EXPERIMENTAL STUDY

The effects of zinc sulfate mineral supplementation on Achilles tendon healing in rats

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ABSTRACT

BACKGROUND: The Achilles tendon is the most commonly injured and ruptured tendon in the body and typically occurs during participation in sports or recreational activities in men between 30 and 50 years of age. Treatment options for Achilles tendon rupture include conservative and surgical approaches. Conservative treatment is associated with a higher risk of rerupture, while surgical treatment carries a risk of wound site complications. Generally, both methods result in a prolonged tendon healing time. Studies are ongoing to identify biomolecules that aid tendon repair. The main objective of our study is to investigate the effects of zinc sulfate (ZnSO4) mineral supplementation on Achilles tendon healing in rats.

METHODS: Forty-eight female Sprague-Dawley rats were divided into four equal groups (C-15, C-30, ZnSO4-15, and ZnSO4-30) after standard Achilles tendon repair surgery. The ZnSO4-15 and ZnSO4-30 groups received an oral zinc sulfate monohydrate solution (50 mg/kg/day) for 15 and 30 days, respectively. The C-15 and C-30 groups were given 1 mL of distilled water per day orally during the experimental periods. Rats were sacrificed on the 15th and 30th day depending on their groups, and the healing of the operated tendons was evaluated using Movin and Bonar histopathologic scoring. For biomechanical analyses, the operated and intact Achilles tendons of all groups were removed, and tensile tests were performed to determine the tensile strength and toughness values for each tendon.

RESULTS: Movin and Bonar scores were significantly lower in the ZnSO4-15 group than in the C-15 group and in the ZnSO4-30 group than in the C-30 group ($p<0.05$). Although we did not find the biomechanical results statistically significant, the intact tendons of the ZnSO4-15 group exhibited higher toughness than those of the C-15 group, and the tensile strength and toughness values of the operated and intact tendons of the ZnSO4-30 group were also higher than those of the C-30 group.

CONCLUSION: Zinc sulfate monohydrate mineral supplementation had histopathologically positive effects on the proliferation and remodeling stages of Achilles tendon healing and may biomechanically benefit both operated and intact tendons.

Keywords: Achilles tendon healing; biomechanical examination; Experimental study; histopathological examination zinc sulfate; mineral supplementation.

INTRODUCTION

The Achilles tendon (AT) is the most commonly ruptured tendon in the human body. AT injuries are typically more common in men between the ages of 30 and 50 years, and participation in sports or recreational activities is the most common cause of injury.[1,2] It is clear that extrinsic variables such as altered training habits, improper technique, previous injury, poor footwear choice, and training on rough, slippery, or inclined

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surfaces dominate in acute injuries, whereas intrinsic variables such as gastrocnemius-soleus dysfunction, tendon vascularity, age, gender, body weight, height, pes cavus, forefoot varus, and lateral ankle instability often play a role in chronic conditions.[3] Corticosteroids and fluoroquinolones have also been shown to be risk factors for tendinopathy.[4,5] Treatment for Achilles tendon rupture has conservative and surgical options. While conservative treatment has been associated with a higher risk of tendon re-rupture, surgical treatment carries the risk of wound complications, and overall, both methods require a long healing time.[6] Efforts are now underway to identify biomolecules that aid in tendon repair. Less than 50 mg/kg of zinc, an important micronutrient, is present in the human body. It is of vital importance to human health and disease, as it plays an essential role in growth and development, bone metabolism, the central nervous system, the immune system, and wound healing. Numerous physiological functions, such as regulation of transcription, DNA repair, apoptosis, metabolic processing, modulation of the extracellular matrix (ECM), and antioxidant defense, depend on zinc-dependent proteins.[7] Our hypothesis was to investigate the benefits of zinc sulfate supplementation for Achilles tendon healing based on the aforementioned data.

