Effects of laser, ultrasound and electrical stimulation on the repair of achilles tendon injuries in rats: a comparative study

Araújo, RC.[1]*, Franciulli, PM.[1], Assis, RO.[1], Souza, RR.[1] and Mochizuki, L.[2]

[1]São Judas Tadeu University – USJT, Department of Physical Therapy, 546, CEP 03166-000, Taquari, SP, Brasil
[2]University of São Paulo – USP
*E-mail: prof.raraujo@usjt.br

Abstract

The study was established to compare the effects of laser, ultrasound and electrical stimulation on the healing of the Achilles tendon injury. Twenty-eight rats underwent suture repair of a tenotomy of the left Achilles tendon. The animals were randomly separated into four groups of seven animals each as follows: control group, laser group, ultrasound group, and electrical stimulation group. Following sacrifice at 14th, 23rd and 34th days, postoperatively, the retrieved tendons were quantitatively evaluated by histological techniques. The increase in the number of capillaries in the 14th day after injury and the increase of the number of fibroblasts in the 23rd day after injury suggest that the electrical stimulation accelerates collagen synthesis. At the early stages of tissue repair and considering the doses which were used, the electrical stimulation showed more increase in capillaries and fibroblasts number than the other agents for the tendon tissue healing.

Keywords: capillaries, fibroblasts, lasers, ultrasonics, electric stimulation therapy.

1 Introduction

Tendon healing of acute injuries occurs in three stages: inflammation, proliferation, and remodeling (GROSS, 1992). During the first stage, fibroblasts migrate to the injured site. In the second one, the proliferative stage, fibroblasts increase in number and synthesize collagen. The last step involves cell and capillary number reduction and collagen fibers realignment. So, we access the healing stages through the measurement of the number of capillaries and fibroblasts, since that both are increased in the early stages of the process and decreased along the remodeling phase.

Connective tissue injuries, specifically tendon sprain, may require long-term rehabilitation. Although the early soft tissue healing phase requires seven to ten days, complete tendon healing can take several weeks or months (ENWEMEKA, 1989). One of main topics about rehabilitation is how to improve the injured connective tissue healing process (ENWEMEKA, 1989). In general, the wound repair process is complex (HUNT, 1979) and scars are often found at the end of the process. To avoid such problems, different techniques are applied during rehabilitation. The use of physical agents and procedures to assist the tendon injuries healing were studied on numerous occasions (OWOEYE and SPIELHOLZ, 1987; WILLIANS, 1984).

Several investigators have reported a beneficial effect from the connective tissue healing process acceleration by low-intensity laser (BECKERMAN, BIE, BOUTER et al., 1992; BLIDDAL, HELLESEN, DITLEVSEN et al., 1987; VECCHIO, CAVE, KING et al., 1993), therapeutic ultrasound (GUM, REDDY, STEHNO-BITTEL et al., 1997; SPEED, 1992) and electrical stimulation (NESSLER and MASS, 1987; REICH and TARJAN, 1990). As well, low level laser therapy applied on injured tissues improves skin regeneration, due to improvement of epithelial cells mitotic activity shows better capillary density distribution, and more granulation tissue (MESTER and YÁSZAGI-NAGY, 1973; MESTER, NAGYLUCSKAY, TISZA et al., 1977; MESTER and YÁSZAGI-NAGY, 1973; MESTER, 1976; MESTER, 1981).

About the effect of ultrasound therapy on wound healing, it was reported an increased rate in the Achilles tendon repair (JACKSON, SCHWANE and STARCHER, 1991). Byl et al. (1992) reported that such repairement issue and the ultrasound effectiveness is still controversy.

Carley and Winapel (1985) compared low intensity direct current to conventional wound therapy, (gauze dressings). Wounds under electrical stimulation healed twice as fast as those in the control group. Although these studies contributed for the understanding of the effect of each one of these therapeutic agents, we have not found investigations about different methods effect under the same methodological procedure.

