week six, surgical sites were opened, and Achilles tendons were evaluated in terms of peritendinous adhesion formation before sampling. According to Tang et al., adhesion was assessed qualitatively on the 0 to 6 scale as 0 = none, 1-2 = mild, 3-4 = moderate and 5-6 = severe adhesion.

Histopathological evaluation. Longitudinal 5.00 μm thick sections of tendons (n = 6) from both groups were stained with hematoxylin and eosin and Masson’s trichrome and evaluated quantitatively for the following parameters: Number of inflammatory cells (including neutrophils and macrophages), fibroblasts (ovoid-shaped tenoblasts), and newly formed vessels (neovessels) in 0.25 mm² of granulation tissue of each section. Collagen intensity obtained from the average distance between the adjacent collagen bundles with longitudinally oriented fibers was measured in 10 microscopic fields, where a lower value represents a higher density. Image analysis was done with FIJI software (version 2.0.0; National Institutes of Health, USA), and 3D plots were drawn using Image-pro Insight software (version 8.0; Media Cybernetics Inc., Rockville, USA).

Mechanical evaluation. Six samples from each group were subjected to mechanical evaluations. Before testing, suture materials were removed, and tendons were mounted on STM-20 tensile device, including a 50.00 kg load cell. The constant velocity of 60 mm min⁻¹ was used for the tensile test. During each test, a force-displacement curve was displayed in real-time, and the following parameters were recorded: Maximum load (N), load at yield point (N), energy absorption (J), maximum stress (MPa), maximum strain (%), and stiffness (MPa mm⁻¹).

Statistical analysis. Two-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni’s post hoc test was used for AFI data. Adhesion scores were analyzed with the Mann-Whitney test. Quantitative histopathological parameters and mechanical properties of repairs were compared using a Student t-test. All

Fig. 1. A) Rat Achilles tendon was exposed via a longitudinal incision on the posterior aspect of the left hind limb; B) Modified Kessler suture was used to repair the transected tendon.
analyses were conducted using Statistical Package for Social Sciences (SPSS) software (version 20, IBM Corp., Armonk, NY, USA), and \( p \) values less than 0.05 were considered as significant. All quantitative data are presented as mean ± standard deviation.

**Results**

**Macroscopic evaluation.** Gross examination revealed a lower amount of adhesion formation in the treatment group than the controls (\( p < 0.05 \)). In the treatment group, samples were harvested by slight blunt dissection, while due to the severe adhesions, sharp dissection was needed to expose and detach the Achilles tendon in the control group (Fig. 2). The mean score for adhesion formation in the treatment group was 0.40 compared to 2.40 in the control group.

![Fig. 2. A) Sharp dissection using a scalpel was required to release the adhesions surrounding the Achilles tendon in the control group; B) Adhesion formation was significantly lower in the silymarin treated group, and tendons were harvested by slight blunt dissection.](image)

**Histopathology.** Figures 3 and 4 demonstrate the results of histopathological evaluations after six weeks. Accordingly, a reduced number of fibroblasts was observed in the silymarin treated group compared to control samples; however, no significant difference was found (\( p > 0.05 \)). However, a significant decrease in the number of inflammatory cells was seen in samples with silymarin treatment compared to controls (\( p < 0.05 \)). The number of neovessels was also significantly lower in the treatment group (\( p < 0.05 \)) than control one.

Measuring the distances between collagen bundles indicated that silymarin administration resulted in the formation of more dense and well-organized granulation tissue in the repair site. In contrast, in the control group, collagen bundles were sparsely distributed, showing the low organization of granulation tissue. Based on the intensity plot profile, the neotendons in the control group represented loose and irregular collagen expansion and alignment, while in the treatment group, intense collagen alignment was seen, and the collagen fibers were oriented tangentially in 2530×2530 μm of tissue. The mean distances were 126.67 ± 4.80 μm and 155.33 ± 6.53 μm in treatment and control groups, respectively (\( p < 0.05 \)).

