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Role of a combination dietary supplement containing mucopolysaccharides, vitamin C, and collagen on tendon healing in rats

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ABSTRACT

Objective: The aim of this study was to investigate the effect of mucopolysaccharide, vitamin C, and collagen supplementation on the healing of Achilles tendon in rats.
Methods: Sixteen rats were separated into 2 groups. Both Achilles tendons of all rats were transected 5 mm above the insertion and repaired using a Kessler suture. After the surgical repair, the study group received the daily recommended amount of the supplement by gastric gavage, while the control group received a placebo. At the end of the third week, the animals were sacrificed. The biomechanical properties of the groups were compared with ultimate tensile strength and stiffness tests. The biological properties of the 2 groups were assessed with a histomorphometric comparison to determine the amount of collagen type I (COL1), proliferating cell nuclear antigen (PCNA), and transforming growth factor β1 (TGF-β1) expression in 3 different tissue subgroups (collagen matrix, tenocytes, and endotenon fibroblasts).
Results: Analysis of histomorphometric results revealed that the rats receiving dietary supplements demonstrated a significant increase in PCNA (mean value of 86 in the control group and 168.85 in the trial group; $p < 0.05$) and TGF-β1 (mean value of 87.57 in the control group and 161.85 in the trial group; $p < 0.05$) in the endotenon fibroblasts of the repair site. However, there was no difference between the groups in PCNA or TGF-β1 when the collagen matrix and the tenocytes of the repair site were examined. Furthermore, no significant difference could be found between groups in COL1 in any of the 3 tissue subgroups (collagen matrix, tenocytes, and endotenon fibroblasts). The statistical analysis also indicated that the rats receiving supplements did not demonstrate a significant increase in the ultimate tendon tensile strength or stiffness.
Conclusion: The results of this study revealed no advantage to the oral administration of the trial supplement in collagen synthesis or biomechanical properties in rats after 3 weeks using the presented study design. However, the increased expression of PCNA and TGFβ1 seen in the endotenon fibroblasts of the repair site might play a role in the continuum of tendon healing.

Introduction

Tendon injury is a common problem with a wide range of clinical and pathological presentations. The spectrum of tendon injury includes simple synovitis of the tendon sheet at 1 end to complete rupture from degeneration at the other end. Nevertheless, the main pathology is believed to be physical strain surpassing the regenerative capacity of the tendon.[1] The treatment approach ranges from simple rest, ice, and anti-inflammatory medication, to splinting or surgical repair, mainly depending on the amount of

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degeneration and the tendon's physical integrity level. Physical therapy plays an important role in some resistant cases and after surgical treatment of tendon ruptures. Steroid injections have been employed for tendonitis cases with varied results and with drawbacks. In the last decade, some new treatment options have been advocated, such as platelet-rich plasma injections, prolotherapy, and dry needling, with very controversial results.[2–6] Along with these minimally invasive, yet debated new treatment options, a variety of dietary supplements have also been advocated in recent years for a variety of orthopedic disorders.[7–13] The most renowned are mucopolysaccharides and collagen supplements.

Unlike the well-established and accepted effects of vitamin and mineral supplements like vitamin D, vitamin C, and calcium on the musculoskeletal system, the literature focusing on musculoskeletal wellbeing is not very strong in legitimate data on dietary supplements. To our knowledge, there is no report of regarding a deficiency of mucopolysaccharides, but it is known that the level of some subgroups decreases with aging.[14,15] There are also studies that report favorable outcomes for tendon pathologies with oral supplementation of mucopolysaccharides.[16–18] Since collagen is a protein widely available in a standard Western diet, supplementation in someone who is on a regular diet can be argued. There is also some literature evidence correlating supplementation of collagen with endogenous upregulation of collagen synthesis. Some studies even report that collagen administered with vitamin C might have synergistic benefits on mesenchymal tissue and collagen synthesis.[19–21]

Nevertheless, the literature is still weak in providing crystalized data regarding the benefits of these supplements to mesenchymal tissue metabolism and there seems to be very little interest in providing further data in the research community. Since many physicians, including orthopedic surgeons, do not hesitate to ask their patients to use some sort of supplement for conditions including tendon pathologies, the problem also becomes an ethical issue. The lack of solid evidence in these arguments should provoke the interest of orthopedic community toward research of musculoskeletal dietary supplements.

