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The effect of low-molecular-weight heparin on rat tendon healing

Düşük molekül ağırlıklı heparinin sıçan tendon iyileşmesi üzerine etkisi

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Amaç: Düşük molekül ağırlıklı heparinin (DMAH) tendon iyileşmesi üzerindeki etkileri araştırıldı.

Çalışma planı: Çalışmada, ağırlığı yaklaşık 300 gram olan 45 adet erişkin Wistar tipi sıçan eşit sayıda üç gruba ayrıldı. Tüm gruplarda Aşil tendonu kesildi ve primer tamir uygulandı. Grup I ve II'deki deneklere (grup I' e yüksek doz, 6 mgr/kg, 170 IU AXa; grup II'ye düşük doz, 3 mgr/kg, 85 IU AXa) dört hafta süreyle, günlük, subkutan, tek doz olarak DMAH (nadroparin kalsiyum) uygulandı. Grup III'deki deneklere DMAH uygulanmadı, kontrol grubu olarak değerlendirildi. Histolojik olarak, elektron ve ışık mikroskobunda, iyileşen tendonlardaki fibriler kollajen sentezi, mitokondri dejenerasyonu ve ekstraselüler matriksteki kollajenin fibriler veya homojen yapıda olup olmadığı değerlendirildi. Biyomekanik olarak da en büyük yüklenme kuvvetleri ve bu sıradaki uzama miktarları belirlendi.

Sonuçlar: Histolojik olarak, kontrol grubu ile karşılaştırıldığında, DMAH verilen iki grupta, grup I'de daha fazla olmak üzere, fibroblast sayısında, ekstraselüler matrikste fibriler kollajen oluşumunda, fibroblastların sitoplazmik içeriklerindeki granüllü endoplazmik retikulum sayısında artış, dejenerasyon göstergesi olarak da mitokondri vakuolizasyonunda azalma görüldü. Biyomekanik çalışmada, grup I'de ölçülen en büyük yüklenme ve uzama değerleri (31 N ve 25 mm) grup II (24.6 N ve 19.6 mm) ve grup III'e (23.1 N ve 17.3 mm) göre anlamlı farklılık gösterdi ($p<0.05$). Grup II ile III arasında ise bu açıdan anlamlı fark görülmedi ($p>0.05$).

Çıkarımlar: Günlük olarak tek doz şeklinde DMAH uygulaması, fibroblast sayısı ile fibriler kollajen sentezini artırıp, mitokondri dejenerasyonunu azaltarak tendon iyileşmesini olumlu etkilemektedir.

Anahtar sözcükler: Aşil tendonu/yaralanma; heparin, düşük molekül ağırlıklı; sıçan; yara iyileşmesi.

Objectives: We investigated the effect of low-molecular-weight heparin (LMWH) on the healing of tendons.

Methods: Forty-five adult Wistar rats weighing 300 g were randomized into three groups equal in number. All the rats underwent full-thickness surgical incision of the Achilles tendon followed by primary repair. After the operation, two groups received daily subcutaneous LMWH injections (nadroparin calcium) for four weeks at high or low doses (group I, 6 mg/kg, 170 IU AXa; group II, 3 mg/kg, 85 IU AXa). Group III remained untreated as the control group. Histologically, the specimens were examined under light and electron microscopy with regard to the amount of fibrillar collagen synthesis, mitochondrial degeneration, and the composition of the extracellular matrix collagen. Biomechanically, maximum load to failure and correspondent elongation of the tendons were measured.

Results: Compared to the control group, histologically, both LMWH-treated groups exhibited increased number of fibroblasts, increased fibrillar collagen formation in the extracellular matrix, and higher counts of granular endoplasmic reticula in cytoplasmic contents of fibroblasts as well as decreased mitochondrial vacuolization and degeneration. Biomechanical assessments showed that tendons in group I had significantly higher maximum load to failure and elongation values than group II and III (31 N *vs*. 24.6 N and 23.1 N; 25 mm *vs*. 19.6 mm and 17.3 mm, respectively; $p<0.05$). Group II and III did not differ significantly in this respect ($p>0.05$).