MATERIALS AND METHODS

Approval was obtained from the regional ethics committee for animal research on September 23, 2021 (No. 2021/16). We conducted the study in accordance with the unified ethical principles and the Declaration of Helsinki on animal research as the foundations of international cooperation. Forty-eight adult female Sprague-Dawley rats aged 12 months with an average weight of 400-450 g were used. The animals were kept in a room at 22°C with a 12-hour light/dark cycle. They had unrestricted access to water and were fed standard rat pellets. Four groups of rats were formed by blind randomization, with groups 1 and 2 serving as controls and groups 3 and 4 receiving zinc sulfate (ZnSO4) (n=12 for each group). Gentamicin (8 mg/kg) was administered before the surgery as a prophylactic antibiotic, and ketamine-xylazine anesthesia was used to induce surgery. Under aseptic conditions, a conventional posterior longitudinal incision of 2 to 3 cm was performed. Using a No. 11 scalpel (Interlab, Istanbul, Türkiye), a full transverse incision was made at a distance of 5 mm on the proximal side of the tendon insertion on the calcaneus. The modified Kessler technique PDO II 4/0 (Meril, Istanbul, Türkiye) was used to atraumatically suture the end edges of the tendon. Under sterile conditions, the incision site was sutured with four polypropylene sutures 3/0 (Meril, Istanbul, Türkiye) at regular intervals, and a povidone-iodine dressing (Batticon, Adeka, Samsun, Türkiye) was applied. No immobilization techniques were used in the postoperative period.[8] An appropriate dose of paracetamol was mixed into their drinking water for 48 hours to reduce their postoperative pain. Groups 1 (C-15) and 2 (C-30) were control groups, and Groups 3 (ZnSO4-15) and 4 (ZnSO4-30) were experimental groups. During their respective periods, Groups 1 and 2 received only 1 milliliter (mL) of distilled water daily, while Groups 3 and 4 received 1 mL of a 50 mg/kg ZnSO4 monohydrate solution. Groups 1 and 3 were sacrificed on day 15, and Groups 2 and 4 on day 30. In all rats in the control and experimental groups, the right or operated AT was removed for histopathological examinations. The femoral condyle and part of the calcaneus were removed together with the operated ATs. The plantaris tendon was left in place so as not to interfere with the measurements. For histopathological examination, the samples were preserved in 5% formic acid and fixed in a 10% neutral formaldehyde solution, then sectioned and embedded in paraffin blocks. Sections were stained with hematoxylin and eosin (H&E), Masson's trichrome, and Alcian blue (pH: 2.5) (Figure 1a, b, c, d). A bone and soft tissue pathologist assessed the specimens histopathologically in a blinded fashion using an Olympus BX51 light microscope (Olympus, Tokyo, Japan). The semi-quantitative scores of Movin and Bonar were used to evaluate the results. The examination of tenocytes, ground substance, collagen, and vascularity is part of Bonar's scale.[9] Each variable was given a score from 0 to 3 on a scale of 0 for normal, 1 for slightly abnormal, 2 for abnormal, and 3 for markedly abnormal. The total score ranged from 0 to 12 (normal tendon to most abnormal tendon).[10] Analysis of fiber structure, fiber arrangement, rounded nuclei, regional differences in cellularity, increased vascularity, decreased collagen stainability, hyalinization, and glycosaminoglycan (GAG) content are all part of Movin's semiquantitative scale. Each variable was given a score from 0 to 3 on a scale of 0 for normal, 1 for slightly abnormal, 2 for abnormal, and 3 for clearly abnormal. The total semiquantitative score ranged from 0 to 24 (normal tendon to most abnormal tendon).[11] The same histologist assessed the histopathology of all samples twice at one-month intervals to determine the reliability of the observers. Excellent intra-observer reliability was found (Intraclass Correlation Coefficient, ICC=0.986-0.996). In addition to the operated tendons, the left or non-operated (intact) ATs were also removed from all rats in the control and experimental groups for biomechanical analysis. The tendon samples were frozen at -20 °C until the day of examination and then thawed to room temperature.[8] The cross-sectional dimensions and cross-sectional area of each tendon were measured prior to tensile testing to quantify the tensile strength of each tendon. Tensile tests were carried out on a universal electromechanical tester with class 1 calibration (Alşa, Istanbul, Türkiye). The tensile strength tests were performed at a strain rate of 5 mm/min to better understand the mechanical behavior of the tendons. The tendons were gripped from the sides of their origo and inserted with grippers specially made for this purpose (Fig. 2). The maximum force that a comparable tendon could withstand was divided by the cross-sectional area of that tendon to determine its tensile strength. The toughness values were derived by integrating the stress-strain curve and determining the area under the curve.

