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ORIGINAL ARTICLE

Evaluation of low-level laser therapy, platelet-rich plasma, and their combination on the healing of Achilles tendon in rabbits

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Abstract Tendon repair is still one of the challenges for rehabilitation. Various treatments for tendon injuries have been used in recent decade. This study was established to investigate the effects of low-level laser therapy (LLLT), platelet-rich plasma (PRP) treatment alone, and using combined method on the healing of Achilles tendon in rabbits. Seventy-two healthy mature male white New Zealand rabbits were divided randomly into four groups of 18 animals each: control: partial tenotomy with no treatment, only 1 mL normal saline was injected on days 1, 8, and 15 at the site of splitting; PRP: partial tenotomy with PRP treatment on days 1, 8, and 15 at the site of splitting; LLLT: partial tenotomy with LLLT (K30 hand-held probe, AZOR, Technica, Russia, 650 nm, 30 mW, surface area=1 cm^2, 60 S/cm^2, energy density=1.8 J/cm^2) for 15 consecutive days; LLLT+PRP: partial tenotomy with LLLT+PRP. At the end of trial, the rabbits were euthanatized and tendon specimens were harvested and were submitted for histopathological evaluation, hydroxyproline levels, and biomechanical measurement. The Tukey post hoc test was performed. The results for these parameters showed that 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. There was no significant difference ($P>0.05$) between the two groups of LLLT and PRP. However, the treatments combining PRP and LLLT showed significant results in comparison of PRP or LLLT alone ($P<0.05$). Our results demonstrate that the healing time of injured tendon decreases by using the two therapies combined.

Keywords Low-level laser therapy · Platelet-rich plasma · Achilles tendon · Tensile strength · Hydroxyproline · Histopathology

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Introduction

Tendon injuries account for approximately 30 to 50 % of all injuries happening in sports [1]. Compared with other soft tissues, tendon is poorly vascularized and heals slowly. As a result, treatment often tends to be lengthy, outcomes tend to be variable, and usually re-injury happens [2–5].

Tendon healing process, includes several overlapping phases: an inflammatory phase, which extends from 1 to 7 days after tendon injury; a proliferative phase, which begins

around day 8 and extends up to 14 days; and at the end, remodeling phase, which begins near day 14 and reaches its peak around day 21 [6].

Return to normal tensile strength is the main goal for treatments in injured tendons. The most abundant protein in tendons is collagen. Collagen type I provides tensile stiffness to the tissue, and type III is distributed between collagen I bundles. Both collagens are fibril forming with the ability to assemble into highly orientated supramolecular aggregates that are responsible for the properties of the tissue [7–9].

In recent years, a variety of treatments for tendon lesions is used or has been trialed such as low-level laser therapy (LLLT) and platelet-rich plasma (PRP) that showing interesting results in modulation of Achilles tendon repair [10]. Most studies have demonstrated that LLLT is effective in reducing inflammatory processes after injury and accelerating soft tissue healing. Previous studies showed that LLLT help to create new blood vessels, increasing collagen fiber deposition, and cause to promote higher fibroblast cell proliferation in the site of the lesion [11].

On the other hand, PRP, an autologous concentrate of blood platelets, has been introduced as a one of new therapies for tendon injury treatment. Platelets by delivering growth factors to the site of injury have a crucial role in the cascade of tissue healing [12]. Upon activation, platelets release growth factors, such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1, and platelet-derived growth factor (PDGF) from their granules [13]. Based on the findings, PRP treatment in tendon lesions results in better mechanical, biochemical, and histological properties of the repaired tissue [14].

However, some studies demonstrated the positive effect of PRP or LLLT on tendon regeneration, but using the combination of these methods have not been addressed yet. The aim of this study was to investigate the effects of LLLT (λ=650 nm) and PRP alone and using combined method on healing processes after partial tenotomy. The results of the present study may be a valuable option in clinical setting.

Materials and methods

Design of experiment

This study has been conducted according to the guidelines of the animal care review board of the Islamic Azad University Department of Specialized Veterinary Sciences and adhering to the guide for care and use of laboratory animals, and has been approved by the ethics committee in Islamic Azad University. Seventy-two healthy mature male white New Zealand rabbits (20-week-old) with body weight varying between 2.5 and 3.5 kg were purchased from the Pasteur Institute of Iran.

The animals were kept in standard cages under constant room temperature of 18–22 °C, and humidity of 40–50 %, 12 h/12 h light/dark cycle, with ad libitum access to standardized food (ration for rodents) and filtered tap water.

