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Effect of platelet-rich plasma, low-level laser therapy (650 nm) or their combination on the healing of Achilles tendon in rabbits: a histopathological study

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ABSTRACT

Tendon repair is still a challenge for rehabilitation. Various treatments for tendon injuries are currently used or have been trialed. This study was established to investigate the effects of low level laser therapy (LLLT) or platelet-rich plasma (PRP) treatment alone or using combined method on the healing of Achilles tendon. Twenty-four male white New Zealand rabbits were divided randomly into four groups of six animals each: GI: partial tenotomy with no treatment, only 1 ml normal saline was injected weekly for 3 weeks consecutive at the site of splitting; GII: partial tenotomy with PRP treatment; GIII: partial tenotomy with LLLT (P= 100 mW, WL= 650 nm, A= 1cm^2, T= 1min) for 15 consecutive days; GIV: partial tenotomy with LLLT + PRP. Histopathological parameters such as inflammatory reactions, adhesion formation and collagen synthesis were measured. In the present study, the results showed that the treatment of rabbits with PRP or LLLT alone has significant advantages over untreated animals (P<0.05). Furthermore, it was found that the combined treatment with PRP and LLLT is even more efficient than when each of the two treatments is used alone but there was no significant difference (P>0.05) between the two groups of laser and PRP. However, the treatments combining PRP and LLLT showed significant results (P<0.05). Our results demonstrate a decrease in the time of tendon regeneration by using the two therapies combined, accelerating the healing process.

Keywords: Low level laser therapy, Platelet-rich plasma, Achilles tendon, histopathology, Rabbit.

INTRODUCTION

Tendon healing of acute injuries occurs in three stages: inflammation, proliferation and remodeling. 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 [1].

Connective tissue injuries, specifically tendon injury, 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 [2].

Tendon matrix is rich in collagens, such as types I and III collagens. Type I collagen is responsible for the mechanical strength of the tendon tissue and type III collagen has an important role in the healing process [3].

Type I collagen is the primary collagen incorporated in the tendon structure, and increasing the production of type I collagen may enhance tendon healing [4].

Recently, a variety of treatments for Achilles tendon lesions is used or has been trialed. However, there is little evidence that any conventional therapies are effective.

In the last years, low level laser therapy (LLLT) and platelet-rich plasma (PRP) have been used in orthopedics, traumatology, and sports medicine showing interesting results in modulation of Achilles tendon repair. However, optimal parameters and mechanisms behind these effects are not fully understood [5].

A series of studies have demonstrated that LLLT is effective at reducing post injury inflammatory processes and accelerating soft tissue healing. LLLT at the cellular level produces increased ATP synthesis, increased mitochondrial respiration, and increased production of molecular oxygen, thus stimulating DNA synthesis and cell proliferation. LLLT can accelerate the healing process of tendinous tissue after injury. LLLT seems to create new blood vessels, to increase collagen fiber deposition, and to promote higher fibroblast cell proliferation in the site of the lesion [6].

On the other hand recently, PRP, an autologous concentrate of blood platelets, has been introduced as a possible new therapy for the treatment of tendon Injuries. Platelets are known to play a crucial role in the cascade of tissue healing by delivering growth factors to the site of injury [7].

Upon activation, platelets release growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-b, vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF)-1, from their a-granules [8]. PRP treatment in tendon lesions results in better biochemical, mechanical, and histological properties of the repair tissue [9].

The aim of this study was to investigate the effects of LLLT (λ = 650nm) and PRP alone and using combined method on the healing of Achilles tendon. The semi- quantitative assessment will be carried out aiming to analyze the presence inflammatory reactions, adhesion formation and collagen synthesis in the histological slides.

MATERIALS AND METHODS

All rabbits of the present research were cared according to the norms of the Islamic Azad University College of Specialized Veterinary Sciences, Tehran, Iran, laboratory of animal experimentations; this investigation was approved by the Committee of Ethics in Research with Animals in Islamic Azad University.

