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Effects of boric acid on the healing of Achilles tendons of rats

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Abstract
Purpose Tendinous lesions are among the most frequent pathologies encountered in sportsmen. The objectives of new treatments are to improve the healing process and reduce the recovery time. Boron plays an important role in the wound repair process by increasing components of extracellular matrix and angiogenesis. This animal study aimed to investigate the effect of boric acid on healing of the Achilles tendon.
Methods The right Achilles tendons of 40 rats were completely sectioned, and the rats were randomly divided into five groups. Each group consisted of eight rats. Groups 1 and 2 were oral boric acid groups with the doses of 4 and 8 mg/kg/day boric acid, respectively. Group 3 was the local boric acid group (8 mg/kg boric acid intratendinous injection). Group 4 was administered both oral and local boric acid (8 mg/kg/day orally and 8 mg/kg boric acid intratendinous injection), and group 5 was the control group with no boric acid application. At the end of the fourth week, all the rats were killed and histopathological examination of the Achilles tendon repair site was made.
Results Histopathological examination of the tissue sections revealed more properly oriented collagen fibres, more normal cellular distribution of tenocytes and more properly organized vascular bundles in group 1 and group 2, which were the groups administered oral boric acid. Pathological sum scores of groups 1 and 2 were less than those of the other groups, and the differences between the oral boric acid groups (group 1 and group 2) and the other three groups (groups 3, 4 and 5) were statistically significant ($p = 0.001$).
Conclusion As boric acid is safe and toxicity even after very high doses is unusual, oral boric acid may be used as an agent to improve the healing process of tendon injuries. However, biomechanical tests should also be performed to show the effect of boric acid on strength and endurance of the tendon before it can be used in clinical practice.

Keywords Boric acid · Achilles tendon · Tendon healing · Rats · Collagen fibres · Angiogenesis

Introduction

Tendinous lesions are among the most frequent pathologies encountered in sportsmen and physical workers [25]. Improving the healing process and reducing the recovery time are the objectives of new treatments. Many studies have been conducted on compounds that would accelerate the healing process. Boric acid (BA) has been shown to be beneficial in wound healing [28]. BA affects the synthesis of the extracellular matrix (ECM), which plays an important role in wound repair process by increasing the release of proteoglycans, collagen and proteins, components of the ECM required for the anchorage of cells moving into the wound space [5, 28]. It also stimulates the synthesis and

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release of tumour necrosis factor (TNF α). TNF α regulates the activity of cells participating in normal wound healing, including the proliferation of fibroblasts. TNF α also stimulates angiogenesis and activates the expression of the genes encoding various inflammatory mediators [4, 5]. BA seems to be effective in wound healing, but the effect of BA on tendon healing is still unknown although it is nontoxic even with very high doses and can safely be used in clinical practice. Also it is an inexpensive agent. Therefore, it was speculated that the healing process of sectioned Achilles tendons of rats could be improved by BA. Histological analyses were performed to evaluate the ECM remodelling and collagen deposition in the newly formed matrix.

Materials and methods

Forty male Sprague–Dawley rats weighing 250–300 g were used in the study. During the experimental procedure, all rats were housed under standard laboratory conditions with an artificial 12-h light/dark cycle. They were caged individually in controlled temperature (22 ± 1 °C) and relative humidity. All rats were allowed to have free access to food and water in polycarbonate units. Rats were observed for 7 days in the animal care laboratory to exclude any possibility of underlying diseases before the start of the experiment.

Anaesthesia/analgesia

Before the surgical intervention, each animal was injected with intramuscular cefazolin (10 mg/kg) for prophylaxis against infection. Surgical anaesthesia was administered using intramuscular injection of ketamine and xylazine (35 and 5 mg/kg, respectively). The animals were kept in a dry and quiet recovery area during the first 24 h following the procedures, and subcutaneous injections of ketoprofen (5 mg/kg) were given for postoperative analgesia.

Surgical technique

The animals were divided into five groups, and each group consisted of eight rats.

Group 1: (oral 4 mg/kg/day BA, $n = 8$)
Group 2: (oral 8 mg/kg/day BA, $n = 8$)
Group 3: (local 8 mg/kg BA, intratendinous injection of 0.5 % BA solution $n = 8$)
Group 4: (oral 8 mg/kg/day + local 8 mg/kg 0.5 % BA, $n = 8$)
Group 5: (control, no BA administration, $n = 8$).