Experimental groups

The animals were allocated randomly into 4 experimental groups of 18 rabbits each:

Control: animals submitted to partial tenotomy with no treatment, only 1 mL normal saline was injected on days 1, 8, and 15 at the site of splitting and sham laser;
PRP: animals submitted to partial tenotomy with PRP treatment on days 1, 8 and 15 at the site of splitting and sham laser;
LLLT: animals submitted to partial tenotomy with LLLT (P=100 mW, WL=650 nm, A=4 J/cm^2, T=1 min) for 15 consecutive days;
LLLT+PRP: animals submitted to partial tenotomy with LLLT+PRP (in the same way).

Anesthesia

The animals were anesthetized via intramuscular injection of ketamine hydrochloride 10 % (35 mg/kg) and xylazine hydrochloride 2 % (8 mg/kg). For maintenance, inhalation machine and isoflurane with tracheal tube size of 2 mm were used.

Surgical procedures

All procedures on animals were performed at the Laboratory of Experimental Surgery, Department of Specialized Veterinary Sciences. After anesthesia, the animals were placed on a table, in ventral recumbency position, with rear and forelimbs immobilized by thin ropes gently. After clipping, disinfecting with antiseptic povidone-iodine solution, and draping, a 2-mm skin incision was made in the location of the Achilles tendon; the right Achilles tendon of each rabbit was freed from surrounding tissues, and sharply, longitudinally, and full-thickness incision was made ten times with the blade number 11, midway between its calcaneal insertion and the musculotendinous junction. Then subcutaneous tissue and skin were sutured with 4–0 absorbable sutures (vicryl) and 3–0 nonabsorbable suture materials (nylon), respectively.

Postoperative considerations like analgesic drugs (tramadol, 4 mg/kg IM) and antibiotics (pantrisol, 30 mg/kg IM; enrofloxacin, 10 mg/kg SC) were used to reduce pain and prevent infection. After 30 days, all rabbits were euthanized and tissue specimens were harvested and were submitted to lab. From each groups, six specimens were used for histopathological assessment, six specimens for tensile strength

measurement, and six specimens for hydroxyproline levels. We also used contralateral tendons for comparison of hydroxyproline levels and tensile strength of normal tendons with healed ones.

Laser therapy

All patients underwent the application of laser device with GaAlInP diode laser (K30 hand-held probe, AZOR, Technica, Russia): λ=650 nm, radiation mode=continues, power=30 mW, surface area=1 cm^2, time=60 S/cm^2, and energy density=1.8 J/cm^2. LLLT was started immediately after skin suturing (day 0) and continued for 15 consecutive days, at the same times. Treatments were made through the noncontact technique, at one point for 60 s to irradiate the injured area. Before starting the irradiation, animals were sedated by 1/2 dose of anesthetizing drugs and were kept in a special container from which their hind limbs and forelimbs were extended by extension at the knee joints and plantar extension in the ankle joints. In the control and PRP groups, the equipment remained turned off throughout the application, while in the LLLT and LLT+PRP groups, it remained turned on.

PRP

After the induction of anesthesia, 10-mL of autologous blood was drawn from each rabbit for PRP preparation. The blood was centrifuged in two steps, step 1: 15-min centrifugation at 2000 rpm; step 2: 20 min centrifugation at 3000 rpm. Calcium chloride (5 %) activator was added in a ratio of 1:10 for obtaining the total volume of PRP. Platelet counts were performed to determine the PRP concentrate, which was around 400 % of the peripheral blood platelet count. PRP for each rabbit was stored at 20 °C until the exact time for use at the site of surgery.

Each animal in PRP and LLLT+PRP received a single dose of PRP (1 mL) on days 1, 8, and 15 directly into the surgical site, on top of the tenotomy. The injection of PRP in the PRP group was performed immediately after suturing the lesion and was repeated on days 8 and 15 at the site of splitting. In group LLLT+PRP, the injection of PRP was applied after LLLT.

Hydroxyproline content measurement

To extracting tendons, each rabbit was euthanized by intracardiac injection of anesthetic sodium thiopental (crystal) at a dose of 0.05 mL/100 g body weight, followed by intracardiac injection of 19.1 % potassium chloride, with a single dose of 0.4 mL/100 g body weight. After euthanasia, six Achilles tendons from each group with their contralateral tendons were harvested to determine hydroxyproline levels. High-performance liquid chromatography (LC 10AD, Shimadzu, Tokyo, Japan) equipped with a reverse-phase column (4.6 mm internal diameter×250 mm length; TSK-gel, ODS-80TM) was used to determine hydroxyproline content. The column was eluted with 0.4 % ammonium acetate (pH 7.4) and 75 % acetonitrile at a flow rate of 2 mL/min through an isocratic pump. Using a fluorescence detector (RF-10AXL, Shimadzu, Tokyo, Japan) and integrated from the retention time and area under the eluting peak, the concentration of hydroxyproline in each specimen was detected. Results were normalized with protein concentration in each sample.