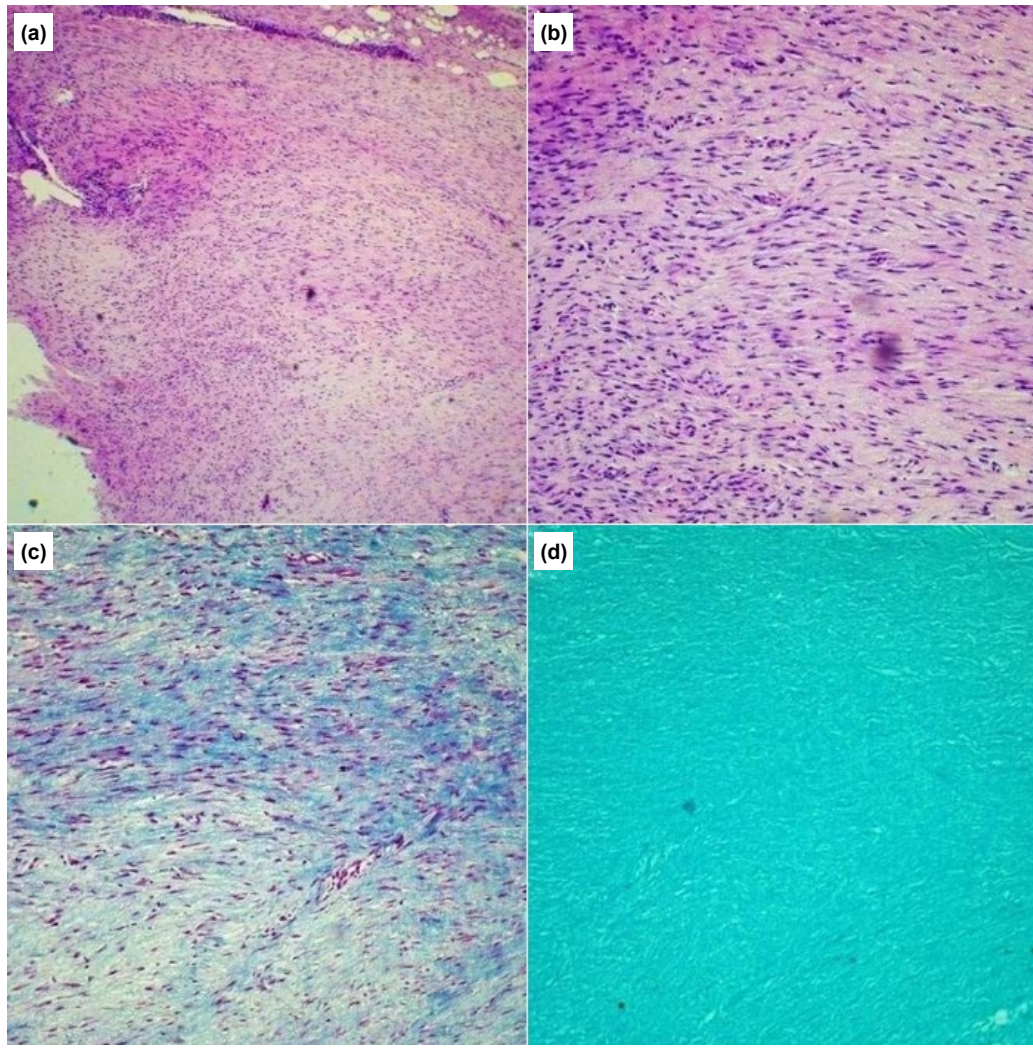

Figure 1. Histologic microscopic images: ZnSO4-30th day. Bonar Score 5. **(a)** Hematoxylin and Eosin (H&E) stain, magnification x40. **(b)** Nuclei in tenocytes become more oval to round in shape without prominent cytoplasm, H&E stain, magnification x100. **(c)** Features include 1-2 capillary clusters per 10 high power fields, separation of fibers with loss of bundle boundaries, and marked loss of normal polarization pattern, Masson's Trichrome Stain, magnification x100. **(d)** Stainable mucin between fibers with bundles remaining discrete, Alcian Blue Stain, magnification x40.

Table 1. Intragroup and intergroup comparisons of histopathologic findings

	Groups	Control	ZnSO4	p value
Total Bonar Score	Day 15	10.67±1.5	7.67±1.4	0.008
		11.0 [9-12]	8.0 [5.0-9.0]	
	Day 30	7.17±0.4	5.5±0.8	0.016
		7.0 [7.0-8.0]	5.0 [5.0-7.0]	
	p value	0.002	0.029	
Total Movin Score	Day 15	20.33±2.3	14.5±1.8	0.001
		21.0 [17.0-23.0]	14.0 [12.0-17.0]	
	Day 30	14.5±1.1	10.0±1.5	0.002
		14.5 [13.0-16.0]	9.0 [9.0-12.0]	
	p value	0.001	0.008	

Statistical Analysis

GraphPad Instat (Boston, USA) was used to perform the sta-

Figure 2. Achilles tendon placed in the testing machine for biomechanical analysis.