This study was conducted with 24 healthy adult male white New Zealand rabbits (20 week-old) with body weight varying between 2.5 to 3.5 kg. The animals were kept in four standard cages under constant room temperature of 18–22°C, relative humidity of 40–50%, 12h/12h light/dark cycle, with *ad libitum* access to filtered tap water and standardized food (ration for rodents).

Experimental groups
The animals were allocated randomly into four experimental groups of six rabbits each:
GI: animals submitted to partial tenotomy with no treatment. Only 1 ml normal saline was injected weekly for 3 weeks consecutive at the site of splitting;

GII: animals submitted to partial tenotomy with injection of 1ml PRP weekly for 3 weeks consecutive at the site of splitting;

GIII: animals submitted to partial tenotomy with LLT (P = 100 mW, WL= 650 nm, A = 1cm^2, T = 1min) for 15 consecutive days;

GIV: animals submitted to partial tenotomy with LLT + PRP (in the same way).

Anesthesia
The animals were anesthetized with ketamine hydrochloride 10% (35 mg/kg) and xylazine hydrochloride 2% (8 mg/kg) via intramuscular injection. For the maintenance inhalation machine and isoflurane were used.

Surgical procedures
After shaving and cleaning the skin nearby, the right Achilles tendon of each rabbit was freed from surrounding tissue, sharply, longitudinally and full-thickness lacerated midway between its calcaneal insertion and the musculotendinous junction 10 times with number 11 surgery blade. Then Subcutaneous tissue sutured with 4-0 absorbable sutures (vicryl) and skin closed with 3-0 non-absorbable suture materials (nylon). After surgery, the animals were kept in four standard cages under constant room temperature of 18–22°C, relative humidity of 40–50%, 12h/12h light/dark cycle, with *ad libitum* access to filtered tap water and standardized food (ration for rodents).

Postoperative considerations like using analgesic drugs (tramadol) and antibiotics (Enrofloxacin 10 mg/kg SC, Pantrisol 30 mg/kg IM) redirected to prevent infection in animals. After 60 days, the rabbits were euthanized and tissue samples were used for histopathological study.

PRP preparation
Animals from GII and GIV after being anesthetized were subjected to a puncture of the central auricular artery, and then 10 ml of blood was removed from each animal for PRP preparation. It is suggested in the literature that the amount of blood withdrawn should be not more than 6.4% of the animal body weight [10]. Most of the protocols for PRP production used a small fraction of blood (0.3–0.5 ml). This blood is first subjected to a 15-min centrifugation at 2000 rpm, followed by another 20min at 3000 rpm. A 5% calcium chloride activator was added in a ratio of 1:10 for obtaining the total volume of PRP. Platelet counts were performed to calculate the PRP concentrate, which should be around 400% of the peripheral blood platelet count [11]. The platelet concentrate was stored at 20°C until the exact time for use at the surgical site.

PRP treatment
The animal in GII and GIV received treatment with PRP. Each animal received a single dose of 1ml directly into the surgical site, on top of the tenotomy. The application of PRP was performed immediately after injury before suturing the lesion and was repeated weekly for 3 weeks consecutive at the site of splitting.

Laser therapy
We used low level laser device with 650 nm wavelength, 100 mW average power, and A = 1cm^2. LLLT was started immediately after surgery and skin suturing and continued for 15 consecutive days. Treatments were made through the contact technique, at one point for 60 seconds, on the injured area. All the procedures were carried out in a stipulated period of the day. During the treatments, irradiated animals were sedated by 1/2 dose of anesthetizing drugs and they were kept in a special container from which their forelimbs and hind limbs were extended by extension at the knee joints and plantar extension in the ankle joints.

