The effect of electroacupuncture on tendon repair in a rat Achilles tendon rupture model

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ABSTRACT

Objective To examine the effect of electroacupuncture (EA) on early post-rupture tendon repair in a rat model of Achilles tendon rupture using histological and mechanical evaluation.

Methods An Achilles tendon rupture model was prepared in 90 Wistar rats, which were randomly assigned to EA, manual acupuncture or control groups. Rats in the EA group received EA (pulse width 5 ms; stimulation frequency 50 Hz; stimulation strength 20 μA; stimulation time 20 min) daily from 1 day following model preparation until the day of assessment (either 7 or 10 days after model preparation), when the region of interest was sampled to assess tendon repair using *in vitro* methods. Total cell count and the number of cells staining positive for transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (b-FGF) were measured. Tension tests were performed 10 days after model preparation to measure the maximum breaking strength of the repaired tendon.

Results Both the total cell count and the number of cells positive for b-FGF were significantly higher in the EA group ($p<0.05$). In the EA group only, immunostaining showed strong expression of TGF-β1 7 days after model preparation ($p<0.05$). Maximum breaking strength of the repaired tendon 10 days after model preparation was significantly higher in the EA group ($p<0.01$).

Conclusions The marked increase in cell count and growth factor expression as well as increased tendon strength in the EA group suggest that EA may be a useful method for promoting tendon repair.

INTRODUCTION

Achilles tendon rupture is a frequently occurring injury. Problems associated with conservative management of Achilles tendon rupture include adhesion of surrounding tissue, muscle atrophy and reduced range of motion due to long-term fixation. Furthermore, regardless of the method of treatment, the biggest problem remaining is re-rupture after tendon repair.[1]

Against this background, studies have been conducted using electrical stimulation[2] and pulsed electromagnetic fields (PEMFs)[3] to promote tendon repair. Electrical stimulation, PEMFs and other methods of physical stimulation have been reported to influence the expression of growth factors, resulting in the proliferation of fibroblasts which, in turn, increase tendon strength.[2 3] However, existing electrical stimulation techniques require an invasive procedure in which electrodes are embedded. Thus, there is a risk of infection and the possibility of adhesion of surrounding tissue to the embedded electrodes. In addition, these methods are not suitable for conservative treatment. By contrast, PEMFs can be used with either surgical or conservative treatment. Since the stimulation covers an extensive area, however, there is a higher risk of adhesions in the surrounding tissue. For this reason, the method is not presently being used clinically.

We have examined the effect of direct current electroacupuncture (EA), which is a type of physical therapy, on peripheral nerve regeneration[4 5] and bone healing.[6 7] Favourable results were obtained in both cases and progress is already being made towards the clinical application of EA for peripheral nerve regeneration.[5] Since EA has a reparative effect on peripheral nerve, bone and other tissue, it has the potential to be useful for tendon repair. When using EA, even for deep tissue, there is no need to embed the electrodes. Furthermore, the

area of stimulation can be localised. If the usefulness of EA for tendon repair can be verified, it could become a valuable complementary therapy that might solve some of the problems associated with existing methods of physical stimulation. In this study we used histological and mechanical assessment to examine the effect of EA on tendon repair in a rat model of Achilles tendon rupture.

It has been shown that tendons have the ability to repair themselves through proliferation of fibroblasts.[8] Platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF) and other growth factors accelerate the tendon repair process.[9] For histological assessment, we have focused on the expression of two growth factors: TGF-β1 and b-FGF. Both of these are considered important, especially in the early stage of the repair of the ruptured tendon.

METHODS

Experimental animals

This study was conducted with the approval of the Experimental Animal Ethics Committee of Meiji University of Integrative Medicine (authorisation no. 2010004). Except during examinations, experimental rats were allowed to eat, drink and move freely in a 12 h light/dark cycle environment. A total of 90 male Wistar rats (weighing 270–290 g) were used to prepare a model of Achilles tendon rupture. Rats were placed under general anaesthesia with pentobarbital (50 mg/kg) administered intraperitoneally. The right hind leg was used for model preparation in all rats. To prevent movement of the ankle, a 23G needle (Terumo Corporation, Japan) was inserted in full planter flexion from the tibial tuberosity through the bone marrow space up as far as the talus and calcaneus as a means of fixation. A longitudinal incision of about 10 mm was made in the skin of the posterior surface of the lower limb to expose the Achilles tendon. After separating the Achilles tendon and plantar muscle, a complete transverse incision was made in the tendon with a surgical knife at a point 5 mm from where the tendon is attached to the calcaneal bone. Under a stereomicroscope, with both cut ends of the Achilles tendon touching, the skin was sutured and the wound site was sterilised with povidone iodine solution. After preparing the Achilles rupture model, the rats were randomly assigned to one of three groups.

