



Tranexamic acid has positive effect in early period of tendon healing by stimulating the tumor necrosis factor-alpha and matrix metalloproteinase-3 expression levels

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Pro-inflammatory tumor necrosis factor-alpha (TNF- α), matrix metalloproteinase-3 (MMP-3), and transforming growth factor-beta (TGF- β) are associated with many diseases in the literature.^[1] MMP-3 belongs to the MMP family and is induced during development, wound repair, inflammation, and cancer.^[2,3] MMP-3 activates cytokines, growth factors, and other MMP members (e.g., the collagenases MMP-1, MMP-8, and MMP-13) and gelatinase B (MMP-9) and cleaves the extracellular matrix proteins, e.g., E-cadherin, laminins and type IV collagen. However, MMP-3 is incapable of cleaving native type I collagen during wound repair.^[1] TNF- α

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ABSTRACT

Objectives: This study aims to evaluate the effect of tranexamic acid (TXA) application in tendon healing by using its immunohistochemical effects on tumor necrosis factor-alpha (TNF- α), matrix metalloproteinase-3 (MMP-3), and transforming growth factor-beta (TGF- β) expression; and to identify if TNF- α , MMP-3, and TGF- β can be used to monitor and evaluate tendon healing or not in tenotomized rat Achilles tendons.

Materials and methods: Twelve male Wistar-Albino rats (age 6-7-month-old; weighing 300-350 g) were used in this retrospective study conducted between November 2016 and May 2017. The rats were divided into two groups with similar weights. The right legs of the rats were determined as the study group (TXA), and the left legs as the control serum physiologic (SP) group. Under anesthesia, bilateral Achilles tenotomy was performed and surgically repaired. 1 mL of TXA was applied locally for the right side and 1 mL of SP was locally applied for the left side. Half of the rats were sacrificed at the third week (right leg-TXA3, left leg-SP3) and the other half at sixth week (right leg-TXA6, left leg-SP6) and tendon samples were taken from the extremities. Immunohistochemical findings of TNF- α , MMP-3, and TGF- β were evaluated on the basis of the frequency and intensity of staining.

Results: In TNF- α and MMP-3 and TXA groups, there was a significant difference in staining compared to SP groups ($p<0.05$). Regarding TNF- α expression, the total index score in the TXA6 subgroup was higher than the TXA3, SP6, and SP3 subgroups (8, 7, 3, and 4, respectively). Overall scores of TNF- α showed that TXA groups had significantly higher scores when compared to SP groups ($p<0.05$). In addition, total MMP-3 expression scores were significantly higher in TXA groups than in SP groups, respectively; TXA3: 14, TXA6: 11, SP3: 10, and SP6: 9 ($p<0.05$). However, the degree of staining with TNF- α was found to be significantly lower than MMP-3 ($p<0.05$). Immunohistochemical reactivity was not observed with TGF- β .

Conclusion: Tranexamic acid has positive effect in early period of tendon healing by stimulating the TNF- α and MMP-3 expression levels. TNF- α and MMP-3 can be used to monitor and evaluate tendon healing.

Keywords: Matrix metalloproteinase-3, tendon healing, tranexamic acid, tumor necrosis factor-alpha, transforming growth factor-beta.

is an important mediator of inflammatory processes. TNF- α promotes collagenolysis causing massive tissue destruction and collagen degradation during the wound repair. TNF- α induces a variety of MMPs and also MMP-3 was shown to activate TNF- α .^[4-6] In recent studies, MMP and TNF- α levels in intraarticular synovial fluid of anterior cruciate ligament (ACL) ruptured knees were found to be increased.^[7] After an injury, TGF- β 1 secreted by platelets, keratinocytes, and macrophages is rapidly upregulated in the wound microenvironment and also TGF- β 1 stimulates myofibroblast activation, leading to increased matrix synthesis and decreased matrix remodeling.^[8,9] Studies on the healing of tendon in chicken demonstrated that TGF- β 1 is highly expressed throughout the early healing period (the inflammatory phase of wound healing), particularly in the peritendinous region, as evidenced immunohistochemically.^[8,10] Thus, TGF- β 1 has been identified as a therapeutic target for adhesions.

