Acupuncture increases the diameter and reorganisation of collagen fibrils during rat tendon healing

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ABSTRACT

Background Our previous study showed that electroacupuncture (EA) increases the concentration and reorganisation of collagen in a rat model of tendon healing. However, the ultrastructure of collagen fibrils after acupuncture is unknown.

Objectives To assess the effect of acupuncture protocols on the ultrastructure of collagen fibrils during tendon healing.

Methods Sixty-four rats were divided into the following groups: non-tenotomised (normal group), tenotomised (teno group), tenotomised and subjected to manual acupuncture at ST36 (ST36 group), BL57 (BL57 group) and ST36+BL57 (SB group) and EA at ST36+BL57 (EA group). The mass-average diameter (MAD) and the reorganisation of collagen fibril diameters were determined during the three phases of tendon healing (at 7, 14 and 21 days).

Results The MAD increased during the three phases of healing in the SB group. In the EA group, MAD increased initially but was reduced at day 21. The reorganisation of collagen fibrils was improved in the EA and SB groups at days 14 and 21, respectively. EA at day 21 appeared to reduce the reorganisation.

Conclusions These results indicate that the use of EA up to day 14 and manual acupuncture at ST36+BL57 up to day 21 improve the ultrastructure of collagen fibrils, indicating strengthening of the tendon structure. These data suggest a potential role for acupuncture in rehabilitation protocols.

INTRODUCTION

Tendons are the structures responsible for transmitting mechanical force generated in the muscles to the bones, thus enabling mobility and joint stability.[1] The incidence of tendon injuries has increased substantially over the past few decades owing to increasing recreational physical activity and complications of diabetes and other metabolic diseases.[2 3] The professionally active middle-aged population is the hardest hit, leading to an economic impact due to work absenteeism.[3] In this context, the main objectives of current regenerative treatments are to improve the quality of scar tissue and accelerate healing.[3 4]

Tendons consist mainly of collagen fibres composed of collagen fibrils that are damaged by injury. After injury (experimental section), tendon healing occurs in three distinct but partially overlapping phases (inflammatory, proliferative and remodelling), during which fibroblasts synthesise collagen molecules that join to form thin fibrils (collagen fibrillogenesis).[5] The population of these thin collagen fibrils increases after tendon injury with a consequent loss of fibril organisation.[6–8] There is a positive correlation between the diameter of collagen fibrils and the biomechanical properties of tendons.[9 10] The organisation or distribution of the fibril diameters is important for the strength (thick fibrils) and elasticity (thin fibrils) of the tendons.[11] Thus the scar tissue formed by thinner and less organised fibrils, relative to that found in normal tendons, may have important consequences for patients with reduced performance and the risk of re-rupture.[8] The aim of this study was to evaluate the effect of different protocols of acupuncture on collagen fibril diameter and organisation.

The clinical use of acupuncture is becoming increasingly popular worldwide and it is the most commonly used complementary and alternative therapy

today.[12] Acupuncture involves the insertion and manipulation of thin needles in the skin and subjacent tissues at specific sites, termed acupuncture points, for preventative and curative purposes. After insertion, the needles may be stimulated manually or with a low-voltage electrical current, termed electroacupuncture (EA).[13]

In a previous laboratory study, it was shown that EA at ST36 (*Zusanli*) and BL57 (*Chengshan*) increased the concentration of collagen and induced better molecular organisation of the collagen fibres in rat tendons during the proliferative phase of healing.[14] Based on these results, we hypothesised that acupuncture would increase the diameter of collagen fibrils and improve reorganisation of their diameters in rat tendons during the healing phases. To test this hypothesis, we used the Achilles tendon injury in rats as an experimental model and evaluated the effect of different acupuncture protocols on the diameter and reorganisation of the collagen fibrils during the inflammatory (7th day), proliferative (14th day) and remodelling (21th day) phases. These protocols included the isolated and combined use of the ST36 and BL57 points with and without electrical stimulation.