tistical analyses. The Kolmogorov-Smirnov test was used to examine the normality of quantitative variables. The Paired/Unpaired t-test was used to compare quantitative variables with normally distributed distributions that were expressed as the mean standard deviation (SD). The Mann-Whitney test and the Wilcoxon Paired Samples test were used to examine quantitative variables with an abnormal distribution. Median values were provided (interquartile range: IQR). Variables from the statistical analysis were considered significant if they had a p-value of less than 0.05.

RESULTS

The Movin and Bonar scores of the ZnSO4-30 and C-30 groups were significantly lower than those of the ZnSO4-15 and C-15 groups, respectively ($p<0.05$). The Movin and

Table 2. Intragroup comparison of biomechanical findings

	Tensile Strength (mPa)			Toughness (J^*m^{-3})		
Groups	**Right**	**Left (Intact)**	**p value**	**Right**	**Left (Intact)**	**p value**
C-15	5.2±3.8	23.8±6.9	0.002	1.56±1.59	3.21±1.59	0.145
	3.4 [1.9-11.5]	23.5 [16.5-34.3]		0.6 [0.18-3.9]	3.31 [1.64-5.65]	
C-30	7.4±3.5	35.3±17.9	0.016	1.89±1.69	6.44±4.8	0.116
	7.3 [3.7-13.1]	31.2 [18.2-60.5]		1.25 [0.58-4.76]	4.43 [1.15-11.98]	
p value	0.375	0.413		0.762	0.227	
ZnSO4-15	5.3±2.3	18.7±10.6	0.008	0.84±0.43	8.54±6.0	0.016
	5.9 [1.3-6.9]	18.6 [8.7-33.9]		0.96 [0.11-1.26]	8.23 [2.18-15.5]	
ZnSO4-30	13.6±9.9	34.0±15.1	0.045	4.70±3.0	12.75±5.6	0.034
	10.0 [5.5-29.4]	35.2 [17.9-52.5]		4.43 [1.85-8.11]	11.0 [6.34-19.4]	
p value	0.222	0.106		0.016	0.413	