The combination of mucopolysaccharides, vitamin C, and collagen is an example of such supplementation that is widely recommended and prescribed, yet there are insufficient data confirming its effects. It is offered under many different generic names and in varied doses. The effects of a generic preparation combining mucopolysaccharides (220 mg), vitamin C (30 mg), and hydrolyzed collagen type I (COL1) (40 mg) in the severed and repaired tendon of a rat model was analyzed in this study.

Materials and methods

Approval to perform the study was obtained from the local ethics committee.

Sixteen skeletally mature male Wistar albino rats (aged 6 months and weighing 250–300 g), obtained from the direct source of the local animal experiments laboratory were used in this study to decrease genetic variability. After the proper induction of ketamine (100 mg/kg; Alfasan International, Woerden, Holland) anesthesia, both Achilles tendons of all animals were transected 5 mm proximal to the point of insertion to the calcaneus (Figs. 1 and 2). The Achilles tendon was repaired using 6/0 Prolene monofilament polypropylene (Ethicon, Inc., Somerville, NJ, USA) sutures with a modified Kessler method (Fig. 3). The plantaris tendon was protected to act as an internal splint to decrease tension at the repair site. The wound was closed with 3/0 Vicryl glycolide/L-lactide (Ethicon, Inc., Somerville, NJ, USA) absorbable uninterrupted sutures. No wound dressing or casting was used after the operation. All of the subjects were left to mobilize freely and were fed with

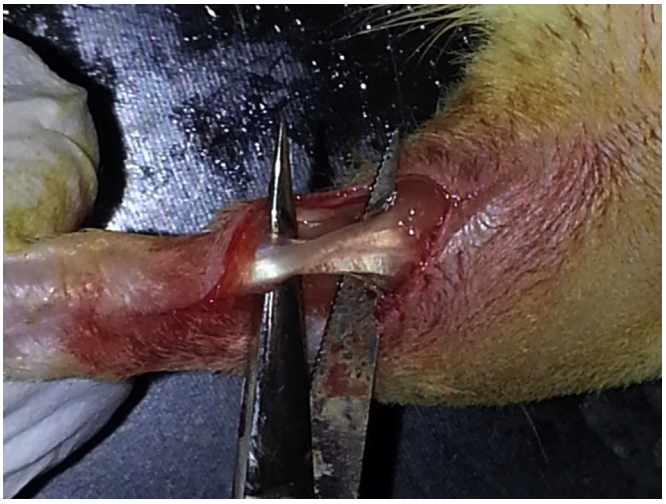

Fig. 1. Photograph of the Achilles tendon before transection.

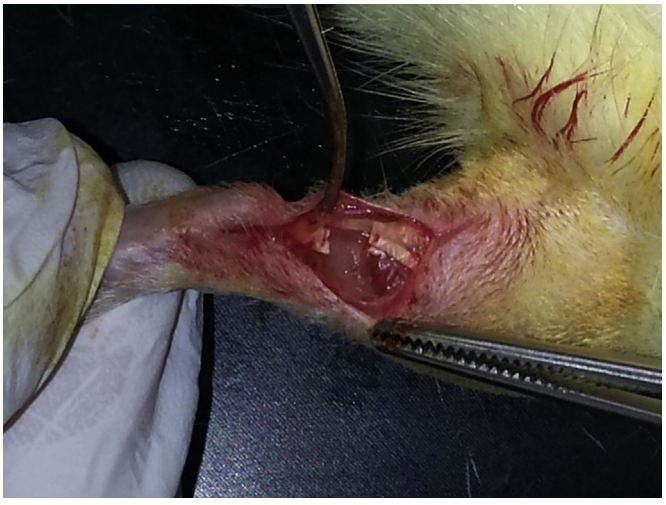

Fig. 2. Photograph of the Achilles tendon after transection.

