Photobiomodulation of Matrix Metalloproteinases in Rat Calcaneal Tendons

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Abstract

Objective: The main objective was to verify the modulatory effects of MMP-1, MMP-3, and MMP-13 levels on the partially injured calcaneal tendons of rat exposure to photobiomodulation.

Background: Photobiomodulation has been shown to have anti-inflammatory and regenerative effects on tendon injuries. However, there is still uncertainty regarding the beneficial effects in matrix metalloproteinase (MMP) levels, especially MMP-1, -3, and -13.

Materials and methods: Sixty-five male Wistar rats were used. Sixty were submitted to a direct trauma on the calcaneal tendons and were randomly distributed into the following six groups: LASER 1, 3, and 7 (10 partially injured calcaneal tendons in each group treated with photobiomodulation for 1, 3, and 7 days, respectively) and Sham 1, 3, and 7 (same injury, with simulated photobiomodulation). The remaining five animals were allocated to the normal group (no injury or treatment procedure). The 780 nm low-level laser was applied with 70 mW of mean power and 17.5 J/cm2 of fluency for 10 sec, once a day. The tendons were surgically removed and analyzed for MMP-1, MMP-3, and MMP-13 through immunohistochemistry.

Results: MMP-3 levels remained close to normal in all experimental groups (p > 0.05); however, reductions (p < 0.05) in MMP-1 and MMP-13 levels were detected in the groups submitted to one, three, and seven low level laser therapy applications.

Conclusions: The photobiomodulation protocol was able to reduce MMP-1 and MMP-13 levels in injured calcaneal tendons.

Keywords: calcaneal tendon, matrix metalloproteinases, inflammation, photobiomodulation, tendinopathy

Introduction

The acute and degenerative calcaneal tendon injuries demand deeper understanding and greater precision in the measurement of various aspects of associated dysfunction.1 This injury presents a significant burden in clinics, representing 45% of musculoskeletal lesions.2–4 There are conditions that make this repair process slow and difficult, one of which is the presence of several pro-inflammatory and degenerative factors in injured tendon tissue,5–8 such as matrix metalloproteinases (MMPs), that are attracted and activated by other pro-inflammatory factors and may further aggravate the injuries and perpetuate the changes in the injured tendon.8–14

MMPs are a family of zinc- and calcium-dependent endopeptidases active at a neutral pH. It has important functions in the maintenance, remodeling, and degradation of the tendon matrix and its activity is inhibited by tissue inhibitors of metalloproteinases (TIMP).15 Further, the synthesis of MMPs may change substantially in the presence of inflammatory processes in injured tendons. This can be the case especially in the presence of primary pro-inflammatory factors seen in the early stages of calcaneal tendon injuries, which stimulate the increase in MMPs’ synthesis.12,16–20

In addition, MMP-1 and MMP-13 levels are upregulated in injured tendon tissue, especially those submitted to shear stress21; therefore, MMP-3 may play a role in the normal maintenance and remodeling of tendons.15 The presence of

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MMP-1, MMP-3, and MMP-13 also promotes degradation of type I and III collagen, the main collagen types that make up the calcaneal tendon. The uncontrolled presence of these agents can make the injured tendon become functionally weakened and susceptible to ruptures. Thus, it becomes important to control these MMPs to improve the repair process of calcaneal tendon injuries.

There are some reports showing that photobiomodulation controls the presence of primary pro-inflammatory factors, stimulates collagen proliferation and realignment, and elicits angiogenesis in tendon injury models. However, there is still a lack of research investigating the photobiomodulation anti-inflammatory mechanisms at this experimental model on the control of secondary pro-inflammatory factors. Thus, the objective of this study is to further clarify the modulatory effects of photobiomodulation on the MMP-1, MMP-3, and MMP-13 levels. This study will provide information that demonstrates possible specificities on the use of this resource for the repair of calcaneal tendon injuries.

Materials and Methods

The Research Ethics Committee of Universidade Federal de São Paulo evaluated and approved the present study, under protocol no. 0074/2011.

A total of 65 male rats (Rattus norvegicus)—lineage: Wistar; var. albinus, order: Rodentia, class: Mammalia, age 12 weeks, and body mass 270–300 g, were used. The animals were housed in standard polypropylene cages (five per cage), in 12-h light/12-h dark cycles, temperature around 20°C, humidity of 65%, and water and food ad libitum.

Injury

Sixty animals were randomly anesthetized with intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (50 mg/kg).