Table 3. Comparison of biomechanical findings between groups

	Tensile Strength (mPa)		Toughness (J^*m^{-3})	
Groups	**Right**	**Left (Intact)**	**Right**	**Left (Intact)**
C-15	5.2±3.8	23.8±6.9	1.56±1.59	3.21±1.59
	3.4 [1.9-11.5]	23.5 [16.5-34.3]	0.6 [0.18-3.9]	3.31 [1.64-5.65]
ZnSO4-15	5.3±2.3	18.7±10.6	0.84±0.43	8.54±6.0
	5.9 [1.3-6.9]	18.6 [8.7-33.9]	0.96 [0.11-1.26]	8.23 [2.18-15.5]
p value	0.841	0.401	0.384	0.191
C-30	7.4±3.5	35.3±17.9	1.89±1.69	6.44±4.8
	7.3 [3.7-13.1]	31.2 [18.2-60.5]	1.25 [0.58-4.76]	4.43 [1.15-11.98]
ZnSO4-30	13.6±9.9	34.0±15.1	4.70±3.0	12.75±5.6
	10.0 [5.5-29.4]	35.2 [17.9-52.5]	4.43 [1.85-8.11]	11.0 [6.34-19.4]
p value	0.261	0.916	0.111	0.098

Bonar scores of the ZnSO4-15 group were significantly lower than those of the C-15 group ($p<0.05$). The Movin and Bonar scores of the ZnSO4-30 group were significantly lower than those of the C-30 group ($p<0.05$) (Table 1).

The operated side in the ZnSO4-15 and ZnSO4-30 groups had significantly lower tensile strength and toughness than the intact side ($p<0.05$). The operated side in groups C-15 and C-30 had significantly lower tensile strength than the intact side ($p<0.05$), but there was no difference in toughness ($p>0.05$). There was no significant difference between the ZnSO4-15 and ZnSO4-30 groups in terms of tensile strength of the operated side ($p>0.05$), but in terms of toughness, the ZnSO4-30 group was significantly higher than the ZnSO4-15 group ($p<0.05$). In terms of tensile strength and toughness for the intact side, there was no significant difference between the ZnSO4-15 and ZnSO4-30 groups ($p>0.05$). In terms of tensile strength and toughness, the operated and intact sides did not differ significantly between the C-15 and C-30 groups ($p>0.05$) (Table 2). For tensile strength and toughness, there was no significant difference between the C-15 and ZnSO4-15 groups for the operated and intact sides ($p>0.05$). There was no significant difference between the C-30 and ZnSO4-30 groups for the operated and intact sides in terms of tensile strength and toughness ($p>0.05$) (Table 3). Summarized results are presented in Table 4.

Table 4. Summary of all findings

HHS	
ZnSO4-30 R > ZnSO4-15 R	(according to HHS)
ZnSO4-15 R > C15 R	(according to HHS)
ZnSO4-30 R > C-30 R	(according to HHS)
C-30 R > C-15 R	(according to HHS)
BS	
ZnSO4-15 L > R	(tensile strength and toughness)
ZnSO4-30 L > R	(tensile strength and toughness)
ZnSO4-15 R ~ ZnSO4-30 R	(tensile strength)
ZnSO4-15 R < ZnSO4-30 R	(toughness)
C-15 L > R	(tensile strength)
C-15 R ~ L	(toughness)
C-30 L > R	(tensile strength)
C-30 R ~ L	(toughness)
C-15 R ~ C-30 R	(tensile strength and toughness)
C-15 L ~ C-30 L	(tensile strength and toughness)
ZnSO4-15 R ~ C-15 R	(tensile strength and toughness)
ZnSO4-15 L ~ C-15 L	(tensile strength and toughness)
ZnSO4-15 L ~ ZnSO4-30 L	(tensile strength and toughness)
ZnSO4-30 L ~ C-30L	(tensile strength and toughness)
ZnSO4-30 R ~ C-30R	(tensile strength and toughness)

• HHS: Histological healing scores and BS: Biomechanical scores, L for left and R for right, ~ means: not statistically different, < means: lower, > means: higher.