Conclusion: Daily administration of single dose LMWH improves tendon healing through increasing the number of fibroblasts and fibrillar collagen synthesis and decreasing mitochondrial degeneration.

Key words: Achilles tendon/injuries; heparin, low-molecular-weight; rats; wound healing.

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Tendons are vulnerable to various types of acute or chronic injures. [1,2] After the injury, tendons heal with a fibrotic scar tissue. The structural and biomechanical properties of scar tissue is known to be inferior than that of the original tissue.[3-5] Different methods have been investigated in hope of obtaining better healing, including high-end genetic and tissue engineering processes such as, fibroblast containing scaffolds, application of growth factors and cytokines.[6-13] There are various studies blaming heparin for tendon degeneration and peritendinitis and condemning its usage as a tendon healing modulator.[14-19]Angiogenetic properties of heparin have been promoted for a better tendon healing.

Thrombin is a serine protease. Its protease activity is responsible for the transformation of fibrinogen to fibrin. Thrombin is also a principal factor in blood clot formation following tissue injury. Furthermore, it has many biological properties in common with growth factors, such as chemotaxis of neutrophils, monocytes, and macrophages, and stimulation of fibroblast proliferation. Thrombin accelerates wound healing. Many thromboprophylactic treatments aim to reduce thrombin activity.

Thrombin itself is also activated by proteolysis of prothrombin by factor Xa, as a result of the coagulation cascade. Factor Xa activity is therefore the target of most thromboprophylactic drugs. Low molecular weight heparins (LMWHs) inhibit coagulation factor Xa and hence the generation of thrombin.[20,21]

Low molecular weight heparin derivatives (LMWH) have been used for a long time as antithrombotic agents. LMWH reduces adhesions, promotes wound healing, decreases intraabdominal adhesions, stimulates macrophages, inhibits enflamation.[12,14,16,17,19,21-24]

The degree of thrombin suppression, during continuous or daily single dose application of low molecular weight heparin is not well known. This study is designed to test the efficacy of systemically administered single dose LMWH preparation on tendon healing via prevention of thrombin formation.

Materials and methods

Forty five adult Wistar type rats, weighing 300±20 grams (Ministry of Health, Refik Saydam National Hygiene Center, Ankara, Turkey) were used. Three to four rats were hosted per cage, at Gazi University, Experimental Research Unit, Ankara, Turkey. As the LMWH, we used Nadroparine Calcium (Fraxiparine, Sanofi- Aventis). The rats were given free access to food and water, and free movement within their cages. All surgical procedures were performed after administration of anesthesia. The animals were sacrificed by high dose thiopental (I.E. Ulagay Pharmaceuticals Topkapi, Istanbul, Turkey). After the surgical procedures, all rats were transferred to their cages and followed in the animal care unit. We did not encounter losses due to deaths or auto mutilation. All procedures were approved by the Gazi University Ethical Committee (G.U.ET-03.007).

Study groups

The rats were randomized (n=15)into three groups. Group I received daily high dose (6mg/kg, 170 IU AXa), group II received half dose according group I (3 mg/kg, 85 IU AXa) LMWH. Group III remained as the control group and did not receive LMWH injections. Drug administration started twelve hours prior to surgery and went on for four weeks as daily subcutaneous injections for groups I and II. Injections were all applied by the same researcher in the dorsal region of the rats. End of the study, eight rats were grouped for biomechanics assesment and seven rats were for histological evaluation.

Surgical procedure

All procedures were done by the same surgeon under intramuscular administration of ketamine-HCl (50 mgr/ml)+ Benzetonyum-HCl (Ketalar-IM)anesthesia. After extremity preparation and draping, a posterior midline longitudinal incision was used to expose the Achilles tendon. The Achilles and the plantaris tendons were stripped from the surrounding fascia. The achilles tendon was transected five millimeters proximal to its insertion to the calcaneus. The plantaris tendon was also transected to prevent internal splint effect. The achilles tendon was repaired by using 6/0 ethilon monofilament nylon (Ethicon, USA) sutures with

modified Kessler method. The wound was closed with 3/0 ethilon monofilament uninterrupted sutures. No wound dressing or casting was used after the operation. All subjects were let to mobilize freely and were fed with standard laboratory food and tap water.