Tendon healing involves processes similar to healing of other soft tissues. Inflammation occurs in the first stage, followed by a restorative/proliferative phase, remodeling and eventually tissue maturation takes place.^[11] The remodeling of the matrix after tendon rupture is not fully understood because the processes and timing of its formation are intertwined. Tranexamic acid (TXA) is a synthetic amino acid derivated plasminogen inhibitor and reduces fibrinolysis and as an antifibrinolytic agent is used to reduce bleeding and the need of postoperative blood transfusion in orthopedic surgery both intravenously or locally.^[12-14] A recent study of us which was also the prototype of this current study revealed that TXA negatively affected tendon healing in the late period after local administration.^[15] In this current study, our hypothesis was that the effect of TXA on tendon healing could be demonstrated by the change in tissue levels of TNF- α , MMP-3, and TGF- β and that also tendon healing could be followed or demonstrated by using expression of these cytokines. As a consequence, in this study, we aimed to evaluate the effect of TXA application in tendon healing by using its immunohistochemical effects on TNF- α , MMP-3, and TGF- β expression; and to identify if TNF- α , MMP-3, and TGF- β can be used to monitor and evaluate tendon healing or not in tenotomized rat Achilles tendons.

MATERIALS AND METHODS

The retrospective study was conducted with approvals of the Ordu University Ethics Committee of experimental animals dated 12.05.2016, number

4/decision 8, and 30.01.2018, number 1/decision 5, respectively, between November 2016 and May 2017. The study was conducted in accordance with the principles of Care and Use of the Laboratory Animals and the animal rights were protected. Twelve male Wistar-Albino rats (age 6-7-month-old; weighing 300-350 g) were divided into two groups so that the weights of the animals were close to each other. The right legs of the rats were determined as the TXA group and the left legs as the serum physiologic (SP) group. Half of the rats were sacrificed at the third week (right leg-TXA3, left leg-SP3) and the other half at sixth week (right leg-TXA6, left leg-SP6) and tendon samples were collected from the extremities. The effect of TXA on tendon healing was evaluated by TNF- α , MMP-3, and TGF- β immunohistochemically from tendon samples obtained from this study.

Operations were conducted under general anesthesia with intraperitoneal injection of ketamine 90 mg/kg (Ketalar; Eczacıbaşı, Istanbul, Turkey) and xylazine hydrochloride 3 mg/kg (Rompun; Bayer, Leverkusen, Germany). After appropriate anesthetic conditions, animals were shaved with care to avoid damaging the skin with a razor blade and disinfected with polyvinylpyrrolidone-iodine (Batticon[®], Adeka, Samsun, Turkey). The surgical field was covered with sterile compresses. Skin and subcutaneous tissues were passed through the incisions of the right and left Achilles tendons with a 2 cm incision. Bilateral Achilles tendons were tenotomized with no. 11 scalpels at approximately 0.5 cm proximal to the insertion to the calcaneus. All tendons were repaired with 4/0 polypropylene suture (Propilen, Dogsan Medical Equipment, Trabzon, Turkey) using a modified Kessler-type technique. The surgical wounds were closed primarily with 3/0 polypropylene skin sutures (Propilen, Dogsan Medical Equipment, Trabzon, Turkey). On surgical sites, 1 mL of TXA (Transamine 250 mg/5 mL, Actavis Pharma Inc., Istanbul, Turkey) was applied to the right leg and 1 mL isotonic SP was applied to the left leg locally.

Wound dressing was performed. During the experiment, rats were kept at normal room temperature and humidity by feeding with standard pellet feed and tap water for 10/14 hours in a light/dark cycle of light, three subjects per cage. After the surgical procedure, all animals were regularly treated with wound dressing. At third and sixth weeks, the rats were sacrificed.

All tendons were removed with the bone tissue adjacent exposing suture area and fixed in 4% buffered formalin and embedded in paraffin. Five-micrometer-

TABLE I

Immunohistochemical results of matrix metalloproteinase-3

Group	Index score			
	0	1	2	3
SP3 (n=6)	-	2	4	-
SP6 (n=6)	-	3	3	-
TXA3 (n=6)	-	-	4	2
TXA6 (n=6)	-	1	5	-

SP: Serum physiologic; SP3: Tendons of serum physiologic group in third week; SP6: Tendons of serum physiologic group in sixth week; TXA: Tranexamic acid; TXA3: Tendons of tranexamic acid group in third week; TXA6: Tendons of tranexamic acid group in sixth week.

