Effects of Low-level Laser Therapy on Fibroblast Density in Achilles Tendon Rupture Healing

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Abstract

Objective: To investigate the effect of low-level laser therapy (LLLT) on fibroblast proliferation as a part of the tendon healing cascade.

Methods: This was an unpaired comparative experimental animal laboratory study with one control groups and two experimental groups, each consisted of 10 Sprague Dawley rats. The experimental groups 1 and 2 were given infrared irradiation for 15 minutes and 30 minutes per day, respectively, after having their achilles tendon partially cut. Histological assessment was carried out to assess the fibroblast density in healing site after three weeks on intervention.

Results: The median values of fibroblast density in group 1, group 2, and control group were 1, 2, and 1, respectively, with a p-value of 0.014. No significant difference (p=0.123) was identified on Mann-Whitney test between the fibroblast density of group 1 and group 2. The same was also true forgroup 1 and control group (p=0.315). A significant difference was found between group 2 and control group (p=0.005).

Conclusions: A regime of LLLT irradiation of 30 min/day for two weeks (1080 J cm-2) improves the fibroblast proliferation amidst tendon healing in a partially injured achilles tendon in a rat model, which is not seen in the regime with a 15 min/day duration. This emphasizes the significance of irradiation time to improve tendon healing, despite the deficient understanding of the mechanism.

Keywords: Achilles, fibroblast, laser, rupture

Introduction

Achilles tendon injuries are common in athletes, due to high stress with jumping and landing, or by overuse. The overall incidence of Achilles tendon rupture is on the rise recently. Nevertheless, controversy has surrounded the optimal treatment of acute Achilles tendon rupture.[1] Nonoperative treatment as an alternative to operative treatment, is a cost-effective option and could be especially suitable for the general population that is not that active.[2,3] In 2005, Ingvar, Tagil, and Eneroth had reported 7% re-rupture rate of 198 consecutive Achilles tendon rupture patients which treated nonoperatively.[4] Thus, surgical intervention as a primary treatment became questionable when taking cost and surgical complications into consideration, such as infection, pain, adhesion, and post-operative scarring.

One of the therapeutic modalities that can be done to enhance the healing of musculoskeletal injuries including tendon rupture is low-level laser therapy (LLLT) such as infrared therapy. LLLT is a general

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term describing a treatment method based on photobiomodulation principles, that induce a biological effects on organisms, due to the interactions of photons with molecules in the cells or tissues. This process is referred to as "low-level" because its low energy compared to other form of laser therapy such as ablation, cutting, or coagulation. LLLT triggers the release of nitric oxide (NO), a small endogenous molecule with many physiological effects on the body systems.[5] Bokhari and Murrell stated that in animal models, competitive inhibition of nitric oxide synthases (NOS) resulted in reduced tendon healing, whereas the addition of NO resulted in enhanced tendon healing.[6]

The results of research and clinical investigations into the effects of infrared, among others, show a potentially effective clinical outcome and a low risk.[7,8] Joensen *et al*. had reported a significant differences in tendon thickness in rats which received LLLT when compared to placebo group.[9] In one study, low-level laser irradiation applied to a cell culture was also known to increase fibroblast cell proliferation and reduces cell death in a dose-dependent manner. In patients, LLLT promotes tendon healing, alleviates the pain, and assists flexibility of soft tissue and joints, thus serves as a proper adjuvant therapy in tendon repair.[11] The applications of LLLT, in contrast with the past-well established terminology linked to the use of lasers, is now performed with a wide variety of different light sources, such as LEDs and lamps.

Although scientific data in relation to infrared application are increasing, the understanding of its effect to injured tendon healing in cellular level is still limited. LLLT remains somewhat controversial for two principle reasons. First, there are uncertainties about the fundamental molecular and cellular mechanisms responsible for signals transduction. Second, there are significant variations in terms of dosimetry parameters: wavelength, irradiance or power density, pulse structure, coherence, polarization, energy, fluence, irradiation time, contact vs non-contact application, and repetition regiment.[12]

It is important to assess the optimal dose of light for any specific use. The aim of this study was to investigate effects of infrared interaction in different dose to the tendon fibroblast proliferation shown histologically.