Histological evaluation

Glutheraldehide - paraphormaldehide solution was used to fix the tendon samples. After the standard procedures for electron microscopy were completed, the blocks were obtained by araldite + DDSA (Dodesenil süksik anhidrate) + BDMA (Benzile dimethilamine) mixture.

One micron thick sections were stained with toluidine blue and examined under light microscopy in order to decide which areas were suitable for electron microscopy. Afterwards, 300-700Aº thick sections were obtained with the help of an ultramicrotome and were stained with acetate + lead citrate stain for electron microscopy. The sections were examined with a Carl-Zeiss EM-900™ electron microscope and toluidine blue stained sections were examined with Olympus-BH2 light microscope. From the electron microscope sections, six images were obtained from each group. The number of granulated endoplasmic reticulum, fibriller collagen density, mitochondrial crista lysis, and homogenus collagen density were analyzed. These data were statistically analyzed.

Biomechanical evaluation

The amount of peak force that led to tendon rupture was analyzed Middle East Technical University Engineering Faculty by the same engineer. The achilles-calcaneus complexes were stretched with a force of 5N per second, using a mechanical test device (LR5K Lloyd Instruments, Ametek, USA). Peak forces between the groups were tested in order to reveal if any statistically meaningful difference existed.

Statistical analysis

Peak force (maximal load) and elongation parameters were tested with Mann-Whitney U and Wilcoxon tests (Wilcoxon non-parametric two related samples test) with SPSS 10.0 for Windows(SPSS, Chicago, IL, USA).

Results

Histological results

Electron microscopy

Group I samples showed increase in rough endoplasmic reticulum quantity and newly synthesized cytoplasmic procollagen in fibroblasts. Samples from group I did not show any mitochondrial crest deletion, which is a finding of normal mitochondrial morphology (Figure1a). Other visible nuclear and cytoplasmic parameters in group I were defined as healthy. Extra cellular matrix examination showed up dense, highly oriented fibrillar collagen (Figure 1a).

Examination of group II revealed higher number of fibroblast counts and more collagen synthesis than group III. But it was inferior to the findings of group I. Extra cellular matrix of the samples from group II showed rare loses of fibrillar orientation (Figure Ib). Rough endoplasmic reticulum numbers in fibroblasts were inferior to that of group I and crest deletion in mitochondria were almost non existent.

Group III showed decreased number of fibroblast combined with lesser cytoplasmic content and collagen synthesis along with loss of fibrillar orientation and transverse banding of the newly synthesized collagen compared to group I and II. This resulting in a homogenous collagen look rather than fibrillar. Greater magnification revealed crest deletion of the mitochondria, collagen synthesis lacking fibrillar orientation (Figure Ic) and vacuolized rough endoplasmic reticulum showing large cisterns, which is a finding of insufficient collagen synthesis.

Light microscopy

Groups I and II were similar in fibroblasts' increased cytoplasmic content and extra cellular matrix collagen's fibrillar orientation (Figure 2a,b). On the other hand, group III showed decreased cytoplasmic content and homogenous collagen look rather than fibrillar (Figure 2c).

Histologically fibroblasts containing healthy looking nucleus and cytoplasms, without crest loss in their mitochondria and increased quantity of rough endoplasmic reticulum along with fibrillar formati-

