The effect of dehydration and irrigation on the healing of Achilles tendon: an experimental study

Dehidrasyon (kuruluk) ve irigasyonun (yıkama) Aşil tendon iyileşmesi üzerine etkileri: Deneysel çalışma

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BACKGROUND

Air exposure is a factor that inhibits *in vitro* cellular proliferation and matrix synthesis in tendons. Aim of this experimental study was to evaluate effect of dehydration and irrigation on healing of Achilles tendon.

METHODS

Achilles tenotomy was done in forty-five Sprague-Dawley rats. In control group, tendon was sutured immediately. In the remaining two groups, the Achilles tendons were allowed to direct exposure of air. Irrigation of Achilles tendon was performed in one of exposed groups, while irrigation was not done in other group. After 60 minutes, tendons of both groups were sutured same as control group. Rats were sacrificed at postoperative day 28. Achilles tendons were dissected and histological and biomechanical evaluations were performed.

RESULTS

Histological evaluation revealed intense fibrosis formation with adhesion of tendon to surrounding tissues in the air exposed groups. The quantity of angiogenesis and inflammatory reaction were also higher in experimental groups regardless of irrigation. Air exposed tendons had higher tensile strength however lower stiffness than control group in biomechanical evaluation.

CONCLUSION

Air exposure decreases quality of healing by increasing fibrosis and adherence formation. These negative effects of exposure to air were not counteracted by irrigation. However, air exposure didn't affect tensile strength of the healing.

Key Words: Achilles tendon; air exposure; dehydration; tendon healing; irrigation; rats, Sprague-Dawley.

AMAÇ

Hava teması tendonlarda canlı-dışı *(in vitro)* ortamda matriks sentezini ve hücre ilerlemesini azaltabilir ve hatta önleyebilir. Bu çalışmanın amacı, dehidrasyon ve irigasyonun Aşil tendon iyileşmesi üzerine etkisinin canlı-içi *(in vivo)* hayvan modeli üzerinde gösterilmesidir.

GEREÇ VE YÖNTEM

Kırk beş adet Sprague-Dawley cinsi sıçanın Aşil tenotomisi yapıldı. Kontrol grubunda, tendon hemen dikildi. Diğer iki grubun cilt ve cilt altı dokuları ekarte edilerek Aşil tendonlarının doğrudan hava ile teması sağlandı. Bu gruplardan birine irigasyon uygulanırken diğerine uygulanmadı. 60 dakikalık hava temasını takiben, her iki grubunda tendonları kontrol grubuna da yapıldığı gibi dikilerek yaraları kapatıldı. Operasyon sonrası 28. günde sıçanlar öldürüldü. Aşil tendonları parçalara ayrılarak histolojik ve biyomekanik incelemelere alındı.

BULGULAR

Hava teması sağlanan grupların histolojik incelemesinde, tendonun çevre dokulara yapışmasıyla birlikte yoğun fibröz doku oluşumu olduğu görüldü. İrige edilmeyen ve edilen her iki deney grubunda da yüksek oranda damarsallaşma ve enflamatuvar reaksiyon saptandı. Biyomekanik çalışma da, hava temasına uğrayan tendonlarda kontrol grubuna göre daha yüksek gerilme gücü buna karşın daha düşük bükülebilme olduğu görüldü.

SONUÇ

Hava teması fibroz doku ve yapışıklık oluşumuna sebebiyet vererek iyileşme kalitesini düşürmektedir. İrigasyon, bu istenmeyen etkileri ortadan kaldırmamaktadır. Buna rağmen gerilme kuvveti hava temasından etkilenmemektedir.

Anahtar Sözcükler: Aşil tendonu; dehidrasyon; hava teması; irigasyon; sıçan, Sprague-Dawley cinsi; tendon iyileşmesi.

