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**Original Article**

# Effects of marine collagen and ascorbic acid on the tendon repair in the rat model: a biomechanical and histopathological study

**Avazzadeh, B.[1]; Meimandi-Parizi, A.[2*] and Khodakaram-Tafti, A.[3]**

[1]Ph.D. Student in Veterinary Surgery, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [3]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

[*]***Correspondence:*** *A. Meimandi-Parizi, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: meimandi@shirazu.ac.ir*

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## Abstract

**Background:** Tendon injuries are common and can lead to significant morbidity. Marine collagen and ascorbic acid have shown potential for promoting tendon repair and regeneration. **Aims:** This study investigated the effects of marine collagen, ascorbic acid, and their combination on tendon healing in a rat model over 6-week duration. **Methods:** Sixty adult male Wistar rats were randomized into four equal groups: control, collagen + ascorbic acid, collagen, and ascorbic acid. A controlled full-thickness transverse incision was made in the mid-portion of the Achilles tendon to induce injury, and the defects were sutured following treatment. The treatments were administered orally for 30 days. Tendon recovery was evaluated through histopathological assessments after 30-day treatment at 2-, 4-, and 6-weeks post-operation and biomechanical properties analyzed at 6 weeks. **Results:** Histological evaluation revealed significantly higher connective tissue and collagen accumulation in the collagen + ascorbic acid group (75-90%) compared with the collagen (50-75%), ascorbic acid (25-50%), and control groups (25-50%) ($P<0.05$). Biomechanical analyses showed at 6 weeks after surgery that the collagen + ascorbic acid group had significantly higher maximum force (28.6 ± 3.2 N), maximum stress (4.8 ± 0.6 MPa), work (90.4 ± 11.3 N.mm), and yield points (18.9 ± 2.1 N) compared to the collagen, ascorbic acid, and control groups ($P<0.05$). **Conclusion:** The combined supplementation of marine collagen and ascorbic acid significantly enhanced tendon healing, as evidenced by improved histopathological and biomechanical parameters. These findings suggest that this combination is a promising therapeutic strategy for tendon repair and regeneration.

**Key words:** Ascorbic acid, Biomechanics, Histopathology, Marine collagen, Tendon healing

## Introduction

Tendon injuries are common orthopedic problems affecting both humans and animals, often resulting from trauma or overuse. Tendons play a crucial role in connecting muscles to bones and transmitting forces necessary for joint movement. Tendon damage can lead to pain, swelling, and loss of function in the affected limb (Sharma and Maffulli, 2005). If left untreated, improper healing of the damaged tendon ends may result in decreased range of motion, weakness, and chronic pain (Liu *et al*., 2013). Therefore, the primary goal of tendon repair techniques is to restore normal tendon structure and function. In cases of severe tendon retraction or degeneration, primary tendon repair by suturing the torn ends together may not be feasible (Matthews *et al*., 2006). These tendon defects may require grafting or augmentation techniques to bridge the gap and promote healing. Ideal biomaterials for tendon regeneration should possess properties such as biocompatibility, biodegradability, bioactivity, successful integration with host tissue, support for cell growth, and suitable mechanical characteristics (Linderman *et al*., 2015). Collagen-based scaffolds are widely used because of their similarity to the native tendon extracellular matrix composition (Caliari *et al*., 2011).

Recent studies have explored the potential of marine collagen derived from fish skin and scale as an alternative collagen source for tissue engineering applications (Silva *et al*., 2014). Additionally, ascorbic acid has been shown to enhance tendon healing in animal models, owing to its essential role in collagen synthesis (Lima *et al*., 2009). This study aimed to investigate the

effectiveness of a composite scaffold comprising marine collagen and ascorbic acid in repairing tendon lacerations in a rat model. The tendon regeneration process was assessed at various time points post-repair through histopathological analysis and biomechanical testing.

## Materials and Methods

### Study design

This experimental study was conducted to evaluate the effects of marine collagen, ascorbic acid, and their combination on tendon healing in rats. The animals were housed individually in standard rat cages under standard conditions of temperature (20-25°C) and humidity 60%, with free access to food and water throughout the study period. The rats were subjected to a photoperiod of 12 h light/dark cycle. All animals were cared for and maintained in accordance with international protocols and the principles of laboratory animal care.

### Animals and grouping

Sixty male adult Wistar rats with an average weight of 300 ± 25 g were purchased from a standard laboratory. Using simple random sampling, the rats were divided into four groups of 15 animals, including:

A: Control group
B: Marine collagen + ascorbic acid
C: Marine collagen group
D: Ascorbic acid group

Animals were evaluated at 2-, 4-, and 6-weeks post-operation. In the clinical evaluation, activity levels, infection, bleeding, and postoperative wound dehiscence were assessed and monitored daily.

### Anesthesia and surgical intervention

Animals were anesthetized by intramuscular injection of a mixture of 40-90 mg/kg ketamine (Alfasan, Netherlands) and 5-10 mg/kg xylazine (Alfasan, Netherlands). Figures 1A-E illustrates the critical stages of the surgical intervention and subsequent recovery of the rats. Panel A preparation of the surgical site on the left leg of the rats where the skin of the calcaneal bone to the stifle joint was shaved. Panel B presents the initial exposure of the Achilles tendon prior to the incision. Following the skin incision, the Achilles tendon was exposed, and the section between the gastrocnemius muscle and calcaneal tuberosity was identified. Panel C shows the Achilles tendon immediately after the incision. The standard No. 10 surgical blade was used to perform a complete transverse incision at the middle part of the Achilles tendon. Panels D and E demonstrates the sutured tendon that the severed tendon was sutured with 910 polyglycolic acid absorbable multifilament suture (Vicryl, Supa Medical Device, Iran, 0-6), and the skin was closed with 0-2 silk (Supa Medical Device, Iran). The animals were then kept in the recovery room until they regained consciousness (Kizilkaya *et al*., 2018). The operated left foot was bandaged, and the rats were kept in a controlled restricted environment.