TABLE II

Immunohistochemical results of tumor necrosis factor-alpha

Group	Index score			
	0	1	2	3
SP3 (n=6)	2	4	-	-
SP6 (n=6)	3	3	-	-
TXA3 (n=6)	-	5	1	-
TXA6 (n=6)	-	4	2	-

SP: Serum physiologic; SP3: Tendons of serum physiologic group in third week; SP6: Tendons of serum physiologic group in sixth week; TXA: Tranexamic acid; TXA3: Tendons of tranexamic acid group in third week; TXA6: Tendons of tranexamic acid group in sixth week.

thick sections were placed on polylysine-coated slides and stained with hematoxylin and eosin. The slides were evaluated under light microscopy (Nikon Eclipse Ni-U; Nikon, Tokyo, Japan) at 40× magnification.

The sections for immunohistochemical study were stained with Leica Bond-Max IHC staining device (Vision Biosystems, Melbourne, Australia) using anti-TNF- α antibody and MMP-3 antibody. Anti-TNF- α antibody (1 mL, lyophilized, Santa Cruz-sc130349, Oregon, USA) was diluted at a ratio of 1:250, and MMP-3 antibody (100 μ g, GTX103647) was diluted at a ratio of 1:250. Reactivity could not be observed with TGF- β ; therefore, it was excluded from the study. Immunohistochemical findings were evaluated on the basis of the frequency and intensity of staining. Staining intensity was scored as 0-3. Staining was as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Staining frequency was graded between 0 and 4. Grade 0 indicated a staining of <3%, grade 1: 3-25%, grade 2: 26-50%, grade 3: 51-75%, and grade 4: >75%. The index score was calculated by multiplying the intensity and frequency scores. The results were shown respectively: index score 0=0, index score 1=1-2, index score 2=3-5, and index score 3=6-7.

Statistical analysis

The SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Descriptive statistics were given as median \pm interquartile range (ICR) (minimum-maximum). The staining difference between the three groups was compared with Tukey's analysis and post hoc test. A value of $p \leq 0.05$ was considered as the statistical significance level.

RESULTS

There was a significant difference between TXA and SP groups in terms of staining with TNF- α and MMP-3 where reactivity was not observed with

TGF- β immunohistochemically. Total index score of expression in TXA6 subgroup was higher than TXA3, SP6, and SP3 subgroups in terms of TNF- α (8, 7, 3, and 4, respectively). Overall scores of TNF- α showed that TXA groups had significantly higher scores compared to SP groups ($p < 0.05$). Besides, total MMP-3 expression scores were significantly higher in TXA groups than SP groups: TXA3: 14, TXA6: 11, SP3: 10, and SP6: 9, respectively ($p < 0.05$). TNF- α and MMP-3 staining intensities of the tendons in the groups are shown in Tables I and II. The degree of staining with TNF- α was found to be significantly lower than MMP-3 ($p < 0.05$) (Figures 1 and 2).

DISCUSSION

The most important finding of this study was that TNF- α and MMP-3 staining were significantly higher in the TXA groups compared to the SP groups. This positive effect in tendon healing was more evident in the early period. TNF- α and MMP-3 staining were correlated with the histopathological evaluation of tenocyte cell morphology findings with our recently reported study.^[15]

The MMPs are a family of proteolytic enzymes that destroy the extracellular matrix components of the tendon. Expanding knowledge on MMP function and its impact on physiological and pathological processes has increased the interest in MMP substrates and led to further research.^[4] MMP-3 belongs to the MMP family and is induced during development, wound repair, inflammation, and cancer.^[2,3] MMP-3 has been implicated in many inflammatory diseases. Moreover, numerous bone pathologies including arthritis, osteoporosis, osteonecrosis, periodontitis, sinonasal osteitis, degenerated lumbar disk tissues, and bone cancer metastasis were found to be contributed by MMP activity.^[16,17] MMP-3 deficient mice show diminished inflammatory responses to various