Experimental groups

Electroacupuncture group (n=30)

EA was performed after anaesthetising the rats with pentobarbital (50 mg/kg, 0.1 mL) administered intraperitoneally and restraining all four legs. Two acupuncture needles (30 mm in length, 0.25 mm in diameter; Seirin, Shizuoka, Japan) were inserted, with the tip of one touching the ruptured tendon on the outer side and the tip of the other touching it on the inner side. Intermittent direct current EA was performed using the electrode at the inner side as the cathode and the other as the anode. Using an electric stimulator (SEN-3301; Nihon Kohden, Tokyo, Japan) and isolator (SS-104J; Nihon Kohden), 5 ms square pulses of 50 Hz were delivered at 20 μA for 20 min. EA was performed every day from the day after preparation of the model until the day of assessment.

Manual acupuncture group (n=30)

After anaesthetising the rats with pentobarbital (50 mg/kg, 0.1 mL) administered intraperitoneally and restraining all four legs (as per the EA group), two acupuncture needles were inserted at the same locations, to the same depth and for the same period of time, but electrical stimulation was not performed. Like the EA group, the manual acupuncture (MA) procedure was performed every day from the day after model preparation until the day of assessment.

Control group (n=30)

From the day after model preparation, the rats were anaesthetised with pentobarbital (50 mg/kg, 0.1 mL) administered intraperitoneally every day until the day of assessment and all four legs were restrained for the same periods of time as for rats in the EA and MA groups.

Assessment

Histological evaluation

On days 7 and 10 after model preparation, 10 rats from each group were euthanised using an overdose of pentobarbital (50 mg/kg) administered intraperitoneally. A 6 mm long sample of repaired tendon was harvested from each rat and fixed in 20% formalin solution (4°C, 7 days) before being embedded in paraffin. Then, 4 μm serial longitudinal sections of the central part of the repaired area of the tendon were prepared and, following deparaffinisation, either stained with H&E or subjected to immunohistochemistry (see below) before being examined with a light microscope (Nikon Model Eclipse E600; Nikon Corporation, Japan) and photographed with a digital camera for microscopy (Nikon Digital Camera Dxm1200: Nikon Corporation, Japan). The images were stored on a PC pending quantitative evaluation of samples using image analysis software (Image J V.1.42: National Institutes of Health, USA).[10] H&E staining was used to count the total number of cells including inflammatory cells and fibroblasts within set regions of interest—namely, both ends of the repaired part of the tendon (0.43×0.34 mm^2). Immunohistochemical staining was used to count the number of cells positive for TGF-β1 and b-FGF in the same regions of interest. All cell counts were performed by an investigator who was kept blind to treatment allocation.

Immunohistochemical staining

The sections were immersed in 0.01 M citrate buffer solution (pH 6.0) and antigen activation was performed in an autoclave at 121°C for 15 min. The sections were then treated in 0.3% H_2O_2 solution for 5 min to block endogenous peroxidase activity, and washed in 0.05 M phosphate buffered saline (PBS; pH 7.6). Following treatment at room temperature with a protein-blocking agent for 5 min, anti-human TGF-β1 rabbit polyclonal antibody (#Y241: Yanaihara, Shizuoka, Japan) at a dilution of 1:200 and anti-human b-FGF rabbit polyclonal antibody (#SC-79: Santa Cruz Biotechnology, California, USA) at a dilution of 1:500 were used as the primary antibodies, and samples were left at a temperature of 4°C to react overnight. After washing with PBS, anti-rabbit horseradish peroxidase (HRP)-conjugated Envision antibodies (#K4003; Dako Cytomation, Glostrup, Denmark) were used as secondary antibodies and left to react for 30 min. The colour was developed using 3-3′-diaminobenzidine-4HCL (Dako Cytomation) and nuclear staining was performed with Mayer's haematoxylin.