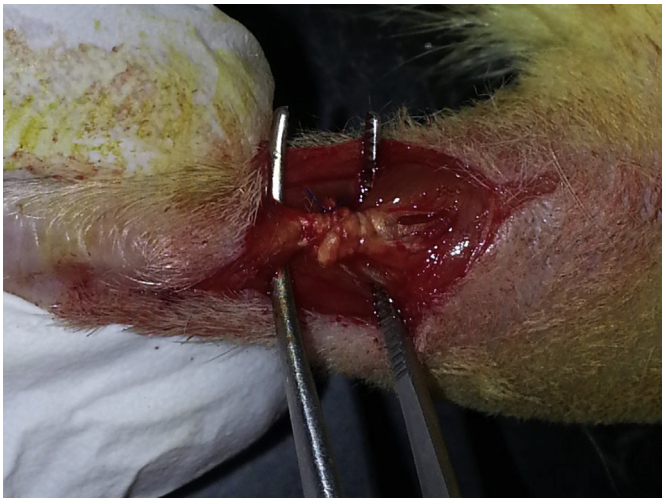

Fig. 3. Photograph of the Achilles tendon after surgical repair.

standard laboratory food and tap water. One rat from the control group was lost right after the operation, probably due to an anesthesia-related complication.

All 8 rats in the study group were administered 2.4 mg of a trial supplement (7.2 mg/kg) by means of gastric gavage every day for 3 weeks. The dose was calculated based on the daily recommended dose for a person weighing 80 kg (2 capsules of 290 mg is recommended for adults with no adjustment for weight). On the fourth day after the surgical procedure, 1 rat from the study group was lost, leaving both groups with equal sample size of 7 rats. The control group received gastric gavage with an equal amount of tap water to control bias for any possible nutritional effects that the long-term gavage might cause. After the third week, all of the animals were sacrificed with a lethal dose of ketamine, which was administered intraperitoneally. Above-knee amputation was performed and the right legs of the animals were fresh frozen and reserved for biomechanical testing. The left legs of the animals were kept in 10% formalin solution and were reserved for histomorphometric tests.

Histological method

After extraction of the Achilles tissues from the preserved left legs, the tissues were fixed using neutral formalin solution for 24 h. Following routine histological procedures, the tissues were embedded in paraffin blocks. A total of 105 sections were obtained from each sample, each 5 μm in thickness. Hematoxylin-eosin stain was applied for regular examination and Masson's trichrome stain (Bio-Optica Milano S.p.A, Milan, Italy) was applied for better visualization of collagen fibers.

To assess the tendon healing process with objective criteria, immune histochemical staining for COL1, proliferating cell nuclear antigen (PCNA), and transforming growth factor β 1 (TGF-β1) was performed.[22,23] To further assess the possible location of biological activity related to the test variable, the strength of the staining was recorded in 3 different tissue areas: the collagen matrix, tenocytes, and endotenon fibroblasts. An Olympus microscope (CX31; Olympus Corp., Tokyo, Japan) with an attached camera and image analysis software (Leica Q Win V3 Plus Image; Leica Camera AG, Wetzler, Germany) was used for immune histochemical quantification. Every 10th section of the 105 serial sections was reserved for quantitative histological analysis (a total of 10 sections for each dye). First a semi-quantitative H-score ($\sum$ Pi (i + 1)) was calculated for each section by defining the density and percentage of expression for COL1, PCNA, and TGFβ1 in the different tissue subtypes (collagen matrix, tenocytes, and endotenon fibroblasts). In this equation, i is the intensity of labeling (1, 2, or 3 for weak, moderate, or strong, respectively) and Pi is the percentage of labeled cells (0%–100%). The H-scores were summed to get a final, single value for each dye.