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In recent decades, spontaneous ruptures of the Achilles tendon have become more prevalent in professional and recreational athletes as a result of overuse and acute injuries.[1] The controversy regarding the pathophysiology and treatment of this injury continues. Although there are advocators of non-operative approach,[2,3] the choice of treatment for patients less than fifty years is primarily operative.[4,5]

The repair phases of the Achilles tendon during the healing period were investigated extensively in experimental studies.[6-8] Tendon inflammation subsides before the 7th postoperative day[9,10] and the phase of fibroplasia begins between the second and third weeks of the healing that precedes the longitudinal alignment of the collagen formation.[8] Both intrinsic and extrinsic factors can be influential on the healing process of the tendon. Variations in the distribution and duration of forces within tendons, trauma and ischemia have been shown to be associated with variations in the histology and the tensile strength of tendon healing. Drying was noted as one of the factors detrimental to some features of the tendon. Freeze dried allografts lost more than half of their initial tensile strength relative to the control value.[10] The initial ultimate tensile stress and longitudinal strain were found to be greater for frozen allografts than for freeze-dried tendons.[11]

Connective tissues tend to get dry as they are exposed to air. Dehydration of the Achilles tendon due to air exposure is frequently encountered during the open surgical repair. The sensitivity of tendon tissue to dehydration should be considered during tendon surgery. Irrigation is one of the measures commonly utilized to prevent dehydration of the tendon assuming to counteract the deleterious effects of drying. Tendon exposure to air could have cellular and biomechanical effects on tendon healing which may have clinical implications. Thus, it is essential to be cognizant about the consequences of tendon drying on tendon healing.

In this study, the effects of dehydration and irrigation on the healing of the Achilles tendon were examined histologically and biomechanically in an *in vivo* animal model.

MATERIALS AND METHODS

The experimental protocol was approved by the authors' Institutional Ethics Committee. All procedures were performed under sterile conditions in an animal operating facility at a room temperature of 21 °C and a relative air humidity of approximately 60-65%. Forty-five female Sprague-Dawley rats, aged seven months, and weighing an average of 280 grams, were used in the experiment. The animals were anesthetized by a mixture of ketamine (35 mg/kg) and xylazine hydrochloride (5 mg/kg) administered intraperitoneally. The animals were given an intraperitoneal injection of cefazolin 30 mg/kg. The skin over and around the right Achilles tendon was shaved and thoroughly scrubbed. The tendon was approached by a medial skin incision and freed from the surrounding tissue. After resecting the tendon of the plantaris muscle, the Achilles tendon was transected 5 mm above its insertion into the calcaneum. The animals were then randomly divided into 3 groups. In the control group (group 1), the severed ends of the tendon were approximated by utilizing no. 3/0 Ethibond suture with Kessler technique and the skin was closed. In the other 2 groups, the skin over the Achilles tendon was retracted and pinned to a plate of cork, allowing the tendon direct exposure to room air. In one of the exposed groups, the Achilles tendon was irrigated with 3 drops of physiological saline solution every five minutes (group 2), while irrigation was not done in the other group (group 3). Rats were kept warm during the exposure period. After sixty minutes of exposure, wounds were irrigated diffusely and tendons were sutured, using the same method as the control, in both groups. To control the movement in the talocrural joint, a suture was inserted through the tibiofibular fork and placed between the calcaneum and the plantar aponeurosis in all groups.[7] The suture was tightened to fix the talocrural joint in the equinus position. Before closure of the wound, a bacteriological culture was obtained by rolling a swab over the tendon and the surrounding soft tissues. Animals received intraperitoneal cefazolin 30 mg/kg twice daily for a week. They were housed in plastic cages where they could move about freely, and received food and water ad libitum. Daily inspections of the animals and the wound area were performed in order to check for signs of any infection.

The rats were sacrificed at postoperative day 28. Under sterile conditions, the wound was opened and the bacteriologic culture was obtained. The immobilization suture was removed and the Achilles ten-

don was exposed. After careful dissection from the surrounding tissues, the tendon was excised from the musculotendinous junction proximally and through the calcaneus distally. In each group, 8 of the specimens were prepared for biomechanical investigation and 7 for histological evaluation. The specimens for mechanical testing were stored in plastic tubes and kept at –22 °C until tested.