After anesthesia, the areas corresponding to the calcaneal tendon of the right hind paw of the animal were shaved. The animal was then positioned at the base of a small guillotine device designed to induce direct trauma injury to the calcaneal tendons with the right hind paw positioned at the designated site of the equipment; a dorsiflexion of the ankle was exerted until the dorsal region touched the base of the equipment. Finally, a weight of 186 g was released from a height of 20 cm above the central part of the calcaneal tendon of each animal, corresponding to an energy potential of 364.9 mJ at the time of the trauma. The five remaining animals were not exposed to any procedure.

Groups

After the partial injury to the calcaneal tendon, the 60 animals were randomly allocated into the following six groups: LASER 1, 3, and 7 (10 partially injured calcaneal tendons in each group treated with photobiomodulation for 1, 3, and 7 days, respectively) and Sham 1, 3, and 7 (same injury, with simulated photobiomodulation).

The remaining five animals (10 tendons) were allocated to the normal group (no injury or treatment procedure).

Photobiomodulation

A low-level laser device (model: Twin Laser/MMOptics—São Carlos, São Paulo, Brazil) with active semiconductor (AsGaAl) was used in this study. Table 1 presents the photobiomodulation parameters.

The animals received daily exposures of photobiomodulation (a single application per animal), in a continuous way, with fluence equal to 17.5 J/cm², for 10 sec and total final energy of 0.7 J. The protocol started 1 min after the injury was induced. The animals were stabilized in a foam cage during the photobiomodulation applications, and the laser irradiations were employed using the contact technique covering all calcaneal tendon injured sites. The animals were treated for 1 day (LASER 1), 3 consecutive days (LASER 3), and seven consecutive days (LASER 7). The animals of Sham groups (1, 3, and 7) received simulated photobiomodulation applications with the device switched off; however, the equipment remained in contact with the injured area following the same procedures as the LASER groups.

Euthanasia

The animals of LASER/Sham groups were euthanized (anesthetic overdose) 24 h after the last photobiomodulation exposure with the exception of normal group animals that were euthanized on the last day of the experiment (eighth day).

Sample procedures

The calcaneal tendons of both hind paws of the normal group were collected, along with the 60 tendons (right hind paw) of the animals belonging to the other groups. Therefore, 70 (65 animals) calcaneal tendons were surgically removed and immediately washed in 0.9% saline and then fixed in 4% paraformaldehyde in 0.1 M Millonig buffer (pH 7.2–7.4) for 12 h. After fixation, the tendons were dehydrated, diaphanized, and embedded in paraffin. The sections were made with longitudinal cuts obtained in a microtome (model: RM2155; Leica) with a thickness of five microns (5 μm) for the immunohistochemical technique. For the adhesion of the cuts referring to the immunohistochemical analyses, the slides were dipped in silane (3-aminopropyl-triethoxysilane; Sigma).

Table 1. Photobiomodulation Dosimetric Parameters

<table>
<thead>
<tr>
<th>Photobiomodulation parameters</th>
<th>AsGaAl (LASER diode)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Light source</strong></td>
<td>780</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>780</td>
</tr>
<tr>
<td>Mean power (mW), mW</td>
<td>70</td>
</tr>
<tr>
<td>Beam area, cm²</td>
<td>0.04</td>
</tr>
<tr>
<td>Power density (ΔP), W/cm²</td>
<td>1.75</td>
</tr>
<tr>
<td>Energy density (ΔE), J/cm²</td>
<td>17.5</td>
</tr>
<tr>
<td>Length of each treatment session, sec</td>
<td>10</td>
</tr>
<tr>
<td>Dose of each treatment session, J</td>
<td>0.7</td>
</tr>
<tr>
<td>Cumulative dose (LASER 1, 3, and 7, respectively), J</td>
<td>0.7/2.1/4.9</td>
</tr>
</tbody>
</table>
Therefore, for each animal studied, four nonconsecutive sections were obtained and randomly allocated—one for hematoxylin–eosin (H&E) staining and three for immunohistochemistry (MMP-1, MMP-3, MMP-13).

**Immunohistochemistry**

The slides were dewaxed and exposed to specific antibodies kits for MMP-1 (monoclonal anti-MMP-1, brand: ABCAM-AB52915, dilution 1:200), MMP-3 (polyclonal anti-MMP-3, brand: ABCAM-AB38929, dilution 1:50), and MMP-13 (polyclonal anti-MMP-13, brand: ABCAM-AB39012, dilution 1:100).

Antigen retrieval was performed with porcine Trypsin in phosphate-buffered saline (PBS, code: T7409; Sigma-Aldrich). Blocking of endogenous peroxidase was performed with 10% hydrogen peroxide (3%), then washed in tap water and deionized water, and left in phosphate buffer pH 7.4 (PBS). The nonspecific proteins were blocked by immersing the slides in casein (Synth, São Paulo, Brazil) diluted in phosphate buffer pH 7.4.