Histological examination

After confirmation of euthanasia by absence of reflexes and verification of vital data, Achilles tendon was transected below its musculotendinous junction and above its calcaneal attachment. Six harvested tendons from each groups was washed in physiological solution and fixed using 10 % formalin and embedded in paraffin, in preparation for histopathological analysis. Thin sections (5 μm) were cut and stained using hematoxylin–eosin (HE) for histopathological study. Under light microscopy, five fields were randomly chosen for each stained section. For histopathological assessment inflammatory reactions, adhesion formation and collagen arrangement 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), and severe inflammation (+3); arrangement of collagen fibers: complete regularity (0), mild regularity (+1), intermediate regularity (+2), and woven bundles (+3); adhesion formation: no adhesion (0), mild adhesion (+1), intermediate adhesion (+2), and severe adhesion (+3).

Biomechanical testing

After euthanasia, six Achilles tendons from each group with their contralateral tendons were harvested for biomechanical study. The tendon specimens were immersed in 0.9 % saline solution for sending to the lab and thawed them before biomechanical testing. The two ends of the tendon were tightly secured by clamps, and the specimen was aligned before the test. The specimen was kept moist during the test in order to avoid tensile strength changes associated with drying. Breaking strength tests for the tendon specimens were performed at a constant speed of 0.03 mm/s, using material testing machine (Zwick/Roell, Ulm, Germany). Tensile loading was applied to the tendon specimen until occurrence of Achilles tendon rupture was observed. Measurements of the repair site were made using a vernier caliper. Data collected from software were used to compute the structural properties of each specimen. We used collected data to compare tendons load at break (Newton (N); breaking strength), and then

converted them to megapascals (MPa) using a defined formula to determine tensile strength: Newton per square millimeter (N/mm^2)×1=MPa.

Statistical analysis

Statistical analysis was performed using SPSS software (version 18). One-way analysis of variance (ANOVA) followed by Tukey post hoc test were employed to analyze the results. All statistical tests were performed at a significance level of $P<0.05$. Results were expressed as mean±standard deviation.

Results

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

Hydroxyproline results

The results indicated that the amounts of hydroxyproline in healthy and healed tendons had a significant difference with untreated ones ($P>0.05$), and the results also showed a significant difference between healthy tendons and treated ones ($P>0.05$). The highest levels of hydroxyproline in treated groups are respectively related to LLLT+PRP, laser, and PRP groups. The results showed that hydroxyproline content in the laser and PRP groups had significant difference with the LLLT+PRP group ($P>0.05$), but there was no significant difference between the laser and PRP groups ($P<0.05$). Figure 1a shows comparison of hydroxyproline content in healed tendons with hydroxyproline content in healthy tendons (contralateral tendons), and Fig. 2b shows the comparison of hydroxyproline content between groups.

Histological findings

After reviewing the pathology slides, three factors of inflammation degree, arrangement of collagen fibers, and the degree of adhesion were studied. For each factor, a specific grading was considered, as previously described in "Materials and methods." To achieve acceptable and reliable results, all the scores for each factor in each group was collected, and the average for each factor was obtained, then the mean of three factors in each group was added together, and a final result is obtained. The final result relating to each group was used for statistical analysis.

Statistical analysis showed that the treatment of rabbits with LLLT or PRP alone has significant advantages over untreated animals ($P<0.05$), but there was no significant

