Original Article

Anti-Fibrotic Effect of Tacrolimus on Preventing Adhesion after Achilles Tendon Surgery in a Murine Model

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What's Known

- Peritendinous adhesions are the major complication after tendon surgeries.
- Despite advances in medical knowledge, the management of tendon injuries continues to present a strong challenge.

What's New

- Tacrolimus has a protective effect in preventing post-operative adhesion band formation by reducing the expression of pro-inflammatory mediators.
- Our results showed that tacrolimus has anti-fibrotic effects by reducing collagen deposition at the injured site in rats.

Abstract

Background: Peritendinous adhesion as a common post-surgical complication usually occurs as a result of a large portion of sports and hard job-related injuries. This study aims to evaluate the repurposed potential of the immunosuppressive approved drug, tacrolimus, in decreasing adhesion band formation post-Achilles tendon surgeries in an animal model.

Methods: Rats were randomly assigned to four groups: control, sham with surgical intervention but no adhesion, positive control group with surgical transection and adhesion received no treatment, tacrolimus group was the same as the positive control group except that rats were treated with 2 mg/Kg/day tacrolimus orally for 21 days. The anti-inflammatory and fibrinolytic properties of oral tacrolimus treatment in attenuating the formation of adhesion bands were analyzed by One-way ANOVA or the Kruskal-Wallis test.

Results: Tacrolimus decreased the length (P=0.001), density (P=0.001), grading (P=0.023), severity (P=0.001), and thickness (P=0.008) of post-surgical adhesion bands compared to the untreated group. Histopathological changes and recruitment of inflammatory cells to the tendon tissue sections were attenuated in the tacrolimus-treated group (P=0.001) in comparison with the positive control group. Compared to the untreated group, tacrolimus treatment decreased the expression of IL-1β (P=0.059) in the tendon tissue, but the difference was not statistically significant. Moreover, tacrolimus elicited anti-fibrotic responses by reducing the expression of tissue growth factor-β (TGF-β) in the tendon and inhibiting collagen deposition, fibrosis quantity (P=0.001), fibrosis grading (P=0.001), and total fibrosis scores (P=0.001), as visualized by Masson's trichrome staining.

Conclusion: These results support the protective properties of tacrolimus in decreasing post-operative adhesion band formation in the animal model.

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Keywords ● Tacrolimus ● Fibrosis ● Inflammation

Introduction

Following injury, a localized inflammatory reaction is triggered by the infiltration of inflammatory cells that secrete various growth factors and pro-inflammatory cytokines. This will result in fibroblast proliferation and collagen deposition, leading to adhesion band formation in the injured area.[1-4] Since the current therapeutic agents are ineffective in suppressing adhesion bands,

it is necessary to identify the exact mechanism of adhesion formation for developing novel and effective therapeutics. Various substances have been utilized to regulate the fibrosis process with diverse efficiencies, but treatment with immunosuppressive agents is effective in preventing the formation of post-operative adhesion bands.[5-7]

Tacrolimus, as a potent immunosuppressive and anti-inflammatory agent, has been effectively used for patients with renal, liver, and lung transplantation or patients with autoimmune disorders.[8, 9] Tacrolimus was shown to reduce the activity of T and B lymphocytes through inhibiting calcineurin and downregulating some transcription factors, including the nuclear factor of T cells (NF-AT) and nuclear factor of kappa B (NF-kB).[10-12] Decreased expression of NF-AT results in the downregulation of various inflammatory cytokines, including Interleukin (IL)-2, IL-3, IL-4, and tumor necrosis factor-alpha (TNF-α). Tacrolimus also reduces inflammatory responses by inhibiting leukocyte infiltration into the injured tendon area through downregulation of several proinflammatory cytokines.[13, 14] Moreover, treatment with tacrolimus was found to attenuate oxidative stress by decreasing reactive oxygen species and free radical levels in animal tissues.[15, 16]

Despite recent improvements in therapeutic methods and the development of non-surgical methods with a low incidence rate of adhesion bands, including flexion, physiotherapy, and wave therapy, the formation of adhesion bands during tendon healing remains a major challenge after tendon surgeries. Tacrolimus was found to have a critical inhibitory effect on suppressing collagen synthesis and downregulation of inflammatory cytokines in different human diseases. Recent studies have shown the importance of fibrosis and inflammation processes in the formation of adhesion bands, but the therapeutic effects of tacrolimus on the formation of adhesion bands after tendon surgery have not been fully investigated. The present study aimed to evaluate the anti-inflammatory, fibrinolytic, and protective effects of tacrolimus on post-surgical tendon adhesions in a rat model.