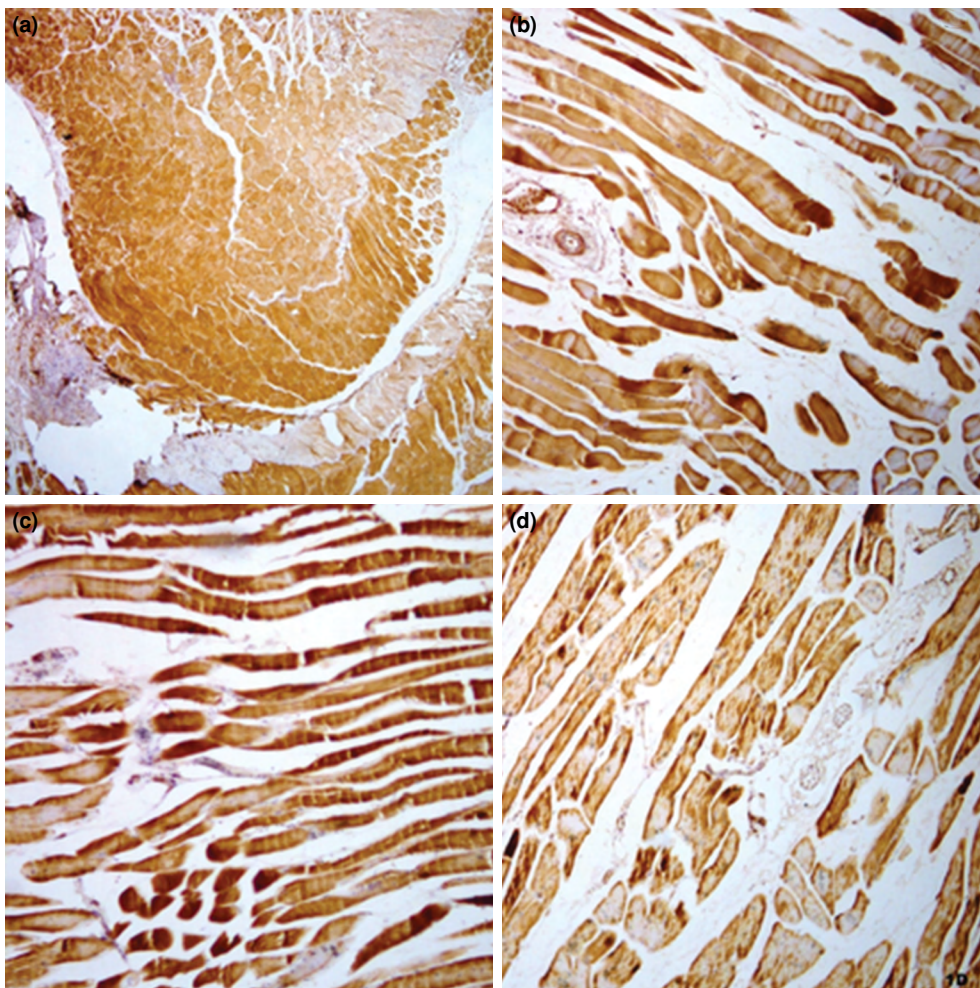


FIGURE 1. Matrix metalloproteinase-3 staining was observed in cytoplasm and extracellular matrix of striped tendons. (a) Score 1 staining of striped tendons (SP3 groups) (MMP-3 $\times 400$). (b) Score 2 staining of striped tendons (SP6 groups) (MMP-3 $\times 100$). (c) Score 1 staining of striped tendons (TXA6 groups) (MMP-3 $\times 100$). (d) Score 2 staining of striped tendons (TXA3 groups) (MMP-3 $\times 100$).

MMP-3: Matrix metalloproteinase-3; SP: Serum physiologic; TXA: Tranexamic acid.

stimuli, and reduced cutaneous wound contraction.^[1] There is a constant balance between MMP activity and their specific endogenous tissue inhibitors (TIMP) in maintaining physiological events.^[18] MMPs and TIMPs are involved in many biological processes.^[19] Higuchi et al.^[20] measured the cytokine, MMP, and TIMP levels in intraarticular synovial fluid of ACL ruptured knees. They found that TIMP and MMP-3 levels increased in knees with ACL rupture and also the balance between TIMP and MMP-3 was largely disrupted in favor of MMP-3. This increase was thought to be due to an increase in the cytokine level. According to Higuchi et al.,^[20] in this current study, we also found the expression of MMP-3 in tenotomized rat Achilles tendons in healing process

and increased expression by the local application of TXA. We thought that as an inflammatory cytokine, MMP-3 might be used in evaluating tendon healing.