Histological studies

The entire Achilles tendon was fixed by immersion for at least 2 days in 4% formaldehyde solution. Subsequently, specimens were dehydrated and embedded in paraffin wax in order to prepare 4 sections at a thickness of 5 µm from the region of the tenotomy site and the surrounding area. Sections were stained with *Ehrlich's* haematoxylin/eosin and were evaluated under a light microscope by the following criteria:[7,12]

- intensity of fibrosis
- density of blood capillaries in tendon tissue
- distribution of inflammatory cells (i.e. granulocytes, macrophages and lymphocytes)
- cartilaginous metaplasia

Inflammation, fibrosis and vascularization is graded as 0 (no), 1 (minimal), 2 (moderate) or 3 (severe). Cartilaginous metaplasia is graded according to their presence: 0 (no) or 1 (present). Four of the sections were analyzed per sample and the average data was reported.

Biomechanical investigations

On the day of mechanical testing, the specimens were thawed in physiological saline at room temperature for two hours. During the mechanical testing, specimens were kept moistened with normal saline and the room temperature was controlled at 25 °C. Biomechanical measurements were carried out using a materials testing machine (model 1321B, Instron, Canton, MA, USA). The musculotendinous end was fixed between two paper strips to the upper and the calcaneum was mounted to the lower jaws of the machine. Tensile load was then applied at a displacement rate of 20 mm/min. Load characteristics were directly plotted on an X-Y chart recorder and a force displacement graph was obtained. All strength characteristics of the tendons were calculated from that part of the load-displacement curve. All specimens displayed a typical load-displacement curve, with an upward linear slop and a failure response at the point of failure. *Load to failure* is defined as the ability of the tendon to resist a tensile load before rupture. *Tendon stiffness* is calculated by dividing the load to failure by displacement to failure [N/mm].[7]

The received data was analyzed by a computer statistics program (SPSS 11.5 for Windows®). The presence of a significant difference between multiple groups was tested by Kruskal Wallis Analysis of Variances. If a significant difference was found, groups were compared by Tukey-Kramer multiple comparison test. In all the tests, a value of $p<0.05$ was considered significant.

RESULTS

There was no sign of infection (abscess formation, erythema, swelling or warmth around the wound) during the follow-up period in any of the animals. Bacteriological cultures obtained at the end of the operation and after sacrifice of the animals were negative for inoculations.

Gross examination of the tendons at the time of harvest revealed a dense peritendinous scar and adherence to the overlying skin in the groups exposed to air. A well-defined demarcation area was apparent in the control group. Dehiscence of the suture with gap formation between the tendon stumps was not detected in any of the groups.

Histological results

In the control group, the tendon tissue was clearly visible with its wavy pattern having collagen bundles. Although there were some scattered areas of fibrosis along the tendon, the boundary between the muscles and the tendon was clearly visible (Fig. 1). Blood capillaries and inflammatory cells were rarely observed among the tendon tissue. Cartilaginous metaplasia was not present in any of the specimens in the control group (Table 1). The mean grade of fibrosis, vascularization and inflammation was 1.25, 1.0, and 1.50 respectively (Table 2).

There was an intense fibrosis formation in the irrigated group (group 2). The collagen bundles of the tendon tissue had lost the closely packed architecture characteristic of the control group. It was difficult to visualize the main tendon structures. Fibrosis was enclosing the tendon tissue and invading the tendon substance diffusely. Massive infiltra-

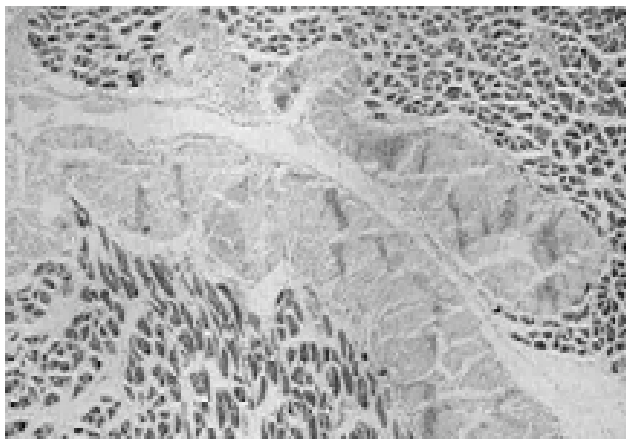

Fig. 1. Normal appearance of the tendon structure in the center of the muscle fibres in the control group (H-E x 100).