Mechanical evaluation

Maximum breaking strength was measured by tension testing (EZ Graph; Shimadzu Corporation, Japan). On day 10 after model preparation, the remaining 10 rats from each group were euthanised. The repaired tendon, together with the triceps surae muscle and calcaneal bone, was harvested. Since the cross-sectional area of each of the tendons must be known when measuring maximum breaking strength, the anteroposterior and lateral diameters of the repaired part of the tendon were measured when the sample was taken. To fix the tendon in a clamp, centric muscle tissue from the repaired area was bluntly exfoliated from the tendon fibre. The sample was then covered in cloth soaked in saline solution and preserved at −20°C. On the day of testing, the sample was thawed to room temperature and the repaired tendon was fixed in a clamp and extended until rupture. Measurements were taken with a maximum load capacity of 100 N and a test speed of 0.05 mm/s.

Statistical analysis

All values are presented as mean±SD. One way analysis of variance was performed to compare differences between the three groups for all parameters, as well as differences between day 7 and day 10 for selected parameters (total cell counts after H&E staining and number of cells positive for TGF-β1 and b-FGF after immunohistochemical staining). Post hoc testing was performed using Bonferroni/Dunn multiple comparison tests where appropriate. Statistical analyses were performed using Statview V.4.5 (SAS Institute, Tokyo, Japan) and data were considered significant when the p value was <0.05.

RESULTS

Histological and immunohistochemical evaluation

The results from the quantitative analysis of total cell counts and the number of positive cells in H&E and immunostained sections, respectively, are shown in figure 1. Representative images of staining within each group are shown in figure 2.

Following H&E staining, a large number of cells resembling fusiform fibroblasts were observed near the ruptured part of the tendon 7 days after model preparation in all groups (figure 2A). Total cell count was significantly greater in the EA group at day 7 compared with the other two groups (figure 1A). On day 10 after model preparation, obscure tissue images of the rupture site were observed in all groups (figure 2A) and the total cell count remained significantly greater in the EA group than in the other two groups (figure 1B). No significant differences in total cell count were observed between the MA and control groups at day 7 or day 10.

As shown in figures 1C, D and 2B, a greater number of TGF-β1 positive cells were observed in the EA group at day 7 after model preparation than at day 10 (54.9±35.3 vs 25.4±13.2, $p<0.05$). There were no such differences in staining between day 7 and day 10 in the other groups. On day 7 after model preparation, a significant increase in the number of TGF-β1 positive cells was observed in the EA group compared with the control group only (figure 1C). At day 10 there were no significant differences between any groups.

No significant differences in the b-FGF positive cell counts were observed at day 7 vs day 10 after model preparation in any of the three groups; however, there were significantly more b-FGF positive cells in the EA group than the other two groups on both day 7 and day 10 (figures 1E, F and 2C). No significant differences between the MA and control groups were observed at either time point.

Mechanical evaluation

The maximum breaking strength, as determined by tension tests, and the cross-sectional area of the repaired part of the tendon on day 10 after model preparation are shown in figure 3. Maximum breaking strength in the EA group was significantly higher than in the other two groups ($p<0.01$ and $p<0.001$ vs control and MA groups, respectively). The values for the MA and control groups were not significantly different. The cross-sectional area calculated from the anteroposterior diameter and lateral diameter of the repaired part of the tendon measured at the time of the tension test did not differ significantly between groups.

DISCUSSION

In general, the tendon repair process can be divided into inflammatory, proliferative and remodelling phases. In this study, the days selected for assessment