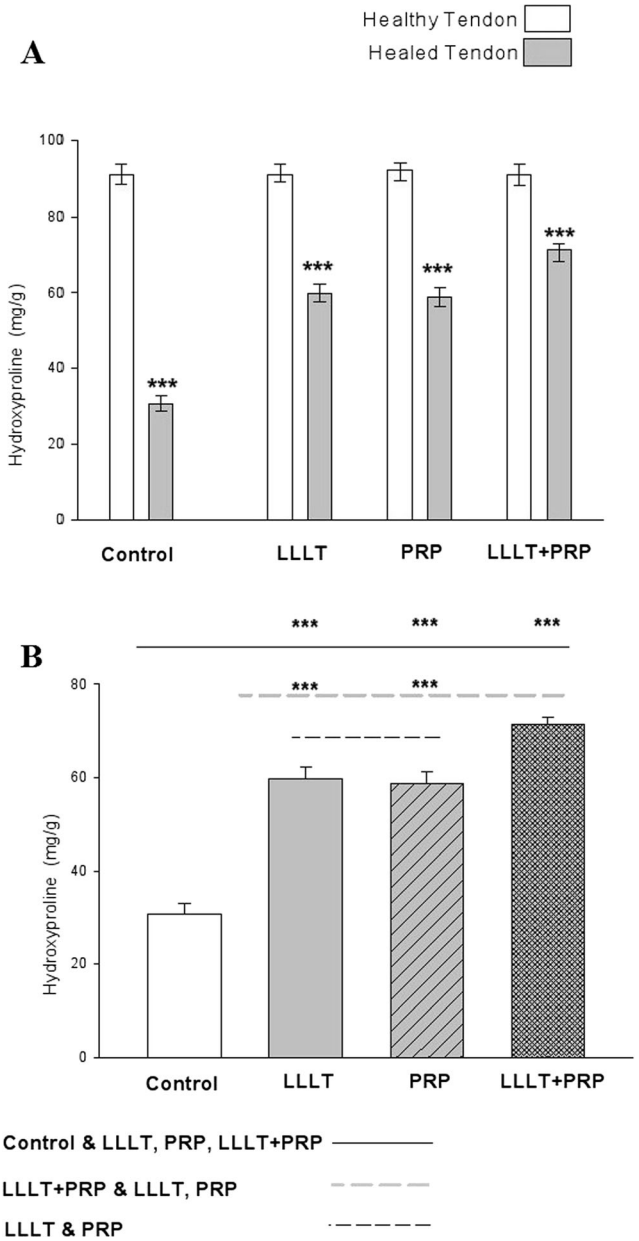

Fig. 1 **a** Comparison of hydroxyproline content in healed tendons with hydroxyproline content in healthy tendons (contralateral tendons); **b** the comparison of hydroxyproline content between groups. Tukey post hoc test was performed. ***$P<0.00$

difference ($P>0.05$) between the PRP group and LLLT group, although the PRP group results were slightly better compared with the laser group. Furthermore, it was found that the combined treatment with LLLT and PRP is even more efficient ($P<0.05$) than when each of the two treatments is used alone. Table 1 shows the mean and standard deviation of the experimental groups. Figure 2 shows histological changes using the hematoxylin and eosin staining. Sections of Achilles tendons with partial tenotomy showing the presence of collagen fibers are thin and red. Collagen fibers were more frequent in treated groups than in the untreated (control) group. Both groups that

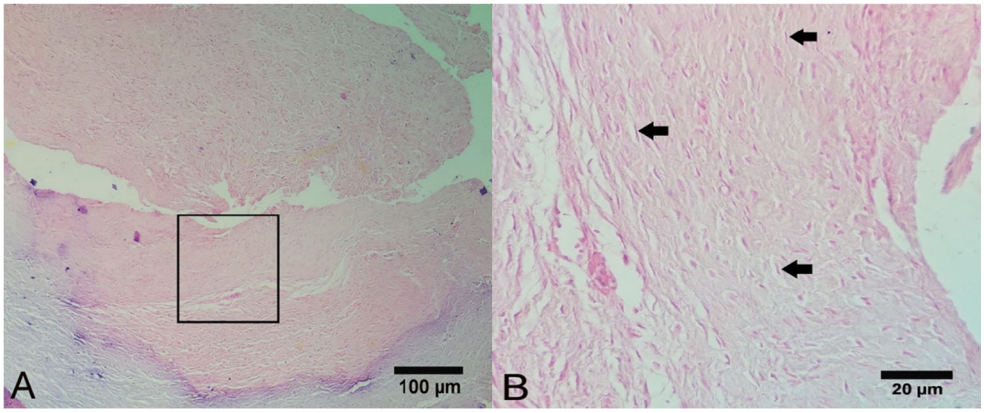

Fig. 2 Cross section of Achilles tendon with partial tenotomy; **a** control group. **b** Note the higher magnification with less regular arrangement of collagen fibers and low proliferation of the fibroblasts (*arrows*), which are thin and red. Hematoxylin and eosin staining. **a** ×100; **b** ×400

associated PRP and LLLT treatments presented higher proliferation of fibroblasts and better fiber arrangement (Figs. 3 and 4). Additionally, the LLT+PRP group showed higher fibroblasts proliferation and higher fiber arrangement compared with the PRP or laser groups alone (Fig. 5). However, no significant difference was found between the laser and PRP groups.