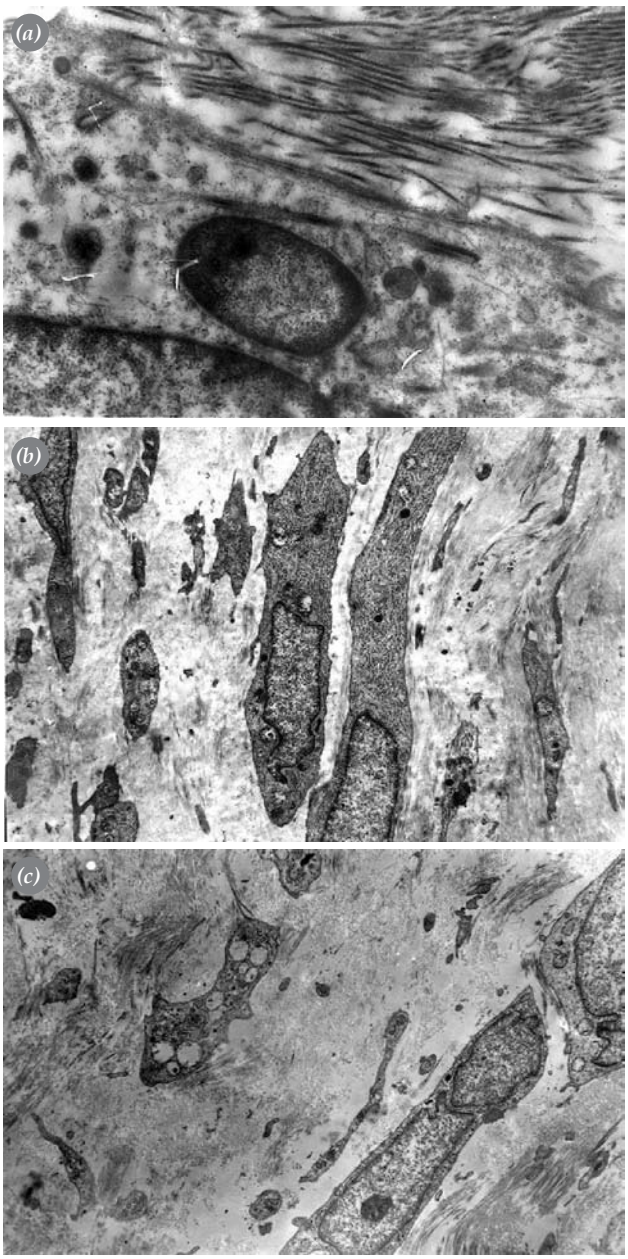

Figure 1. Electron microscopy images after uranyl asetat and lead citrate staining. **(a)** Group I (x3600); **(b)** group II (x9000); **(c)** group III (x9000).

on of extracellular matrix collagen are considered to be histologicaly positive healing marks, as seen in groups I and II. Lower fibroblast counts with less rough endplasmic reticulum and homogenous extra cellular collagen formation, rather than fibrillar collagen, indicates that there is inefficient tendon healing in control sampes. We used the fibriller collagen formation and collagen arrangement, cellular function and level of cellular synthesis as his-