Materials and Methods

Materials

Tacrolimus and other experimental reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). ELISA (Enzyme-linked immunoassay) kits were bought from ZellBio GmbH (Lonsee, Germany). RNA extraction kit and complementary DNA (cDNA) synthesis kit were obtained from Pars Tous (Iran) and Yekta Tajhiz (Iran), respectively.

Animals

Twenty-four male Wistar rats (weighing 230-250 g) were purchased from the Pasteur Institute (Tehran, Iran) and housed based on the Institutional Animal Care Guidelines. The study was reported following the ARRIVE guidelines. All the animal experiments were conducted according to the guidelines for the Care and Use of Laboratory Animals approved by Mashhad University of Medical Sciences (Ethics committee approval number: IR.MUMS.MEDICAL.REC.1400.546). Six rats were kept in each cage and given *ad libitum* access to food and water at a room temperature of 22–25 °C, humidity of about 60%, and a light-dark cycle of 12 hours.

The Induction of Adhesion Post-Tendon Surgery

Adhesion formation at the site of tendon injury was induced according to an established model. Briefly, rats were anesthetized, and a 2 cm longitudinal incision was made on the right Achilles tendon under a control tourniquet, and the transected tendon was sutured using a modified Kessler technique. Twenty-four animals were randomly assigned to the following four groups (six rats in each group): 1) Rats with no surgery receiving normal saline (control), 2) Rats exposed to surgery but no tendon transection performed and received normal saline (sham), 3) Rats exposed to surgery and tendon transection performed received normal saline (positive control), and 4) The tacrolimus group was the same as the positive control group except that rats were treated with 2 mg/Kg/day tacrolimus orally for 21 days. The animals that had less appetite or weight loss of more than 20% after surgery were excluded. A schematic representation of the overall experimental procedure is demonstrated in figure 1. Finally, the animals were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (Alphasan Co. Woerden, Netherland) into the right side of the abdomen and sacrificed. After sacrifice, the samples were dried at -80 °C for future biochemical analysis.

Scoring of Adhesion Formation in Tendon Tissues

Ishiyama and colleagues' scoring system was used to grade the adhesion severity as described in table 1.[17] Furthermore, the Tang score was employed to grade tendon tissues macroscopically (table 2).[18]

Figure 1: A schematic illustration of the study design is presented.

Table 1: The severity of tendon adhesions based on Ishiyama and colleagues's scoring system

Grade 1	No adhesion formation
Grade 2	Adhesion could be separated by blunt dissection alone.
Grade 3	Sharp dissection was needed to separate no more than 50% of adhesive tissues.
Grade 4	Adhesion could be separated by sharp dissection was required to separate 51-97.5% of adhesion tissues.
Grade 5	Sharp dissection was required to separate > 97.5% of adhesion tissues.

Table 2: Macroscopic grading system for adhesions based on Tang and colleagues' scoring system

Criteria	Point	Adhesion appearance
Length	0	No adhesion
	1	Localized, <5 mm longitudinal
	2	10–15 mm
	3	Intense, >15 mm
Density	0	No adhesion
	1	Loose, elastic, and mobile
	2	Moderate mobility
	3	Rigid, dense, and immobile
Grading	0	No adhesion
	1-2	Inferior
	3-4	Medium
	5-6	Severe

Table 3: Inflammatory Grading Scale based on Moran and colleague's scoring system

Grade 0	None
Grade 1	Leukocyte infiltration within fibro-osseous sheath
Grade 2	Infiltration of synovium and epitenon
Grade 3	Infiltration of endotenon
Grade 4	Diffuse inflammation extending within the tendon and beyond the sheath

Histological Assessment of Tendon Tissue Sections

Hematoxylin and eosin (H&E) staining was performed as described previously.[2,4]The severity of inflammation in tissue sections was analyzed by using the Moran and colleagues' scoring system (table 3).[19] Masson's trichrome staining was used to evaluate fibrosis in tendon samples and quantified by using Image J software (version: 1.53t, National Institutes of Health (NIH), Maryland, USA).

Measuring the mRNA Expression by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

A total RNA extraction kit (Pars Tous, Iran) was used to extract RNA from tissue samples. Next, the cDNA was synthesized using a cDNA synthesis kit according to the manufacturer's guidelines (Yekta Tajhiz, Iran). The gene expression of TNF-α, IL-1β, and tissue growth factor-β (TGF-β) was evaluated by quantitative real-time PCR (qRT-PCR) using Amplicon SYBR Green PCR Master Mix and specific primers (Macrogene Co., Korea), as described previously.[2] All the data were normalized according to the glyceraldehyde□3□phosphate dehydrogenase (*GAPDH*) gene as the internal housekeeping control gene. The primers' sequences are presented in table 4.