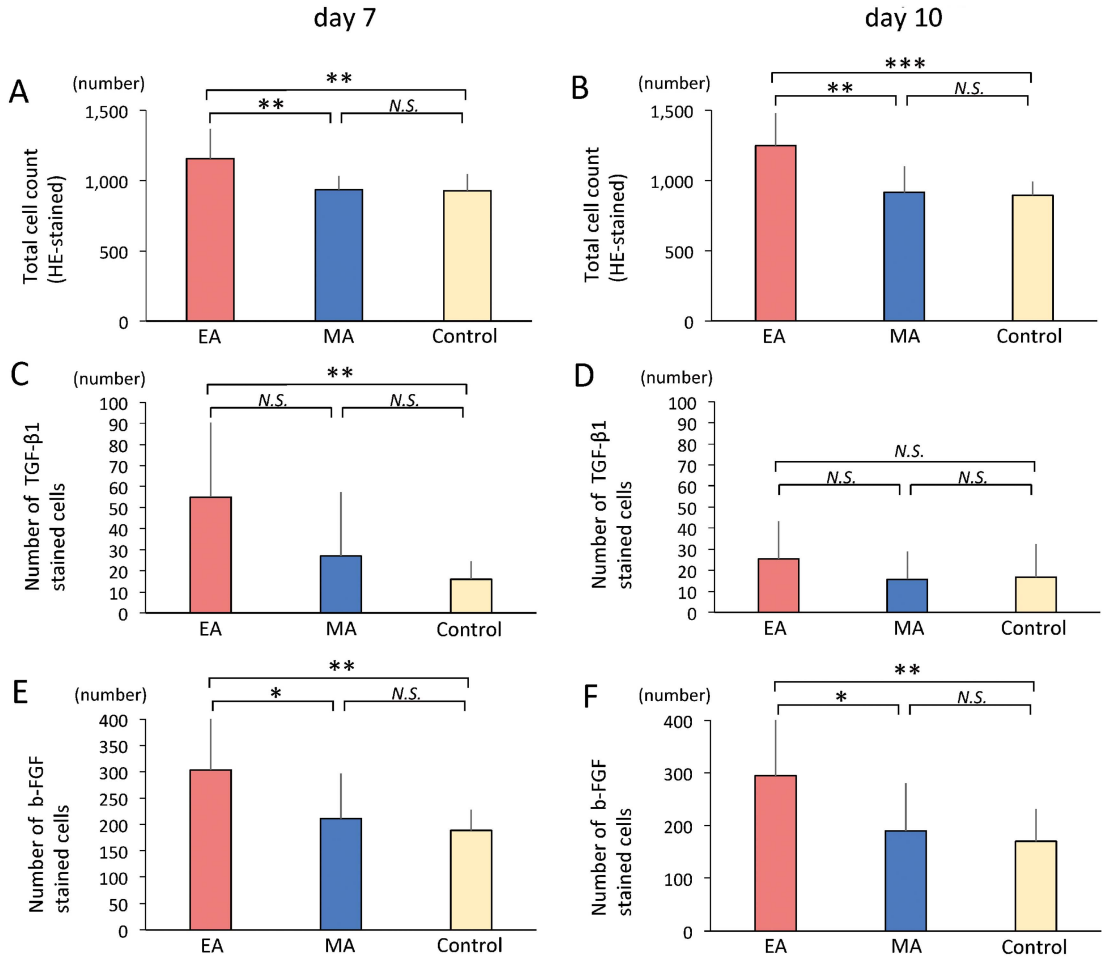

Figure 1 Total cell counts in H&E stained sections (A, B) and numbers of cells positive for transforming growth factor-β1 (TGF-β1) (C, D) and basic fibroblast growth factor (b-FGF) (E, F) assessed by immunohistochemistry in the Achilles tendon of rats 7 and 10 days following tendon rupture (n=10 per group at each time point). Rats were left untreated (control group) or received treatment with manual acupuncture (MA group) or electroacupuncture (EA group). Data are expressed as mean±SD. ***p<0.001, **p<0.01, *p<0.05. NS, not significant.