Biomechanical method

The fresh frozen right legs of the animals were thawed at standard room temperature. The mounting of the Achilles tendons to the test device was performed using the method described by Probst et al.[24] The muscles proximal to the Achilles tendon are scraped from the tendon's fascia with a scalpel. The tendon fibers are then fixed between strips of blotting paper to prevent slippage and clamped with a pair of solid blocks, which are later mounted on the mechanical test device. The distal calcaneus is osteotomized to leave the tuber calcaneus intact. A solid block with a pit just big enough to house the osteotomized calcaneus is prepared. Later, the tuber calcaneus is placed into the pit of the block, which is then mounted on the test device (Fig. 4). An Acumen 1 Electrodynamic Test System (MTS Systems Corp., Eden Prairie, MN, USA) mechanical testing device was used for the biomechanical assessment. A preload of 2 N was applied to adjust for equal pretension between all tendons. Later, continuous traction with 10 mm/min constant speed was applied to the tendons to define stiffness and ultimate tensile strength (Fig. 5).

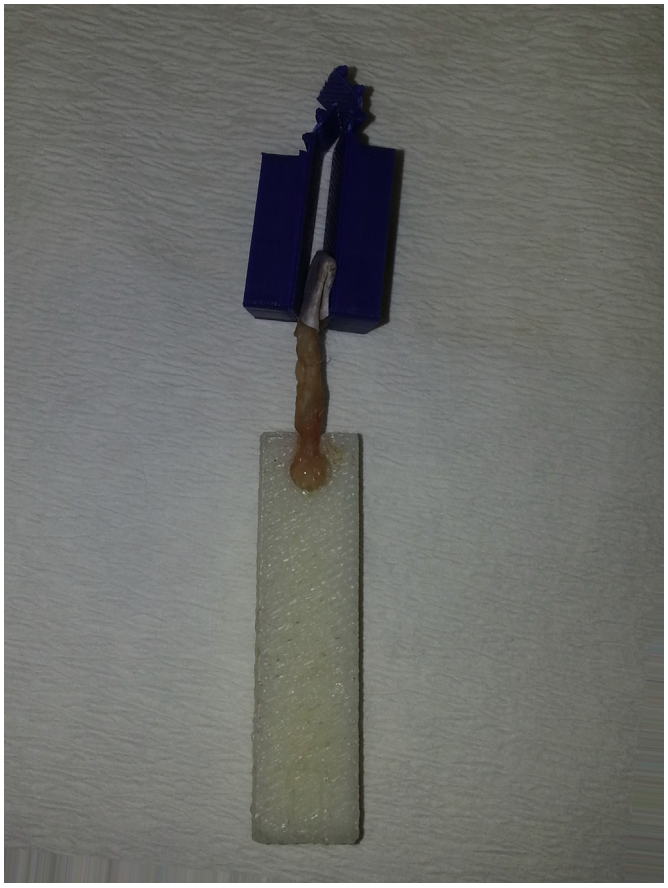

Fig. 4. The Achilles tendon and the mount used for mechanical testing.

Statistical method

All statistical analysis was performed with IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA). Distribution of the data was tested using the Shapiro–Wilk test, skew, and kurtosis values, and was determined to be normal. Since the sample sizes were too small to be analyzed with a parametric test, the Mann–Whitney U test was used to statistically test the null hypothesis with a 95% confidence interval.

Results

The mean value for the ultimate tensile strength was found to be 25.29 ± 8.43 N for the study group and 22.18 ± 6.78 N for the control group. The mean stiffness value was 13.06 ± 4.69 N/mm for study group and 12.40 ± 4.50 N/mm for the control group. Statistical analysis of the biomechanical testing revealed that there was no significant difference between the study group, which was administered the trial supplement, and the control group in terms of ultimate tensile strength ($p = 0.48$) or stiffness of the tissues ($p = 0.65$). Table 1 summarizes these data for easy visualization.