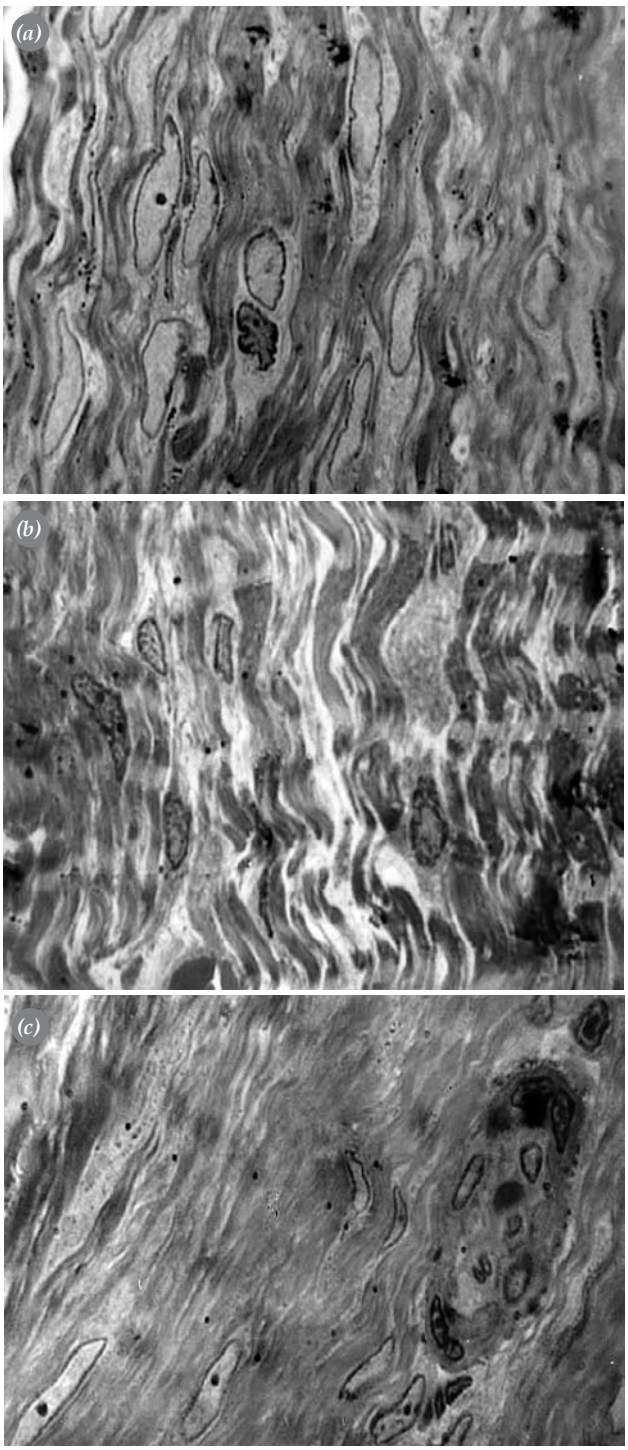

Figure 2. Light microscopy images after toluidin blue staining (x1000). **(a)** Group 1; **(b)** group II; **(c)** group III.

topathologic evaluation parameters. Histologically, collagen synthesis was better and more organized in group I, compared to group II. No significant difference was observed between Groups II and