DISCUSSION

The use of nutritional supplements appears to be widespread in the healing of AT.[12] The majority of current zinc treatments are aimed at the supportive management of patients with serious conditions such as burns, surgical and traumatic wounds, diabetic ulcers of the lower limbs, and skin wounds.[7,13,14] This is the first study we know of that shows the effect of zinc sulfate mineral supplementation on AT healing. We concluded that zinc sulfate mineral supplementation has positive effects on the proliferation and remodeling phases in histopathology and may be biomechanically advantageous for both surgically repaired and intact ATs. We used the scales developed by Movin and Bonar to analyze the histological results. In both scoring systems, lower scores represent better tendon healing. The ZnSO4 groups had significantly lower Movin and Bonar scores than the control groups. As an expected improvement in tendon healing, Movin and Bonar scores were lower on the 30th day than on the 15th day in both the control and experimental groups.

Guerquin et al.[15] investigated the role of the zinc finger transcription factor early growth response protein-1 (EGR-1) in tendon development, healing, and repair using rodent models and mesenchymal stem cells (MSCs). The research results showed that EGR-1 is crucial for the development, healing, and repair of tendons. On the other hand, collagen and other structural molecules in the ECM are degraded by zinc-dependent endopeptidases called matrix metalloproteinases (MMPs), which are involved in the degradation and modification of the ECM.[16] This may be the main reason for the positive effects in the remodeling phase of tendon healing in our study. In addition, researchers have shown that zinc oxide nanoparticle-loaded chitosan scaffolds increase lubricity and histologic expression while reducing adhesion formation in rabbit deep digital flexor tendons.[17] All these studies suggest a function of zinc in tendon healing or remodeling.

Another important issue is the relationship between inflammation, immunological response, wound healing, and zinc. Zinc plays a key role in controlling the wound healing process, including membrane repair, oxidative stress, coagulation, inflammation, immunological defense, tissue re-epithelialization, angiogenesis, and fibrosis or scarring. Zinc promotes wound healing, and a delay in wound healing has also been associated with zinc deficiency.[7,18] The inflammation or oxidative stress that develops in the injured tendon as a result of acute trauma has been shown to worsen within 24 hours and manifest as peritendinous adhesions 48 hours later. Therefore, precautions should be taken before damage occurs.[19] Superoxide radicals generated by the mitochondria, which include reactive oxygen species (ROS) and reactive nitrogen free radical species (RNOS), are different types of oxidative stress.[20,21] Biomolecules such as DNA, proteins, and lipids can be oxidatively damaged by superoxide radicals, which impairs their function. At this point, we must mention the antioxidant effects. Firstly, zinc is a redox-inert molecule;

it inhibits the uptake and activity of redox-active transition metals such as copper and iron.[22,23] Metallothioneins (MTs) provide protection against oxidative stress and heavy metal toxicity. Zinc, copper, and selenium in diets impact the ability of MTs to synthesize, and zinc supplementation increases MT expression.[24,25] Numerous studies have shown that oxidative stress is increased by zinc deficiency,[26,27,28] and antioxidant therapy may be helpful to control oxidative stress and promote tendon repair.[29]

Toughness is the ability of a material to absorb energy until failure and is characterized by the area under the stress-strain curve, while tensile strength is the highest stress a material can withstand before breaking. These properties define the performance of tendons under mechanical conditions and can be used to determine the effectiveness of the treatments applied. The operated side was weaker than the intact side in terms of tensile strength and toughness in all ZnSO4 groups. The operated side was also weaker in terms of tensile strength than the intact side in all control groups. Even though the tensile strength of the repaired injured tendon increases over time, it does not reach the level of the uninjured tendon.[30] Note that the intact ATs in the ZnSO4-15 group had higher toughness values than those in the C-15 group. In addition, the tensile strength and toughness values of the operated and intact tendons of the ZnSO4-30 group were all higher than those of the C-30 group (except for the tensile strength of the intact side). Although the biomechanical results were not statistically significant, supplementation with zinc sulfate may be biomechanically beneficial for both operated and intact ATs.