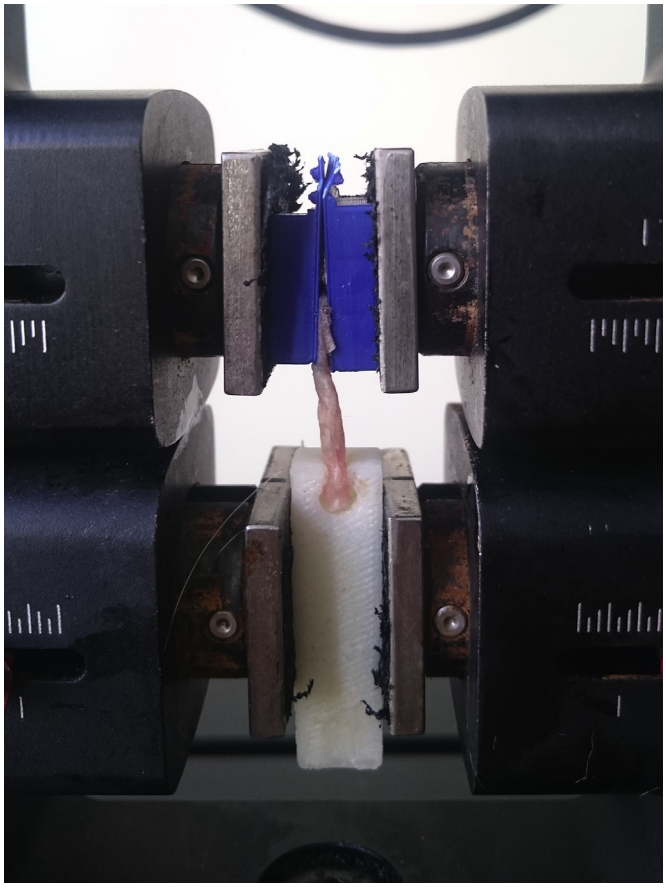

Fig. 5. The specimen during the mechanical test.

Subjective microscopic examination of the hematoxylin-eosin-stained tendon sections revealed that both groups were similar in the number of new blood vessels, fibroblasts, and quantity of connective tissue within the endotendineum. Gross examination also indicated that the quantity of tenocytes and collagen fibers within the fascicules of the tendon was similar (Fig. 6). Examination of the Masson's trichrome sections revealed that both groups were comparable in collagen fiber density and configuration (Fig. 7).

The quantized results of the immune histochemical staining performed for COL1, PCNA, and TGFβ1 in the collagen matrix, tenocytes, and endotenon fibroblasts are summarized in this paragraph. The mean value of COL1 in the collagen matrix, tenocytes, and endotenon fibroblasts in the study group was 93.85 ± 14.87, 114.85 ± 13.04, and 113.42 ± 19.76, respectively, and for the control group it was 98 ± 13.95, 112.14 ± 12.85, and 130 ± 12.31, respectively. The mean value of PCNA in the collagen matrix, tenocytes, and endotenon fibroblasts in the study group was found to be 62.14 ± 8.27, 140.28 ± 9.56, and 168.85 ± 14.84, respectively, and 63 ± 3.91, 134 ± 10.77, and 86 ± 7.07, respectively, in the control group. Finally, the mean value of TGFβ1 in the collagen matrix, tenocytes, and endotenon fibroblasts in the study group was determined to be 63 ± 9.39, 94.85 ± 16.30, and 161.85 ± 11.95, respectively, and for the control group it was 62.71 ± 8.69, 91.85 ± 12.33, and 87.57 ± 17.11, respectively (Table 1).

The Mann–Whitney U test interpretation revealed no significant difference between the study group, which was given the trial supplement, and the control in any of the histological subgroups (p value of 0.14 for collagen matrix, 0.94 for tenocytes, and 0.09 for endotenon fibroblasts) in COL1 expression values. The comparison of PCNA expression showed a significant difference between the study and control groups in the endotenon fibroblasts but not in the tenocytes or collagen matrix (p-value for endotenon fibroblasts <0.05, p-value for collagen matrix = 0.74, and p-value for tenocytes = 0.24). Finally, analysis of TGFβ1 expression between groups revealed a significant difference in the endotenon fibroblasts, but no difference in the collagen matrix or tenocytes (p-value for endotenon fibroblasts <0.05, p-value for collagen matrix = 0.89, and p-value for tenocytes = 0.65). Table 1 summarizes these data for easy visualization.