![Fig. 3. A) Granulation tissue in the control group, including randomly scattered fibroblasts. Arrows indicate newly formed vessels, while in the treatment group; B) Fewer levels of angiogenesis (arrows) were observed among fibroblasts with elongated and parallel nuclei, which were in line with collagen fibers in the treatment group. The parallel orientations of the cells indicate improved remodeling of collagen fibers; C) Edema in granulation tissue and haphazardly oriented collagen fibers in the control group; D) Dense and organized collagen bundles in the treatment group. (A and B: H&E staining; C and D: Masson’s trichrome staining, Scale bars = 200 μm). The 3D plots illustrates E) Weak expansion and condensation of collagen fibers in the control group; F) Condensed granulation tissue including well-oriented intense collagen fibers in silymarin treated tendons.](image)

![Fig. 4. Histopathological characteristics of tendon repairs after six weeks from surgery. No significant difference was observed in terms of fibroblast count between the two groups. While the numbers of inflammatory cells and newly formed vessels were significantly decreased in the treatment group. Asterisk indicates a significant difference between control and treatment groups (\( p < 0.05 \)).](image)
Achilles functional index. Figure 5 demonstrates the AFI from the control and treatment groups at the end of each week until the end of the experiment. Repeated measure ANOVA indicated no significant improvement in the control group until the 4th week. At week four, a gradual increase in AFI value was observed in this group; however, regular significant improvements were seen in silymarin-treated rats from week 1 to 6 (p < 0.05). According to the paw prints analysis, there was a significant improvement in AFI from the 4th week in the treatment group compared to the control group (p < 0.05).

Mechanical evaluation. The tensile test showed increased mechanical properties of repairs in terms of ultimate load, yield load, stress, and energy absorption in the treatment group compared to control samples (p < 0.05). However, no significant differences were noted in strain and stiffness between the groups (p > 0.05). The results of mechanical evaluations are presented in Table 1.

Discussion

Different strategies have been developed to improve tendon healing. Despite the cost of sophisticated methods such as cell- and gene therapies, significant progress has not been reported yet. It is believed that the hypocellular and hypovascular nature of tendon tissue makes its repair challenging. Low metabolic rate and limited blood flow eventuate the slow and inadequate healing of the tendon after injury. The present study aimed to investigate the potential effects of orally administered silymarin on the healing of the Achilles tendon after complete transection and anastomosis in rats. Based on the anti-inflammatory and anti-oxidant properties of silymarin, it was hypothesized that silymarin could improve tendon histological and mechanical properties after healing.

Alaseirli et al. have reported that to increase the quality of the repairs, it is essential to decrease the inflammation in the early stages of wound healing. Silymarin possesses potent anti-inflammatory and anti-oxidant effects, and it increases the activity of free radical scavenging enzymes, which help prevent damage from free radicals. Silymarin exerts its anti-inflammatory activity through inhibition of interleukin-1 and prostaglandin E2. In this study, a significant decrease in the infiltration of inflammatory cells was observed in the treatment group. According to the histopathological studies at six weeks post-operation, the neotendons in the silymarin treated group demonstrated characteristics of the maturation phase of tendon healing in which vascularity declines and dense collagen bundles line up in the direction of tension. Lower number of capillaries and dense and organized deposition of collagen are illustrated in Figure 2. The maturation phase occurs after 10 weeks from a tendon injury in normal condition. Based on these histopathology findings, it can be postulated that silymarin accelerates the healing process through its anti-inflammatory and anti-oxidant properties by preceding the repair events from 6 to 10 weeks post-injury. In this study, no significant difference was observed in the number of fibroblasts between the two groups. Although, it is in agreement with Sharifi et al. obtaining similar results in an in vitro study on human skin treated with silymarin. Ashkan-Esfahani et al. have reported that silymarin enhances fibroblast proliferation in rat dermal wound healing after 15 days. Considering the dense, elongated, and parallel nuclei of fibroblasts in the treatment group, it can be believed that silymarin increased fibroblasts population in earlier stages of Achilles tendon healing. Then, due to the accelerated maturation phase, they progressively disappeared from granulation tissue.