ELISA Assay

To evaluate the protein concentration of IL-1β and TNF-α in the tendon tissue homogenates

of the rats, the concentrations were evaluated using ELISA kits (ZellBio ELISA kits, Germany) according to the instructions provided by the manufacturer.

Statistical Analysis

We used SPSS version 20 (SPSS Inc., Chicago, USA) for statistical analysis. Results were presented as mean±standard error of the mean (SEM) from three independent experiments. Statistical analysis was conducted using one-way ANOVA, following a *post hoc* LSD test. Differences were considered statistically significant at a P value<0.05.

Table 4: The qPCR primers sequence

Gene	Source	Primer	Sequence
GAPDH	Rat	Forward	CTTCTCTTGTGACAAAGTGGACA
		Reverse	TTGACTGTGCCGTTGAACTTG
TNF-α	Rat	Forward	AGGCTGTCGCTACATCACTG
		Reverse	CTCTCAATGACCCGTAGGGC
IL-1β	Rat	Forward	GACTTCACCATGGAACCCGT
		Reverse	GGAGACTGCCCATTCTCGAC
TGF-β	Rat	Forward	CACTCCCGTGGCTTCTAGTG
		Reverse	CTTCGATGCGCTTCCGTTTC

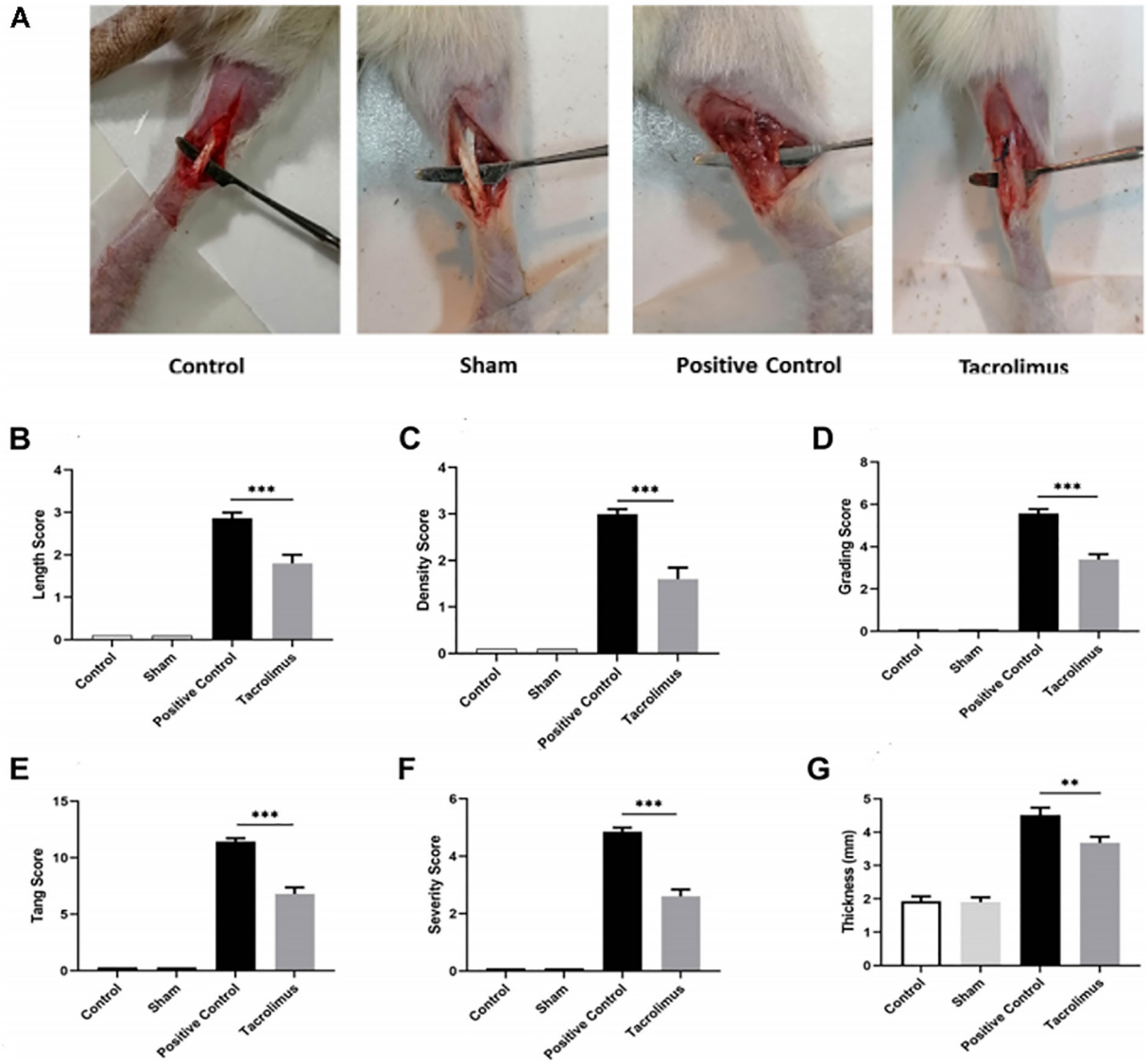

Figure 2: The protective effects of tacrolimus on the formation of adhesion bands and macroscopic grading of tendon adhesion bands are illustrated in this figure. A) Compared to the positive control group, the formation of adhesion bands was reduced in the tacrolimus group (n=6 in each group). Tacrolimus treatment showed protective effects on the (B) length, (C) density, (D) adhesion grade, (E) overall Tang score, (F) severity, and (G) thickness of fibrotic adhesion bands in treated groups. (n=6 in each group) (**P≤0.01, ***P≤0.001).

Results

Tacrolimus Potently Inhibited the Formation of Tendon Adhesions

The post-operative anti-adhesion function of tacrolimus was assessed by adhesion scoring systems. In this line, the tendon tissues were evaluated to determine the severity of adhesion bands and to investigate their macroscopic properties. As shown in figure 2A, all rats from the positive control group suffered from post-surgical tendon adhesions exhibited a higher grading score, while the rats receiving tacrolimus demonstrated a lower adhesion score through reducing adhesion length (P=0.001) (figure 2B), density (P=0.001) (figure 2C), grading (P=0.02) (figure 2D), and overall Tang score (P=0.001) (figure 2E). Moreover, the severity (P=0.001) (figure 2F) and thickness (P=0.008) (figure 2G) of adhesion bands were found to be considerably attenuated by the tacrolimus. Taken together, these findings impressively support the protective effects of tacrolimus on the formation of post-surgical adhesion bands in tendon tissues.