Biomechanical results

In this study, we examined two parameters of breaking strength and tensile strength.

Breaking strength

Significant differences are found between various breaking forces of treated tendon groups with untreated group ($P<0.05$), also the results showed that the breaking strength in the laser and PRP groups has a significant difference with the LLLT+PRP group ($P>0.05$), but no significant difference was found between the PRP and laser groups ($P>0.05$), as well as the fact indicating the importance of proper treatment for Achilles tendon healing. Figure 6a shows comparison of breaking forces in healed tendons with breaking forces in healthy tendons (contralateral tendons), and Fig. 6b shows the comparison of breaking forces between groups.

Table 1 Scores of tendon histological changes

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

Tukey post hoc test was performed

**$P=0.01$; ***$P=0.000$ compared with the control group; ‡ $P=0.046$ compared with the laser+PRP group; † $P=1.00$ compared with the PRP group

Tensile strength

We used defined formula (Newton per square millimeter × 1 = MPa) to convert collected data to megapascals and determine tensile strength. Based on the results, tensile strength in healthy and healed tendons had significant difference with untreated ones ($P>0.05$), and the results also showed a significant difference between healthy tendons and treated ones ($P>0.05$). The highest levels of tensile strength in treated groups are respectively related to the LLLT+PRP, PRP, and LLLT groups. The results showed that tensile strength in the LLLT and PRP groups had significant difference with the LLLT+PRP group ($P>0.05$), but there was no significant difference between the LLLT and PRP groups ($P<0.05$). Figure 7a shows a comparison of tensile strength in healed tendons with tensile strength in healthy tendons (contralateral tendons), and Fig. 7b shows a comparison of tensile strength between groups.

Discussion

Tendon repair begins with the reconnecting of tendon fibers and the gliding mechanism. Injury in tendon initiates several signaling events that recruit fibroblasts and stimulate the tenocytes population in the area to synthesize collagen and some other extracellular components, establishing physical continuity at the site. With more appropriate stimuli, such as mechanical strain and stress, subsequent remodeling restores partial or close-to-normal function. The healing process is slow and characterized by a scar with mechanically inferior tissue, at least initially. To achieve best results from tendon repair, this complex process will require a healing response that is enhanced by a combination of therapies, including tissue engineering, growth factors, mechanical stimulation, physical modalities, and gene therapy. These treatment modalities may improve tissue healing, mechanical strength, tendon gliding, and return to normal function while preventing

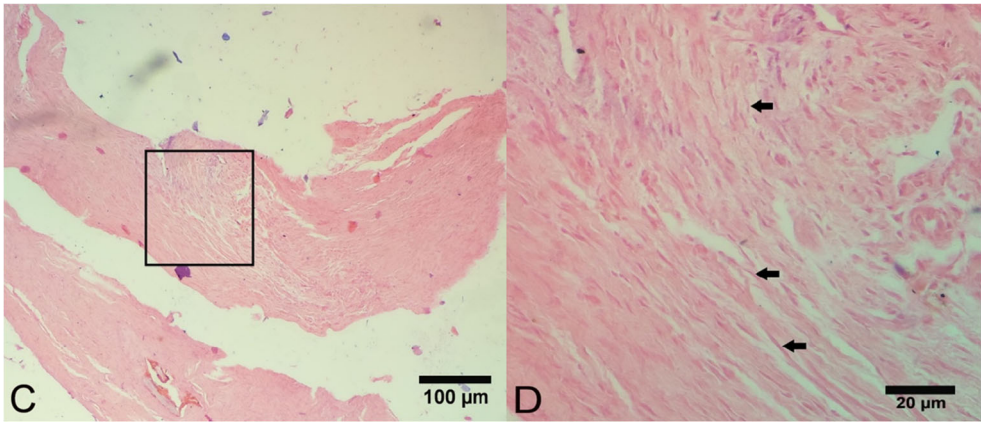

Fig. 3 Cross section of Achilles tendon with partial tenotomy: **c** laser group. **d** Note the higher magnification with regular arrangement of collagen fibers and high proliferation of the fibroblasts (*arrows*), which are thick and red. Hematoxylin and eosin staining. **c** ×100; **d** ×400

tendon gapping or ruptures and extensive adhesions. The proper choice of treatment is very important [15].