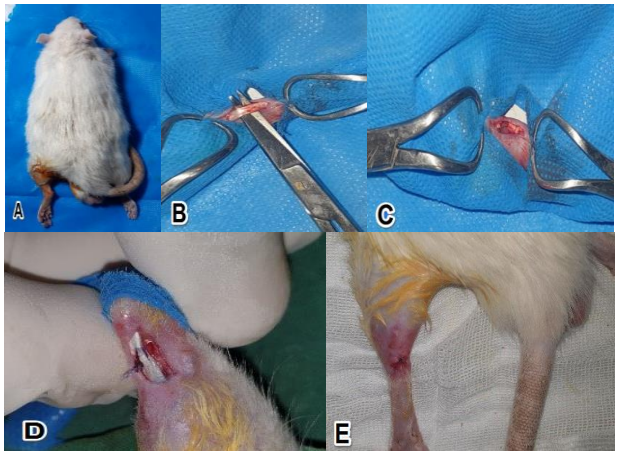


**Fig. 1:** Sequential representation of the surgical process in tendon repair. (**A**) Preoperative skin preparation, (**B**) The initial exposure of the Achilles tendon prior to the incision, (**C**) The Achilles tendon immediately following the incision, (**D**) The sutured tendon, and (**E**) Depicts the immediate and subsequent post-operative states of the rat model's limb

### Treatment regimen

The groups were treated with marine collagen powder (Sports Research, USA) and ascorbic acid (Giti Salamat Aria, Iran) 1000 mg capsules. The control group (A) did not receive any treatment. For the combined treatment group (B), the dissolved marine collagen (4.5 g/kg in distilled water (1.3 g/1 cc)) (Xu *et al*., 2010), 10 times more the recommended amount of human consumption, and 100 mg/kg ascorbic acid (every 12 h) were administered orally (via gavage daily) for 30 days. The marine collagen group (C) received marine collagen at the same dose as group B. The ascorbic acid group (D) received ascorbic acid at the same dose as group B.

### Euthanasia

At the designated time points (2-, 4-, and 6-weeks post-operation), animals were anesthetized by intramuscular injection of a combination of ketamine hydrochloride 10% (40-90 mg/kg) and xylazine hydrochloride 2% (5-10 mg/kg). Under general anesthesia, euthanasia was performed by intracardiac injection of potassium chloride, resulting in cardiac arrest (Gagea-Iurascu and Craig, 2012).

### Sample collection

Sample collection was performed according to the methods described by Oryan *et al*. (2011). At 2-, 4-, and 6-weeks of treatment, 5 rats from each group were randomly selected. The Achilles tendon samples were harvested and sent to the laboratory for biomechanical and histopathological analyses.

### Biomechanical analysis

The operated Achilles tendon, including the calcaneus bone, Achilles tendon, and a portion of the attached muscle tissue, was harvested and stored in a -20°C freezer. On the scheduled testing day, the sample was thawed and transferred to the tensiometry

laboratory. The two ends of the sample were fixed in the stationary and movable clamps of the Zwick material strength testing machine (Zwick Roell, Germany). The movable clamp was withdrawn from the fixed clamp at a rate of 1 mm/s. Deformation load curve of the sample was recorded by the device. The cross-sectional area ($mm^2$) of the sample was determined at the time of sampling. Using a digital caliper, two transverse diameters (right-left and anterior-posterior) of the tendon were measured at the site of reparative tissue and used as an index for the cross-sectional area of the reparative tissue. The maximum stress was calculated by dividing the maximum load by the sample cross-sectional area. The sample's cross-sectional data was input into the computer connected to the testing device. As the movable clamp withdrew from the fixed one, the computer generated a load-deformation curve. The peak of the curve, representing the maximum tissue yield point (the point at which the maximum applied tolerance is reached without macroscopic tear), was considered the maximum force. The work was calculated as the total area under the curve. The upward slope of the curve at the end indicates the yield point of the reparative tendon tissue (Fig. 2).

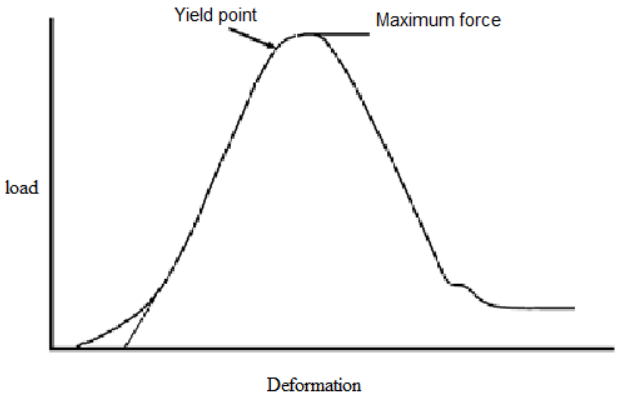


**Fig. 2:** Deformation-load curve and location of biomechanical parameters of maximum force and yield point of tendon

## Histopathological evaluation

The tissue specimens were fixed using 10% buffered formalin. After ensuring tissue stability, processing steps were performed, and the samples were stained with hematoxylin and eosin (H&E) and Masson's trichrome. The histopathological evaluation was conducted according to the scoring system proposed by Moshiri *et al*. (2014), which assessed the intensity of inflammation, the maturity of fibroblasts, the accumulation of connective tissue fibers, the orientation of connective tissue fibers, and the extent of angiogenesis.