Methods

This research was an experimental study with simple random sampling. We divided the animals into three groups, specifically one control group and two experimental animal groups, getting infrared irradiation for 15 minutes (group 1) and 30 minutes (group 2), respectively. The dependent variables in this research was histologically analyzed fibroblast density. This study was approved in advance by Health Research Ethics Committee of Universitas Padjadjaran No. 120/UN6.KEP/EC/2020 (Reg No. 0319121706).

The study animals comprised of 30 male Sprague Dawley rats from Animal Laboratory of Universitas Padjadjaran Medical School, weighing 250-300 g, which had their Achilles tendon partially cut and divided into three groups. Rats were kept at bioterium for one week, placed in a quiet room with adequate lighting and temperature maintained at 20°-25°C, with food and water *ad libitum*.

The experimental procedure was carried out in four steps. First, all animals were anesthetized using 0.5 ml intramuscular ketamine, carried out cleaning and hair removal in the heel area. A longitudinal skin and subcutaneous incision was performed on the posteromedial side of the heel with attention to antiseptic procedure. Deepened incision until the Achilles tendon was done followed by a sharp hemisection to the Achiles tendon with a scalpel blade size no. 15, at 0.5 cm above its insertion to the calcaneus bone (Fig. 1a). The surgical wound was closed layer by layer using polypropylene thread size 4-0 and standard dressing. The limbs were not immobilized due its partial cut design. Other consideration was to prevent inhibition of radiation by the enclosing cast or splint. Second step, animals in experimental group were irradiated starting at 5th day after treatment using a Philips Infraphil 13379F/479, 150W lamp, ranging from 600 to 1500 nm, with a peak at 1000 nm, at a 30 cm distance from the animals. Animals had their affected leg irradiated while fixed on a board. The other body areas were covered with a wet towel. The duration was differed between two experimental groups, specifically 15 min (540 J cm^{-2}) and 30 minutes (1080 J cm^{-2}) a day for fourteen days. To prevent infrared heating, the temperature was controlled by indoors air-conditioning with plenty of ventilation and routine temperature measurement using thermometer (Fig. 1b).

Three weeks after initial treatment, the animals were killed by injection of 1 ml saturated potassium chloride solution to cause cardiac arrest. The tissue around the hemisected tendon were taken parallel to the joint ends *en bloc*, and then fixed in 10%

formaldehyde solution. Tendon specimens were taken and examined histologically under haematoxylin-eosin staining, particularly at hemisection site (Fig. 2). Fibroblast density was evaluated semi-quantitatively using three scales (0: absent, 1: mild appearance, and 2: marked appearance) and recorded for comparison between groups (Fig. 3). Histological analysis was conducted by an independent histopathological expert.

Data analysis was performed using Statistics Social Service Program (SPSS) ver. 25 for Windows (SPSS Inc. Chicago, IL, USA). Descriptive analysis was carried out and data distribution and normality tests were performed to determine whether parametric or nonparametric analysis was used, and comparative hypothesis testing of numerical variables was carried out using One Way ANOVA for parametric testing or Kruskal Wallis test for nonparametric tests.

Results

Table 1 shows the distribution of fibroblast density among three groups. Score maximum of 2 were found in both experimental groups, but not in control group.

Kruskal Wallis statistical test followed by median test was used for analysis because the data were ordinal, abnormally distributed with a significance of 5%. Calculated p value was 0.014, which means that there was a significant difference in the value median fibroblast density between the three groups.

It can be seen that the fibroblast density has the highest median in the experimental group 2. Considering there was a significant difference between groups, a further test was carried out with the median test to compare between the two groups (Table 2). There was no significance difference between fibroblast density between experimental group 1 and group 2, as well as between experimental group 1 and control group ($p>0.05$). Rather, the difference of medians between experimental group and control group was significant ($p=0.005$).

Table 2 Median Test Results for Fibroblast Density Variable

Group Comparison	p value
Experimental Group 1 and Group 2	0.355
Experimental Group 1 and Control Group	0.778
Experimental Group 2 and Control Group	0.005

Discussion

The results of this study were in a concordance with prior basic studies stating that infrared therapy in tissue healing can increase fibroblast proliferation.[10,15] Additional value of this study to the LLLT application is the distinction made by dosage differences, specifically the irradiation time. This study showed histologically an increase of fibroblast cell density following a LLLT. Notable findings here was the different results among experimental groups, thus emphasizing the importance of the dosage setting, specifically different irradiation time of similar energy. In this study, irradiation of 540 J cm^{-2} for two weeks yielded insignificant result difference with control group.

