ORIGINAL ARTICLE

Microcurrent Neuromuscular Electrical Stimulation Helps to Promote Fibroblast and Capillary Formation in the Early Healing Phase of Achilles Tendon Rupture: An Experimental Study on Animal Model

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ABSTRACT

Introduction: Rehabilitation plays a critical role in the treatment of ruptured Achilles tendons. Therapeutic interventions such as neuromuscular electrical stimulation (NMES) could help to enhance tissue healing. There are some controversies on the amount of current needed to optimize the healing process in tendon. There is still limited study on the effect of NMES in Achilles tendon healing, and there has been no study to compare the effect of two different current intensities in Achilles tendon healing. This study aims to compare the effect of microcurrent NMES and motor-level NMES on the early healing phase of the ruptured Achilles tendon model by evaluating fibroblast and capillary formation. **Methods:** Model ruptures were created in the right Achilles tendon of thirty New Zealand white rabbits and randomly divided into three groups: (1) control; (2) treatment with microcurrent NMES; and (3) treatment with motor-level NMES group. After two and four weeks, the tendons were histologically evaluated for fibroblast and capillary formation. **Results:** The microcurrent NMES group demonstrated the highest mean value of fibroblasts count in week 2 and 4, as well as capillary formation in week 2 compared to the other groups. No difference was observed in capillary formation after four weeks. **Conclusion:** Microcurrent NMES could help to facilitate the early healing phase of Achilles tendon rupture.

Keywords: Achilles tendon, Capillaries, Electric stimulation, Fibroblast, Rupture

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INTRODUCTION

INTRODUCTION
Ruptures of the Achilles tendon, being the largest and strongest tendon, may result in devastating and unpredictable outcomes (1). In recent decades, studies showed that acute and chronic ruptures are increasingly common, being responsible for as much as 50% of all sports-related injuries (2–4). Despite the intrinsic capacity of spontaneous healing in ruptured tendons, the exact mechanisms of tendon healing are still not completely understood (5). Our uncomprehending understanding of tendon healing biology hinders us from achieving a clear consensus on the optimal treatment option for Achilles tendon ruptures (2,5). Several meta-analyses of randomized control trials showed that surgical repair significantly reduces the risk of re-rupture, despite higher complication rates such as deep vein thrombosis, nerve injury, and infection (6,7). Nevertheless, no significant differences were found between the surgical and non-surgical groups in the physical activity scale, ankle range of motion, and the number who returned to sport (6,8).

Studies have demonstrated that rehabilitation plays a critical role in ruptured Achilles tendons treatment (9). Unfortunately, the healing capacity is limited by its slow rate of healing; hence it requires prolonged rehabilitation in most cases (5,10). Therefore, to facilitate the healing process, it is important to introduce therapeutic

interventions. It is reported that neuromuscular electrical stimulation (NMES) plays a significant role in enhancing tissue healing (10,11). There are some controversies on the amount of current needed to optimize the healing process. Some studies showed that microcurrent NMES between 1 to 1000 microampere (μA) is effective to facilitate the process (10,12). On the contrary, the more commonly practiced higher current intensity, also known as motor-level current, is used to produce functional isometric contraction to serve as mechanical stimulation to improve the tendon healing.(13,14) Higher current is needed to overcome the impedance of skin and subcutaneous fat before reaching deeper tissues.(15)

To our best knowledge, there is still limited study on the effect of NMES in Achilles tendon healing, and there has been no study to compare the effect of two different current intensities in Achilles tendon healing. This study aims to compare the effect of microcurrent NMES and motor-level NMES on the early healing phase of the ruptured Achilles tendon model by evaluating fibroblast and capillary formation.