The scoring system for the histopathological variables was as follows:

1. Vascularization (scored in the 40 × field): score 1 = 0-3 vessels; score 2 = 3-6 vessels; score 3 = ≥6 vessels
2. Orientation of connective tissue fibers (based on the percentage of fibers perpendicular to the tendon cutting site): score 1 = 0-25%; score 2 = 26-50%; score 3 = 56-75%, score 4 = 76-100%
3. Accumulation of connective tissue fibers (based on the space occupied by collagen fibers): score 1 = 25%; score 2 = 0-50%; score 3 = 51-75%; score 4 = 76-100%
4. Fibroblast maturity: score 1 = most cells are fibroblasts with large round or oval bright nuclei; score 2 = most cells are cigarette-shaped fibroblasts with bright nuclei; score 3 = most cells are elongated and have dark nuclei
5. Inflammation intensity: score 1 = no inflammation; score 2 = moderate inflammation; score 3 = severe inflammation

## Statistical analysis

The obtained data were reported as mean ± standard deviation. The results of the biomechanical evaluation were analyzed using one-way analysis of variance (ANOVA). Data from the histopathological evaluation were analyzed using the nonparametric Kruskal-Wallis and Mann-Whitney U-tests. All statistical analyses were performed using SPSS statistical software (IBM, USA), with a significance level set at $P<0.05$.

# Results

This study investigated the effects of various treatments on connective tissue dynamics in a rat model over a six-week period. The treatments included: group A (Control), group B (Collagen + ascorbic acid), group C (Collagen), and group D (ascorbic acid). Clinical evaluations showed that the animals ambulated without difficulty and gained weight bearing on the surgically treated limb.

## Biomechanical results

The descriptive statistics for the biomechanical variables across the four groups are presented in Table 1. Significant differences in variances across groups were observed for maximum stress ($P=0.023$), maximum force ($P=0.009$), and yield points ($P=0.010$). Subsequent one-way (ANOVA) tests showed significant differences between groups for all biomechanical variables: work ($F=96.17$, $P<0.001$), maximum stress ($F=267.57$, $P<0.001$), maximum force ($F=62.903$, $P<0.001$), and yield points ($F=75.03$, $P<0.001$). Post-hoc comparisons using Tukey's honest significant difference (HSD) test revealed that the group A had significantly lower values compared to the groups B and C for work, maximum stress, maximum force, and yield points (all $P<0.001$). Additionally, the group A had significantly lower maximum force ($P=0.003$) and yield points ($P<0.01$) compared to the group D. No significant differences were observed between the groups B and C for most variables ($P>0.05$). The group D had significantly lower values for work, maximum stress, maximum force, and yield points compared to the groups B and C (all $P<0.001$).

## Histopathological results

The light microscopic examination revealed that the orientation of connective tissue fibers and accumulation

of connective tissue fibers were scored as 1, indicating a range of 0-25% in the 2-week post-injury (WPI). In this week, a lot of inflammatory cells and immature vessels were also observed (Fig. 3). In 4 WPI, the orientation of collagen fibers, and the maturation of fibroblasts improved in the treatment groups (Fig. 4). Inflammation was also slightly reduced, and mature vessels were seen. At 6 WPI, the orientation, density and maturation of collagen fibers were greatly improved compared to 2 WPI. Collagen fibers were well differentiated and their cellularity was reduced. The cells were more mature. Fibers were organized and more distinct. The orientation, density, and maturation of collagen fibers were further improved (Fig. 5).

The groups B and C showed a gradual maturation of tissue, with the presence of fibroblasts, blood vessels, and collagen fibers. The group B exhibited the most promising results, with initially disorganized collagen fibers and increased cellularity, followed by mature vessels, better collagen fibers orientation, and increased connective tissue and collagen accumulation at later stages. The group D demonstrated improvement in cell and connective fiber organization, resembling the control group by the end of the study period.

No significant differences were observed in fiber orientation between weeks 2 and 4. However, at week 6, a significant difference emerged (P=0.043) between the group B (Collagen + ascorbic acid) and groups A and D (ascorbic acid) (Table 2) (Figs. 3, 4 and 5).

Similarly, connective tissue fiber accumulation exhibited a significant difference at week 6 (P=0.043) (Table 2). Pairwise comparisons revealed significant differences between the groups A, B, and D (Fig. 6).