Low-level laser therapy (LLLT) refers to the use of photons at a non-thermal irradiance to alter biological activity. The main medical applications of LLLT are reducing pain and inflammation, augmenting tissue repair, promoting regeneration of different tissues, and preventing tissue damage in situations where it is likely to occur.[12,13] Avci *et al.*[12] has also mentioned that this procedure is referred to as 'low-level' because the energy or power densities employed are low compared to other forms of laser therapy such as ablation, cutting, and thermal tissue coagulation. It can be implied that the effects of irradiation are a response to the light and not due to heat.[14]

The increase in the number of fibroblasts mediates the production of collagen. Infrared can also increase the activity of macrophages in the phagocytic process, secretion of growth

Table 1 Fibroblast Density According to Groups

Groups	N	Min.	Max.	Median	Mean	SD	p-value
Experimental Group 1	10	0	2	1	1.00	0.816	0.014
Experimental Group 2	10	0	2	2	1.60	0.699	
Control group	10	0	1	1	0.60	0.516	

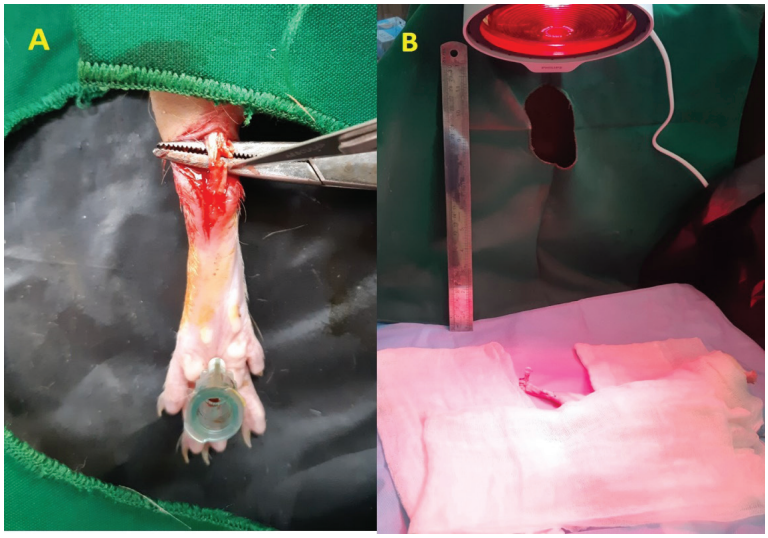

Fig. 1 (A) Partial Achilles Tendon Cutting in the Rat; (B) LLLT Irradiation Set-Up

