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Research Article

Investigation into the effect of systemic single high-dose erythropoietin on the healing of Achilles tendons in rats

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ABSTRACT

Objective: This study aimed to examine systemic erythropoietin's effect on the Achilles tendon's healing in a rat model.

Methods: Twenty-five adult Wistar rats were randomly assigned to one of two groups. The Achilles tendon of each rat was transected 5 mm proximal to its insertion to the calcaneus. All Achilles tendons were then repaired using modified Kessler methods. A single dose (5000 U/kg) of intraperitoneal erythropoietin (EPO) was administered to group I. Group II was a control group and did not receive an EPO injection. Four rats from each group were sacrificed at 1, 3 and 6 weeks after injection. Histopathological assessments were performed by observers blinded to the treatment.

Results: Groups I and II showed a similar increase in fibroblast cytoplasmic content and fibrillar collagen in the extracellular matrix. Collagen deposition, cellular proliferation, number of lipid vacuoles and capillary increases were similar between the groups.

Conclusion: Evidence from this study has shown no direct effect of a single systemic high dose of EPO on the histological properties of the Achilles tendon in rats.

Introduction

Erythropoietin (EPO) regulates the production of red blood cells via its specific interaction with its cell-surface receptor (EPOR).[1] A series of recent studies have provided experimental evidence of the diverse non-hematopoietic biological effects of EPO-EPOR signaling. For example, the expression of EPOR in the kidney, the intestine, and the skeletal muscle is associated with the ability of EPO to induce cellular proliferation.[2,3] Additionally, EPO has been further demonstrated to promote angiogenesis.[4] Thus, it is of major interest to investigate whether treatment with EPO can induce beneficial effects on tendon healing and in particular on angiogenesis.

The Achilles tendon is a common site of acute and overuse injuries, and its healing is a time-consuming process.[5] However, the optimal treatment for rupture of the Achilles tendon remains unclear.[6] The repair process may range from months to years, and the injured ligament never fully regains its original mechanical properties. During tendon repair, the local signaling events leading to inflammation and early proliferative activity remain poorly defined.[7,8] Several studies have been conducted in an attempt to reduce tendon healing time through the use of ultrasound, by applying early controlled motion and tensile stress across the tendon, by galvanic stimulation, and by administering growth factors, bone morphogenetic proteins, chondromorphogenetic proteins, and non-steroid inflammatory drugs.[9-16] Moreover, there is a study examining the effect of EPO healing in rat patellar tendons where they found the additive effect of EPO on tendon healing.[17]

To our knowledge, there is no information on the action of EPO on tendon healing, and this study is the first to investigate the effect of EPO on healing of the Achilles tendon.

Materials and Methods

This study was approved by the local ethics committee (ID number: 6). A total of 25 adult Wistar rats (300 ± 20 g) were used. The rats were randomly assigned into 2 groups. Group I received a single dose of EPO (5000 U/kg). Group II was used as a control group and did not receive EPO injections. All injections were completed by the same researcher and were applied in the intraperitoneal region of the rats. The dose was selected on the basis of previous studies in animals and humans.[18] The time points were selected to establish a foundation for translation to

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treatment of human traumatic tendon injuries. The rats were housed at a temperature of 24°C and humidity of 55% with 12 hours of day/night in a light-controlled room.

Surgical procedures

Anesthesia was induced using an intramuscular injection of ketamine (0.03 mg per 200 g body weight) and xylazine (0.05 mg per 200 g body weight) mixture. All surgical procedures were performed bilaterally. After preparation of the surgical site, 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 horizontally at 5 mm proximal to the point of adherence to the calcaneus (Figure 1). The plantaris tendon was also transected to prevent an internal splint effect. The Achilles tendon was repaired using 5/0 monofilament nylon (Ethicon ®, Johnson & Johnson, Somerville, NJ, USA) sutures using a modified Kessler method. The wound was closed using 3/0 monofilament nylon (Ethicon®, Johnson & Johnson) uninterrupted sutures. No wound dressing or casting was used after the operation. All subjects were allowed to move freely and were provided with standard laboratory food and tap water. Achilles tendons were sent to the pathology department after euthanasia of the rats on the first, third, and sixth weeks from each group.