Fibroblasts secrete collagen fibers, and collagen deposition is essential for increasing the tensile strength of the wound. The most reliable method to assess the tensile strength of connective tissues such as tendons is mechanical evaluation. This method is believed as a gold standard to evaluate the efficacy of any treatment on tendon healing.

The results of mechanical tests in the present study revealed that silymarin significantly improved the tensile strength of tendons, directly linked to collagen deposition and fibers alignment, as approved by the histological assessments. Increased ultimate load indicates that newly repaired tendons could withstand higher tensile forces during physical activities, and increased yield load shows more elastic behavior of tendons after releasing the forces. The lower yield load or the plastic limit load observed in the control group suggests that neotendons are more predisposed to disruption under minor tensile forces. Tendons act as shock absorbers by absorbing energy and protect muscles from injury. It has been reported that the more energy is absorbed, the more resistance to injury occurs. The muscles and the repaired tendons
themselves are prone to injury and re-rupture if they show insufficient energy absorption capacity. In the present study, higher energy absorption in treatment groups was observed compared to a control group.

According to Aparecida et al. and Young et al., the stress parameter reflects the quality of matrix ultrastructure in collagen fibrils alignment. In this study, treatment samples demonstrated a significant increase stress compared to controls supported by microscopic observations. Based on the histopathology results of this study, the collagen bundles were closely packed in the treatment group, and the fibers were longitudinally aligned; thus, dense and uniform granulation tissue was observed as a result of silymarin administration. Moreover, image processing revealed significantly increased collagen intensity and integrity in silymarin treated tendons, supporting silymarin’s therapeutic effects on tendon repair.

Although the ex vivo assessments of repairs (e.g., histological and biomechanical analyses) requiring an invasive tissue sampling provide important data regarding healing in terms of structural and tensile properties, they do not demonstrate the clinical evidence for tissue repair and are not indicative of functional recovery and patient experience during convalescence. The AFI seems to be a more practical and appropriate approach to evaluate the clinical outcomes of any treatment recruited for the Achilles tendon repair. It is known as the most powerful indicator of Achilles tendon function recovery after tenotomy.

Assessment of AFI is non-invasive, reproducible, and inexpensive, which does not require sophisticated instruments to measure the functional performance of the Achilles tendon. In the present study, AFI results showed that Achilles tendon function recovered faster in the treatment group than the control group. During the first three weeks after surgery, no improvement in AFI was observed in the control group, and then a gradual recovery was seen until the end of the sixth week. While, in the treatment group, silymarin administration resulted in a week recovery in the AFI, demonstrating a sharp increase and significant difference in AFI values from the 4th week versus the control group. This improvement indicates that rats receiving silymarin started to use the operated hind limb earlier compared to control rats; therefore, the weight-bearing function of the healing tendon recovered faster over time.

Adhesion formation between the tendon and surrounding connective tissue is known as the most common complication after surgery, limiting the range of motion, and preventing the normal gliding function of the tendon. Post-operative mobilization reduces peritendinous adhesion formation and improves the gliding function after tenorrhaphy. In this study, macroscopic evaluation of repairs indicated a significantly lower adhesion score in the silymarin treated group than the control group. Decreased adhesion and early functional recovery of repairs make tendons experience the tensile loads; thus, increased mechanical properties are achieved.

In conclusion, the present study showed that oral administration of silymarin facilitated tendon healing via improved structural characteristics, earlier recovery, and significantly stronger neotendons formation in rats after experimental Achilles tenotomy and tenorrhaphy.

**Acknowledgments**

The authors would like to thank the Department of Mechanical Engineering of Biosystems, Shahrekord University (Shahrekord, Iran) for their excellent technical assistance. Also, the technical assistance of Dr. Mazdak Rouhani A, Tabrizi A, Ghavidel E. Effects of non-narcotic analgesics on infrapatellar fat pad swelling and pain during knee flexion: An experimental study of rabbit knees. J Orthop Res. 2020;28(3):398-404.

**Conflict of interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

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