Anti-Inflammatory Effects of Tacrolimus in the Tendon Adhesion Model

To explore the protective and anti-inflammatory

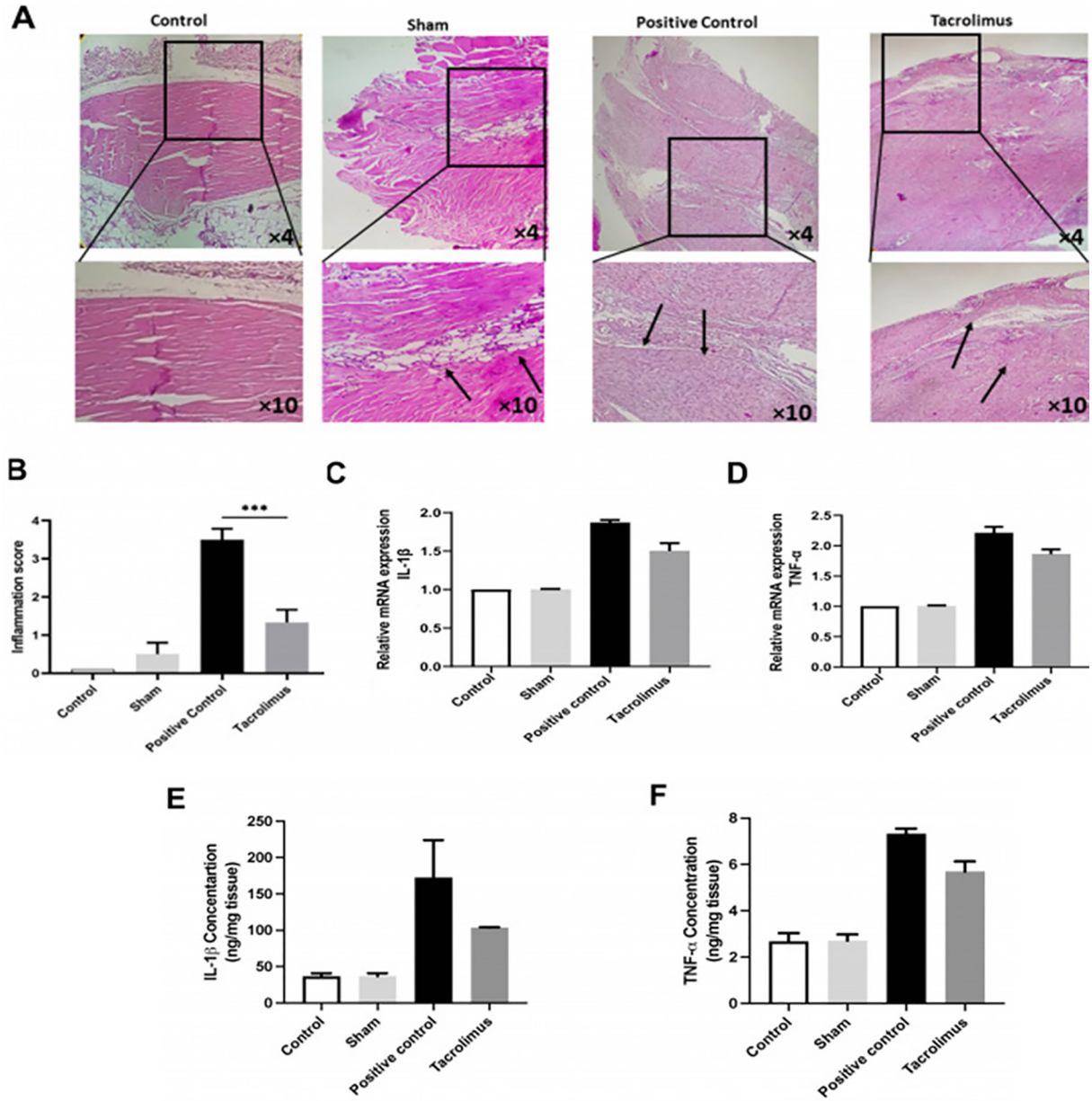

Figure 3: Tacrolimus treatment reduced inflammation scores in the tendon tissues. (A) Administration of tacrolimus could alleviate inflammation in post-surgical tendon tissues. (B) The inflammation score was quantified using the Moran scoring system. The mRNA expression of C) IL-1β and D) TNF-α were decreased in tacrolimus-treated rats. ΔΔCt method was used for Real-Time PCR analysis. Oral tacrolimus decreased the concentration of E) IL-1β and F) TNF-α in the tendon tissue (n=6 in each group). (***P≤0.001).

effects of tacrolimus, the H&E staining was performed to investigate the tissue morphology and infiltration of inflammatory cells in the tendon adhesion tissues. As demonstrated in figure 3A, tacrolimus significantly attenuates leukocyte infiltration in injured sites in adhesion tissues. To further confirm the histological findings, a validated scoring system (Moran scoring system) (table 3) was used. Our results indicated that the inflammation score was reduced in the tacrolimus group compared with the positive control (P=0.001) (figure 3B). Next, the mRNA expression of IL-1β (P=0.0643) (figure 3C) and TNF-α (P=0.0536) (figure 3D) were found to be decreased in tacrolimus-treated animals compared to the control group. However, no statistically significant difference was observed. To further evaluate the immunomodulatory effects of tacrolimus, the tissue concentration of IL-1β (P=0.059) (figure 3E) and TNF-α (P=0.0692) (figure 3F) were also measured by ELISA. Similarly, the transcriptional expression of the mentioned proteins was decreased in tacrolimus-treated rats compared to the positive control group, but the difference was not statistically significant (P=0.062).