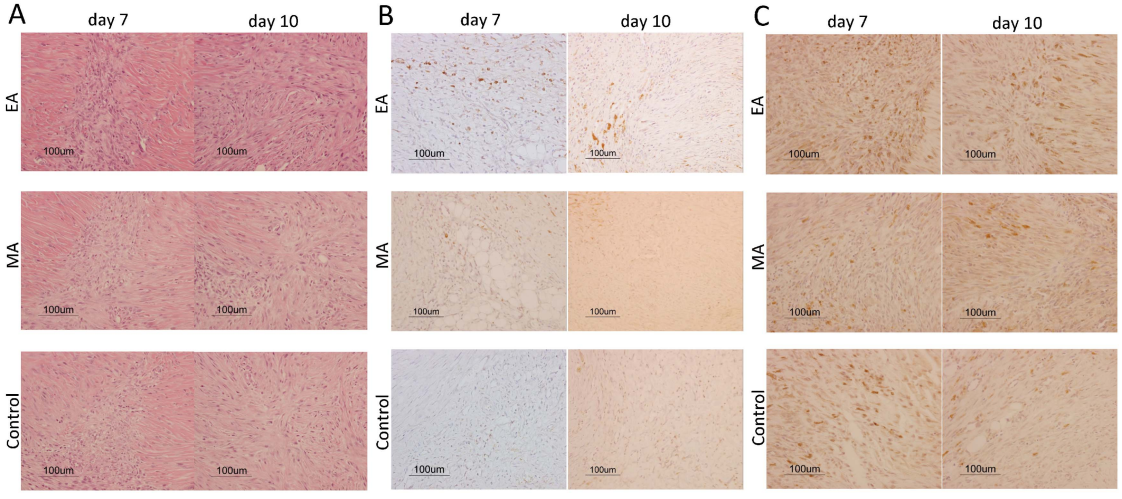

Figure 2 Typical images from (A) H&E stained, (B) transforming growth factor-β1 (TGF-β1) immunostained and (C) basic fibroblast growth factor (b-FGF) immunostained sections of rat Achilles tendon at day 7 and day 10 following tendon rupture. Rats were left untreated (control group) or received treatment with manual acupuncture (MA group) or electroacupuncture (EA group). The junction of the ruptured tendon forms the centre of each picture.

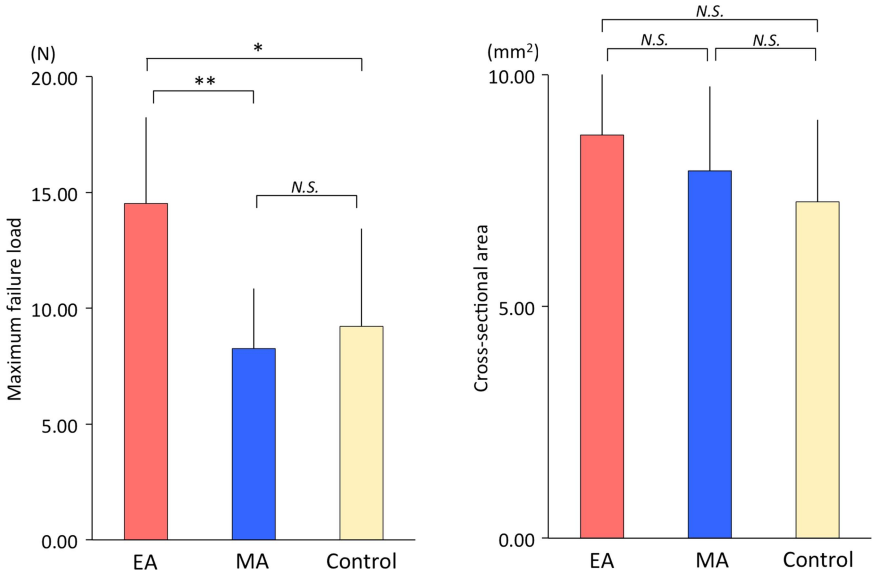

Figure 3 Maximum breaking strength (as measured by tension testing) and cross-sectional area of the Achilles tendon of rats (n=10 per group) 10 days following tendon rupture. Data are expressed as mean±SD. **p<0.001, *p<0.01. NS, not significant.

were 7 and 10 days after model preparation. Both of these time points fall into the latter half of the inflammatory phase and the first half of the proliferative phase of the tendon repair process. In H&E-stained tissue samples, fibroblasts were observed together with inflammatory cell infiltrates. Since fibroblasts near the tendon stumps were fusiform, it may be inferred that, on both day 7 and day 10 after model preparation, fibroblasts were in a state of activity. Furthermore, the number of cells in the EA group had increased significantly more than in the other groups on both assessment days, suggesting that EA promotes cell activity. Since the activated fibroblasts are considered to be TGF-β1 or b-FGF positive cells, the total cell counts may include those positive cells.