Histological analysis
Sixty days after surgery, each rabbit was euthanized with an intracardiac injection of anesthetic sodium thiopental (crystal) at a dose of 0.05 mL per 100 g body weight, followed by 19.1% potassium chloride via intracardiac, with a single dose of 0.4 mL per 100 g body weight. After confirmation of euthanasia by verification of vital data and absence of reflexes, Achilles tendon was transected below its musculotendinous junction and above its calcaneal attachment. The extracted tendon was washed in physiological solution and then fixed using 10% formaline and embedded in paraffin, in preparation for histopathological analysis. Thin sections (5 µm) were cut and stained using hematoxylin-eosin (HE). Under light microscopy, 5 fields were randomly chosen for each stained section. Inflammatory reactions, adhesion formation and collagen synthesis were analyzed and quantified the following histological findings. The data from the means were analyzed according to their grade: Inflammation: No Inflammation=0, Mild Inflammation=+1, Intermediate Inflammation=+2, Severe Inflammation= +3; Arrangement

of collagen fibers: Complete regularity=+0, Mild regularity=+1, Intermediate regularity =+2, Woven bundles=+3; Adhesion formation; No Adhesion=0, Mild Adhesion=+1, Intermediate Adhesion=+2, Severe Adhesion=+3.

Statistical analysis

The data obtained from the histopathological analysis were subjected to statistical treatment. The one-way analyses of variance followed by the Tukey post-hoc test were employed to analyze four groups consecutively. All statistical tests were performed at a significance level of $P<0.05$.

RESULTS AND DISCUSSION

The surgical procedure, PRP treatment and the laser application were well tolerated by the rabbits and no animal died during the experiment. There was no sign of infection and/or suture dehiscence in operated rabbits.

The scores of tendon histological changes from four groups, 60 days after creating the injury, are illustrated in table 1. The results showed that the treatment of rabbits with PRP or LLLT alone has significant advantages over untreated animals ($P<0.05$), but there was no significant difference ($P>0.05$) between the LLLT group and PRP group. Furthermore, it was found that the combined treatment with PRP and LLLT is even more efficient ($P<0.05$) than when each of the two treatments is used alone. Figure 1 shows histological analysis using the Hematoxylin and eosin sections of Achilles tendons with partial rupture showing the presence of collagen fibers, which are thin and red. Collagen fibers were more frequent in all treated groups than in the untreated control group. Both groups that associated LLLT and PRP treatments presented higher proliferation of fibroblasts and fibers arrangement (Figures 2, 3). Additionally, LLT + PRP group showed higher proliferation of fibroblasts and fibers arrangement than PRP or laser alone (Figures 4). However, no difference was found between PRP and laser groups.