MATERIALS AND METHODS

Study Design

A randomized posttest-only control group study was done using New Zealand White Rabbits (*Oryctolagus cuniculus*). The protocol had been approved by the Animal Care and Use Committee, Universitas Airlangga prior to the study (certificate number: 2.KE.018.01.2018). Using the formula to calculate sample size, thirty male New Zealand White Rabbits (*Oryctolagus cuniculus*), 6-month-old, with an average weight of 2500 grams ± 100 grams, were used in this study. All rabbits were randomly and equally divided into three groups: (1) control group (group 1); (2) treatment group of microcurrent NMES (group 2); and (3) treatment group of motor-level NMES (group 3). There were ten rabbits in each group. Each group would be evaluated twice: in the 2nd and 4th week, as this would be sufficient to represent the early healing phase of rabbit tendon(16,17). In each rabbit, a model injury was created on the Achilles tendon of the right hind limb. All rabbits were housed in the animal care laboratory and were well-taken care according to the National Institute of Health standards. The rabbits were housed individually in a separate cage (100 x 60 x 75 cm) with environmental conditions: temperature of 21 C ± 2 C, the humidity of 60% ± 10%, the lighting of 350 lux a dark-light cycle of 12:12. All rabbits were given access to regular and scheduled feeding and water ad libitum. During housing, animals were monitored three times daily for health status. No adverse events were observed.

Surgical Procedure

All rabbits in all groups received a similar surgical hemi-transection injury to the right Achilles tendon of the hind limb to create a partial tendon rupture model. All surgical procedures were carried out under sterile conditions and general anesthesia using a combination of intramuscular injection of ketamine hydrochloride (35 mg/kg body weight) and xylazine hydrochloride (5 mg/kg body weight). Before the surgery, the hair was removed from the surgical site at the posteromedial side of the right hind limb. The animal was put on the surgical table in a side-lying position. The surgical field was disinfected and draped. A three-centimeter straight incision was made on the medial to Achilles tendon, extending from just above the heel to the middle of the leg. The Achilles tendon was exposed and freed from the surrounding tissue. To standardize the injury model, the posterior half of the Achilles tendon was sharply hemi-transected about one centimeter above the calcaneal insertion (see Fig 1a). The anterior half of the tendon was left intact to simulate a partial tendon rupture and prevent retraction of the transected ends. The posterior half of the hemi-transected Achilles tendon was left unsutured (10, 18, 19). Two non-absorbable sutures were placed proximally and distally to the tenotomy site (see Fig 1b) for making it easier to identify during harvesting. The skin wound was then sutured using 3.0 absorbable monofilament. Afterward, the hind limb was immobilized with Plaster of Paris (POP) back slab (applied from the mid-thigh to the foot and secured with adhesive tape) with the knee in flexion and ankle in 45° of plantar flexion as splinting to reduce movement. The immobilization was retained for six days and removed only for wound dressing and treatment procedures. Postoperative analgesic (carprofen, 1.5 mg/kg, twice a

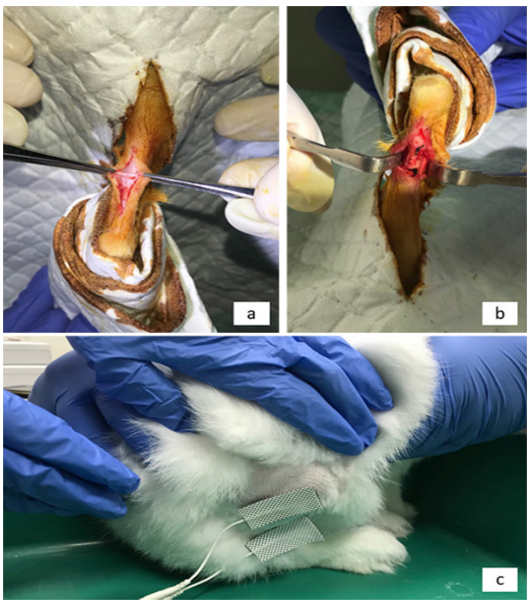


Fig 1. Surgical procedure and neuromuscular electrical stimulation application.
(a) Hemitransection of the posterior half of the Achilles tendon to simulate partial tendon rupture;
(b) Placement of non-absorbable suture as markings to facilitate easy identification during harvesting;
(c) Placement of anode and cathode electrodes