METHODS

Animals

A total of 64 male Wistar rats (60 days old, weighing 250–300 g) were randomly divided into groups of four, with one group for each of three time points for the intervention arms: non-tenotomised (normal group), tenotomised (teno group), tenotomised and subjected to manual acupuncture at points ST36 (ST36 group), BL57 (BL57 group) and ST36+BL57 (SB group) and electrical stimulation at ST36+BL57 (EA group). Rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) of the State University of Campinas (UNICAMP), São Paulo, Brazil. They were maintained with a 12 h alternate light–dark cycle and food and water were freely available. The animal protocols were approved by the ethics committee on animal experiments of UNICAMP, which is in accordance with the guidelines for the care and use of laboratory animals (protocol No 2525-1).

Achilles tendon injury model

Animals were subjected to partial injury (tenotomy) of the right Achilles tendon, a method which has been repeatedly used by our research group.[14–17] Each animal was preweighed and anaesthetised with intramuscular injections of ketamine and xylazine (10%) at doses of 70 and 12 mg/kg, respectively. The skin over the Achilles tendon was incised and the tendon was released from the adjacent tissue through this incision. The tendon was then transected (~50% diameter) midway between the myotendinous junction and the insertion in the calcaneus bone. The sectioned area was not sutured. Finally, the skin incision was closed. All surgical procedures were performed under aseptic conditions.

Acupuncture treatment

Before all sessions, animals were immobilised in a plastic cylinder that allowed access to the right hind limb for the application of acupuncture needles. Sterilised stainless steel needles measuring 0.25 mm×25 mm were placed unilaterally on the side of the lesion. The insertion depth was about 6 mm. The BL57 point was needled midway between BL40 (*Weizhong*), which is located in the centre of the popliteal fossa and BL60 (*Kunlun*), which is located between the lateral malleolus and the Achilles tendon. The ST36 point was needled at a point 5 mm lateral and inferior to the anterior tibial tubercle.[14] Needles were inserted and manually rotated in clockwise and counterclockwise directions for a total of 16 rotations each way.[18] In the EA group, electrical stimulation was given with a device providing asymmetric bipolar Faradic continuous waves (Accurate Microprocessor Pulse 585PRO, Lautz, Rio Claro, Sao Paulo, Brazil). The frequency was set at 2 Hz due its putative anti-inflammatory, compositional and organisational effects.[14 19 20] The stimulation intensity was between 2.0 and 4.0 V so that there was moderate muscle contraction for a period of 20 min. After the procedures, the rats were returned to their cages pending further treatments. Acupuncture was repeated on alternate days up to a maximum of nine times (three times weekly). Rats were killed by an overdose of anaesthetic agents, and tissue samples were collected and analysed at days 7, 14 and 21. The rats killed at days 7, 14 and 21 received three, six and nine applications, respectively.

Transmission electron microscopy

Tendons were removed and fixed for 24 h in Millonig buffer containing glutaraldehyde (5%) and tannic acid (0.25%). The transection region was isolated using a dissecting microscope. A secondary fixation was performed in Millonig buffer containing 0.5% osmium tetroxide for 1 h. Samples were washed in distilled water and dehydrated in acetone then embedded in epon and sectioned (70–80 nm) transversely to the long axis using a diamond knife. Sections were stained with lead citrate and uranyl acetate and examined under a transmission electron microscope (LEO 906, Zeiss). Images (magnification ×60 000) of the tendon collagen fibrils were obtained with five fields for each sample. Areas containing elastic fibres, cellular components, residual dye and other artefacts were avoided.