Anti-Fibrotic Effects of Tacrolimus in the Tendon Adhesion Model

To further evaluate the regulatory function of tacrolimus in the post-operative fibrosis process, trichrome staining was performed to compare the accumulation of collagen fibers between different groups. Our results showed that tacrolimus decreased fibrosis in tendon tissues post-surgery (figure 4). Consistently, quantity (P=0.001) (figure 5A), quality (P=0.069) (figure 5B), and grading (P=0.001) (figure 5C) of tendon tissues were decreased in the tacrolimus-treated group. Moreover, tacrolimus remarkably decreased the overall fibrosis score in the treatment group, as evaluated by the Tang scoring system (P=0.001) (figure 5D). Moreover, in competition with the positive control group, the mRNA expression of TGF-β was also evaluated in the tendon tissue, and a statistically significant reduction (P=0.042) was observed in tacrolimus-treated rats (figure 5E).

Discussion

In the present study, we showed that treatment with tacrolimus reduces the severity, length, and density of tendon adhesion bands. Moreover, the histopathological changes and infiltration of inflammatory cells were significantly decreased in the tacrolimus-treated rats. Tacrolimus treatment also showed modulatory properties on the mRNA expression and protein level of TNF-α and IL-1β in the tendon tissue. Similarly, tacrolimus exerts fibrinolytic properties in tendons via suppressing levels of fibrotic factors, including collagen post-tendon surgery, as well as reducing the mRNA expression of TGF-β.