Early in the tendon repair process, and particularly during the inflammatory phase, TGF-β1, which is produced and released from inflammatory cells invading injured tissue, is at its highest level of expression. In the subsequent proliferative phase, TGF-β1 is produced by fibroblasts and other cells and so continues to be expressed to some degree.[11] Since the level of expression of TGF-β1 on day 7 after model preparation had increased, it is possible that, during the early stages of the repair process when TGF-β1 is expressed, EA promoted that expression. It has been established that b-FGF, together with TGF-β1, PDGF and VEGF, promotes the migration, differentiation and proliferation of fibroblasts and vascular endothelial cells during the proliferative phase, leading to the production of collagen and vascularisation.[12] On both day 7 and day 10 after model preparation, a significantly increased level of b-FGF expression was seen in the EA group. By contrast, no significant differences in the expression of b-FGF on day 7 or day 10 after model preparation were observed in either of the other groups. This suggests that EA has an action in the early stage of b-FGF expression and may promote its continued expression. Fibroblasts, which proliferate and become active due to the action of TGF-β1, b-FGF and other growth factors, have a complex role in the production and composition of collagen and other extracellular matrices.[11 12] Tendon strength is determined by collagen configuration and density. In this study, tension tests showed that maximum breaking strength had significantly increased in the EA group in the absence of any change in cross-sectional area of the repaired portion of the tendon. Taking these results together, it is possible that EA has an action that promotes the expression of growth factors that activate fibroblasts, which are beneficial for the production and composition of collagen, resulting in an increase in the mechanical strength of repaired tendon. There is a report that supports the hypothesis that EA increases the production of collagen, although there are dissimilarities in the site of the stimulation and electrical parameters.[13]

There was no significant difference in tendon repair between the MA and control groups, which indicates that MA had no major effect on acceleration of the healing process within the tendon. Although there are several reports regarding the effect of EA on tendon repair, none of them used EA on the ruptured tendon itself.[13 14] In the present study we applied EA stimulation to the ruptured tendon with the expectation that the stimulation would have similar effects to those seen in our previous studies.[4–7] For the electrical stimulation, two acupuncture needles, acting as electrodes delivering a direct electric current, were in contact with the injured part of the tendon. However, because of the extremely small distance between the two electrodes, it is not clear whether the promotion

of tendon repair in this study was the result of EA-induced changes in blood flow at the injury site or the effect of electrical polarity, as has been shown in the case of peripheral nerve regeneration.[4 5] It has been reported that EA at the Achilles tendon of normal rats increases tendon blood flow,[15] and this is the result of electrical stimulation, regardless of whether it is an alternating or direct current. Electrical polarity has a clear effect on peripheral nerve regeneration and bone union[6 7] and has also been shown to have characteristic effects on soft tissue.[16 17] Consequently, determination of the most appropriate stimulation conditions is required.

Clinical application of this method of stimulation would provide the following advantages. With EA, it is not necessary to embed or surgically remove the electrodes. It can also be used regardless of the surgical treatment or conservative treatment selected. Furthermore, taking into consideration the results of this study, EA might be expected to have a similar effect on ligaments, which are a similar tissue to tendons. Since ligaments are located deep in the body, adherence of surrounding tissue and energy attenuation when using PEMFs and other stimulations applied to the surface of the body are even bigger concerns than with tendons. In cases such as this, using acupuncture needles that can be inserted to any depth could provide an effective treatment with physical stimulation.

This study has shown that EA causes an increase in the positive cell counts of growth factors required for tendon repair and increases tendon strength over a relatively short time period following rupture of the tendon, suggesting that it could be a useful complementary therapy for tendon repair. Acquisition of mechanical strength in the early stage of tendon repair might enable weight bearing and exercise at an early stage following rupture. It may also help to prevent muscle atrophy and functional disorders due to a restricted range of motion. If it can be confirmed that EA increases tendon strength after long-term follow-up, it might also prevent re-rupture after repair. We aim to further examine the effects of EA at an earlier stage after tendon rupture and its effects after long-term follow-up, as well as its potential for clinical application.

CONCLUSIONS

Our key findings were that the application of EA to a tendon rupture model increased total cell counts, TGF-β1 and b-FGF positive cell counts, and also the mechanical strength of repaired tendon compared with control groups receiving MA or no treatment. These results suggest that direct current EA promotes Achilles tendon repair and could be an effective complementary treatment for tendon rupture.

Summary points

- We examined the effects of electroacupuncture (EA) and manual acupuncture compared with no treatment on early post-rupture tendon repair in 90 Wistar rats subjected to surgical Achilles tendon injury.
- Maximum breaking strength, total cell counts and the number of cells staining positive for transforming growth factor-β1 and basic fibroblast growth factor were significantly higher in the EA group.
- EA may have a role in the promotion of tendon repair.

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Contributors MI: Study design, conducted research, data analysis and wrote the manuscript. MN and YO: Conducted research and acquisition of data. TH: Analysis and interpretation of data. MI: Data analysis and supervision of the study. HK: Critical revision of the article for important intellectual content and overall control.

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