Collagen fibril diameter measurement

Collagen fibril diameters were measured using Image-Pro Plus V.6.3 image analyzer software (Media Cybernetics, Inc, Silver Spring, Maryland, USA). A

region of interest of 3 μm^2 was fixed for each field (15 μm^2 in total) and the fibrils contained in these fields were marked and their diameters measured. A total of 58 476 fibrils were measured across all samples. These measurements were used to prepare histograms of the diameter distribution for the collagen fibrils, and the mass-average diameter (MAD) was calculated using the following formula: $MAD=(\Sigma [n_i \times d_i^3])/(\Sigma [n_i \times d_i^2])$, where n_i is the number of fibrils with a specific diameter and d_i is the specific diameter.[11 21]

Statistical analysis

MADs of the collagen fibrils are shown as mean±SD and were compared using the Kruskal–Wallis test and Dunn's multiple comparison post hoc test. The Kolmogorov–Smirnov test for two samples was used to compare the frequency of collagen fibril diameter distributions between the groups. The underlying distributions were considered statistically different when $p<0.05$. Statistical analyses were performed using Minitab V.16 Statistical Software (State College, Pennsylvania, USA).

RESULTS

Acupuncture increases the collagen fibril diameters

Representative electron micrographs from the different groups are shown in figure 1. The collagen fibril diameters of normal tendon are characterised by a mixture of large and thin fibrils (figure 1A). After injury, only thin fibrils were seen in the three analysis periods in the teno group (figure 1B: day 7; figure 1C: day 14; figure 1D: day 21). Application of acupuncture at ST36 did not increase the collagen fibril diameters (figure 1E: day 7; figure 1F: day 14; figure 1G: day 21) relative to the teno group. However, when the BL57 point was stimulated and the tendons examined at days 14 and 21 after injury, large fibrils were visualised between the small ones (figure 1I, J). A similar pattern was visualised in the SB group (figure 1L: day 14; figure 1M: day 21). In the EA group at day 7, there was an increase in the fibril diameters (figure 1N) relative to all other groups on the same day (figure 1B, E, H, K). These large fibrils were visualised again at day 14 (figure 1O) but not at day 21 (figure 1P) in the EA group.

The MAD values of the collagen fibrils are shown in table 1. At day 7, MAD increased in SB and EA groups relative to both teno ($p<0.001$ and $p<0.05$, respectively) and ST36 ($p<0.01$ and $p<0.05$, respectively) groups. There were no significant differences between the SB and EA groups. At day 14, the increase in MAD was evident in BL57, SB and EA groups relative to teno ($p<0.01$, $p<0.05$ and $p<0.001$, respectively) and ST36 ($p<0.001$, $p<0.01$ and $p<0.001$, respectively) groups. There were no significant differences in MAD between the BL57, SB, EA and normal groups. At day 21, MAD was increased in SB versus teno ($p<0.05$) and ST36 ($p<0.05$) groups. In the EA group at day 21, MAD was lower than the SB group on the same day ($p<0.05$) and the EA group on day 14 ($p<0.001$). Together the data suggest that manual acupuncture at ST36+BL57 increased collagen fibril diameters during all three phases of tendon healing, as did EA at the same points during the first two phases only (days 7 and 14). By contrast, EA at day 21 appeared to decrease the collagen fibril diameters.

Acupuncture increases reorganisation of collagen fibrils

After transection, the frequency of thin fibrils increased in all groups subjected to transection, whether treated or not, at days 7, 14 and 21 after injury (data not shown). The predominance of thin fibrils in the transected groups (disorganisation) did not match the pattern of collagen fibril diameter distribution (thick and thin fibrils) found in normal tendons (figure 2). Therefore, the pattern of collagen fibril diameters distribution was different ($p<0.05$, Kolmogorov–Smirnov test) in all groups in relation to the normal group, with the single exception of the EA group at day 14 after injury. In this group the pattern did not differ from the normal group or from the SB group at day 21 (figure 2). However, the EA group at day 14 and the SB group at day 21 both differed from the teno group at day 21 ($p<0.05$). Thus, these data suggest that EA at ST36+BL57 up to day 14 (proliferative phase) increased the reorganisation of collagen fibrils. Moreover, fibril reorganisation increased after manual acupuncture at the same points up to day 21 (remodelling phase).