The purpose of this study was to determine whether there is any difference among therapeutic ultrasonics (TUS), electrical stimulation for tissue repair (ESTR) and low level laser therapy (LLLT) effects on the tendon healing. If one of these agents induces capillaries and fibroblasts at the early stage and decreases of them at the late stage, we can propose that this agent can accelerate the healing process. And so, this agent would have a better prescription than other agents.

2 Material and methods

2.1 Animals

Twenty-eight female Wistar rats (three months old, 0.250-0.300 kg weight) were kept in a vivarium. They were

randomly distributed in 4 groups: (control, LLLT, TUS and ESTR) and placed apart in differents cages. Temperature (22 °C) and the ratio of daylight hours to non daylight hours (12/12 hour light/dark) were constant. Animals remained in their cages, but during treatment. Food and water were available *ad libitum*.

2.2 Surgical procedures

This study was submitted and approved by the Research Ethics Committee. The animals were anesthetized with Sodium Phenobarbital (30 mg/50 mL) via IM injection.

After shaving and cleaning the skin nearby, the left Achilles tendon of each rat was freed from surrounding tissue, sharply and transversely lacerated midway between its calcaneal insertion and the musculotendinous junction. Then, the severed extremities of the tendon were joined and sutured with two loops of 2.0 non-absorbable suture. After surgery, the ankle joint was immobilized at approximately 90° with aluminum plate and adhesives tapes to reduce the tension at the repair site. The immobilization was used during three weeks following the surgery, and it was removed only for treatment procedures.

2.3 Treatment

The LLLT group was treated with 4 J.cm^{-2} energy density during 90 seconds in one unique point on the incision scar.

The TUS group was submitted to 1 MHz frequency, 1 W.cm^{-2} intensity, 100 Hz pulsed frequency by 50% duty cycle, during 60 seconds. Since the TUS treated area was very small, considering the animal's limb size and the incision size (1 cm), it was used a latex glove full of water in order to improve the sound transmission. Then, the scar surface was ointed with a hydro soluble gel to improve ultrasound transmission (KITCHEN, 2002).

The ESTR intensity was established at the sensorial level: the current intensity was previously set (pilot study performed in a healthy animal) and it was progressively increased up to motor level (4 mA, t = 100 µs, F = 50 Hz, pulsed biphasic symmetric rectangular current). So, we decided to use 2 mA intensity because a motor level stimulation would provoke tension in the wound, causing damage on the scar tissue formation. This group was submitted to 10 minutes treatment.

The Figure 1 illustrates the LLLT, TUS and ESTR treatment conditions.

The treatments were applied at the same day, five sessions/week, for five weeks. Healed tendon samples were collected at the 14th, 23rd and 34th days after injury (9, 16 and 24 sessions, respectively). To extract those samples, two rats of each group were sacrificed at 14th and 23rd. At the 34th day after injury, three rats of each group were sacrificed.

The Table 1 sumarizes the doses that were used for each agent in this study, and they were choose to reproduce the doses that are usually adopted in the clinical practice (KITCHEN, 2002).

2.4 Histological analysis

The animals were killed by ether ethylic inhalation. Then, the tendon suture site was localized, removed, placed in *Bouin's* fixative solution for 6 hours and embedded in paraffin. Three equidistant longitudinal sections of 10 µm for each animal were stained with hematoxylin and eosin (HE) (JUNQUEIRA, BIGNOLAS and BRETANI, 1979). Under light microscopy (1000x), 12 fields were randomly chosen for each stained section (Figure 2). In these three stained sections the numbers of capillaries and fibroblasts nuclei were counted for each field. Then, the means ± standard error of means was calculated for each group. So, the measurements were made in the following samples: 28 rats, 4 groups, 3 post injury dates (14th, 23rd and 34th days), 3 equidistant histological sections of each tendon and 12 fields for each section. This procedure allowed us to analyze the granulation and fibroblastic stages of the scar tissue formation. Three

Table 1. Characteristics of the therapeutic agents used in animals groups.