In terms of fibroblast maturation, significant differences were observed across groups at weeks 4 (P=0.041) and week 6 (P=0.021) (Table 2). Pairwise comparisons showed significant differences between the groups A and B at weeks 4 and 6, the groups B and C at week 6, and the groups B and D at weeks 4 and 6, suggesting that the combination of collagen and ascorbic

**Table 1:** Descriptive statistics of biomechanical variables assessed for the groups

| Group | n | Force max mean (±SD) (N) | Stress mean (±SD) (N/mm$^2$) | Work mean (±SD) (N/mm) | Yield point mean (±SD) |
|---|---|---|---|---|---|
| A | 5 | 21.98 (±1.81)$^a$ | 7.25 (±0.45)$^a$ | 16.83 (±1.25)$^a$ | 20.27 (±1.82)$^a$ |
| B | 5 | 81.65 (±4.80)$^b$ | 24.08 (±1.23)$^b$ | 83.03 (±2.85)$^b$ | 78.16 (±2.70)$^b$ |
| C | 5 | 70.40 (±10.19)$^b$ | 19.68 (±0.75)$^c$ | 82.65 (±6.44)$^b$ | 66.98 (±9.35)$^b$ |
| D | 5 | 42.36 (±10.12)$^c$ | 7.74 (±1.77)$^a$ | 27.24 (±14.45)$^a$ | 39.01 (±9.32)$^c$ |

$^{a, b, c}$ Different letters assigned to P-values (P<0.05) indicates statistical significance

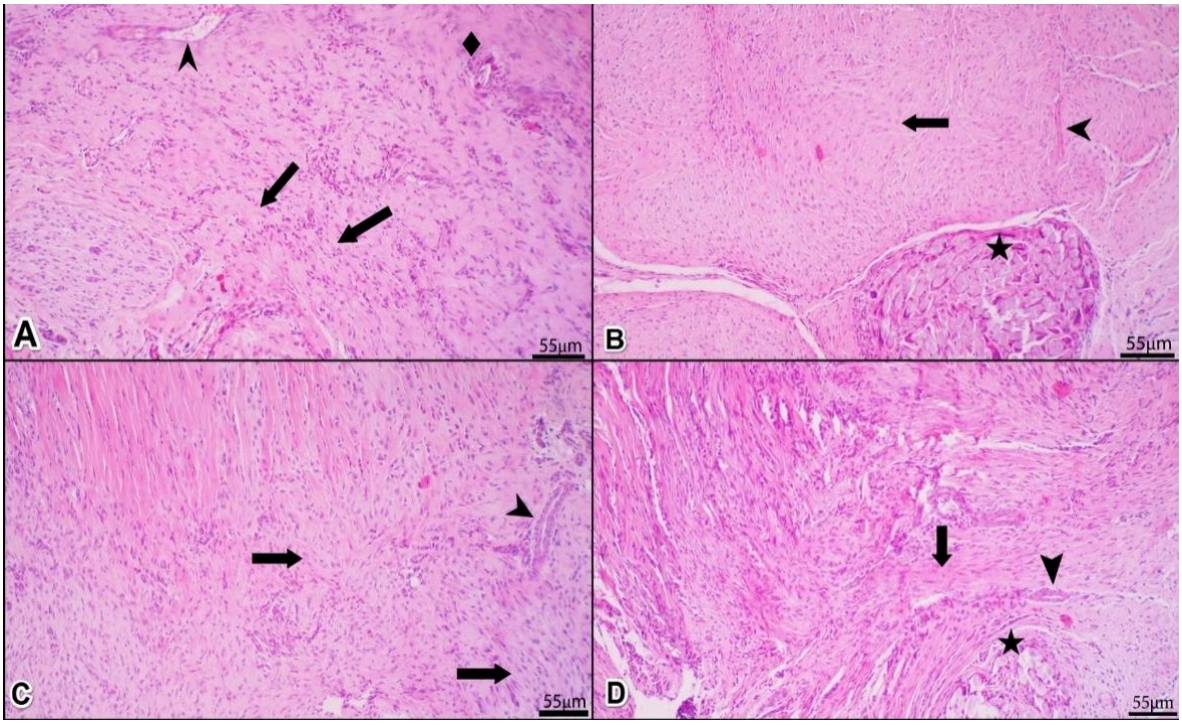


**Fig. 3:** Histopathological process of the groups at two weeks post-injury (complete tendon incision). (**A**) (group A): Abundant vessels (arrow head) and proliferating cells, scant collagen, collagen fibers in various directions (arrow), and presence of inflammatory cells (star) around vessels, (**B**) (group B): Suture thread (star) present with inflammatory cells surrounding the suture and abundant fibroblast cells (arrow), as well as the formation of highly cellular blood vessels (arrow head), (**C**) (group C): Abundant vessels (arrow head), many proliferating cells, increasing connective tissue fibers but lacking proper organization (arrow), (**D**) (group D): Abundant vessels (arrow head), increased cells, disorganized collagen fibers (arrow), few inflammatory cells, and presence of suture thread (star), (scale bar, 55 µm)