DISCUSSION

Collagen fibrils are composed of individual triple helical collagen molecules arranged in a pseudo quarter-staggered array to produce fibrils with a 67 nm periodic axial repeat.[22] The formation of fibrils (fibrillogenesis) occurs in three distinct steps. First, small collagen fibril intermediates nucleate outside fibroblasts in distinct extracellular compartments. Second, these intermediates assemble lengthwise into long, thin collagen structures. Third, these chains fuse laterally to form the thicker fibrils seen in fully developed tissue.[23] However, the diameter of these fibrils decreases after tendon injury.[8]

Our results indicate that acupuncture at ST36 +BL57 increased the collagen fibril diameters (expressed as MAD) and improved the reorganisation of collagen fibril diameters in rat tendons after injury. An increase in MAD indicates strengthening of the tendon structure, increasing its resistance to injury and/or re-rupture.[24] The MAD is defined as the diameter of a fibril that contains the average mass present in the distribution.[21] The MAD takes into account the fact that a small number of collagen fibrils with a large diameter contribute significantly to the

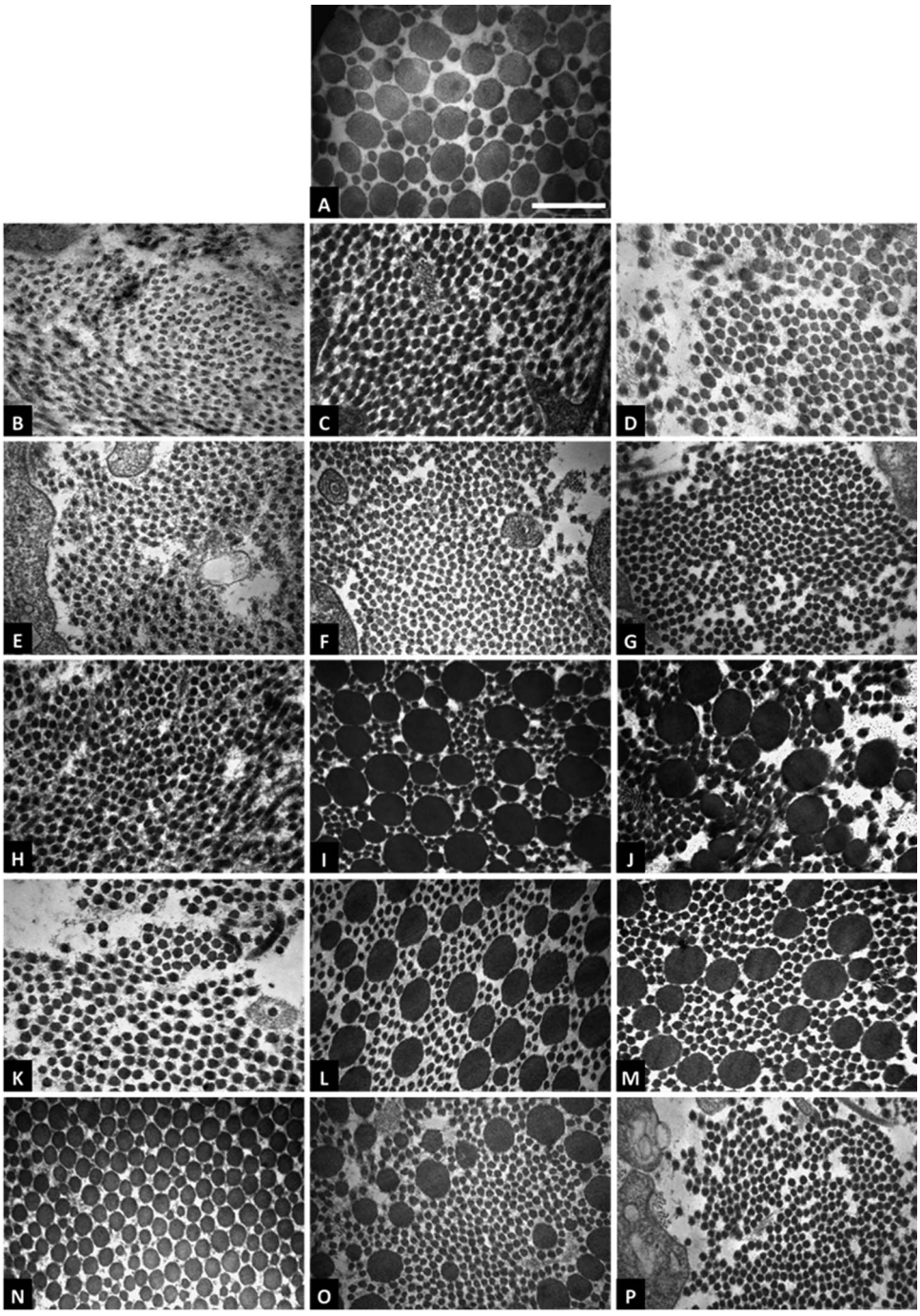

Figure 1 Electron micrographs demonstrating collagen fibril diameters in the different experimental groups (n=4 each): normal (A); tenotomised at day 7 (B), day 14 (C) and day 21 (D) after injury; manual acupuncture at ST36 at day 7 (E), day 14 (F) and day 21 (G); manual acupuncture at BL57 at day 7 (H), day 14 (I) and day 21 (J); manual acupuncture at ST36+BL57 at day 7 (K), day 14 (L) and day 21 (M); and electroacupuncture at ST36+BL57 at day 7 (N), day 14 (O) and day 21 (P). A mixture of large and thin fibrils is apparent in the normal group. After injury, the collagen fibril diameters decreased in the tenotomised and ST36 groups. Large fibrils were present in the BL57 and ST36+BL57 groups at days 14 and 21 and in the electroacupuncture group at day 14. By contrast, these large fibrils were not seen in the electroacupuncture group at day 21. Bar=500 nm.

biomechanical characteristics of the tendon.[25] A greater fibril diameter results in a higher concentration of covalent cross-links compared with smaller fibrils because these fibrils present a low surface area to volume ratio.[25] Thus, the greater the MAD, the higher the tensile strength that the tendon can withstand. The MAD increases from birth to maturity and it is believed that this increase occurs owing to the application of mechanical stimuli to the tendon.[11] In human Achilles tendons, the MAD increases in adulthood and declines with ageing as a result of the loss of larger collagen fibrils.[26]