Pro-inflammatory TNF- α is an important mediator of inflammatory processes. TNF- α induces a variety of MMPs.^[4] MMP-3 was shown to activate TNF- α .^[5,6] Activated TNF- α induces the downstream expression and secretion of MMP-3.^[21,22] In a recent study, TNF- α was found to enhance collagenolysis via the up-regulation of MMP-3 in murine skin.^[1] Cameron et al.^[23] found an increase in the levels of inflammatory cytokines TNF- α and interleukins (ILs) in intraarticular synovial fluid in the weeks after ACL rupture. Elsaid et al.^[7] found that particularly in the early period, there was

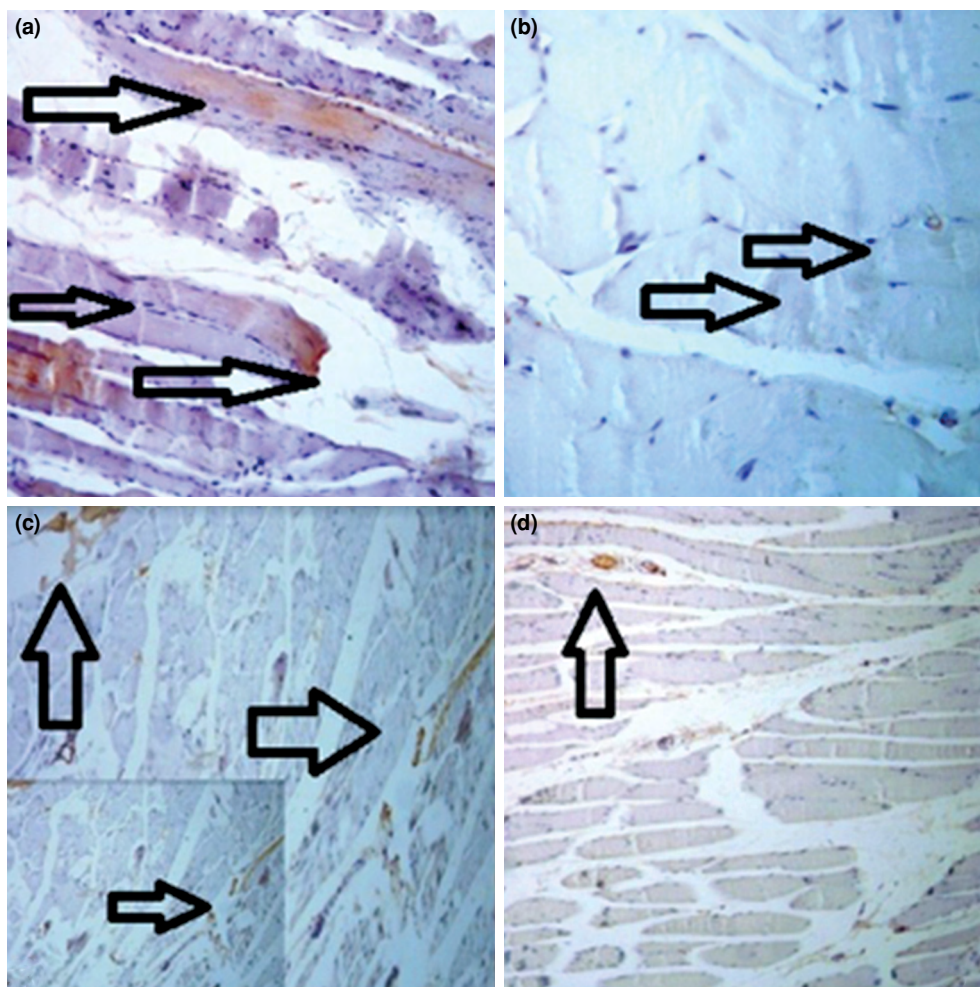


FIGURE 2. Tumor necrosis factor-alpha staining was observed in cytoplasm of striped tendons (arrow). (a) Score 2 staining of striped tendons (TXA6 groups) (TNF- α $\times 200$). (b) Score 1 staining of striped tendons (TXA3 groups) (TNF- α $\times 100$). (c) Score 1 staining of striped tendons (SP3 groups) (TNF- α $\times 400$). (d) Score 1 staining of striped tendons (SP6 groups) (TNF- α $\times 100$).