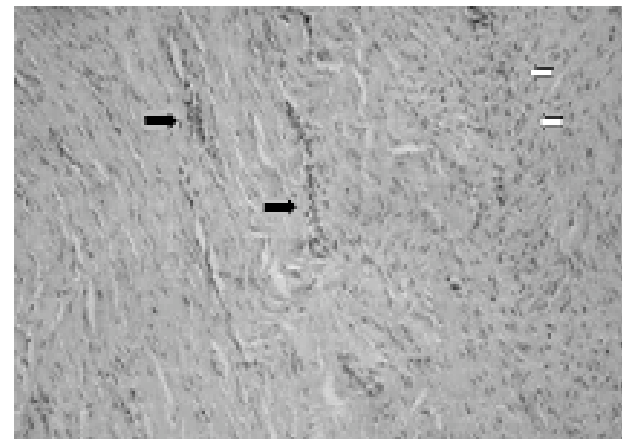

Fig. 2. Intense amount of fibrosis,vascularity and inflammatory cells in the irrigated group. Black arrows showing the angiogenesis and white arrows indexing the boundary between the intense fibrosis and the tendon tissue (H-E x 100).

tion of inflammatory cells with predominantly polymorphonuclear neutrophil granulocytes was clearly visible within the fibrosis and the tendon tissue (Fig. 2). Many blood capillaries were observed that were indicative of intense angiogenesis. Although many of them were in the tendon tissue, they were also occasionally observed within the fibrosis. Fibrocartilage formation was present in almost every specimen. The mean grade of fibrosis, vascularization and inflammation was 3.0, 2.4 and 2.4 respectively.

Fibrosis formation was also the most prominent feature in the non-irrigated group (group 3). However, the fibrosis was dispersed more uniformly and the intensity was more moderate with regard to the irrigated group (Fig. 3). The blood capillaries were also disseminated among the fibrosis and the tendon tissue; however they were larger with comparison to the irrigated group. Cartilaginous metaplasia was present in almost all of the specimens. The mean grade of fibrosis, vascularization and inflammation was 2.6, 2.0 and 2.2 respectively in the non-irrigated group.

There was no statistically significant difference between the air exposed groups with regard to the degree of fibrosis, inflammation and vascularization. However, a significant difference was present between the experimental groups and the control group in comparison of histological findings (fibrosis, inflammation and vascularization) ($p<0.05$).

Biomechanical results

The average load to failure was 11.8 N (±1.8 N) for the control group. The repair strength was 14.2 N (±1.5 N) for the irrigated group and 13.8 N (±2.5 N) for the non-irrigated group. No statistically significant difference was detected with comparison of the non-irrigated group to the irrigated group. However, there was a statistically significant differ-

Table 1. Description of the histological findings in the groups

	Control	Group 2	Group 3
Fibrosis	Scattered; clear boundary between tendon and muscles	Intense; invading	Moderate; intensity
Blood capillaries	Rarely observed	Within both fibrosis and tendon tissue	Within both fibrosis and tendon tissue
Inflammatory cells	Rarely observed	Massive infiltration	Infiltrative
Cartilaginous metaplasia	None	Approximately in all specimens	Approximately in all specimens

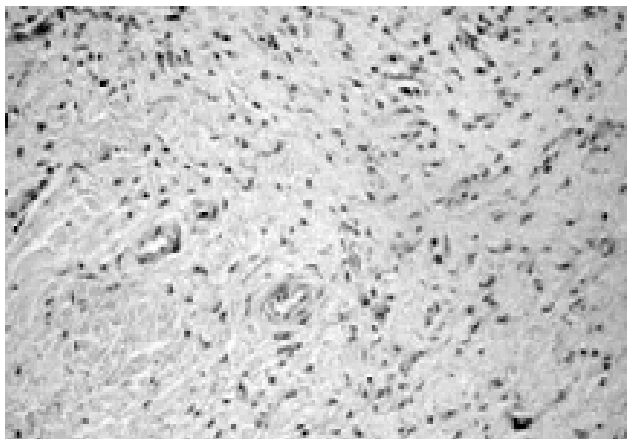

Fig. 3. Fibrosis intervening with the collagen fibers of the tendon in the non-irrigated group (H-E x 400).

ence between the control group and the air exposed groups ($p<0.05$).