Table 1 Mass-average diameter (nm) of collagen fibrils

	7 day	14 day	21 day
Teno	46.6±9.7*	56.2±16.1*	61.6±26.3*
ST36	51.0±18.2*	61.7±38.9*	55.5±6.6*
BL57	63.3±15.6*	135.8±80.0$^{\#\ a}$	105.2±59.0
SB	84.9±17.7*$^{\#\ a}$	105.3±50.5$^{\#\ a}$	107.1±34.5$^{\#\ a}$
Electroacupuncture	89.0±28.7*$^{\#\ a}$	134.8±47.0$^{\#\ a}$	56.2±10.0*$^{b\ A}$
Normal		179.4±31.3	

All data are presented as mean±SD (n=4 per group). Statistical significance is indicated by p<0.05.
*Indicates values which differ significantly relative to the normal group (bottom row, single time-point). *Within column comparisons*: #, significantly different from the tenotomised group; a, significantly different from ST36 group; b=significantly different from SB group. *Within row comparisons:* A, significantly different from the 14-day time point in the electroacupuncture group.

To our knowledge, this is the first study examining the effects of acupuncture on the ultrastructural characteristics of collagen fibrils after tendon injury. In this study, acupuncture at ST36 alone did not increase MAD during the three periods analysed. Several previous studies have shown that acupuncture at ST36 has systemic anti-inflammatory effects via the inhibition of synthesis of proinflammatory mediators such as interleukin (IL)-1β, IL-6, tumour necrosis factor α (TNFα), interferon γ (IFNγ), cyclo-oxygenase (COX)1, COX2, inducible nitric oxide synthase (iNOS) and prostaglandin E_2 (PGE_2).[19 27 28] TNFα has a detrimental effect on healing due to its regulatory effects on macrophage differentiation, which subsequently increases synthesis of collagenase and reduces collagen production.[29] IFNγ decreases collagen production and increases the time for lesion healing.[30] PGE_2 is a potent proinflammatory molecule and is also associated with decreased collagen production by fibroblasts.[31] Accordingly, these cytokines affect the presence and activation of fibroblasts and their role in tendon regeneration and may influence the formation and organisation of collagen fibrils in the extracellular matrix. Indeed, it has been shown that the reduction of the inflammation in the early stages of tendon regeneration promotes precocious synthesis of collagen, accelerating the repair process.[32] Despite the potential effects of ST36 acupuncture on collagen synthesis and reorganisation, needling at this point did not result in an increase in MAD relative to the teno group.