TNF- α : Tumor necrosis factor-alpha 3; TXA: Tranexamic acid; SP: Serum physiologic.

an increase in TNF- α levels in addition to other inflammatory cytokines in intraarticular synovial fluids aspirated by arthroscopy from knees with ACL rupture and this increase led to a decrease in lubricin concentration. In our study, TNF- α levels increased during the tendon healing period and we saw that this was more expressed with TXA application; from this point of view, we think that TNF- α can be used to evaluate tendon healing as MMP-3.

The TGF- $\beta 1$ stimulates myofibroblast activation, leading to increased matrix synthesis and decreased matrix remodeling.^[8,9] Studies on the healing of tendon in chicken demonstrated that TGF- $\beta 1$ is highly expressed throughout the early healing period,

particularly in the peritendinous region, as evidenced immunohistochemically.^[8,10] Thus, TGF- $\beta 1$ has been identified as a therapeutic target for adhesions. However, in this study, we observed no reactivity with TGF- β during the entire tendon healing period with or without TXA.

The TNF- α , MMP-3, and TGF- β seem to affect each other in physiological and pathological processes. MMP gene expression is stimulated with inflammatory cytokines such as TNF- α , IL-1, and IL-6, and many growth factors and hormones. TGF- β , heparin, corticosteroids, retinoids, and prostaglandin E2 act by inhibiting MMP gene transcription.^[19] Although MMP-3 and TNF- α staining was found to be significantly higher in the

TXA group, immunohistochemical reactivity with TGF- β was not observed. Although we observed that TNF- α and MMP-3 affected each other in physiological and pathological processes during tendon healing period, we noted that TGF- β did not comply with this process.

According to our findings, TNF- α and MMP-3 expression was higher in the TXA group compared to the SP group, and this increase was evident in the TXA6 group. In six-week groups, when the outcomes were evaluated together with our recent study, histomorphological analysis of collagen disorganization (Borman score mean: 2.167 ± 0.408) was correlated with TNF- α expression; however, despite this increase in TNF- α , MMP-3 expression decreased compared to TXA3 group. This finding was in line with the literature showing that TNF- α suppressed MMP-3 in hypertrophic scar.^[24] Negative histomorphological effect of TXA in TXA6 group is also correlated with the increase in MMP3 and TNF- α expressions immunohistochemically. In our previous study, the TXA3 group showed a positive effect compared to both the SP3 and TXA6 groups histomorphologically. However, in this study, an increase in TNF- α and MMP-3 expression was observed in TXA groups. In addition, in the TXA3 group, TNF- α expression was observed at score 1, while MMP-3 was observed at scores 2 and 3. With these findings, it can be suggested that TNF- α increases the expression of MMP-3 in the TXA group in the early period but decreases it in the long term. Histomorphologically, disorganization in collagen and cell morphology deterioration is a finding that supports this result.^[15] These findings support the idea that TNF- α and MMP-3 markers may be used particularly in the early period for follow-up in the administration of TXA for tendon rupture and hemorrhage control during the healing process.

The limitation of the current study is the lack of biomechanical analyses. Although immunohistochemical evaluation is important for tendon healing process, biomechanical test results are also important for the evaluation of tendon repairs.

In conclusion, TXA has a positive effect in the early period of tendon healing by stimulating the TNF- α and MMP-3 expression levels. Thus we suggest that TNF- α and MMP-3 may be used to monitor and evaluate tendon healing. Evaluating the level of these markers as an important laboratory parameter can be a guide about healing, follow-up, and treatment stage. Further larger studies of this outcome can be illuminating.

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Declaration of conflicting interests

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