The mean amount of displacement to load was 1.4 mm (±0.2 mm), 2.1 mm (±0.3 mm) and 2.2 mm (±0.2 mm) in group 1, group 2 and group 3, respectively (Fig. 4). There was no statistically significant difference between group 2 and group 3 however both are statistically different from group 1 ($p<0.05$).

The mean stiffness was not statistically different between group 2 (6.2±2.2 N/mm) and group 3 (6.5±0.5 N/mm) (Fig. 5) and both was lower than the control group (8.5±2.0 N/mm) ($p<0.05$).

DISCUSSION

We investigated the effects of air exposure and irrigation on the healing of Achilles tendon in an *in vivo* experimental study. Air exposure of the Achilles tendon caused an intense fibrosis formation and inflammatory reaction. Irrigation didn't

Table 2. Distribution of the histological grading among the groups

	Control	Group 2	Group 3
Fibrosis	1.25	3	2.6
Vascularization	1	2.4	2
Inflammation	1.5	2.4	2.2

counteract the deleterious effects of dehydration; on the contrary increased the severity of fibrosis formation and inflammation. Although the ultimate repair strength was higher in the air exposed tendons, the stiffness of the construct was lower than the control group. Our data suggest that air exposure of the Achilles tendon causes a low quality healing both at the cellular and biomechanical level.

In a study done by Abrahamsson et al.,[13] even brief exposure to air was found to markedly inhibit *in vitro* matrix synthesis and cellular proliferation in rabbit flexor tendons. Deep flexor tendons were reported to lose half of their cellular capacity to synthesize matrix components after 45 minutes of exposure to air, and all of it within 120 minutes, indicating a time-dependent correlation between exposure to air and subsequent decrease in the mitotic and metabolic activity of tendon cells. The detrimental effects of air exposure were proposed to be counteracted by moistening of the tendon with physiologic saline.[4]

Unlike results reported by Potenza, which documented the inability of tendon tissue to regenerate and contribute to the repair process,[14] tendon cells have been shown by Mathews and Richards to have a marked reparative and regenerative activity.[15]

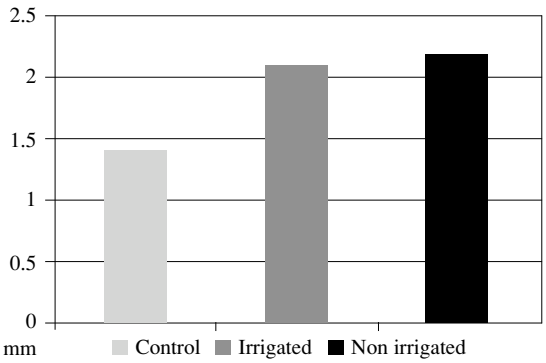

Fig. 4. Amount of tendon displacement to load in mm.

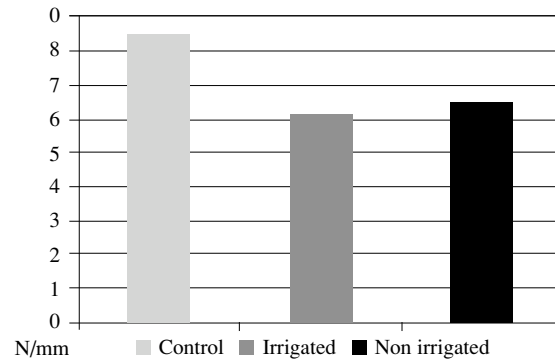

Fig. 5. Result of biomechanical test for stiffness according to groups.

Although some factors, like aridity, may have temporary adverse affects on tendon cellularity, the proliferation capacity of the tendon cells may have a compensatory activity during the healing period. *In vitro* studies are inadequate to display the active response of the tendon metabolism during this dynamic period.

Experimental studies have shown that wounding and inflammation provoke the release of growth factors responsible for inducing neovascularization, fibroblast proliferation and synthesis of collagen. Increased amount of fibrosis due to air exposure is essentially consistent with Abrahamsson's study, where a temporary increase of the mean rate of cellular proliferation was detected at the 60 minutes of exposure to air preceding cell death.[4] However, there is a major controversy between the studies. In the mentioned study, although irrigation was found to be a preventive measure against air exposure for decreasing the in vitro synthesis of collagen and non-collagen, such a clear distinction was not detected histologically among the air exposed groups in the current study. The compensatory mechanisms and regeneration capacity of the tendon during the healing period may be a rationale for this difference.