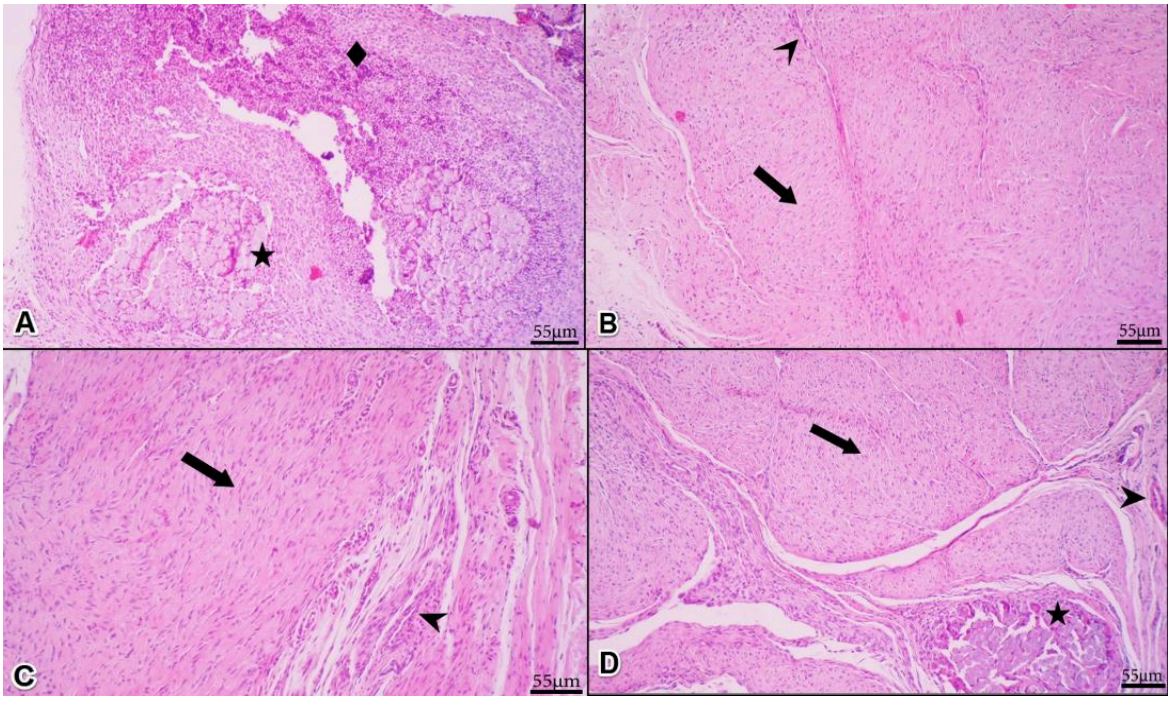


**Fig. 4:** Histopathological process of the groups at four weeks post-injury (complete tendon incision). (**A**) (group A): Suture thread (star) with the presence of inflammatory cells (star) indicative of inflammation, (**B**) (group B): Presence of numerous and disorganized fibroblast cells (arrow) and medium blood vessels (arrow head), (**C**) (group C): Relatively mature vessels (arrow head), presence of few inflammatory cells, and better orientation of collagen cells (arrow), and (**D**) (group D): Presence of inflammatory cells (arrow), lack of organization of collagen fibers, relatively abundant vessels (arrow head), and suture thread (star), (scale bar, 55 µm)

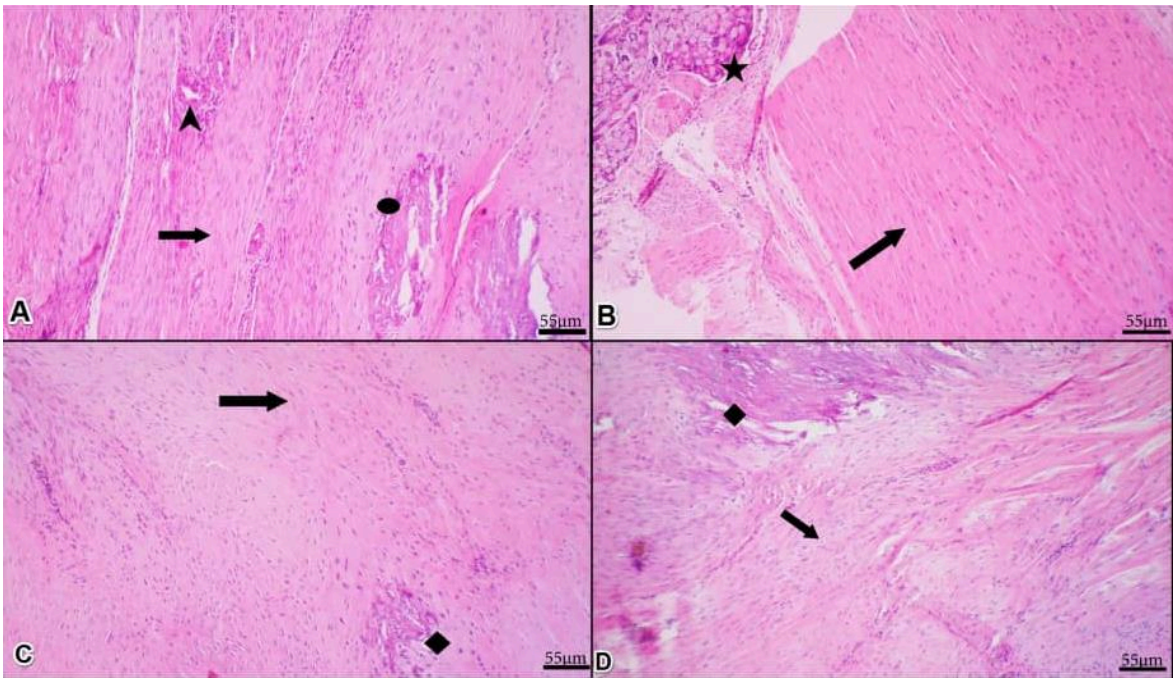


**Fig. 5:** Histopathological process of the groups at six weeks post-injury (complete tendon incision). (**A**) (group A): Chondroid metaplasia (diamond) and presence of mononuclear cells, particularly around vessels (arrow head), with observable collagen fibers (arrow), **B** (group B): Organized collagen fibers (arrow), a few vessels, spindle-shaped cells, and suture thread (star), **C** (group C): Chondroid metaplasia (diamond), increase in organizing collagen fibers, and cellular maturity with cells transitioning from cuboidal to pavement-like in appearance (arrow), **D** (group D): Chondroid metaplasia (diamond), ordering, organization of cells, and connective fibers (arrow) similar to the control group, (scale bar, 55 µm)