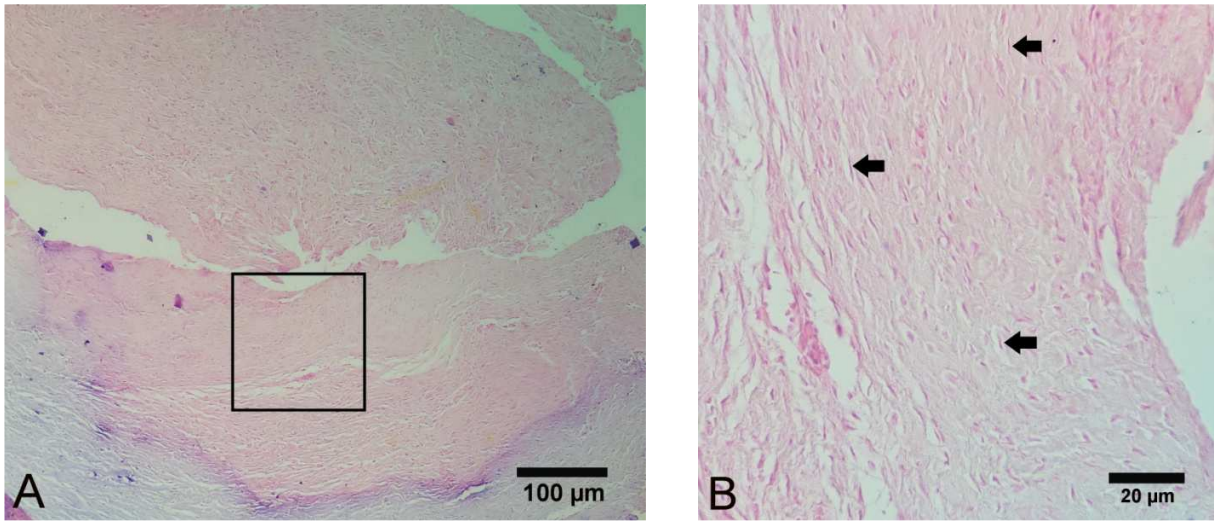

Figure 1: Cross section from the Achilles tendon with partial rupture after 60 days; (A) control group: (B) Note the higher magnification with low proliferation of the fibroblasts (arrows) and less regular arrangement of collagen fibers, which are thin and red. Hematoxylin and eosin staining, A: 100×; B: 400×

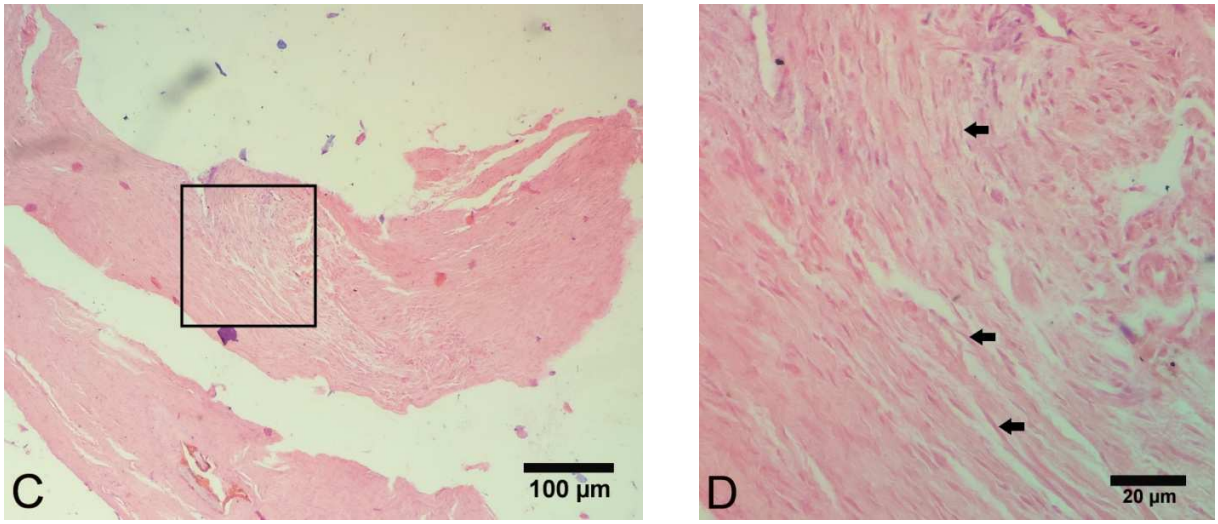

Figure 2: Cross section from the Achilles tendon with partial rupture after 60 days; (C) Laser treated group: (D) Note the higher magnification with highly proliferation of the fibroblasts (arrows) and regular arrangement of collagen fibers, which are thick and red. Hematoxylin and eosin staining, C: 100×; D: 400×