The region between the tendon ends, as demonstrated from histological sections, was filled with inflammatory cells and blood vessels indicating an inflammatory response. One of the early events of tissue healing and remodeling is angiogenesis in which neo-vascularization prompts delivery of inflammatory cells and fibroblasts to the wound site.[16] The most potent angiogenetic factor is the vascular endothelial growth factor (VEGF) which also significantly up-regulates the expression of TGF-β in tendon healing and improve fibroblast proliferation.[10,17] Hypoxia is a well known factor for VEGF secretion.[16] Aridity and irrigation of the tendon may be the other stimulants for expression of VEGF. This may be an explanation for the close relation between angiogenesis and fibroblast proliferation in the air exposed groups. The relevance between angiogenesis and increased immigration of fibroblasts was also reported in previous studies.[7,18,19] However, their relation with VEGF and TGF-β should be investigated with further evaluation.

Cartilaginous metaplasia is an occasional finding encountered during the healing of the Achilles tendon. Absence of fibrocartilage was defined as an indicator of higher grade of restoration and quality of healing.[7] Kraus et al.[20] defined the presence of Achilles tendon calcifications after repair as having a negative effect on clinical outcome. In this study, the presence of cartilaginous metaplasia in almost every specimen in the air exposed groups, regardless of irrigation, was another marker of low quality healing.

Increasing fibroblast proliferation and biosynthesis of extracellular matrix and collagen are crucial stage for the return of normal tendon strength.[8,21] The amount and degree of fibroplasia is a major component of the tensile property in a healing tendon.[8,21,22] Inflammation in tendons subsides in the first few days after injury and fibroplasias and fibrillogenesis begin at around day 5.[7,9,10,12] The new collagen fibrils scatter in the extracellular matrix and start to aggregate into organized bundles by day 21 which increase the biomechanical parameters.[8] Accumulation of larger masses of fibrosis, as detected in the histological evaluation, is probably the cause of a higher failure to load of the air exposed groups relative to the control group. Both the amount of fibroplasia and failure to load were higher in the air exposed groups which indicates a close correlation between the histological findings and the biomechanical evaluation. This also demonstrates that the repair strength of the healing tendons are closely related to the amount of fibroplasia formed at the repair site.

Although failure to load is a measurement of the maximum strength, stiffness is a measurement of the structural properties at submaximal loading. Many factors are effective on stiffness of a healing tendon. Among them, increase of the volume fraction of the collagen fibers and change in the orientation of these fibers are the significant factors that increase the tendon stiffness. Although the higher failure to load in the air exposed groups is due to high amount of fibrosis resulting a bigger construct, relatively higher stiffness of the control group is probably related to the more mature healing tissue with a more oriented collagen fiber bundles. It has previously been demonstrated that, stiffness returns relatively faster than the strength in normally healing tendon.[6,8,21] It can be considered that, the failure to load in the control group is likely to progress during the prospective healing period.

In summary, air exposure of the Achilles tendon during the surgical repair was found to have no detrimental effect on the failure to load of the tendon in an animal model. However, air exposure was a cause of low quality healing with extensive fibrosis formation and cartilaginous metaplasia. Restoration of normal function following tendon injury requires reestablishment not only of the continuity of the tendon fibers, but also of the gliding mechanisms between the tendon and surrounding structures. So that an overwhelming fibrosis and adhesion formation will adversely effect the tendon gliding and the excellence of healing. Achilles tendons, with an average open repair time between 45-100 minutes[23,24] are prone to form adhesions following the surgery which may impair the clinical outcome.[9,20] These negative effects of air exposure were not counteracted by irrigation with saline solution. The reasonable solutions may be to shorten the operation time as much as possible or to prefer percutaneous techniques instead of open repair. The interaction between air exposure and certain growth factors (i.e. TGF-β,VEGF) on fibrosis formation needs further evaluation.

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