Following tissue injury, a localized inflammatory response is triggered by the infiltration of inflammatory cells that secrete different growth factors and proinflammatory cytokines, including TNF-α, IL-1β, and IL-6.

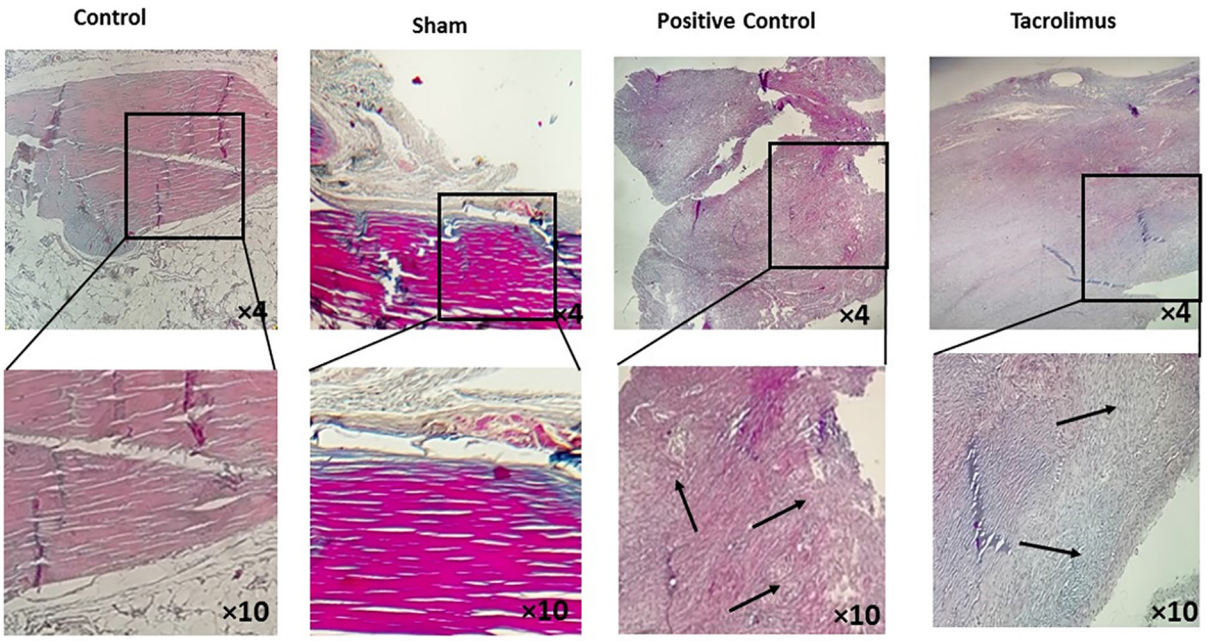

Figure 4: Tacrolimus elicits potent anti-fibrotic properties post-operative tendon adhesion bands. Administration of tacrolimus decreased deposition of collagen post-surgery. Arrows in this figure show fibrosis (n=6 in each group).