Based on these results, at the end of the third week, the level of PCNA and TGF-β1 were greater in the endotenon fibroblasts but not in the collagen matrix or the tenocytes of the repair zone. The amount of COL1 was not significantly different between the study and the control group in any tissue/cell subgroup. Similarly, there was no difference in the ultimate tensile strength or stiffness between the group that was administered the trial supplement and the placebo group.

Discussion

Successful treatment of tendon pathologies remains challenging even in the modern era. New techniques, materials, and stem cell therapies are still being researched with the goal of achieving better outcomes. Along with these efforts, the medical community should also pay attention to dietary supplementation options. This area has been neglected by the research community. Most of these supplements lack robust scientific data to support their effects. The

Table 1
Table summarizing biomechanical and immune histochemical results and statistics.

Dependent variable	Study group		Control group		Mean difference	Mann–Whitney U Sig. (2-tailed)
	Mean value	Standard deviation	Mean value	Standard deviation		
UTS (N)	25.29	8.43	22.18	6.78	3.11	0.48
Stiffness (N/mm)	13.06	4.69	12.4	4.5	0.66	0.65
Collagen matrix						
COL1	93.85	14.87	98	13.95	−4.15	0.14
PCNA	62.14	8.27	63	3.91	−0.86	0.74
TGFβ1	63	9.39	62.71	8.69	0.29	0.89
Tenocytes						
COL1	114.85	13.04	112.14	12.85	2.71	0.94
PCNA	140.28	9.56	134	10.77	6.28	0.24
TGFβ1	94.85	16.3	91.85	12.33	3	0.65
Endotenon fibroblasts						
COL1	113.42	19.76	130	12.31	−16.58	0.09
PCNA	168.85	14.84	86	7.07	82.85	**0.002**
TGFβ1	161.85	11.95	87.57	17.11	74.28	**0.002**

Bold defines statistically significant results.

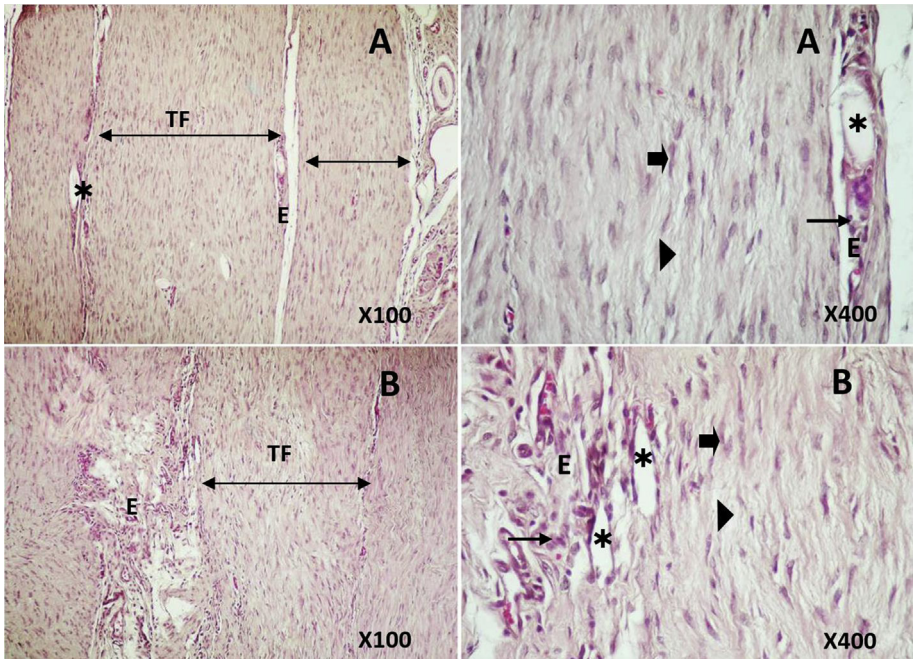

Fig. 6. Hematoxylin-eosin staining of the repair site. Control group (A); Study group (B). ▸: Collagen fiber; ➡: Tenocyte; ✱: Blood vessel; →: Fibroblasts; **E**: Endotendineum; **TF**: Tendon fascicule.