The slides were incubated with the primary antibodies and specific diluent (Spring Bioscience, Pleasanton, CA) for 24 h at 4°C and subsequently incubated with the secondary antibody Reveal (Spring Biosciences) for 45 min at 37°C. After that procedure, the slides were revealed in diaminobenzidine (Spring Biosciences) for 10 min at room temperature. Finally, the slides were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany) and mounted with Entellan (Merck).

The photobiomodulation anti-inflammatory response was analyzed in three calcaneal tendons’ microscopic fields (proximal: the region just under the myotendinous junction, intermediate: the area between these two regions, and distal: the region just above the osteotendinous junction). Those microscopic images were previously obtained at 400× magnification (objective of 40×) with a camera (DFC420; Leica Microsystems, Switzerland) coupled to a DMLB microscope (Leica Microsystems). After that, the positive cells for MMP-1, MMP-3, and MMP-13—marked in brown shades—were quantified with image software LAS version 4.5.0 (Leica Microsystems).

**Statistical analysis**

The data normality distribution has been verified using the Shapiro–Wilk test and homogeneity of variances by Levene test. For each animal studied, four nonconsecutive sections were obtained and randomly allocated—one for hematoxylin–eosin (H&E) staining and three for immunohistochemistry (MMP-1, MMP-3, MMP-13).

Table 2. MMP-1, MMP-3, and MMP-13 Positive Cells (Mean ± SD) of the Three Tendon Regions Analyzed (Proximal, Intermediate, and Distal).

<table>
<thead>
<tr>
<th>Tendon Region</th>
<th>Proximal</th>
<th>Intermediate</th>
<th>Distal</th>
<th>Mean ± SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18.9 ± 5.6</td>
<td>15.7 ± 6.0</td>
<td>40.3 ± 10.7</td>
<td>22.3 ± 7.3</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Sham 1</td>
<td>18.4 ± 11.4</td>
<td>16.6 ± 6.2</td>
<td>14.9 ± 8.1</td>
<td>14.2 ± 4.3</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>LASER 1</td>
<td>16.5 ± 4.6</td>
<td>10.5 ± 3.5</td>
<td>18.9 ± 4.1</td>
<td>11.6 ± 4.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>LASER 2</td>
<td>16.5 ± 4.6</td>
<td>10.5 ± 3.5</td>
<td>18.9 ± 4.1</td>
<td>11.6 ± 4.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>LASER 3</td>
<td>15.6 ± 7.7</td>
<td>14.8 ± 6.0</td>
<td>14.2 ± 4.9</td>
<td>10.2 ± 3.4</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Sham 7</td>
<td>12.9 ± 4.6</td>
<td>11.7 ± 5.4</td>
<td>18.9 ± 8.1</td>
<td>13.1 ± 3.0</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>LASER 7</td>
<td>13.0 ± 4.6</td>
<td>10.3 ± 2.5</td>
<td>10.3 ± 2.5</td>
<td>11.3 ± 4.5</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

MMP, matrix metalloproteinase.
Results

There were no differences \( p > 0.05 \) between the three tendon regions analyzed (proximal, intermediate, and distal) for MMP-1, MMP-3, and MMP-13 mean number of positive cells (Table 2), and the regression model proved to be efficient \( p < 0.05 \) to predict the outcome for MMP-1 and MMP-13, but not for MMP-3 \( p > 0.05 \).

Although the regression model did not prove to be efficient to predict MMP-3 outcomes, the experimental LASER 3 group presented lower levels of MMP-3 than the normal group \( p < 0.05 \). However, the other experimental groups (LASER 1, LASER 7, and Sham 1, 3, and 7) did not present modulations at MMP-3 levels when the means of the analyzed areas were compared with those of the normal group \( p > 0.05 \). Differences were identified between the experimental groups and the normal group \( p < 0.05 \) for MMP-1 and MMP-13 levels. LASER 1, LASER 3, LASER 7, and Sham 7 had lower MMP-1 levels than the normal group, and LASER 1, LASER 3, LASER 7, Sham 1, and Sham 7 had lower MMP-13 levels than the normal group (Table 3).

Figure 1 shows photomicrography of tendon morphology (H&E) and immunohistochemistry positive cells \( (\times) \) for MMP, both acquired with 40× objective lens.

Discussion

The main objective of this animal controlled trial was to evaluate the photobiomodulation of MMP-1, MMP-3, and MMP-13 levels in injured calcaneal tendons of rats. The most significant findings of this study were the non-modulation of MMP-3 levels and reductions in MMP-1 and MMP-13 levels in the groups submitted to one, three, and seven photobiomodulation applications.