day) and antibiotic (enrofloxacin, 10 mg/kg, twice a day) were given orally with a dropper for two days. The back slab would limit the hind limb movement to minimize interference to the tendon healing process, but the general activity and movement of the rabbits within the cages were still permitted to ensure the rabbit's well-being. All wounds were inspected to observe any surgical site infection during wound dressing change every two days. No suture removal was required since the wound was sutured using absorbable monofilament. No wound complications such as infection or dehiscence were observed in any rabbit.

Neuromuscular Electrical Stimulation Application

The treatment started on the first-day post-surgery until the end of four weeks (28 days). Group 1 received no electrical stimulation. Group 2 and 3 received the regiments of 6 sessions/week daily, except on Sunday. Each rabbit was positioned relaxed on its side after being sedated with intramuscular injection of ketamine hydrochloride (35 mg/kg body weight). Before treatment, the skin on the area of electrode placement sites was prepared and cleaned from any hair. Two disposable electrodes were used. The anode was placed on the lateral to Achilles tendon The cathode was placed proximally on the gastrocnemius muscle belly, approximately one centimeter apart (see Fig 1c). (10,12,20) An Endomed 482 electric stimulator (Enraf-Nonius, Rotterdam, The Netherland) was used for the treatment. The device was calibrated using EZ Digital 60Mhz Analog Oscilloscope OS 5060A (EZ Digital Co. Ltd., Korea). The microcurrent NMES group received the stimulation as the following: intensity 100 μA/cm^2, pulse frequency 10 Hz, pulse width 50 ms, voltage 2.5 V with a duration of 30 minutes.(10,12,21,22) The motor-level NMES group received similar electrical stimulus properties except for the intensity. The rabbits were given the initial intensity of 1 mA to determine the motor-level current intensity. No gastrocnemius muscle contraction was observed at this current intensity level. The intensity was progressively increased by 0.5 mA until gastrocnemius muscle contraction was observed. The motor-level intensity was determined as the current intensity that sufficiently caused visible and palpable gastrocnemius muscle contraction without excessive ankle joint movement.(23–25) Based on that, we found the intensity of 4 mA/cm^2 causes just enough gastrocnemius muscle contraction without causing restlessness to the rabbits. Hence, the motor-level NMES group was given the following stimulation: 4 mA/cm^2, pulse frequency 10 Hz, pulse width 50 ms, voltage 2.5 V with a duration of 30 minutes.

Histological Evaluation

Since the evaluation was carried out in weeks two and four, five rabbits from each group were sacrificed after two and four weeks. After the designated time, the samples were collected from the hind limb and processed for histological examination. Three equidistant longitudinal sections of 10 μm for each rabbit were stained with hematoxylin-eosin (HE). Ten fields were randomly chosen for each stained section. In each section, the number of fibroblasts and the capillaries were evaluated under a light microscope (Nikon H600Lm with digital camera DS Fi2 200 mp and Nikon Image System Software). The average number of both fibroblast and the capillaries were calculated after evaluating in ten different fields (magnification of 1000x). Under the microscope and HE stain, fibroblasts are purplish-blue spindle-shaped cells. The new capillary formation was observed by counting the number of lumina of vessels in each field.(20,21,26) All examination was done in a blinded manner by two different observers.

Statistical Analysis

The data collected were analyzed using the Shapiro-Wilk test which verified the normal distribution of the data sets. After that, a comparison between groups was performed using the ANOVA test. All significant results from ANOVA ($p<0.05$) were analyzed further using the Bonferroni post-hoc test. All analyses were performed using the statistical software package SPSS version 21. A p value of 0.05 was considered significant. All data are presented as mean ± SD with 95% confidence intervals.