Group	Doses
Laser AsGa	Energy density 4 J.cm^{-2}, t = 90 seconds
Ultrasound - 1 MHz	Peak intensity 8 W = 1,0 W.cm^{-2}, pulsed at 50%, Freq = 100 Hz, t = 60 seconds
Electrical stimulation	T = 100 µs, freq = 50 Hz, I = 2 mA, t = 10 minutes

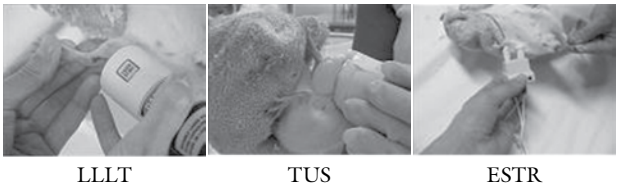

Figure 1. Illustrative pictures of the Low Level Laser Therapy (LLLT), Therapeutic Ultrasound (TUS) and Electrical Stimulation for Tissue Repair (ESTR) application conditions.

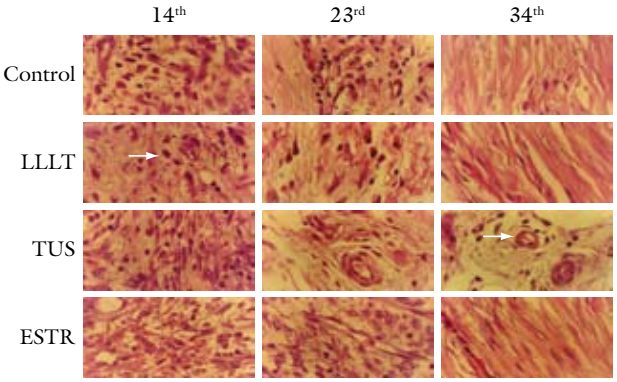

Figure 2. Light photomicrographies illustrating the histological sections of the tendon stained with hematoxylin-eosin. The results are presented for different groups (control, low level laser therapy - LLLT, therapeutic ultrasound - TUS and electrical stimulation for tissue repair - ESTR, from top to bottom) and for different phase (14th, 23rd and 34th post injury days). Note the decrease in fibroblast numbers along the days in all groups. Also, note that fibroblast number for the ESTR group, are higher at the 14th day and lower at the 34th day. The white arrow shows one fibroblast and the black arrow shows one capillary; 1000x.

independent examiners blind counted the cells and capillaries. The repeatability of their measurements was accurate enough to not affect the variability within groups.

2.5 Data analysis

Two-way analysis of variance (ANOVA) was applied to account the differences across groups (four levels: control, TUS, ESTR and LLLT groups) and phases (three levels: 14th, 23rd, and 34th day). Two variables were analyzed (number of capillaries and number of fibroblasts nuclei). Post hoc analysis included Tukey test. The level of significance was set at $p < 0.05$.

3 Results

Capillary count. The mean number of capillaries across groups and phases are presented in Figure 3. Two way ANOVA, GROUP x PHASE, showed effect of both factors on capillary count (PHASE - $F_{(2,249)} = 47$, $p = 0.001$, and GROUP - $F_{(3,747)} = 234$, $p = 0.001$). Tukey HSD post hoc test indicated that the highest number of capillaries was observed at the 14th day after injury ($p < 0.001$). The same post hoc test indicated that the highest number of capillaries was found for the ESTR group ($p < 0.001$). As well, the GROUP x PHASE interaction effect was significant ($F_{(6,747)} = 164$, $p < 0.001$) on capillary count. Post Hoc analysis showed that the lowest number of capillaries was found at the LLLT group ($p < 0.001$) and the control group ($p < 0.001$) at the 23rd day. In addition, for the 34th day after injury, the number of capillaries for the ES group was the lowest of all groups ($p < 0.001$).