PRP increases the release of various substances such as vascular endothelial growth factor, platelet-derived growth factor (PDGF), transitional growth factor beta 1, fibroblast growth factor, connective tissue growth factor, insulin-like growth factor 1 (IGF-1), epidermal growth factor, platelet thromboplastin, serotonin, calcium, fibrinogen, and hydrolytic enzymes [16]. The exact mechanism of PRP is not known, but elevated levels of PDGF, tumor growth factor beta (TGF-β), and IGF-1 may play a positive role in tendon healing. PDGF stimulates the production of other growth factors in the acute phase of tendon injury. TGF-β inhibits MMP activity in the inflammatory phase and has a lot of effects on cell proliferation and migration. IGF-1 enhances cell migration and proliferation and collagen production and improves healing [17]. Hapa et al. found PRP injection, reduce the degree of inflammation at the site of injury, and after 2 weeks led to increase of tendon integration and tendon strength that these results correspond with our findings [18]. Many studies have reported that using PRP treatment causes tendon healing acceleration, raising quality of repair, and better organization in fibroblasts and collagen bundles.

Another therapeutic method for accelerating tendon healing is LLLT. LLLT accelerates the process of tendon healing and causes fibroblast cell proliferation and collagen synthesis increment. LLLT also causes reduction of inflammation after injury. At the cellular level, LLLT causes increment of ATP synthesis, cellular respiration, and production of molecular oxygen, resulting in increment of DNA synthesis and cell proliferation as well [11, 19]. Some of the papers indicate that laser irradiation increases cell growth via changes in mitochondrial physiology as well as the effect on RNA synthesis. LLLT may help more deposition of collagen and collagen reorganization in the affected area by stimulating the release of fibroblast growth factor and increasing the fibroblast cells. It seems that laser therapy can stimulate angiogenesis, restoration of blood flow to the injury site and thus limiting ischemic necrosis, and accelerate tissue repair [19]. The authors obtain different results from molecular, biomechanical, and histological changes after irradiation with different wavelength. Most of these studies have shown that LLLT is associated with positive outcomes in the healing tendon. LLLT increases GAGs and collagen types I and III and improves the healing process; it also increases migration of tenocytes to the injured area [20, 21]. One of the main requirements for the

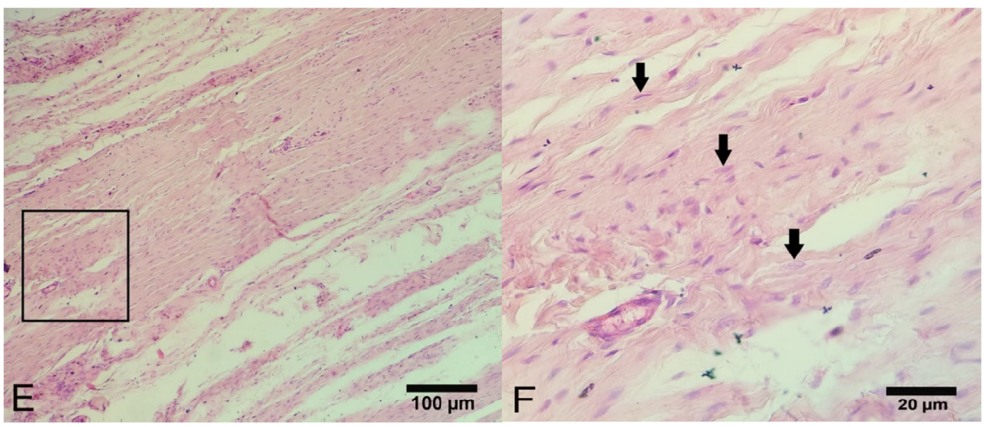

Fig. 4 Cross section of Achilles tendon with partial tenotomy; **e** PRP group. **f** Note the higher magnification with regular arrangement of collagen fibers and proliferation of the fibroblasts (*arrows*), which are thick and red, similar to laser group. Hematoxylin and eosin staining. **e** ×100; **f** ×400