Preoperatively, the posterior part of the right legs of the rats was shaved and scrubbed with Betadine (povidone–iodine, 10 % solution) to achieve aseptic conditions. The skin of the right Achilles tendon was incised laterally, and tendon was exposed after dissection of the subcutaneous tissue. The Achilles tendon was sectioned 5 mm proximal from the insertion to the calcaneus transversely. Then, the tendon was repaired by using 4/0 ethylon monofilament nylon (Ethicon, USA) sutures with modified Kessler method, and also for group 3 and group 4 (total 16 rats), 1 ml intratendinous injection of 0.5 % BA solution (8 mg/kg) was administered to the repair site. The wound was closed with 4/0 silk sutures in a continuous fashion. No wound dressing or casting was applied (Fig. 1). The animals were kept in separate cages after the surgery and were fed under standard laboratory conditions. In oral BA groups, BA (99.99 % pure, in powder form; National Boron Institute of Turkey) was dissolved in distilled water and the solution (4 mg/kg/day BA for group 1 and 8 mg/kg/day BA for groups 2 and 4) was added to daily water of the rats and the animals were checked to see whether they had finished the water every day for 4 weeks. Boron dose was determined based on the knowledge that 3–4 mg/kg/day is a supranutritional amount for rats [1, 12], so that 4 mg/kg/day boron was determined as a therapeutic dose and twice

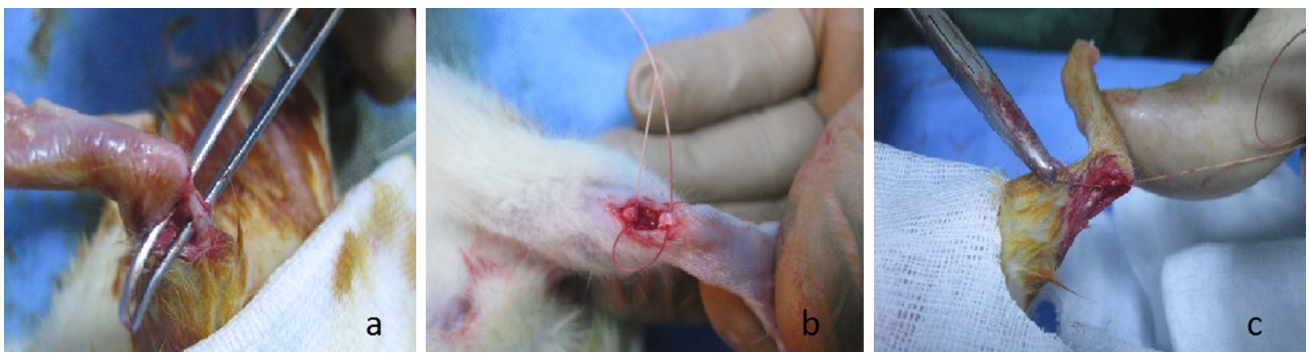

Fig. 1 Photographic appearance of Achilles tendon dissection (**a**), transection (**b**) and after the repair (**c**)

this amount (8 mg/kg/day) was determined as a high-level amount which was still not toxic.

At the end of the fourth week, all the rats were killed and 1 cm lengths of the right Achilles tendons, including the repair site, were harvested and embedded in 10 % formalin solution for 48 h for fixation. The formalin-fixed tendons were embedded in paraffin, and 5-μm-thick longitudinal sections were obtained. The sections were stained with Masson's trichrome, and microscopic evaluation was carried out under 100× and 200× magnification. Alterations to tenocyte morphology, ECM and vascular proliferation were noted as well. To assess the slides, we used a semiquantitative grading scale ranging from 0 to 21, which considers fibre structure, fibre arrangement, rounding of the nuclei, regional variations in cellularity, increased vascularity, decreased collagen stainability and hyalinization. Each variable is scored from 0 to 3 (0 normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal), and a pathological score was calculated from the sum of these scores [22–24, 26]. All of the evaluations were performed by the same pathologist to increase the reliability.

All procedures of the current study were reviewed and sanctioned by Çanakkale 18 Mart University Animal Research Ethical Committee (2013/10-01). All of the animal experiments were conducted in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (revised 1985).

Statistical analysis

Kruskal–Walllis test was used in order to compare the treatment groups in terms of histological grading. Differences were considered to be significant at $p < 0.05$ for all tests (two tailed). In order to identify the differences between groups, the Mann–Whitney *U* test was performed, and the Bonferroni correction was used to account for multiple comparisons. The *p* value was determined as $p = 0.005$. Minitab statistical package program (version 17.0) was used to determine suitable sample size for each treatment group, and it was seen that eight rats would be enough for achieving at least 80 % test power.