trial supplement used in this study was one of these diet supplements that have been suggested to enhance tendon healing in both tendinitis-related conditions and after tendon surgery. However, this claim is very poorly demonstrated in the literature. As a serious, randomized, controlled, in vivo animal trial, we believe that our study adds some of the missing data regarding this issue. We believe this is one of the strengths of our research.

PCNA is a nuclear protein that is known to affect DNA replication and repair and the cell cycle. Therefore, expression of PCNA is known to increase during tissue repair, where cellular proliferation and reproduction takes place.[25,26] It has been demonstrated in other studies that synovial border paratenon, endotenon, and aggressive fibroblasts play a critical role in tendon healing.[27,28] It has also been shown that the number of PCNA-positive cells increases in the tendon repair zone. These PCNA-positive cells are thought to be cells differentiated from mesenchymal stem cells that migrate from the paratenon's synovial border to more central healing zones in the tendon tissue.[29,30] TGF-β is accepted as one of the key proteins that seem to regulate tissue repair and collagen synthesis in connective tissue, though TGF-β1 expression is

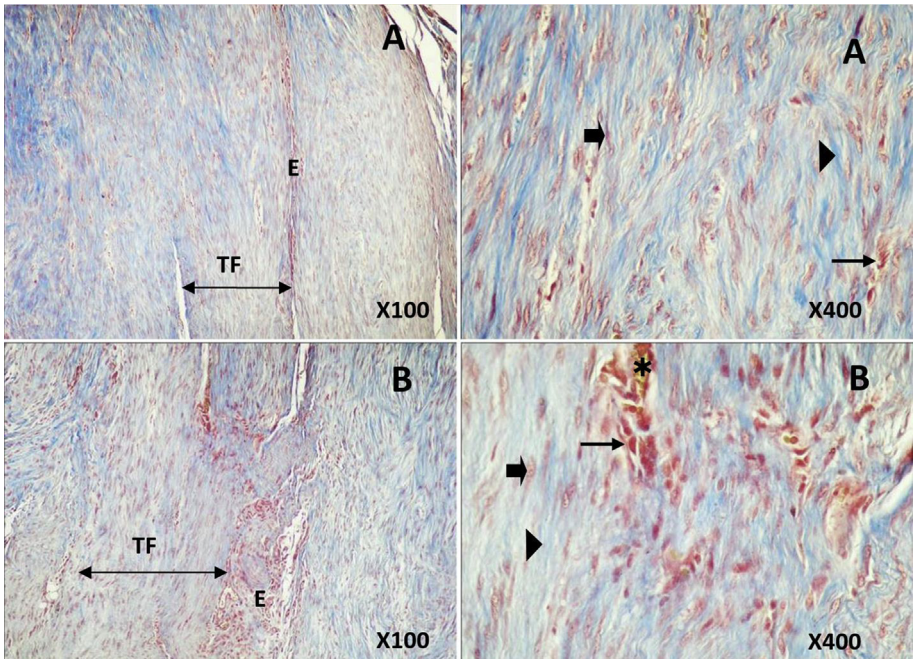

Fig. 7. Masson's trichrome staining of the repair site. Control group (A); Study group (B). ▸: Collagen fiber; ➡: Tenocyte; ✱: Blood vessel; →: Fibroblasts. **E**: Endotendineum.

affected by many factors and displays variability throughout the healing continuum.[31–33]