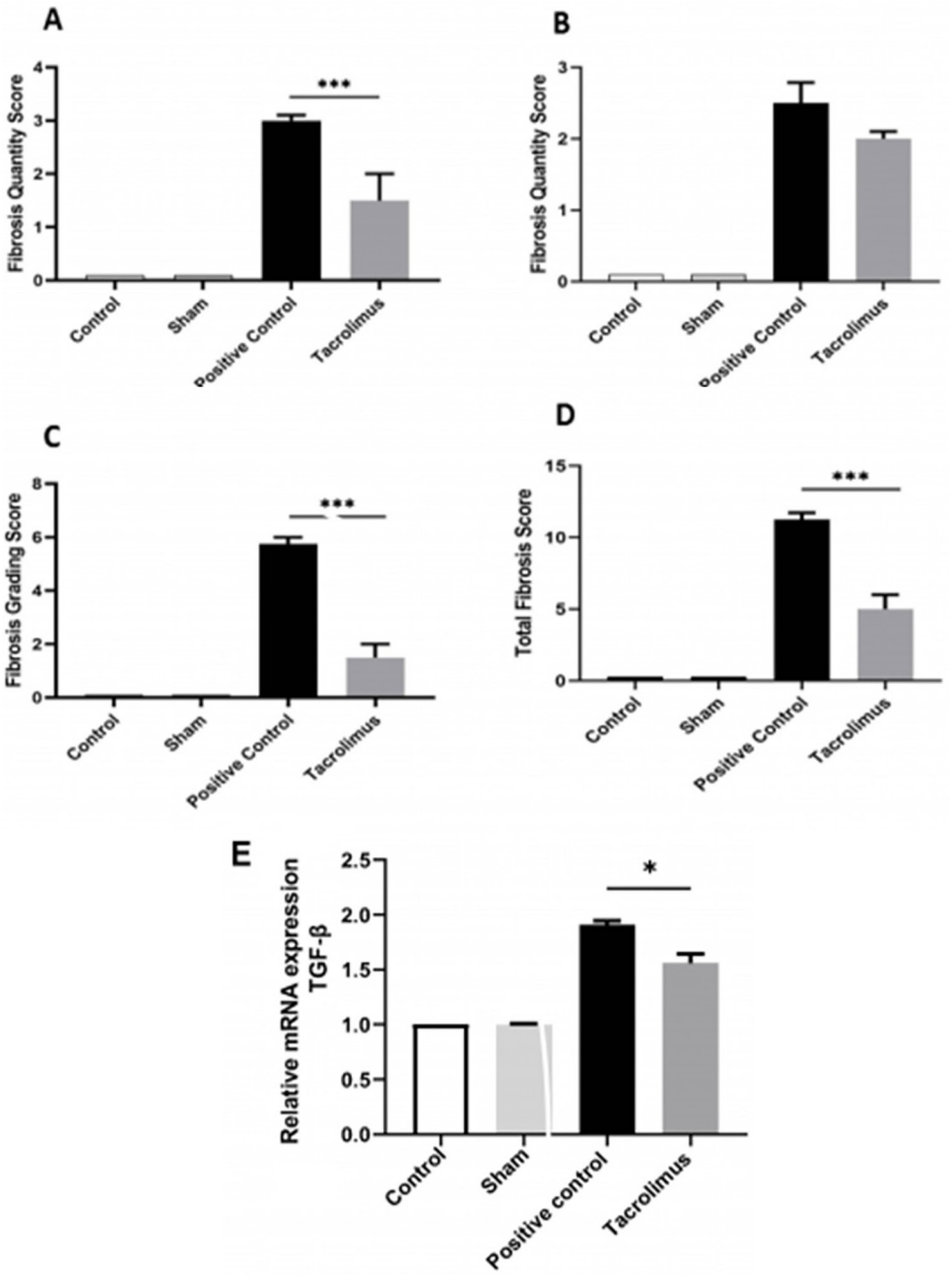

Figure 5: Quantification of the anti-fibrotic properties of tacrolimus using the histological scoring system is presented in this figure. Tacrolimus reduces (A) the quantity, (B) quality, (C) fibrosis grading score, and (D) overall histological Tang score in Achilles tendon tissues. E) tacrolimus treatment caused a significant downregulation in the expression of TGF-β gene. ΔΔCt method was used for Real-Time PCR analysis (n=6 in each group) (*P≤0.05, ***P≤0.001).

Enhanced expression of these cytokines and growth factors induces fibroblast proliferation and collagen deposition resulting in the formation of adhesion bands in the injured area.[20, 21] TGF-β, which is one of the main growth factors released by the activated fibroblast, is shown to have a prominent role in fibrogenesis and the formation of adhesion bands by promoting extracellular matrix deposition and tissue remodeling.[2, 3] Recent findings showed that tacrolimus, as a potent immunosuppressive agent, has a significant protective effect against renal fibrosis by suppressing TGF-β in the sample tissues.[22, 23] Consistent with these findings, Ren and others reported that treatment with tacrolimus inhibits pulmonary fibrosis by alleviation of TGF-β, small worm phenotype Mothers Against Decapentaplegic (SMAD3), and connective tissue growth factor (CTGF) in rat alveolar epithelial cells.[16] In another study, the protective effects of tacrolimus-loaded nano-micelles against kidney injury were evaluated in a lupus nephritis mouse model. Results showed that the administration of tacrolimus reduces renal dysfunction and histological injury by regulating the fibrosis-associated signaling axis.[24] To the