Results

Histopathological examination of the tissue sections revealed more properly oriented collagen fibres and more normal cellular distribution of tenocytes in group 1 and group 2, which were the groups administered oral BA. Additionally, vascularity was decreased in these groups, but more properly organized vascular bundles were apparent compared to the other groups (Fig. 2).

The pathological sum scores of the groups averaged 2.5 ± 2.2, 1.6 ± 1.9, 12.7 ± 3.2, 14.7 ± 1.1 and 16.5 ± 3.5, respectively (Table 1). A statistically significant difference was determined between the groups with regard to the mean histopathological scores (Kruskal–Wallis test, $p < 0.0001$). A paired comparison was made with the Mann–Whitney test to detect the group/groups from which the difference originated. Pathological sum scores of groups 1 and 2, which were the oral BA administered groups, were less than those of the other groups, and the differences between the oral BA groups (group 1 and group 2) and the other three groups (groups 3, 4 and 5) were statistically significant ($p = 0.001$) (Fig. 3).

Variables

- *Fibre structure*: The normal tendons show parallel, closely packed collagen fibres and slight waviness of the fibre. Increased waviness and separated fibres are typical of mild–moderate changes, where loss of the structure and marked hyalinization are considered as marked abnormalities. The median scores were 0.5 and 1 in groups 1 and 2, respectively, which meant nearly normal, and 2, 3 and 2 in groups 3, 4 and 5, respectively, which meant markedly abnormal
- *Fibre arrangement*: Fibres are organized parallel in normal tendons. Disorganized or differently arranged fibres are markers of pathological changes. Median scores of the groups were 0.5, 1, 2, 2.5 and 2, respectively, indicating nearly normal fibre arrangement in the oral BA groups.
- *Tenocyte nuclei*: Normal tenocytes have flattened or spindle-shaped nuclei. Abnormal tenocytes have round and large nucleus with abundant cytoplasm. The median scores were 0.5, 1, 2, 2 and 2 for the groups, respectively, which meant better tenocyte morphology in the oral BA groups.
- *Cellularity*: Abnormal tendons contain areas of increased cellularity compared to normal architecture. Median scores of the groups were 1, 1, 2, 2 and 2, respectively, which meant the oral BA groups showed more normal cellular architecture.
- *Vascularity*: Normally, vascular bundles run parallel alongside collagen fibres. In abnormal tendons, the number of vessels is significantly greater than normal. Median scores of the groups were 0.5, 0.5, 2, 2 and 3, respectively, which meant vascularity in the oral BA groups was more normally organized.
- *Collagen stainability and hyalinization*: The normal collagen colour is pink red. In degenerative conditions, the stainability of collagen was reduced, and the collagen looked paler (from 0 to 3). The median values of the groups for collagen stainability were 0.5, 0.5, 1, 2