Histological evaluation

Achilles tendons were fixed in 10% formalin and routinely processed to perform a histological examination. Thick sections of 5-6 μm were cut from the paraffin blocks, stained with hematoxylin-eosin stain (H&E), and examined under the light microscope. The degree of inflammatory cell infiltration, vascularization, organization of connective tissue proliferation, and lipid vacuole formation were all evaluated separately. Each item was graded by 2 pathologists based on a semiquantitative scale as absent (0), mild (1), moderate (2), moderate to severe (3), or severe (4). Each specimen was graded based on a minimum of 10 sections.

Statistical analysis

The Statistical Package for the Social Sciences 20.0 for Windows software (IBM SPSS Corp., Armonk, NY, USA) was used for statistical analysis. When evaluating the study data, definitive statistical methods were expressed as means. For quantitative data, Student's *t* tests were used for comparisons of variables that exhibited normal distributions. $P < .05$ was accepted as statistically significant.

Results

In both groups I and II, when the histological examination of the Achilles tendons removed at the end of a 1-week period, there was connective tissue proliferation consisting of a pretty dispersed, loose web of dense reticular fibers of collagen and fibroblasts. Besides this, dense mononuclear cell infiltrations accompanied by a few neutrophils drew attention in the examined microscopical fields. In the third week, the connective tissue proliferation in the tendons wascomposed of irregular collagen fibers along with lymphocytes and lymphoblasts. The mononuclear cell infiltrations were seen also on this week. In the sixth week, the collagen fibers were more regular. The inflammatory changes mentioned in other groups were seen as less common but still existed. It was noticed that the vascularization was intense in the first week but lesser in the third and sixth weeks. Along with vascularization, lipid cells related to healing were seen to be intense in the first week and less in the third and sixth weeks (Figure 2).

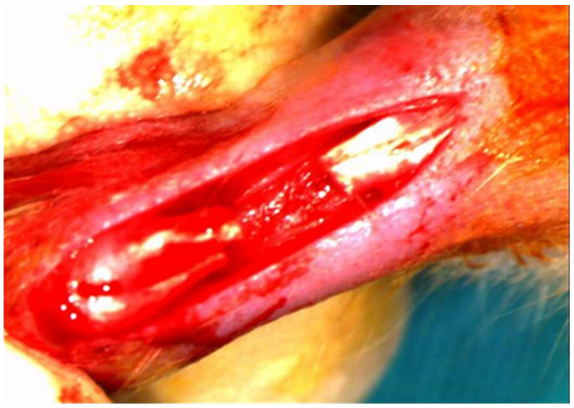

Figure 1. Horizontally transected Achilles tendon.

The evaluation of the data from the Achilles tendons of groups I and II rats on the first, third, and sixth weeks are given in Tables 1, 2, 3 respectively. No significant differences were observed between groups I and II ($P > .05$). Similar healing was observed in both groups.

Discussion

The most important finding of this study was that single high-dose systemic EPO did not affect histological parameters of tendon healing in rats.

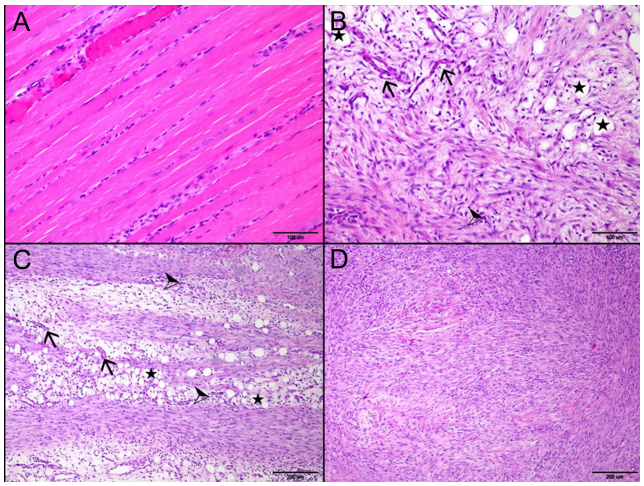

Figure 2. A-D. Photomicrographs of Achilles tendon sections. (A) The histology of Achilles tendon, (B) Group I (first week), inflammatory cells (arrow head), vascularization (arrows), lipid vacuoles (stars), (C) group II (third week), inflammatory cells (arrow heads), vascularization (arrows), lipid vacuoles (stars), (D) group II (sixth week) showing healing with connective tissue proliferation, H&E.