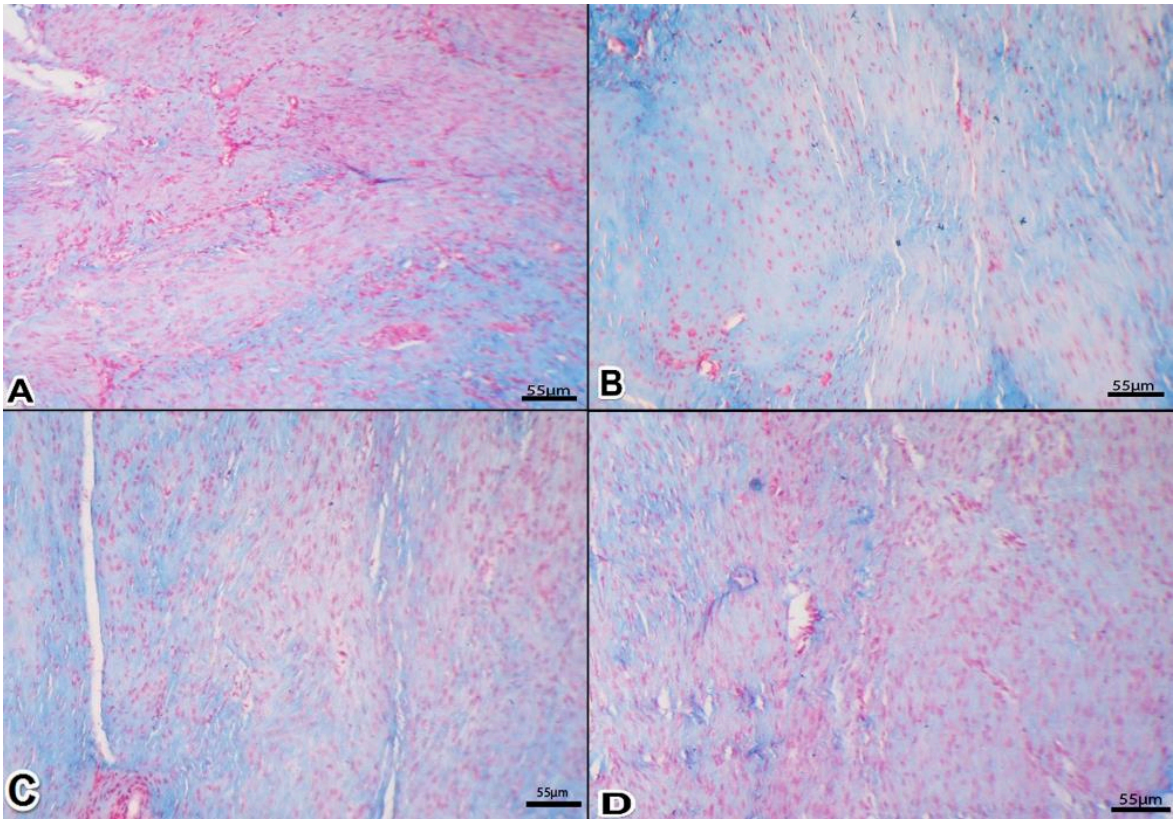


**Fig. 6:** Histopathological results in different groups with Masson's trichrome staining. (**A**) (group A): Irregular orientation of connective tissue fibers with very sparse accumulation of collagen fibers, (**B**) (group B): Increased and organized accumulation of connective tissue fibers and collagen at the repair site is around 75-90% (score 4), (**C**) (group C): Appropriate connective fibers approximately 50-60% (score 3) and collagen fibers, and (**D**) (group D): Increase and organization of collagen fibers approximately 20-25% (score 1), (scale bar, 55 µm)

**Table 2:** Descriptive statistics of histopathological variables assessed for the groups in different weeks

| Histopathological variables | Groups | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Group A Med (min-max) | | | Group B Med (min-max) | | | Group C Med (min-max) | | | Group D Med (min-max) | | |
| | 2nd week (n=3) | 4th week (n=3) | 6th week (n=3) | 2nd week (n=3) | 4th week (n=3) | 6th week (n=3) | 2nd week (n=3) | 4th week (n=3) | 6th week (n=3) | 2nd week (n=3) | 4th week (n=3) | 6th week (n=3) |
| Accumulation of connective tissue fibers | 1 (1) | 1.3 (1-2) | 2 (2)$^{a}$ | 1 (1-2) | 2 (2) | 3.3 (3-4)$^{a}$ | 1 (1) | 1.6 (1-2) | 2.3 (2-3)$^{a}$ | 1 (1) | 1.3 (1-2) | 1.6 (1-2)$^{a}$ |
| Orientation of connective tissue fibers | 1 (1) | 1.3 (1-2) | 2 (2)$^{b}$ | 1 (1-2) | 2.6 (2-3) | 3.6 (3-4)$^{b}$ | 1.3 (1-2) | 2.3 (2-3) | 3 (2-4)$^{b}$ | 1 (1) | 1.6 (1-2) | 2 (2)$^{b}$ |
| Fibroblast maturity | 1 (1) | 1 (1)$^{c}$ | 1.3 (1-2)$^{c}$ | 1.3 (1-2) | 2 (2)$^{c}$ | 3 (3)$^{c}$ | 1 (1) | 1.3 (1-2)$^{c}$ | 2 (2)$^{c}$ | 1 (1) | 1 (1)$^{c}$ | 2 (2)$^{c}$ |
| Vascularization | 3 (3) | 3 (3)$^{d}$ | 2.6 (2-3)$^{d}$ | 2.6 (2-3) | 1.6 (1-2)$^{d}$ | 1 (1)$^{d}$ | 3 (3) | 2 (2)$^{d}$ | 1.6 (1-2)$^{d}$ | 3 (3) | 3 (3)$^{d}$ | 2.3 (2-3)$^{d}$ |
| Inflammation intensity | 3 (3) | 2.6 (2-3) | 2.3 (2-3)$^{e}$ | 2.3 (2-3) | 1.6 (1-2) | 1 (1)$^{e}$ | 2.6 (2-3) | 2 (2) | 1.6 (1-2)$^{e}$ | 3 (3) | 2.6 (2-3) | 2 (2)$^{e}$ |