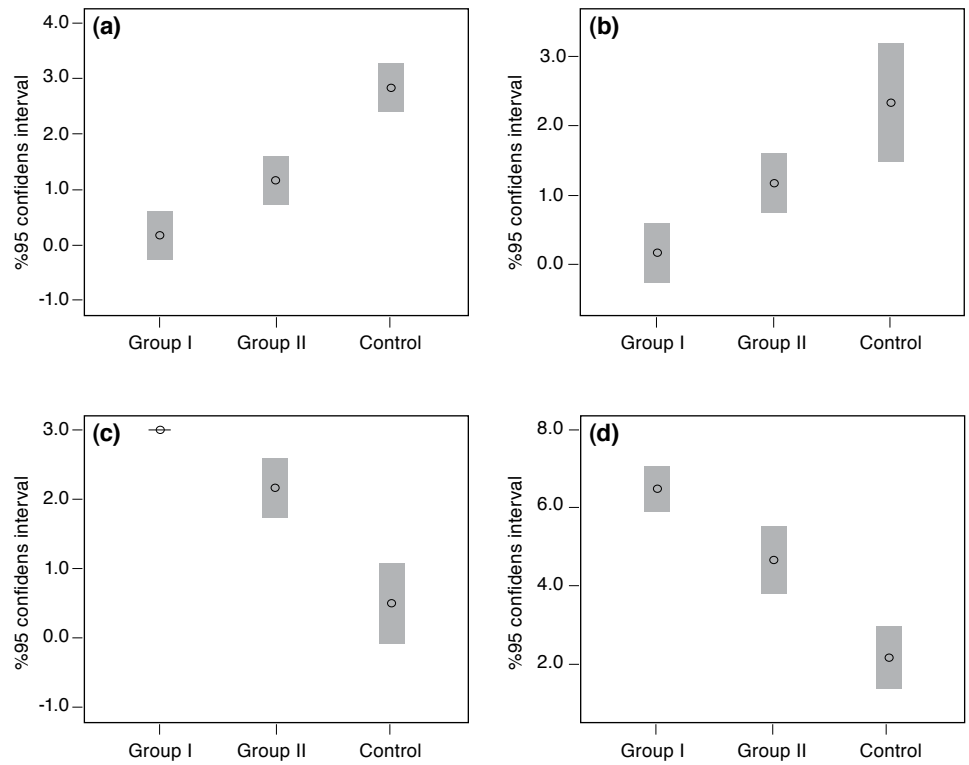

Figure 3. Histological differences amount the groups. **(a)** Homogen collagen density; **(b)** Mitochondrial crista lysis; **(c)** Fibrillar collagen density; **(d)** Count of granular endoplasmic reticulum.

III ($p>0.05$). There were marked mitochondrial degeneration and less fibrillar collagen synthesis in Group III. A better healing was observed in Group I (6 mg/kg sc injection), compared to Groups II (3 mg/kg sc injection) and group III (control). Beside the increased number of healthy energy source mitochondrial amounts, the increasing amounts of granulated endoplasmic reticulum have been associated with less number of cellular apoptosis.

Biomechanical results

Eight calcaneus-achilles complexes from each group were examined for peak loading force before rupture and maximal elongation values.(Figure 4). It was noted that ruptures in groups I and II were distal to the surgical splitting zone, close to the calcaneal insertion, whereas rupture was through the split zone or in close proximity in the control group samples. The mean peak loading forces for groups I, II and III were found to be 31 N, 24.6 N and 23.1 N respectively The mean maximal elongation values for groups I, II and II were found to be 25 mm, 19.6 mm and 17.3 mm. respectively.

Discussion

Tendon and soft tissue injuries constitute an important part in orthopedic surgeons' daily working activity. Achilles tendon ruptures are particularly encountered in middle-aged patients with degenerative changes and vascular insufficiency.

All healing mechanisms including that of tendons consist of a common pathway. Primarily, a fibrin clot is formed in the injury zone and; blood cells, fibronectin and platelets are captured and degraded in this clot. Healing process is thus, initiated with chemotactic factors released from degraded cells and local growth factors in the zone.[12, 16, 19] Thrombin formation, an effective pathway in clotting, is inhibited by Heparin and its derivatives, LMWH (Low Molecular Weight Heparin). Early phases of healing are thought to be inhibited by LMWH with its mechanism of action. In contrast, Heparin and its derivatives are reported to stimulate wound healing by decreasing cell degradation in tissues, increasing neoangiogenesis and, nourishing revascularization, granulation and epitheli-

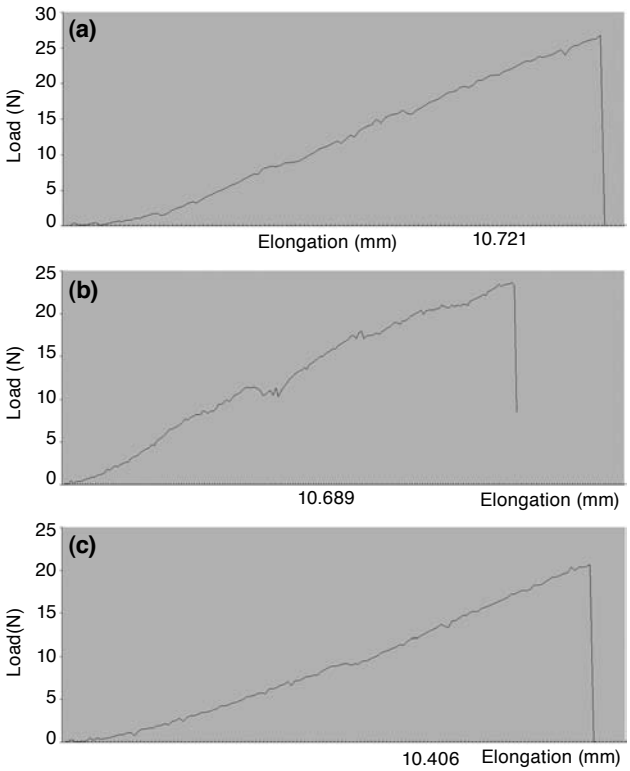

Figure 4.(a) Peak load and elongation curves: **(a)** Normal tendon (contralateral side), **(b)** group 1; **(c)** group III

zation.[21,22] Heparin and its derivatives also reduce cellular degradation by stimulating neoangiogenesis and decreasing the cellular deposits in the ischemic tissues and thus, favor wound healing.[16, 17, 19-21] It is also shown that Heparin accelerates wound healing by stimulating cellular regeneration and formation of new vascular tissue.[15, 19, 21]