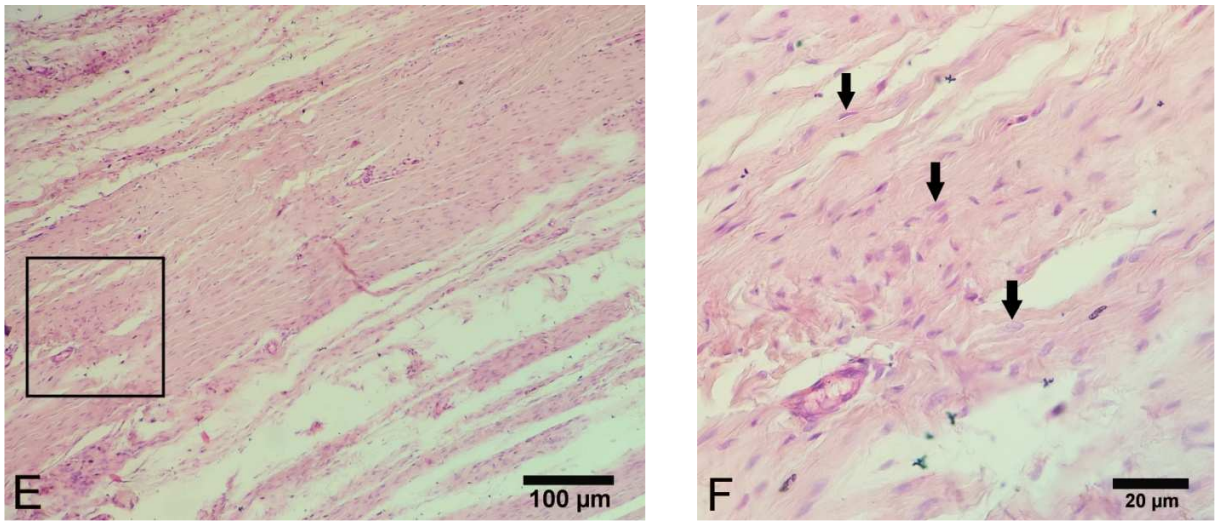

Figure 3: Cross section from the Achilles tendon with partial rupture after 60 days; (E) PRP treated group: (F) Note the higher magnification with proliferation of the fibroblasts (arrows) and regular arrangement of collagen fibers, which are thick and red similar to laser group. Hematoxylin and eosin staining, E: 100×; F: 400×

Table 1 Scores of Tendon histological changes, 60 days after creating the injury

Group	N	Mean ± SD
Control	6	6.66±1.21
PRP	6	3.83±1.69**‡
Laser	6	3.83±1.69**‡†
Larer + PRP	6	2.00±0.89***

***P = 0.01, ***P =0.000 compared with the control group.*
‡P= 0.046 compared with the Larer + PRP group.
†P = 1.00 compared with the PRP group.

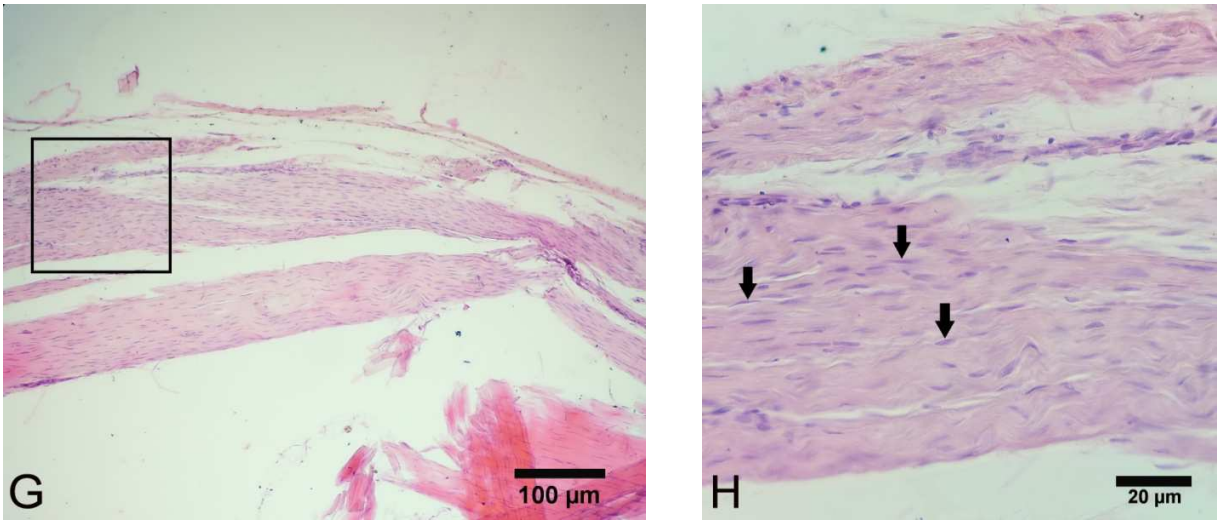

Figure 4: Cross section from the Achilles tendon with partial rupture after 60 days; (G) PRP+ Laser treated group: (H) Note the higher magnification with highly proliferation of the fibroblasts (arrows) and more regular arrangement of collagen fibers. Hematoxylin and eosin staining, G: 100×; H: 400×