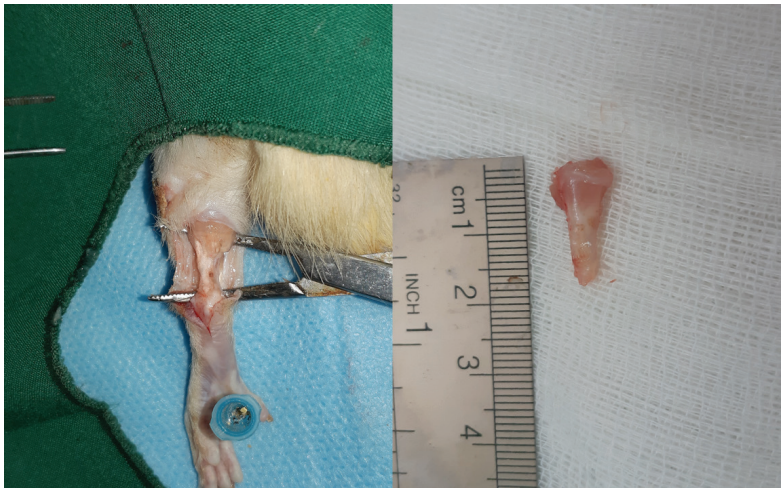

Fig. 2 Macroscopic Finding of Healed Achilles Tendon

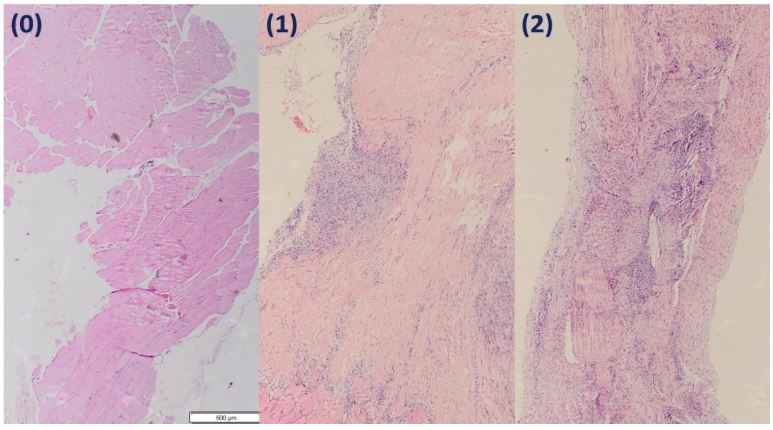

Fig. 3 Histological Findings Showing the Different Result of Fibroblast Density, Specifically Scored as 0 (absent), 1 (mild), and 2 (marked)

factors, and stimulate collagen synthesis. In addition, it can also stimulate the formation of neovascularization, which will support increased perfusion and oxygenation, as well as regeneration of endothelial cells. Fibroblasts are cells that have a significant role in the tendon healing process. Fibroblasts secrete essential substances and collagen, which form scar tissue as a substitute for defects in wounds. The fibroblast proliferation process uses fibrin threads originating from the blood clotting process as a framework, and then the fibrin disappears according to collagen deposition.[16]

Infrared therapy has been shown to stimulate nitric oxide production. Nitric oxide is a small endogenous molecule with multiple effects on the body's systems. Nitric oxide is involved in a wide range of biological functions in the body, including vasodilation, immune response, and neurotransmission as reported by Wink *et al*.[17] The fact is that infrared can penetrate deep into wound tissue and allow non-invasive treatment of the wound healing process. The visible, infrared waves are easily absorbed by the surface components of the blood and muscle surfaces, limiting the penetration of the tissue to <10 mm. Shortwave infrared (810 nm) is not easily absorbed and has a much greater depth of tissue penetration of 30-40 mm or more and thus provides more significant deposition of photons at the site of injury.[18]

The effect of providing heat to the body via infrared can also increase collagen fibers in the tendons and joint capsules, reduce the viscosity of the fluid tissue elements, reduce joint stiffness, reduce muscle spasm, vasodilate blood vessels, and increase metabolism. Some of the positive clinical effects of infrared has been introduced, including those for reducing rheumatic knee pain, as well as its impact on wound healing.[19]

There are some limitations of this study. To date, no proper method for measuring the parameters of the biological effects of LLLT, including the effective wavelength, irradiation time, and intensity has been developed. Tsai and Hamblin correctly noted that if certain parameters such as irradiation type, laser wavelength, continuous vs pulsed irradiation, pulse shape, and target area are changed, it may not be possible to compare between studies.[14] Thus, it is difficult to compare fairly between many LLLT modalities for its efficacy as shown by many studies. However, Hsu *et al.*[20] had reported the effective irradiation time as an important factor for the biological application of LLLT. Among different irradiation times, specifically 15, 30, 45, and 60 minutes, the far-infrared biological index of 30 minutes irradiation was significantly higher than those of the other durations.There was a potential bias of this study as well, resulting of a single observer analysing the histological specimens. However, this study may provide the framework to define the guidelines for LLLT therapy regarding Achilles tendon rupture.

Other problematics included disadvantages of adjusting this study for clinical relevance in human, concerning the age and dosage adaptation. This short-term study was also unable to identify the side effects in rats, both locally and systemically.

In conclusion, low-level laser irradiation at 1080 J cm^{-2} per day, administered for fourteen days appeared to increase the fibroblast density of injured Achilles tendon in rat models, compared with control group and experimental group receiving 540 J cm^{-2}. This experiment is useful for investigating the positive effects of low-level laser therapy to enhance tendon healing and optimal duration of therapy. However, considering the healing cascade, we believe this results is only applicable for acute healing phase. Further studies using this model should explore the maximum duration allowed to avoid harmful effects such as tissue damage.

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