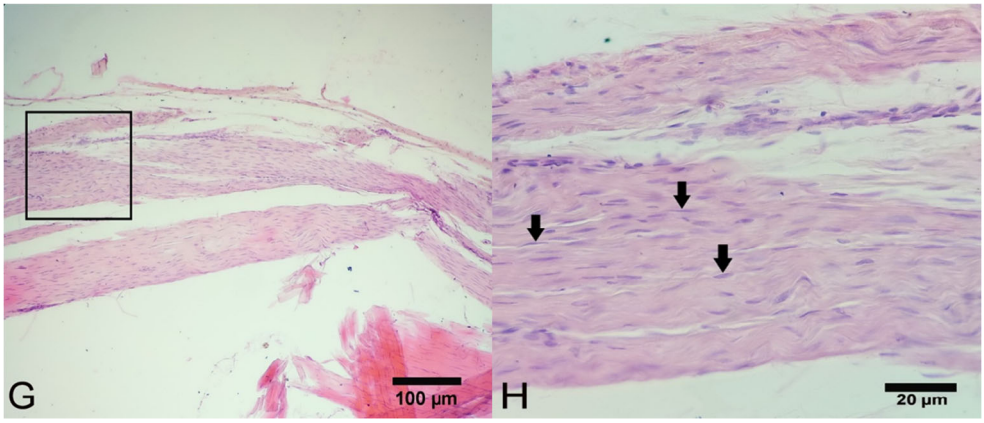

Fig. 5 Cross section of Achilles tendon with partial tenotomy; **g** LLT+PRP group. **h** Note the higher magnification with more regular arrangement of collagen fibers and highly proliferation of the fibroblasts (*arrows*). Hematoxylin and eosin staining. **g** ×100; **h** ×400

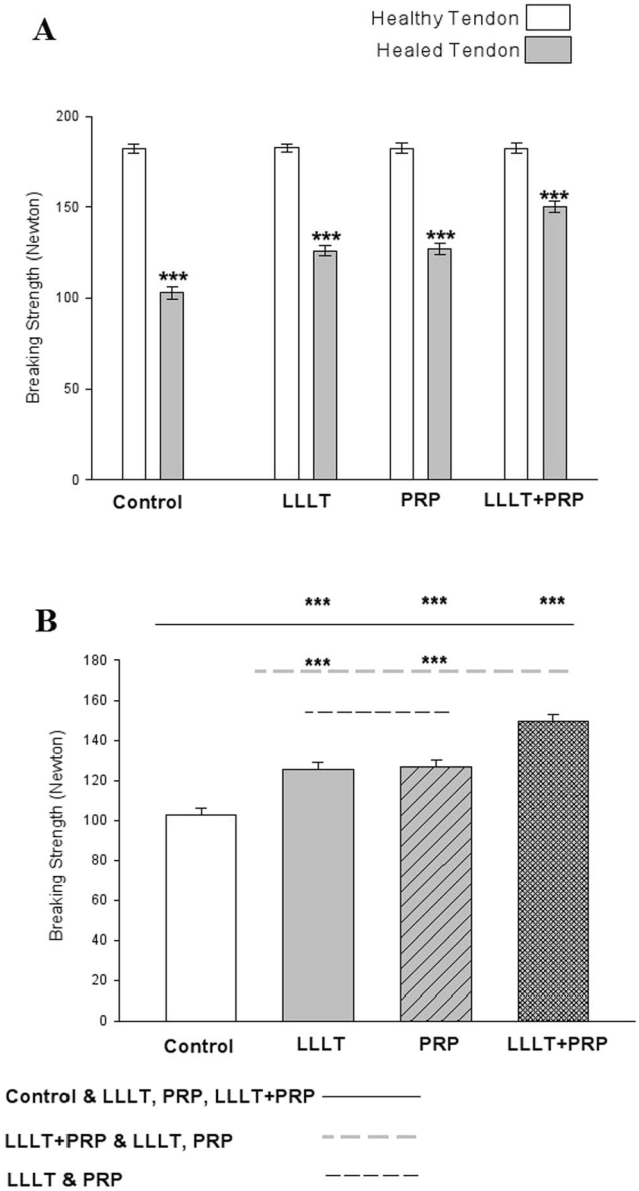

Fig. 6 **a** Comparison of breaking forces in healed tendons with breaking forces in healthy tendons (contralateral tendons); **b** the comparison of breaking forces between groups. Tukey post hoc test was performed. ***$P<0.00$