Our research has some limitations. First, we induced an iatrogenic acute AT rupture through surgery. However, the majority of AT ruptures in humans have a degenerative basis. Second, due to the insufficient number of animals available, we did not study the histopathology of the earliest stages of tendon healing.

CONCLUSION

Zinc sulfate mineral supplementation has histopathologically positive effects on the proliferation and remodeling phases of rat Achilles tendon healing, and both operated and intact tendons may benefit biomechanically. For these reasons, zinc sulfate mineral supplementation may be used to improve and accelerate the quality of healing following tendon injuries.

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DENEYSEL ÇALIŞMA - *ÖZ*

Sıçanlarda çinko-sülfat mineral takviyesinin aşil tendon iyileşmesi üzerindeki etkileri

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AMAÇ: Aşil tendonu vücutta en sık yaralanan ve rüptüre olan tendon olup tipik olarak 30 ila 50 yaş aralığındaki erkeklerde bir spor veya eğlence aktivitesine katılımla ortaya çıkar. Aşil tendonu rüptürü tedavisinin konservatif ve cerrahi seçenekleri vardır. Konservatif tedavi daha yüksek yeniden kopma riski ile ilişkilendirilirken, cerrahi tedavi yara yeri komplikasyonları riski taşır, genel olarak her iki yöntem de uzun bir tendon iyileşme süresine sahiptir. Tendon onarımına yardımcı olan biyomolekülleri tanımlamak için çalışmalar devam etmekte olup çinko-sülfat ($ZnSO_4$) mineral takviyesinin sıçanlarda Aşil tendonu iyileşmesi üzerindeki etkilerinin araştırılması çalışmamızın ana hedefidir.

GEREÇ VE YÖNTEM: 48 adet Sprague-Dawley cinsi dişi sıçan geçirdikleri standart aşil tendon onarımı cerrahisi sonrası dört eşit gruba (C-15, C-30, $ZnSO_4$-15 ve $ZnSO_4$-30) ayrıldı. $ZnSO_4$-15 ve $ZnSO_4$-30 gruplarına sırasıyla 15 ve 30 gün boyunca oral çinko-sülfat-monohidrat (50 mg/kg/gün) çözeltisi verildi. C-15 ve C-30 gruplarına ise deney sürelerince oral 1 ml distile su/gün verildi. Sıçanlar gruplarına bağlı olarak 15 ve 30. günlerde sakrifiye edildi, takiben opere tendonların iyileşmeleri Movin ve Bonar histopatolojik skorlaması kullanılarak değerlendirildi. Biyomekanik analizler içinse tüm grupların ameliyat edilmiş ve sağlam aşil tendonları çıkarılarak gerilme testleri yapıldı ve her bir tendon için gerilme mukavemeti ve tokluk değerleri hesaplandı.

BULGULAR: Movin ve Bonar skorları $ZnSO_4$-15 grubunda C-15 grubuna göre ve $ZnSO_4$-30 grubunda C-30 grubuna göre anlamlı olarak düşüktü ($p<0.05$). Biyomekanik sonuçları istatistiksel olarak anlamlı bulmamamıza rağmen, $ZnSO_4$-15 grubunun sağlam tendonları C-15 grubundan daha yüksek tokluğa sahipti ve $ZnSO_4$-30 grubunun ameliyat edilmiş ve sağlam tendonlarının gerilme mukavemeti ve tokluk değerleri C-30 grubundan daha yüksekti.

SONUÇ: Çinko-sülfat-monohidrat mineral takviyesinin, Aşil tendonu iyileşmesinin proliferasyon ve remodelizasyon aşamaları üzerinde histopatolojik olarak olumlu etkileri olduğu, hem ameliyat edilmiş hem de sağlam aşil tendonlarına biyomekanik olarak da fayda sağlayabileceği öngörüldü.

Anahtar sözcükler: Aşil tendon iyileşmesi; biyomekanik inceleme; deneysel çalışma; çinko-sülfat; histopatolojik inceleme; mineral takviyesi.

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