The effect of subcutaneous Heparin administration on wound healing in rats is evaluated in variable studies. In an experimental study in which burn wounds are created on rats, Heparin administration is shown to decrease the healing time.[24] In a study by Kweon at al.[23], Heparin/Chitosan complex topically administered in the full-thickness wounds on rats' necks is reported to yield near-full wound healing after 15 days of treatment. Kutlay et al.[22] report that subcutaneous LMWH administration after abdominal surgery decreases intra-abdominal cohesions and accelerates healing. Yılmaz et al. [25] also report acquiring tendon compositions closer to normal and, histologically lesser scar formation and cohesions in their study where LMWH is administered locally on rat Achilles tendons after a crush injury.

During tendon healing, more fibrillar than hyaline collagen formation and new vascular tissue formation are considered to be the signs of effective healing.[1, 5, 16, 17, 23] In this study; healthy mitochondriae, an increase in rough endoplasmic reticulum (showing increased cellular synthesis), a decrease in cellular apoptosis and significant fibrillar collagen formation are noted histologically. These findings are consistent with the data in the literature.

In a study by Virchenko et al. [26], the effect of continuous and intermittent (twice a day) LMWH administration on tendon healing is evaluated. The tendon healing is noted to be biomechanically impaired in the continuous LMWH group while, no significant difference is evident between the other two groups (single dose before the operation vs. twice a day). In our study, the effect of single dose LMWH administration daily is evaluated. In today's clinical practice, LMWH is also administered as a single dose daily, unless there is a clinical suspicion or diagnosis of venous thromboembolism or pulmonary embolism. LMWH administration as a single, daily dose is shown to augment healing histologically, as well as biomechanically in both high-dose group (group I) and, low-dose group. In Virchenko et al.[26] study, the rats are sacrificed on the seventh day and early biomechanical results are evaluated; while in our study, the histological and biomechanical evaluations are performed on the fourth week.

Our findings show that daily, single dose LMWH administration has an increasing and accelerating effect on healing in the long term. During intermittent administration (LMWH is generally administered as a daily, single dose in clinical practice), tendon healing is augmented without inhibiton of thrombin formation. A decrease in thrombin and fibrin clot formation in the early phases of healing causes an increase in blood flow and a subsequent rise in the number of local growth factors while; LMWH accelerates the healing rate by nourishing neovascularization. In our study, it is shown that daily, single dose LMWH administered systematically increases collagen synthesis in fibroblasts, fibrillar collagen formation in extracellular matrix and new vascular tissue formation and thus, has a positive effect on tendon healing. As an addition to

this, no macroscopic findings of a haematoma or bleeding is encountered during LMWH administration. Whereas LMWH might be expected to impair the early phases of healing process and ideal healing in the long-term by decreasing clot formation; that it increases and positively effects tendon healing is noted in this study.

As planning, not being based on a context where samples collected in several days of the study (2, 7, 14 and, 28. days) are evaluated on a regular basis; as histologically, unavailability of the collagen staining and collagen subtyping and; as biomechanically, unavailability of tendon pre-stretching and a camera system to monitor the tendon stretch and rupture may be listed as the shortcomings of our study.

We conclude that, by having advantages over the standard Heparin such as causing lesser bleeding and haematoma formation and, not needing a laboratory follow-up; intermittent LMWH administered systematically has a positive effect on tendon healing by increasing fibroblast quantity and fibrillar collagen synthesis and, decreasing mitochondrial degeneration.

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