Our findings revealed potential benefits from the trial supplement, in that we found significantly higher PCNA and TGF-β1 levels in the endotenon fibroblasts in the study group at the end of the third week. Similar elevated levels were not observed in the tenocytes or collagen matrix. To our knowledge, there are no literature data with which to compare these findings, but it is easy to provide a reason for PCNA and TGF-β1 not showing elevated expression in the collagen matrix. Since PCNA is a nuclear antigen and TGF-β1 is predominantly an autocrine protein, they may not have increased expression in the extracellular matrix where collagen is deposited. We believe that indifferent levels of PCNA and TGF-β1 in the tenocytes may have been a result of the yet-hypothetical fact that the trial supplement does not affect the tenocytes, at least at the third week in rats. This needs to be confirmed by additional studies.

The meaning of increased PCNA and TGF-β1 expression in the endotenon fibroblasts has yet to be discovered, since our study did not show a significant difference between groups in COL1 accumulation in the repair zone. This is also confirmed by the insignificant biomechanical test results for ultimate tensile strength and stiffness. The reason for these seemingly conflicting results may be numerous.

First, we cannot be sure that the stimulated endotenon fibroblasts are differentiated from synthesized collagen at the end of the third week. Even though most of the collagen deposition has occurred in the repair zone by the third week, we have no data to show that supplements such as we used in this research increase or speed up collagen synthesis. To better understand this, new studies investigating the trial supplement's effect at different time periods must be conducted to determine PCNA and TGF-β1 behavior throughout the continuum of tendon healing.

Secondly, pronounced expression of PCNA and TGF-β1 might not have resulted in increased collagen synthesis because this study was modeled on healthy, non-deficient rats (not deficient in any of the supplement's ingredients). Biological behavior might be totally different in the case of a deficiency of any of the supplement ingredients. We also do not know if these supplements have any potential to hasten or increase collagen synthesis. To overcome this problem, further studies are needed with deficient animal models, but to our knowledge there are no proven deficient animal models or reported deficiency for any of our trial supplement's ingredients, except vitamin C. It is known that in-vitro fibroblast cultures treated with vitamin C exhibit increased synthesis of COL1.[34,35] It has also been reported in several animal studies that administration of a very high dose of vitamin C results in an increase in collagen density in the mesenchymal tissue.[36–38] But these studies differ from ours in the amount of vitamin C administered, which can be as much as 75–100 mg/kg per day. As a result, we can assume that any supplement targeting an increase in collagen synthesis through the known effects of vitamin C should have meticulous dose-effect studies and possibly intoxication studies as well.

Furthermore, the literature includes complex and confounding proof on TGF-β1 and its role in collagen synthesis. Tsubone et al[31] reported extensive variability in TGF-β1 expression in different time periods in TGF-β inducible early gene (TIEG) knockout mice (TIEG is the first step in the TGF-β1 action pathway). They also showed that, although TGF-β1 expression varied in TIEG knockout mice, COL1 synthesis continued, with a pattern identical to that of healthy mice. TIEG knockout mice do not have any problems regarding soft tissue healing. This indicates that there are other cytokines that regulate collagen synthesis aside from TGF-β1. Other studies have also mentioned that the role of TGF-β1 in collagen synthesis is not very well understood and that there are other responsible cytokines that have regulatory roles in collagen synthesis.[39,40]

Conclusion

Our research on rats revealed no difference in the group given the trial supplement (a combination of mucopolysaccharides, vitamin C, and COL1) in mechanical strength or the amount of collagen deposited at the repair site at the end of the third week. Since these are primary outcomes for tendon pathologies, we can conclude that the trial supplement provided no significant advantage in tendon healing using this methodology. On the other hand, we did note activation of fibroblasts in the repair site through increased expression of PCNA and TGF-β1. We have provided an initial explanation, but we believe further studies approaching this issue with a different methodology are needed to explain the nature of this cellular stimulation throughout the continuum of tendon healing.

Conflicts of interest

None of the authors have any potential conflict of interest to be disclosed.

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