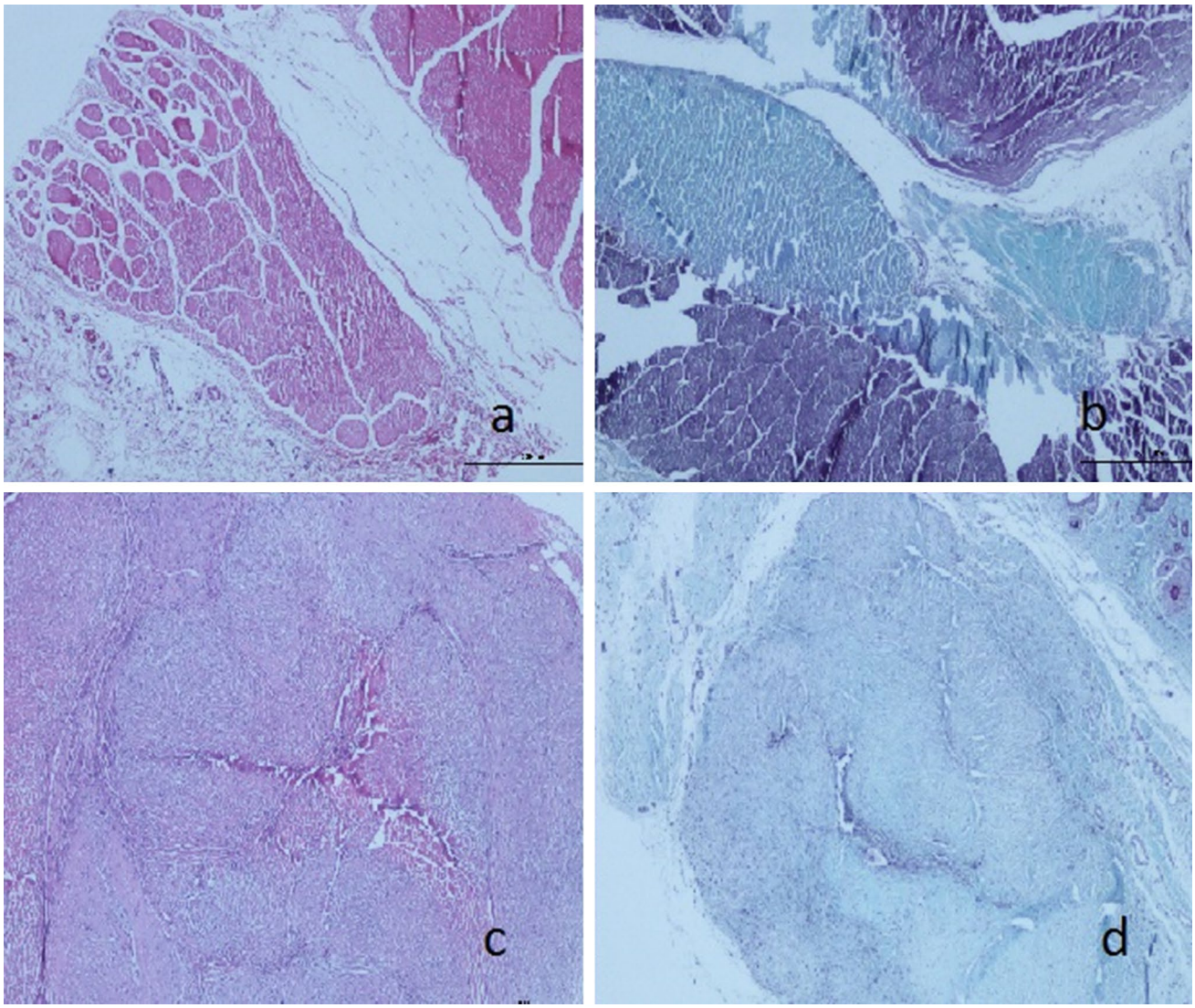

Fig. 2 H and E, ×40 (**a**) and Masson trichrome, ×40 (**b**) stained section and images of the group 2 rat tendon which is oral boric acid (8 mg/kg/day) administered group and H and E, ×40 (**c**) and Masson trichrome, ×40 (**d**) stained section and images of the group 5 rat tendon which is control group. In the **a/b** collagen fibre configuration, staining and matrix architecture is seen as nearly normal tendon though in **c/d** abnormal collagen configuration, staining and matrix morphology is prominent

Table 1 Summary of pathological scores of the groups

Pathologic sum score	Group 1	Group 2	Group 3	Group 4	Group 5
Mean	2.5	1.6	12.7	14.7	16.5
Median	3.0	1.0	13.0	14.5	15.0
SD	2.2	1.9	3.2	1.1	3.5
Range	0–5	0–5	8–18	13–16	12–21

and 2, respectively, and for hyalinization were 0.5, 0.5, 1, 2 and 1.5, respectively. Also this meant that collagen stainability and hyalinization were better in the oral BA groups.

Discussion

The principal finding of this study was that significantly more properly oriented collagen fibres, more normal cellular distribution of tenocytes and more normally organized vascularity were found in the oral boric administered groups. But in the study, local injection of BA solution to the repair site was found to have no beneficial effect on tendon healing. Even local injection of BA to the repair site obscured the positive effect of oral BA in group 4. In the literature, there is no consensus on the dosage of BA for local usage. Blech et al. reported that 3 % BA solution significantly improves the healing of deep wounds [6, 7]. On the other hand, Bendendour et al. reported that

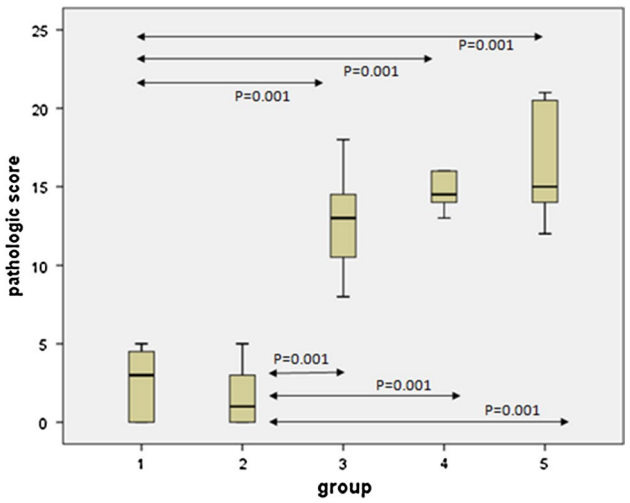

Fig. 3 Schematic illustration of the pathologic sum scores and *p* values between the groups showing statistically significant difference