Fibroblast count. The mean number of fibroblast across groups and phases are presented in Figure 3. Two way ANOVA, GROUP x PHASE, showed significant effects of factors and their interaction on fibroblast count (PHASE - $F_{(2,249)} = 603$, $p < 0.001$, GROUP - $F_{(3,747)} = 298$, $p < 0.001$, and PHASE x GROUP, $F_{(3,747)} = 876$, $p < 0.001$). Tukey HSD test showed that the largest number of fibroblasts was found at the 23rd day ($p < 0.001$). As well, post hoc analysis showed that the number of fibroblasts was the lowest for the control group ($p < 0.001$) and the highest for the ESTR group ($p < 0.001$). For the PHASE x GROUP interaction, the highest number of fibroblasts was accounted for the ESTR group ($p < 0.001$) at the 23rd day, and for the LLLT group ($p < 0.001$) at the 34th day.

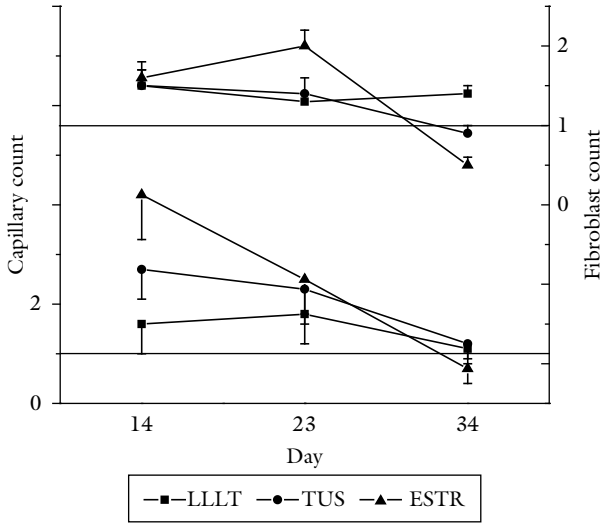

Figure 3. The number of capillaries (bottom) and fibroblasts (top) for the treated groups of rats, after Achilles tendon injury (electrical stimulation for tissue repair – ESTR, therapeutic ultrasound TUS and low level laser therapy – LLLT). All values are normalized in relation to the control group. The left vertical axis shows the capillary number values and the right vertical axis shows the fibroblast number values (data obtained from 12 histological fields for each one of the 28 rats distributed in 4 groups, collected at the three post-injury days: 14th, 23rd and 34th).

4 Conclusion

Several studies were performed in order to analyze the effects of therapeutic agents (ESTR, TUS and LLLT) on wound healing process (BASFORD, SHEFFIEL, MAIR et al., 1987; BOSATRA, JUCCI, OLLIARO et al., 1984; BROWN, McDONNEL and MENTON, 1988; ENWEMEKA, 1986; GUM, REDDY, STEHNO-BITTEL et al., 1997; JACKSON, SCHWANE and STARCHER, 1991; JUNQUEIRA, BIGNOLAS and BRETANI, 1979; KLOTH and FEEDAR, 1988; MULDER, 1991; NESSLER and MASS, 1987). The beneficial effects of each one of these agents have been demonstrated extensively.

As an example of the ES effects, Alvarez et al. (1968) reported collagen synthetic capacity in the dermis of wounds under direct current. They suggest that the increase in collagen biosynthesis is due to an increased number of collagen producing cells at the wound site. Although our methods are different their results are in accordance with ours, since it is well known that increase in capillaries and fibroblasts number precede collagen synthesis. The histological findings also indicated that the healing response was enhanced by electrical stimulation (NESSLER and MASS, 1987). Our results are in accordance with Reich and Tarjan (REICH and TARJAN, 1990), and Mulder (1991) who reported similar improvements in wound healing through the increasing in scar tissue repair by ESTR.