HIGHLIGHTS

- Erythropoietin (EPO) is an agent that is shown to induce cellular proliferation and promote angiogenesis. The data on the effect of EPO on tendon healing is limited. This study aimed to investigate the systemic single high-dose effect of EPO on tendon healing.
- No significant difference was seen in degree of inflammatory cell infiltration, vascularization, organization of connective tissue proliferation, and lipid vacuole formation.
- The results indicate that single high-dose EPO administration does not have a direct effect on the healing of the Achilles tendon. Further clinical and experimental studies, such as adding another angiogenic agent or increasing the administration dose, are required to determine the effect of EPO on tendon healing.

Table 1. Data of histological evaluation at 1 week

Animal Number	Cell infiltration	Vascularization	Connective tissue proliferation	Lipid vacuoles
C1L (1 week)	+++	+++	+	+++
C1R (1 week)	+++	+++	+	+++
C2 L (1 week)	+++	+++	+	+++
C2 R (1 week)	+++	+++	+	+++
C3 L (1 week)	++	++	+	+++
C3 R (1.week)	+++	+++	+	+++
C4 L (1 week)	++	+++	+	+++
C4 R (1 week)	+++	+++	+	+++
E1L (1 week)	+++	++	+	++
E1R (1 week)	++	+++	+	++
E2 L (1 week)	+++	+++	+	+++
E2 R (1 week)	+++	+++	+	+++
E3 L (1 week)	++	++	+	++
E3 R (1 week)	++	++	+	++
E4 L (1 week)	++	++	+	+++
E4 R (1 week)	++	++	+	++

C, control group; E, EPO group; L, left tendon; R, right tendon; EPO, erythropoietin.

Table 3. Data of histological evaluation at 6 weeks

Animal Number	Cell infiltration	Vascularization	Connective tissue proliferation	Lipid vacuoles
C9 L (6 weeks)	-	++	+++	-
C9 R (6 weeks)	++	++	+++	++
C10 L (6 weeks)	+	+	+++	+
C10 R (6 weeks)	+	+	+++	-
C11 L (6 weeks)	+	++	+++	+
C11 R (6 weeks)	++	++	+++	+
C12 L (6 weeks)	+	++	+++	++
C12 R (6 weeks)	++	+	+++	+
E9 L (6 weeks)	+	+	+++	–
E9 R (6 weeks)	++	+++	+++	+
E10 L (6 weeks)	-	-	+++	
E10 R (6 weeks)	+	++	+++	++
E11 L (6 weeks)	++	+++	+++	+++
E11 R (6 weeks)	+	+++	+++	++
E12 L (6 weeks)	+	++	+++	+
E12 R(6 weeks)	+	+++	+++	–

C, control group; E, EPO group; L, left tendon; R, right tendon; EPO, erythropoietin.