Kruskal-Wallis test was used. Group A: Control group (n=9), Group B: Marine collagen + Ascorbic acid (n=9), Group C: Marine collagen group (n=9), and Group D: Ascorbic acid group (n=9). The letters $^{a, b, c, d}$ and $^{e}$ indicate statistical significant across different groups and weeks. P-values (P<0.05)

acid had a notable impact on fibroblast maturation (Figs. 3, 4 and 5).

Vascularization rates also showed significant differences at week 4 (P=0.015) and week 6 (P=0.047). Pairwise comparisons indicated significant differences between the groups A and B at both weeks 4 and 6, the groups B and D at weeks 4 and 6, and the groups C and D at week 4 (Figs. 3, 4 and 5).

Lastly, while inflammation levels did not show significant differences at weeks 2 and 4, a significant difference was observed at week 6 (P=0.048) (Table 2). Pairwise comparisons revealed significant differences between the groups A and B, the groups B and D, and between the groups C and D, suggesting that the combination of collagen and ascorbic acid may have a role in reducing inflammation at later stages of healing (Figs. 3, 4 and 5).

## Discussion

The present study aimed to investigate the effects of collagen, ascorbic acid, and their combination on key

connective tissue parameters in a rat model over a 6-week timeframe. The results provide valuable insights of these treatments into the dynamics and their effects on connective tissue properties, particularly in the context of tendon repair.

Collagen and ascorbic acid have well-established roles in modulating collagen synthesis and structure (Peterkofsky, 1991; Gelse, 2003). Our findings demonstrate that combining collagen and ascorbic acid supplementation results in significant effects on connective tissue fiber orientation and accumulation by week 6. These results are consistent with previous research, indicating the synergistic effects of simultaneous collagen and ascorbic acid provision on supporting collagen-based extracellular matrix development (Kim *et al*., 1988; Franceschi and Iyer, 1992). The improved fiber orientation and accumulation observed in the combined treatment group, suggesting that this approach may be beneficial for tendon repair processes.

Ascorbic acid serves as an essential cofactor in collagen synthesis pathways, enhancing collagen fiber deposition and linear alignment through stimulation of lysyl and prolyl hydroxylation activity (Barnes, 1975). The current study's observations suggest that coupling exogenous collagen supply with ascorbic acid-mediated synthesis pathways promotes favorable structural organization over time in the context of tendon healing. The specific mechanisms underlying these effects may relate to enhanced fibroblast productivity, crosslink formation, secretion of collagen fibrils with improved orientation and packing, and extracellular matrix remodeling dynamics (Liu *et al*., 1997; Brinckmann *et al*., 1999). Further research is warranted to elucidate the precise temporal impacts on these interconnected processes and their relevance to tendon repair. Nevertheless, our findings strongly support the potential of combining collagen and ascorbic acid to optimize structural matrix properties in tendon healing.

Moreover, our results revealed consistent significant differences in fibroblast maturation rates between the collagen + ascorbic acid group and other groups, including ascorbic acid alone. While ascorbic acid availability is necessary for collagen formation by fibroblasts, it is not entirely sufficient for optimal fibroblast function (Tajima and Pinnell, 1996).

Enhanced fibroblast maturation in the collagen + ascorbic acid group may derive from the external collagen promoting fibroblasts' synthesis pathways and secretion of correctly configured endogenously produced collagen (Lee *et al*., 2001). This extracellular scaffolding likely helps spatially and biochemically guide fibroblast performance, which is crucial for effective tendon repair.

The sustained superior maturation of fibroblasts in the collagen + ascorbic acid group suggests that this combination establishes an optimal biochemical and structural environment for tendon healing. Over time, a cyclical positive feedback loop may occur, whereby maturing fibroblasts with improving functional capabilities synthesize matrices that promote further maturation. Elucidating these temporal cell-matrix interactions would provide meaningful insights into the mechanisms underlying the observed beneficial effects. Nonetheless, our findings demonstrate the advantage of the collagen plus ascorbic acid combination in directing productive fibroblast development, which may have significant implications for tendon repair strategies.