The application of photobiomodulation to optimize the repair process of the calcaneal tendon is very promising because this treatment modality has the ability to use the modulatory pathway to control inflammatory processes \(^{8,10,24}\). This ability to control inflammatory processes elucidates the relevance of using this treatment modality because the presence of primary pro-inflammatory factors \(^{8,10,15,21}\) can induce the increase in MMPs’ levels. In addition, the uncontrolled presence of MMP-1, MMP-3, and MMP-13 tends to increase the degradation of extracellular matrix \(^{25}\) as well as of the calcaneal tendon collagen, fragilizing and hampering its repair process. \(^{15,21,26-28}\)

Unlike other studies on the same topic, \(^{10,12}\) there was a decrease in MMP-1 and MMP-13 levels in the experimental groups in the current study, and MMP-3 levels showed no modulations. A previous experimental study by our research group, \(^{9}\) with the same design as the current study, showed that 780 nm low-level laser has the capacity to modulate IL-1\( \beta \), COX-2, and PGE2 levels when the same treatment protocol was implemented. The use of the same design in terms of dose and groups may explain the present results, given that these primary pro-inflammatory factors can trigger the increase in MMPs’ levels. \(^{15,21}\)

Although the inflammatory process may perpetuate and aggravate the calcaneus tendon injury \(^{29,30}\), most of these lesions are caused by repetitive mechanical stress in the tendon and consequently morphological changes. \(^{1,31}\) Because of this and differently from previous studies, we chose to use a well-established injury model that mimicked this characteristic. \(^{5,7,8,14,24}\) It is possible that these other forms of injury (chemical: collagenase \(^{4,12,22}\) and carrageenan \(^{1,25}\)) were more sensitive to cause modulation of MMPs’ levels. Further, MMP-3 is more active in the maintenance of healthy tendons and in later phases of the repair process (remodeling phases). These phases are in contrast to those promoted by the experimental model times of the current study, \(^{15,21}\) which focused on the inflammatory phase of the repair process (1, 3, and 7 days) and which may have influenced the nonmodulation of the levels of this enzyme.

The Sham groups presented some differences in their MMP-1 and MMP-13 levels. These results may be caused by the mechanical stresses imposed by the rat’s walking pattern. There are reports in the literature showing increased MMP-1 levels in tendinopathic tissue submitted to shear stress, unlike the mechanical overload imposed by the rat’s walking pattern. Instead, cyclic strain overload (similar to the rat’s mechanical walking pattern) causes a reduction in MMP-1 levels and may cause an upregulation of MMP-13. \(^{15}\)

The interaction between photobiomodulation and the different pathways of tissue repair processes is dependent on several factors related to wavelength, mean power, dose, and frequency of treatment. \(^{32,33}\) This can be observed in previous studies that used photobiomodulation with \( \lambda = 810 \text{ nm} ^{10,12} \) and \( 830 \text{ nm} ^{11,25} \) mean power of 100 mW \(^{10,12}\) and 40mW \(^{11,25}\) and final energy varying between 1 and 3 J. \(^{10,12,25}\) Although the dosage adjustments and the treatment regimens of these studies differed from those of the present study, reductions in MMP-13 levels were also detected, as well as reductions in COX-2, TNF-\( \alpha \), MMP-2, MMP-3, and MMP-9. Casalechi et al. \(^{22}\) used photobiomodulation with \( \lambda = 780 \text{ nm} \), mean power of 22 mW,
E = 1.54 J, and one, three, four, and seven applications and found similar results to those of the present study, that is, modulations in MMP-1 and MMP-13 levels.

Another study shows specific effects of different wavelengths and dosage adjustments of photobiomodulation for the most diverse factors seen in an injury and consequent repair process of the calcaneal tendon. It is possible that the reduction in MMPs' levels after photobiomodulation was directly and indirectly influenced by the treatment regimen used. This is due to the fact that previous studies demonstrated interactions between the same photobiomodulation protocol applied in this study (λ = 780 nm) and the TIMP and other studies published by our group showing modulation of primary inflammatory factor levels. These effects seem to be achieved by the modulatory pathway.

Although currently photobiomodulatory protocol has presented positive effects for the control of MMPs' levels, new experimental studies involving low level laser therapy effects in all phases of the calcaneal tendon repair process (inflammatory, proliferative, and remodeling phases) are needed.

**Conclusions**

The photobiomodulation protocol applied in this animal model was able to reduce MMP-1 and MMP-13 levels in injured calcaneal tendons in the early stages of injury and the prechronic phase.

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Author Disclosure Statement

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