By contrast, our results showed that acupuncture at BL57 increased MAD values 14 days after injury relative to the teno and ST36 groups. At day 21, the MAD values were similar in magnitude to the normal group but were not significantly different from those of the teno and ST36 groups. The great variability of collagen fibril diameters seen between the groups could be explained by the interindividual variation of

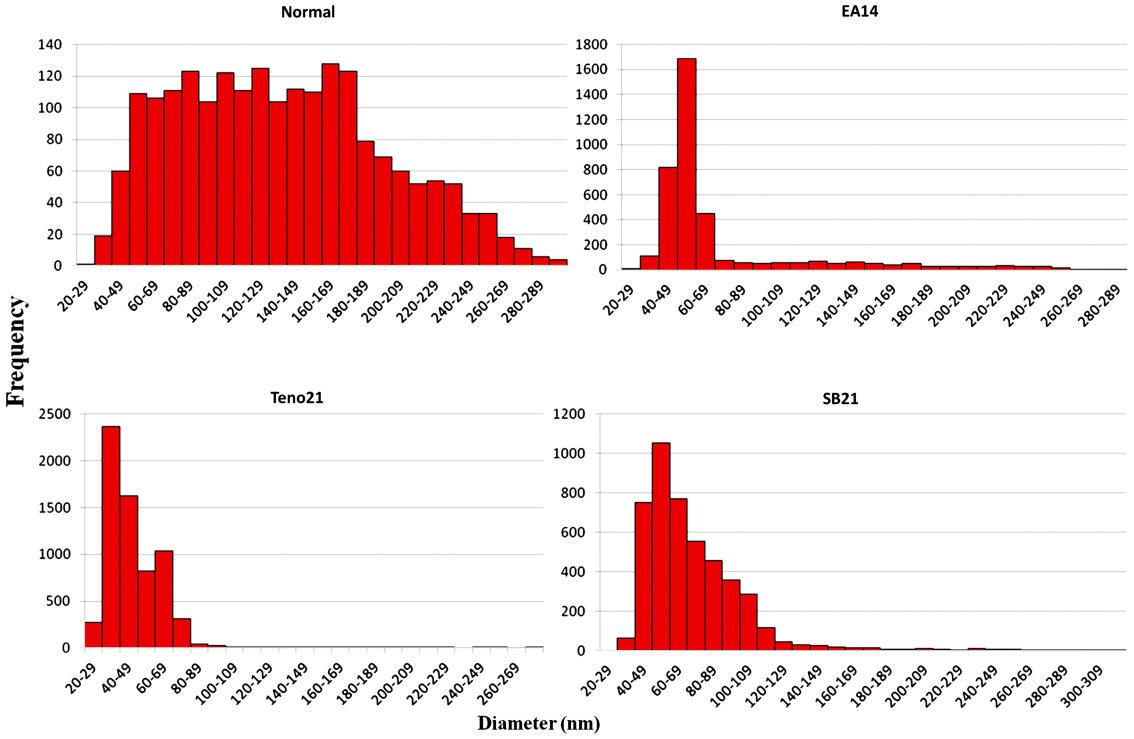

Figure 2 The frequency distributions of collagen fibril diameters in selected experimental groups: normal, tenotomised only at day 21 (teno21), electroacupuncture at ST36+BL57 at day 14 (EA14) and manual acupuncture at ST36+BL57 at day 21 (SB21) after injury. In the groups with injury there was an increase in the frequency of thin (30–69 nm) collagen fibrils. The frequency of thick fibrils (100–259 nm) was increased in the EA14 group and SB21 groups. The reorganisation of collagen fibril diameters was improved in the EA14 group—the values did not differ statistically from those of the normal (non-tenotomised) group.