Vascularization plays a critical role in tissue repair processes, and our results showed that collagen plus ascorbic acid supplementation significantly increased vascularization compared to the control group at weeks 4 and 6. This enhanced revascularization likely supported the tissue accrual and organization processes observed in the combined treatment group. Angiogenesis relies heavily on signaling interactions with fibroblastic cells and the surrounding extracellular matrix environment (Hirschi *et al*., 1999; Stratman *et al*., 2009). Thus, the synergistic provision of collagen substrate and enhanced ascorbic acid-dependent collagen synthesis presumably offered ideal conditions for angiogenesis in the context of tendon repair. Reciprocal fibroblast-endothelial cell signaling induces vessel formation, while vascular cells release matrix remodeling factors (Koike *et al*., 2004; Rajkumar *et al*., 2006). Our findings suggest that the combined collagen and ascorbic acid treatment modulated this fibroblast-endothelium relationship to best stimulate vascularization, which may improve tendon healing observed.

As vascular development permeates the healing tendon, associated permeability factors foster improved transport and mobility of matrix proteins and cells essential to tissue growth (Helm *et al*., 2005). Furthermore, increased vascularity promotes oxygen and nutrient supply to meet the rising metabolic demands of the repair process (Pittman, 2011). Ultimately, the augmented vascularization observed in the collagen + ascorbic acid group likely served to facilitate overall connective tissue accrual and structural enhancements in the healing tendon. Further research should explore the temporal signaling profiles and metabolic activity correlations associated with these vascularization changes to better understand their role in tendon repair.

The results revealed no significant differences in inflammation levels between the treatment groups at 2- and 4-weeks post-injury. However, at 6 weeks, there was a significant difference in inflammation across groups. Specifically, the inflammation levels were significantly lower in the group receiving both marine collagen and ascorbic acid compared to the control group and the ascorbic acid only group. These findings showed that the combination of marine collagen and ascorbic acid may have an anti-inflammatory effect during the later stages of tendon healing in this rat model. However, further investigation, such as measuring inflammatory mediators, is required. The reduced inflammation at 6-weeks in the combination treatment group may indicate a faster resolution of the inflammatory phase of healing and earlier progression to the tissue formation and remodeling phases (Xu and Murrell, 2008). Other studies have similarly demonstrated anti-inflammatory and

antioxidant effects of marine collagen (Gómez-Guillén *et al*., 2011; Hu *et al*., 2017) and ascorbic acid (Duygulu *et al*., 2007; Fu *et al*., 2013) individually in tendon injury models. However, this is the first study to show their combined effects on inflammation during tendon repair, highlighting the potential synergistic benefits of this combination therapy.

The exact mechanisms by which marine collagen and ascorbic acid reduce inflammation in tendon healing is still unclear. Marine collagen contains unique amino acid compositions and sequences that may modulate inflammatory signaling pathways (Hu *et al*., 2017). Ascorbic acid, in addition to its essential role in collagen synthesis, may also have direct anti-inflammatory effects by reducing reactive oxygen species and inhibiting NF-kB signaling (Fu *et al*., 2013). Further studies are needed to elucidate how these two compounds interact to accelerate the transition from the inflammatory to tissue formation phases of tendon healing, as this could have significant implications for optimizing tendon repair strategies.

The biomechanical analysis in our study provides robust evidence that the combination of collagen and ascorbic acid supplementation substantially strengthens the biomechanical properties of healing tendons. By supporting beneficial fiber and matrix accrual, organization, and fibroblast-vascular interactions, the combined treatment led to dramatic improvements in load-bearing capability and resilience. Biomechanical integrity greatly relies on the quality and abundance of deposited structural proteins, crosslinks, and the recapitulation of native-like formation environments (Bielajew *et al*., 2020). Our results suggest that the tested combination enabled optimization of these fundamental elements to distinctly improve force/displacement characteristics and energy absorption capabilities of the healing tendons.

Future research should focus on mapping detailed mechanical profiles over temporal and spatial scales, connecting tissue ultrastructure and cellular activity to the emerging biomechanical response. Such studies would provide highly informative insights into the mechanisms underlying the observed biomechanical improvements (Sarig *et al*., 2018; Mok *et al*., 2020; Song *et al*., 2022; Putra *et al*., 2023). Nonetheless, our current results clearly demonstrate substantial fortification of load-bearing capacity in healing tendons thanks to the synergistic effects of collagen and ascorbic acid supplementation over 6-weeks.

In the context of tendon repair, biomaterials that mimic native tendon properties are crucial for successful outcomes. Native tendons exhibit remarkable tensile strength due to their highly aligned collagen fibers arranged in an intricate hierarchical structure that enables effective resistance to tensile forces (Tang *et al*., 2022). However, regenerated tissue following tendon injury often lacks the strength of native tendons. Incorporating such biomimetic strategies alongside the combined collagen and ascorbic acid supplementation approach investigated in our study may further enhance tendon repair outcomes.

In conclusion, our findings strongly support the combination of collagen and ascorbic acid supplementation as a highly beneficial approach for promoting the healing of connective tissues, particularly tendons. Further investigations into the effects of dosage, ultrastructural interactions, and the application of this combined treatment in the context of pathological injury repair promise to yield additional valuable insights. Ultimately, harnessing the synergistic potential of combined exogenous collagen administration and enhanced endogenous collagen production mediated by ascorbic acid may significantly advance tissue engineering and regenerative medicine approaches for tendon repair and other connective tissue disorders. Future translational studies should focus on optimizing this combined therapy for clinical application and evaluating its efficacy in human patients.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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