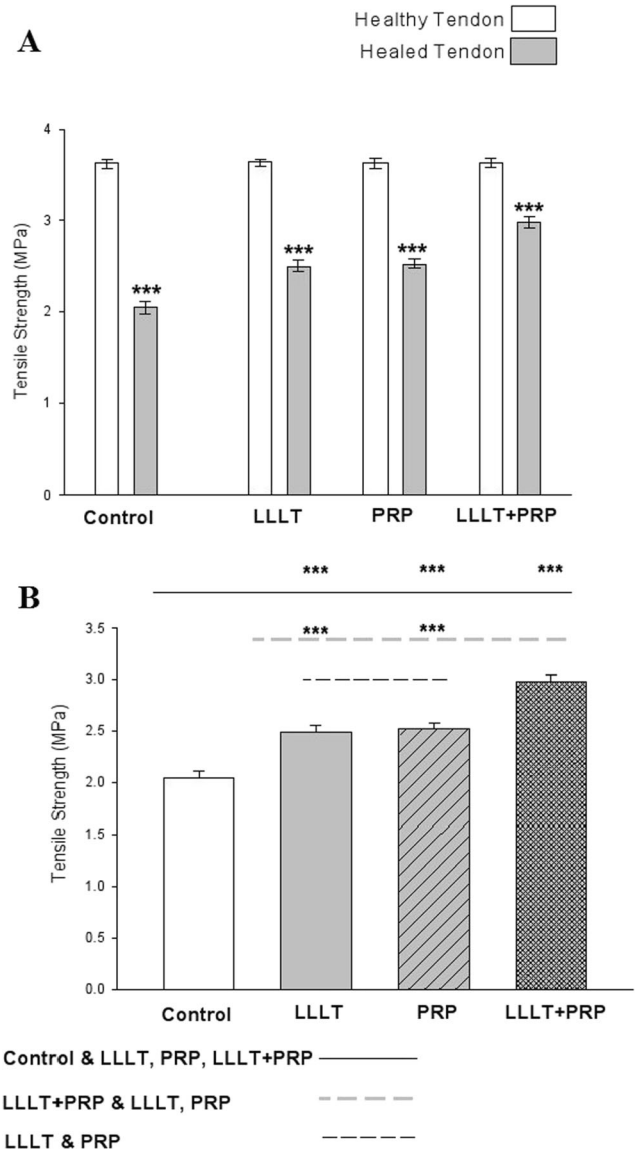

Fig. 7 **a** Comparison of tensile strength in healed tendons with tensile strength in healthy tendons (contralateral tendons) and diagram; **b** the comparison of tensile strength between groups. Tukey post hoc test was performed. ***$P<0.00$

reconstruction of the tendon is to achieve adequate tensile strength; therefore, we determined maximum tensile force before tendon rupture and tensile strength in this study. Measuring the breaking strength of the reconstructed tendons reflects the tensile strength of the newly scarred tissue collagen. In the present study, the results showed that the treatment of rabbits with PRP or LLLT alone has a significant advantage in breaking forces and tensile strength as a biomechanical parameter over untreated animals.

Barbosa et al. have examined effects of LLLT and PRP alone or together; they found that the deposition of collagen type I when the LLLT is done with a combination of PRP is higher, and histological changes in the treatment groups have positive and significant results compared with the control groups [16]. Hydroxyproline is a specific amino acid of collagen which is widely used in biological samples used to estimate the amount of collagen. Few studies have been conducted on the impact of PRP and LLLT on hydroxyproline levels in injured tendon. Studies that evaluated the effects of PRP treatment on the hydroxyproline levels of tendon showed positive results [22–24]. A study conducted by Ibrahimi et al. analyzed data collected from hydroxyproline contents of tendons, showing a significant difference between the treatment groups (PRP) and the control group [23]. A study by Nazhvani et al. in 2013 was conducted to evaluate the effect of PRP on tendon healing; the results showed hydroxyproline content increment by using PRP treatment, also the results showed that the hydroxyproline content in the PRP groups had a significant difference with the control groups. These findings confirm our results about hydroxyproline levels in the PRP group [24]. LLLT for 14 days on experimental defect in canine Achilles tendon after 30, 60, and 90 days resulted in hydroxyproline increment that after 90 days, this amount returned to normal range [25].

The results of this study emphasize the importance of LLLT and PRP treatment. These results resemble those found by other researchers who have reported satisfactory effects from LLLT and PRP treatment during the repair process in the tendon.

The results of this study indicate that combined treatment with PRP and laser has beneficial effects on reconstruction of the Achilles tendon. The results showed that treatment with PRP or laser alone has significant advantages compared with the untreated animals. Moreover, it was found that simultaneous LLLT+PRP treatment is even more effective.

Conclusion

The combination of LLLT and PRP is a very effective way for tendon injury treatment. Especially in cases where obtaining the desired results is necessary, the use of multiple therapies is required. The combination of these two methods enables the patient to recover faster.

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