responses to acupuncture. In the SB and EA groups there was an increase in MAD at all three points of analysis except day 21 for EA only, relative to the teno and ST36 groups. BL57 is located in the myotendinous junction of the triceps surae, which is continuous with the Achilles tendon.[33] We hypothesise that a mechanical stimulus generated at this point through rotation of the acupuncture needle may reach the site of the tendon injury via tension on the collagen fibres forming the conjunctive sheaths of muscle and tendon, modulating synthesis of collagen and other extracellular proteins and thereby collagen fibril diameters.

This hypothesis stems from the following information. A mechanotransductor effect has been attributed to the mechanical stimuli generated by acupuncture needle manipulation,[34] which can be defined as the ability of cells to transform mechanical stimuli into biochemical changes.[35] There is an intimate relationship between mechanical stimulation and the biochemical changes in tendons and these changes appear to be adaptations to the tendons' structural properties.[36] The collagen bundles can act as cellular signalling transducers by shearing electricity, piezoelectricity and helical geometry, which could influence the tendon repair process.[36] The mechanical stimulation may lead to an increase in small leucine-rich proteoglycan (SLRP) synthesis by fibroblasts close to the injury site. SLRPs have been implicated as important regulators of collagen fibrillogenesis.[20] In summary, the acupuncture points ST36 and B57 points were chosen in view of their putative anti-inflammatory and mechanotransductor effects, respectively.[37]

In contrast with the results described above, there was an apparent decrease in MAD in the EA group at day 21 relative to the SB group at day 21 and the EA group at day 14. The cumulative application of electrical stimuli might have activated enzymes such as metalloproteinases in the remodelling phase, which break down the large collagen fibrils into smaller ones.[38]

Previous studies examining the ultrastructural organisation of collagen fibrils in normal tendons have described two discrete populations of fibrils, resulting in a bimodal diameter frequency distribution.[25 39] The bimodal distribution of the fibril diameters is important for the properties of strength (thick fibrils) and elasticity (thin fibrils) of tendons as a whole.[11] After injury, the pattern of collagen fibril diameter distribution changed from bimodal to unimodal (thin fibrils) distribution in all groups studied here, except for the EA group at day 14, which showed a recovery of large fibril populations, leading to a pattern of a collagen fibril diameters that was similar to the distribution seen in normal tendons. In the SB group at day 21, the pattern of collagen fibril diameter distribution was the same as that of the EA group at day 14 but different from the normal group. This improvement in the reorganisation of the distribution of collagen fibrils diameters may be due to both mechanical and electrical stimulation applied at the BL57 point, leading to adaptations in the structural properties of fibrils.[36] In line with our results, a recent review article summarised the evidence for the potential use of acupuncture as a clinical treatment for tendinopathy and suggested that its effects might be mediated through facilitation of tendon blood flow and fibroblastic activity.[40]

In conclusion, our data indicate that manual acupuncture at ST36+BL57 during the three phases of tendon healing, and EA at the same points during the first two phases, improves the ultrastructural properties of collagen fibrils in tendons after injury. These observations suggest strengthening of the tendon structure and resistance to re-rupture. We would suggest that electrical stimulation should be applied carefully, avoiding the remodelling phase, because of a possible detrimental effect on the MAD. Future studies need to investigate further how electrical stimulation itself influences the collagen fibril diameters and distribution. The influence of SLRPs on the modulation of collagen fibril diameters must be also be examined.

Summary points

- We explored the nature of acupuncture's effect on tendon healing.
- Manual and electroacupuncture were used, in an animal model.
- Both given up to 14 days improved fibril diameter and organisation.
- Electroacupuncture after 14 days led to deterioration.

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