boron was not toxic for fibroblasts until 0.5 % but at 1 % the cell viability decreased by 25 % and also reported that the maximum effect of boron on ECM protein release was observed with 0.1 and 0.25 % [4]. In our study, we preferred the dose of 0.5 % (8 mg/kg) for BA local injection because the dose was reported to be a non-toxic dose for fibroblasts. We speculate that this dose may still be high and the reason for the negative effect of BA in our study was dose dependent.

Tendon and soft tissue injuries constitute an important part in orthopaedic surgeons' daily working activity. All healing mechanisms including that of tendons consist of a common pathway. There are three main stages of tendon healing: inflammation, repair or proliferation and remodelling. 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 [15, 32, 34]. Also vascularization seems to have great importance for tendon healing. Gelberman et al. [17] showed an initial vascular response in tendon injury that was profuse and haphazard, and stated that the early stages of repair were characterized by a marked increase in vascularity. After acute response to the injury, vascularization decreases and more properly organized vascular bundles which run parallel alongside the collagen fibres can be seen [23, 29].

Tendon healing depends on several factors such as age, nutrition, hormones, infections, systemic diseases and vitamins, and the aim of the treatment for tendon injuries is to obtain a mechanically and functionally nearly normal tendon. The results of many different treatment modalities for Achilles tendon including open repair and percutaneous repair are reported [9, 10, 13, 14, 18, 19]. Also proprioception levels for the ankle joint after the repair have great importance for the patient's daily life and normal gait. Although mechanically strong enough tendons can usually be obtained after repair, proprioception impairment may be a problem [21].

In the literature, many experimental and clinical studies have been reported, showing that the Achilles tendon healing can be improved by using several methods such as local injection of mesenchymal stem cells [11], platelet concentrate [3], botulinum toxin [33] or growth factors [2, 36] at the repair site or systemic use of some anti-inflammatory drugs [16]. Although the beneficial effects of BA on wound healing have been reported, an experimental or clinical study analysing the effect of BA on the tendon repair process has not been performed yet.

Boron is a mineral that is abundant in soil, air, and the surface water of oceans [35]. The most notable boron compounds are BA and borax. The major source of the boron is diet (e.g. fruits, vegetables and nuts) and water [31]. Dietary boron supplementation may have important effects on various metabolic and physiological systems. Many studies have demonstrated that boron compounds have beneficial effects such as increased vitamin D biosynthesis [20], induction of hematopoiesis [27] and stronger antioxidant defences in animals and humans [30]. Boron limits oxidative damage by enhancing the body's store of glutathione and its derivates, or by inducing other ROS-neutralizing agents [8]. Boron has been also shown to improve the healing of deep wounds, reducing the two-thirds the time required in intensive care [6, 7]. Benderdour et al. showed that boron increases angiogenesis by stimulating TNF α and affects the synthesis of the ECM, which plays an important role in the wound repair process by increasing the release of proteoglycans, collagen and proteins, components of ECM required for the anchorage of cells moving into the wound space [4, 5].

There are some limitations of the study. The main limitation of the study is the lack of biomechanical tests in the study. Although histologically more nearly normal healing processes were determined in the oral BA groups, we still lack knowledge about the tensile strength and the endurance of the tendon. Measurements of the tensile strength of the repaired tendons might be better to demonstrate the effectiveness of the BA treatment. Secondly, although the study shows the positive effects of oral BA on tendon healing, the local effect of BA is still unclear. Thirdly, this study is an experimental study performed on Achilles tendons of rats so that we cannot be sure of the same beneficial effect of BA on human tendons unless we perform a clinical study.

In this study, the effect of BA on tendon healing was aimed to be investigated and the results of the current study

showed that the healing process of the sectioned Achilles tendon could be improved by oral boric administration in rats. In the future, it can safely be used as an additional treatment modality in clinical practice to improve the healing process after tendon injuries which are very commonly seen orthopaedic problem.

Conclusion

Boric acid has positive effects on tendon healing process. Because usage of the BA is safe and toxicity even after very high doses is unusual, we think that oral BA may be used to improve the healing process after tendon injuries; however, biomechanical tests should also be performed before clinical use.

Conflict of interest All authors have no conflicts of interest with respect to the data collected and procedures used within this study.

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