Tendon has unique structure characteristics and function. Injury to tendon caused by trauma is very common and is a problem that requires a repair followed by an early mobilization. According to Enwemeka, healing of the tendon takes weeks or even months to acquire the resistance needed to effectively transmit the force generated by a muscle [12,13]. There is a need for studies focusing in the improvement of tendon repair, reducing recovery time and time required to return to normal activities [14].

Animals are commonly used in tendon disorder research. They have the advantages of incorporating invasive evaluation methods, and the possibility for detailed tissue examination. In animal studies, partial or total surgical tenotomy is the most commonly used technique for inducing injury [15-23]. In the present study, tendon injury was induced by standard partial tenotomy as described in method section.

In recent years, there has been an increased trend in the use of PRP to promote healing in a variety of situations in podiatry. Platelets have been found to possess many important bioactive proteins facilitating tissue regeneration and wound healing. There has been found to be an in vitro dose-response relationship between the concentration of platelets and the production of Type I collagen. When platelets are placed into injured areas, they release growth factors and can aid in activating the healing process. PRP can be very helpful for posterior heel surgery involving the Achilles tendon. In this study PRP was used alone or associated with LLLT. Collectively, the present findings may support the previously proposed effect of PRP that may have a useful role during tendon healing [24-27]. However, the treatments combining PRP and LLLT showed significant results between groups GII (PRP alone) and GIV (PRP+LLLT). It was found that the combined treatment with PRP and LLLT is more efficient than when PRP is used alone.

Laser is one of the physical resources most utilized for the recovery of the tendon. Several studies performed in injured animal tendons are frequent in the LLLT literature. In most of these LLLT studies, tendons were treated daily for 3 to 21 days [28-30]. Observed histopathological changes in tendons receiving LLLT include increased collagen production [15], improved collagen bundle organization [6-17,22], and an increased number of small blood vessels [28]. Some studies have investigated the effect of LLLT in acute inflammation; tissue receiving LLLT exhibited reduced concentrations of inflammatory markers and cells compared to no-treatment controls [31,32]. In the present study, it was decided to work with 650 nm wavelength, 100 mW average power, and A = 1cm^2. The results from this study emphasize the importance of LLLT. These results resemble those found by other researchers who have reported satisfactory effects from LLLT during the repair process in the tendon [27-32], but the LLLT and combined PRP showed significant results between groups GIII (LLT alone) and GIV (PRP+LLLT). From the results obtained from this study, it can be seen that combined treatment with PRP and LLLT after repaired Achilles tendons had favorable beneficial effects on the regenerating neo-tendon.

In the past few years, Barbosa *et al* [5] investigated the effects of LLLT alone or associated with PRP. For that purpose, they induced tendon injury by partial tenotomy with a cut of 2 mm in the middle third of the tendon, the medial to lateral. These authors administered a single dose of 0.2 ml directly into the surgical site. For laser therapy, they used the low-intensity laser device which can be operated in two wavelengths: 660 nm and 830 nm. Laser irradiations were made an interval of 1 day between applications and animals were killed on the 13th day post-tenotomy and their tendons were analyzed using polarization microscopy. They found that the deposition of collagen type I was higher when treatment with PRP and LLLT was combined. In the current study, we tested the histopathological parameters in injured tendon such as inflammatory reactions, adhesion formation and collagen synthesis after using combination of LLLT with PRP. The results showed that the treatment with PRP or LLLT alone has significant advantages over untreated animals. Additionally, it was found that the combined treatment with PRP and LLLT is even more efficient than when each of the two treatments is used alone.

CONCLUSION

The results of this study suggest a decrease in the time of tendon regeneration by using the two therapies combined, accelerating the healing process. Accordingly, this form of treatment, PRP in combined LLLT can be of value after surgical repair of ruptured and injured human tendons.

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