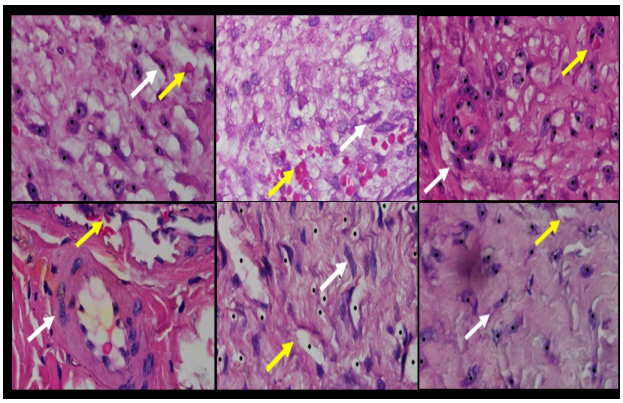
RESULTS

Figure 2 demonstrated the histological examination of all groups. The number of fibroblasts and the capillary count is presented in table I. The microcurrent NMES group demonstrated the highest mean value of fibroblasts count in week 2 and week 4 compared to the other groups (p=0.004 and 0.018, respectively). Furthermore, post-hoc analysis of the number of fibroblasts between the microcurrent NMES and control group showed significant differences in week 2 and week 4 (p=0.004 and 0.009, respectively). No difference was found between the motor-level NMES group and the control group in post-hoc analysis.

Table I. The number of fibroblasts and capillary count after two and four weeks

		Control-Mean±SD	Microcurrent NMES Mean±SD	Motor-level NMES Mean±SD	*p*
Fibroblast	Week 2	143.2±30.79	250.80±42.36	195.20±46.71	0.004
	Week 4	123±53.72	287±49.51	194±65.01	0.018
Capillary	Week 2	144.2±56.58	343.4±66.31	173.8±89.52	0.002
	Week 4	107±47.66	129.2±5.11	122.8±34.21	0.583

NMES, Neuromuscular electrical stimulation; SD, Standard deviation



Fig] 2. Histological examination of the fibroblast (*white arrow*) and the capillary (yellow arrow) under a microscope (HE, 1000x). HE: Histological examination

Microcurrent NMES group only showed the highest number of capillary counts in week 2 (p=0.002), with a significant difference in post-hoc analysis between the group and the control group. (p=0,003). No difference was demonstrated in the number of capillary counts in week 4 among the groups. Post-hoc analysis to compare between microcurrent NMES and motor-level NEMS showed that there were significant different in the number of fibroblasts in week 2 and week 4 (p=0.004 and 0.003, respectively), and the capillary count in week 2 (p=0.009). No significant difference was found between the microcurrent and motor-level NMES groups in the capillary count in week 4. Compared to higher intensity motor-level neuromuscular stimulation, microcurrent neuromuscular electrical stimulation promotes higher fibroblast and new capillary formation in the early healing phase of ruptured tendon healing.

DISCUSSION

This study evaluates the early healing process in tendon. The early healing process in soft tissues involves the initial inflammation followed by proliferation phase. These two phases occur in the first four weeks of the overall healing process as shown by the previous studies. Evaluation of the fibroblast and capillary count in week two and four would most probably provide the necessary information on the healing process (19,20). The most important finding in this study showed that microcurrent NMES promotes higher fibroblast and new capillary formation in the early phase of tendon healing. The early phase of tendon healing involves cell proliferation. Neuromuscular electrical stimulation (NMES) has been considered as a treatment option to optimize soft tissue healing. Several studies on the healing of wounds, muscle, tendons, and ligaments have shown promising results. (10–12,27,28) Although the exact mechanism is still unclear, previously reported studies demonstrated that microcurrent NMES help to promote soft tissue healing by increasing ATP production and amino acid uptake, enhancing active secretion of tenocytes, and facilitating collagen synthesis. These might explain the underlying mechanism of microcurrent NMES in soft tissue healing.(10,22,27,29) Cheng et al demonstrated that the bio-stimulatory effects of NMES occurred in a microcurrent range of 10 to 500 μA.(27) The healing effects of this microcurrent NMES were also supported by two other studies on rat skin and tenotomized rat Achilles tendon.(10,22,30)