On the other hand, previous research showed that LLLT applied on injured tissues improves the skin regeneration, e.g., the mitotic activity of epithelial cells, the distribution density of capillaries, and the granulation tissue (MESTER and YÁSZAGI-NAGY, 1973; MESTER, 1976; MESTER, 1981). It has been suggested that ultrasound interacts with one or more components of inflammation, accelerates fibrinolysis, and increases fibroblast recruitment. Although the ultrasound effect on living tissue was detect for animal models, and focused in particular types of skin wounds and ulcers. Such findings support the use of ultrasound to promote and accelerate tissue healing and repair (SPEED, 1992). Therefore, these three mentioned modalities have shown beneficial effects when studied separately. However, it is hard to compare results of different authors that used different methods to assess the wound healing. In our literature review it was not found a similar study comparing the effectiveness of these three therapeutic agents by one unique evaluation procedure.

The present results showed that after surgery, all treated groups have higher numbers of capillaries at the wound site than the control group. The ESTR group not only showed a

higher number of capillaries at the 14th day after surgery, but also showed a great decrease of them along the 23rd and 34th days after surgery.

The TUS and LLLT groups, in this sequence, also showed higher number of capillaries in the 14th day after surgery than the control group. Then, there was a progressive decrease of capillaries quantity until the 34th day.

Regarding the fibroblast number, the ESTR group also showed a different behavior compared to the other groups: the peak value was found at the 23rd day, followed by a great decrease at the 34th post injury day. Furthermore, all groups showed higher values of the fibroblast number than control group, particularly at the 23rd post injury day, followed by a subsequent fall, mainly in the ESTR and TUS groups. These findings are in accordance with Nessler and Mass (1987), who found less colagen sinthesys at 14th day, which increased along following data collections.

In this study we did not make measurements related to the mechanical behavior of the tissues treated with different therapeutic modalities, but it is known that fibroblasts are stimulated to produce more collagen along the repair phases. And therapeutic modalities such as TUS can promote this synthesis. Besides the collagen production, there is an increasing in the tensile strength after treatment (HARVEY, DYSON, POND et al., 1975). Our study showed that ESTR produces a further increasing in the fibroblast number.

The mechanism that ESTR acts to promote a faster healing is not completely clear. There are some evidence that epithelial cells and connective tissue proliferation and migration involved in the wound healing can be enhanced by electrical field (ALVAREZ, MERTZ, SMERBECK, 1968). It has been related that high voltage galvanic pulsed stimulation induces human fibroblasts enhancement through increasing the DNA and proteins synthesis velocity (VECCHIO, CAVE, KING et al., 1993). While these observations can indicate the way in which normal healing processes can be accelerated, it is still obscure how or why the electrical fields influence in cell activity (LOW and REED, 1999). The most of the studies applied negative electrode at the wound site activity (LOW and REED, 1999).

Becher and Murray, apud Robinson and Snyder-Mackler (1995) propose that tissue polarity reverts after injury. According with this "injury current" theory it is assumed that wound are initially positive related to the surrounding tissue and this can trigger the beginning of the repair process, and if it remains positive it will improve the healing. This theory would be supported by the fact that positive polarity is transitory and chronic open wounds do not show this signal causing error in the trigger (ROBINSON, 1995).

Considering that the enhancement of the granulation and fibroblastic phases has been considered by several authors as an indicator of the acceleration in the wound healing (BOSATRA, JUCCI, OLLIARO et al., 1984; CRAIG, GANION, GEHLSEN et al., 1997; JUNQUEIRA, BIGNOLAS and BRETANI, 1979), our results suggest that the ESTR group performed the best therapeutic effect. Based on the data obtained in the present work, we can conclude that ESTR provoked significant increasing in the process of wound healing. Then, if we consider the doses adopted in the present study, it is possible to conclude that the ESTR would be the best agent to enhance the tissue repair, in relation to the TUS and LLLT considering the doses adopted.

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