best of our knowledge, there are no previous studies particularly exploring the impact of tacrolimus on tendon adhesions, making direct comparisons with the existing research difficult. However, similar to our findings, a case report demonstrated the potential role of systemic tacrolimus in preventing recurrent tendon adhesions in a 27-year-old patient with a wrist injury. Similar to our animal model, this case was treated with systemic administration of tacrolimus, which led to significant improvement in finger motion, which was attributed to tacrolimus's ability to inhibit T and B cell function and downregulate fibroblast activity. These findings align with our results and support tacrolimus efficacy in reducing adhesions by modulating the immune response and fibroblast activity.[25] In another study involving rats, the topical application of tacrolimus after laminectomy was demonstrated to reduce epidural scar adhesions in a dose-dependent manner. Similar to our results, the highest concentration of tacrolimus effectively decreased fibroblast proliferation and reduced the expression of fibrosis-related markers such as IL-2 and TGF-β1, resulting in minimal scar formation. However, while that study focused on scar adhesions in the epidural space, our research focused on the application of tacrolimus to tendon injuries, further presenting its broader potential in preventing adhesions. Though we did not explore different doses of tacrolimus.[26] In line with the previous studies,[27-29] we observed a decrease in mRNA expression of TGF-β and reduced fibrosis in tacrolimus-treated tendon tissue compared to controls, further supporting tacrolimus role in minimizing adhesion formation across different tissue types and injury models. However, our study uniquely contributes to the understanding of tacrolimus effects specifically on tendon healing, an area that remains underexplored.

In addition to anti-fibrotic effects, several studies have investigated the anti-inflammatory mechanism of tacrolimus in cellular and animal models. For instance, the regulatory effect of tacrolimus on the expression of various pro-inflammatory cytokines is investigated in an *in vitro* cholesteatoma model. Results indicated that tacrolimus, as a potent anti-inflammatory agent, significantly reduces IL-1, IL-10, and TNF-α levels in cholesteatoma keratinocytes. Consistent with these findings, it was reported that the inhibitory effect of tacrolimus on the level of proinflammatory cytokines can be mediated by suppressing NF-kB and the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling in keratinocytes.[30] Zhang and others demonstrated that tacrolimus decreases infiltration of leukocytes and downregulates inflammatory cytokines and collagen proteins in diabetic nephropathy by regulating nuclear factor of activated T cell c1, transient receptor potential channel C6)NFATc1/TRPC6(pathway in mouse kidney tissues.[15] Moreover, the therapeutic efficacy of tacrolimus was investigated in patients with autoimmune hepatitis. It has been shown that the over-activation of T cells in liver cells induces various cytotoxic reactions that consequently result in autoimmune hepatitis. As reported by Larsen and others, treatment with a low dose of tacrolimus attenuates the biochemical and histological markers of inflammation and suppresses fibrosis, thereby suggesting the regulatory effect of tacrolimus on the activity of T cells in patients with autoimmune hepatitis.[31] Furthermore, the therapeutic function of tacrolimus on atherosclerotic plaques was investigated in a mouse model of atherosclerosis. Results demonstrated that treatment with tacrolimus reduces the formation of atherosclerotic plaques by decreasing reactive oxygen species (ROS) in macrophages and reducing the level of IL-1β and IL-18.[32]

Despite the significant results, this investigation is not without its limitations. One of the key limitations of this study is that while we observed a reduction in tendon adhesions during the usage of this drug, the potential side effects of this drug were not studied. Additionally, although the intervention successfully decreased adhesions, its impacts on the tendon's healing capacity or the functionality of the repaired tendon were not assessed. Future studies are needed to investigate whether tacrolimus has any adverse effects on the overall healing process or the biomechanical properties of the regenerated tendon tissue.

Conclusion

Consistent with these findings, our results showed that the protective effects of tacrolimus in preventing the formation of tendon adhesion bands can be mediated by its anti-fibrotic effects through downregulation of TGF-β mRNA expression and anti-inflammatory effects via reducing the mRNA expression and protein concentration of TNF-α and IL-1β in the surgical areas. Studies on the molecular mechanisms of tacrolimus in cellular and animal models will advance our knowledge about its regulatory effects on inflammatory and fibrotic disorders for better prevention of post-operative adhesions. Moreover, although tacrolimus is an FDA-approved agent, its safety and efficacy in reducing tendon adhesion must be evaluated

and verified by clinical studies.

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Authors' Contribution

S. E. N., E. G., Designed and performed animal experiments. These two authors made equal contributions to this study. M. M. A., and A. M. A., Performed histological H & E, and trichrome staining in tendon tissue sections. E. V., and S. H. S., As orthopedic surgeons, contributed to the establishment of an animal model of tendon adhesion. F. R. Contributed to conducting the study and wrote the manuscript. A. A., and H. N. Analyzed data and contributed to the interpretation of the results. S. M. H. and M. K. Designed the study plan and supervised the project. All authors discussed the results and contributed to reviewing the final manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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