In contrary to the microcurrent intensity explained earlier, NMES is more commonly given in higher current intensity level, also known as motor-level intensity, to create functional muscle contraction.(24) This is based on the fact that muscles and tendons work as an integrated unit, and there is clear evidence that they depend on each other to develop correctly. The literature on how mechanical stimulation by muscle force plays an important role in tendon development and repair is still scarce. One of the theories from a study by Subramanian et al demonstrated that muscle contraction might contribute to tendon repair by inducing tenocytes. Tenocytes are tendon precursor cells. The study showed that the contractile force produced by muscle regulates a TGF-β signaling pathway in the tenocytes. It is believed that this pathway is responsible to shape the tenocytes, and thus controlling how the cells grow and produce necessary tendon matrix proteins for healing.(31–33)

In this study, microcurrent NMES is more effective than motor-level NMES in promoting fibroblast and new capillary formation in the early phase of tendon healing. The early phase of tendon healing involves cell proliferation. Application of microcurrent electrical stimulation might promote activation of tenocytes that will subsequently promote fibroblast proliferation and collagen synthesis as shown by previous studies. (10,20,27) Another possible way of how microcurrent electrical stimulation might contribute to the healing is that the microcurrent electrical stimulation can increase membrane transport, organization of collagen, contraction of wounds, and stimulation of DNA and protein synthesis during the proliferation phase thus accelerating fibroblast growth.(34) Araujo et al demonstrated that high-intensity electrical stimulation might increase the tension in the injury site, resulting in damage to healing tissue and collagen formation. This might be the reason why the increase in fibroblast and capillary formation in the motor-level NMES group was insignificant.(12,20) Electrical stimulation was also reported to promote the regulation of vascular endothelial growth factor (VEGF). A previous study reported that the VEGF gene expression increase after the application of low-intensity electrical stimulation during the proliferation phase. This would help to promote the new capillary formation.(1,35) No difference was observed in capillary formation after four weeks. This might be due to the reduced number of capillaries formation at the end of the early phase since the proliferation rate might have slowed down.(20)

To our knowledge, the study on how NMES would affect the healing of injured tendons is still limited. This study is the first to compare the effects of microcurrent NMES and motor-level NMES on the early phase of tendon healing. Therefore, these findings may have clinical implications on the application of microcurrent NMES in the early phase for treating tendon injury, especially in partial Achilles tendon rupture. However, this study has several limitations. Firstly, the model used was partially ruptured Achilles tendon instead of that of complete rupture. The reason is that we aim to evaluate the early healing phase. Therefore, a partial tendon rupture model is sufficient to demonstrate the healing process involving fibroblast and capillary formation. Secondly, we only aim to observe fibroblast and capillary formation to represent the early phase of tendon healing. To fully understand the healing process, longer evaluation involving the characteristic of collagen formation and protein secretion would demonstrate how NMES would affect not only the early phase but also the later phase of the healing process. Furthermore, the biomechanical study could also provide additional information on the final functional outcome. Thirdly, we only carried out histological evaluation of the fibroblast and capillary number. Immunohistochemical analysis of the expressed proteins during fibroblast proliferation and new vessel formation would provide more quantitative measurement and biomolecular description to evaluate the healing process. Therefore, further evaluation of the possible biomolecular properties involved in tendon healing would provide valuable clues on the exact mechanism of how neuromuscular electrical stimulation could contribute to tendon healing.

CONCLUSION

Compared to the motor-level NMES, application of microcurrent NMES yields higher fibroblast count in week two and four, and capillary counts in week two. The microcurrent NMES could be an option to facilitate the early healing phase of Achilles tendon rupture.

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