Previous studies have shown that tendon healing can be stimulated by several growth factors (e.g., platelet-derived growth factor, transforming growth factor-beta, insulin-like growth factor-1, vascular endothelial growth factor, or bone morphogenetic proteins, such as growth differentiation factors-5, -6, -7) or by platelet-rich plasma.[19] To date, EPO has been the primary hormone investigated that plays a potential role in musculoskeletal disease and injury.[20] Research has identified EPO as being an important factor for homeostasis of many musculoskeletal tissues; however, these studies have often shown an incomplete understanding of the role that this hormone plays in tissue structure and function since collagen content is known to affect the mechanical properties of some connective tissue.[21] A previous study showed that macrophages express EPOR.[22] Macrophages are responsible for the production of cytokines and growth factors essential for the healing cascade, and they are also the predominant cell type to express inducible nitric oxide synthase in healing wounds.[23,24] Systemic and local EPO administration accelerates the wound-healing response significantly.[25] It has been shown that recombinant EPO is not only capable of promoting angiogenesis but it can also stimulate osteogenesis via the differentiation of endothelial progenitor cells (EPCs) into osteoblastic cells. However, further studies are required to determine the relative proportions of EPCs that differentiate into endothelial cells for vascularization or into osteoblasts for osteogenesis during fracture healing.[26,27] It has been shown that EPO stimulates angiogenesis during the first stage of healing after a fracture.[28,29] Therefore, EPO stimulates bone fracture healing via angiogenesis.[29]

Table 2. Data of histological evaluation at third week

Animal Number	Cell Infiltration	Vascularization	Connective Tissue Proliferation	Lipid Vacuoles
C5 L (3 weeks)	+	+++	++	+
C5 R (3 weeks)	+	++	++	+
C6 L (3 weeks)	+	+	++	+
C6 R (3 weeks)	+	+++	++	++
C7 L (3 weeks)	++	++	++	+++
C7 R (3 weeks)	+	++	++	+
C8 L (3 weeks)	+	++	++	+
C8 R (3 weeks)	++	++	++	+
E5 L (3 weeks)	+	++	++	+
E5 R (3 weeks)	+	+	++	-
E6 L (3 weeks)	+	+	++	-
E6 R (3 weeks)	+	++	++	-
E7 L (3 weeks)	+	++	++	+
E7 R (3 weeks)	+	++	++	++
E8 L (3 weeks)	+	++	++	-
E8 R (3 weeks)	+	++	++	++

C, control group; E, EPO group; L, left tendon; R, right tendon; EPO, erythropoietin.

The biochemical mechanism underlying tendon healing and remodeling remains controversial.[30] Both extrinsic and intrinsic nutrition and tissue repair have been implicated. An understanding of the cellular and molecular pathways that govern the events of tendon healing is critical for the future advancement of tendon treatment.

One of the early events to occur during tissue healing and remodeling is angiogenesis.[31] Erythropoietin can increase angiogenesis.[32] However, localized angiogenic responses have been reported to vary depending on the route of administration of angiogenic molecules.[4] For instance, although bFGF was reported to stimulate angiogenic responses when administered locally, systemic intravascular infusion did not promote endothelial mitogenesis and neovascularization.[33,34] Therefore, as stated in these studies, the reason why we could not observe significant effect of EPO might result from the type of administration route.

There is little doubt that erythropoietin has a wider spectrum of effects on a larger number of tissues than that was previously believed.[18,31,35,36] Recent reviews in the literature have suggested 3 principal areas of erythropoietin-mediated protection: neuroprotection, cardio protection, and erythroid support.[32,37,38] These effects likely point to a possible role of erythropoietin as a pleuriprotective agent.[32,38] Thus, there is an expectation that hormones that are triggered by hemorrhage may play a protective role in many tissue systems. Our hypothesis was that EPO induced tendon healing via angiogenesis and a wider spectrum of effects on a number of tissues. The limitations of this study were that we did not use repetitive administration of EPO to the EPO receiving group. We used a single dose of EPO which was the representative of the human dose. It is well known that every drug has specific side effects, and thus, we avoided using repeated dosing. However, to our knowledge, this study is the first in vivo assessment of EPO in tendon healing. This information on the effect of EPO may be used when deciding whether the

administration of exogenous EPO is indicated for enhancing tendon repair.

This study showed that there was no direct effect of a single high dose of EPO administration in the treatment of Achilles tendon in rat models based on the histological data. Further clinical and experimental studies, such as increasing the administration dose, including another angiogenic agent serving as a positive control or addition of another administration route, are necessary to definitively determine the influence of EPO on tendon healing and tendon mechanical properties.

Ethics Committee Approval: The study obtained its ethical approval by the 6th approval number from KOBAY DHL A.Ş.

Informed Consent: N/A.

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