Rehabilitation Practice and Science

Volume 42
Issue 4 *Taiwan Journal of Physical Medicine and Rehabilitation (TJPMR)*

Article 5

12-31-2014

Effects of Low-Level Laser Therapy on Matrix Metalloproteinase and Collagen Expression of Tendon Cells

Chien-Hung Chen

Shih-Wei Hung

Jong-Hwei S. Pang

Wen-Chung Tsai

Miao-Sui Lin

See next page for additional authors

Recommended Citation

Chen, Chien-Hung; Hung, Shih-Wei; Pang, Jong-Hwei S.; Tsai, Wen-Chung; Lin, Miao-Sui; Yu, Tung-Yang; and Chang, Hsiang-Ning (2014) "Effects of Low-Level Laser Therapy on Matrix Metalloproteinase and Collagen Expression of Tendon Cells," *Rehabilitation Practice and Science*: Vol. 42: Iss. 4, Article 5.
DOI: https://doi.org/10.6315/2014.42(4)05
Available at: https://rps.researchcommons.org/journal/vol42/iss4/5

Effects of Low-Level Laser Therapy on Matrix Metalloproteinase and Collagen Expression of Tendon Cells

Authors

Chien-Hung Chen, Shih-Wei Hung, Jong-Hwei S. Pang, Wen-Chung Tsai, Miao-Sui Lin, Tung-Yang Yu, and Hsiang-Ning Chang

Original Article

Effects of Low-Level Laser Therapy on Matrix Metalloproteinase and Collagen Expression of Tendon Cells

Chien-Hung Chen,[1] Shih-Wei Hung,[1] Jong-Hwei S. Pang,[2] Wen-Chung Tsai,[1,2] Miao-Sui Lin,[2] Tung-Yang Yu,[1,2] Hsiang-Ning Chang[1]

[1]Department of Physical Medicine and Rehabilitation, Linkou Chang Gung Memorial Hospital; [2]Graduate Institute of Clinical Medicine Sciences, College of Medicine, Chang Gung University, Taoyuan

Objective: Low-level laser therapy (LLLT) is commonly used to treat sports-related tendinopathy or tendon injury. However, scientific evidence of the effects and underlying molecular mechanisms of tendinopathy treatment remained limited. Tendon healing requires tenocyte migration to the repair site, followed by proliferation and synthesis of the extracellular matrix, including collagens. The study was designed to determine the effect of laser on the metabolism of collagen for injuried tendon repair, furthermore, become a foundation of the LLLT for tendon repair.

Methods: Tendon cells from rat Achilles tendons were obtained to investigate the effect of LLLT on collagen metabolism in tendon repair. LLLT was performed with a 600-nm laser in continuous mode with an output power of 50 mW at different periods with increasing energy densities. Real-time polymerase chain reaction (PCR) and western blot analysis were used to determine the gene expression and production of matrix metalloproteinase (MMP) and collagen.

Results: The mRNA expression of MMP-9 was down-regulated by LLLT dose-dependently, the mRNA expression of type I collagen was up-regulated at an energy density of 1.0 J/cm^2, and type III collagen was up-regulated dose-dependently. Densitometry analysis using western blot indicated that protein expression of MMP-8 and MMP-9 was dose-dependently down-regulated by laser treatment and type I collagen was up-regulated at an energy density of 1.0 J/cm^2.

Conclusions: LLLT dose-dependently down-regulates the expression of MMP-9 and up-regulates the expressions of type I collagen at an energy density of 1.0 J/cm^2. (Tw J Phys Med Rehabil 2014; 42(4): 215 - 221)

Key Words: laser, tendon, matrix metalloproteinase, collagen

INTRODUCTION

Low-level laser therapy (LLLT) has been used to treat musculoskeletal pain for nearly 3 decades[1]. LLLT has shown to be potential of effectiveness in treating musculoskeletal injuries and chronic pain, and for wound healing[2]. Many studies have shown its usefulness for

Submitted date: 10 February 2015 Revised date: 23 March 2015 Accepted date: 8 April 2015

Correspondence to: Dr. Hsiang-Ning Chang , Department of Physical Medicine and Rehabilitation, Linkou Chang Gung Memorial Hospital, Taiwan ; No.5, Fusing Street, Gueishan Township, Taoyuan County 333, Taiwan

Tel：(03) 3281200 ext 3846 E-mail：lilychang0412@gmail.com

doi: 10.6315/2014.42(4)05

treating patients with tendinopathy[3-6]. Although the widespread clinical application of LLLT suggests its efficacy, scientific evidence of the effects and underlying molecular mechanisms of tendinopathy treatment remained limited.

In-vivo studies have revealed that LLLT can enhance healing of Achilles tendon by improving collagen fiber organization, preventing oxidative stress, and reducing fibrosis[7-9]. The exact mechanism is unclear, but the proposed mechanism is photochemical rather than thermal[10,11]. In the regenerative phase of tendon injury repair, the tenocytes migrate to the repaired site, proliferate actively, and enhance deposition of extracellular matrix (ECM) in the tissue. Laser irradiation has been shown to promote porcine tenocyte proliferation and up-regulation of type I collagen and decorin[12]. Among physical agents, therapeutic ultrasound and electric stimulation have been demonstrated to enhance tenocyte and ligament fibroblast migration, respectively [13,14]. However, to our knowledge, no study has explored the effect and underlying molecular biological mechanism of laser irradiation on collagen metabolism.

Extracellular matrix and tendon

Tenocytes, which are the basic cellular components of tendons, produce collagen, protein mediators for tendon repair, and matrix proteoglycans[15]. The ECM is a complex of collagen, glycoproteins, glycosaminoglycans, and proteoglycans[16]. Approximately 90% of the collagen in normal tendons is type I, and less than 10% is type III [17]. The biomechanical properties of a tendon are primarily a feature of the ECM, which is in a constant state of dynamic equilibrium between synthesis and degradation[18]. Matrix metalloproteinases (MMPs) are a family of ECM-degrading enzymes inhibited by a family of proteins called the tissue inhibitors of MMPs (TIMPs) [19]. MMPs are important regulators of ECM network remodeling, and MMP levels are altered during tendon healing[16,19,20]. MMP-8 and MMP-13 are the collagenases that cleave type I collagen molecules in the ECM[21,22]. MMP-2 and MMP-9 also have collagenolytic activity [22,23]. Previous findings suggest that MMP-9 and MMP-13 only participate in collagen degradation, whereas MMP-2, MMP-3, and MMP-14 participate in both collagen degradation and remodeling[16,24]. Therefore, the activity of MMPs may play an important role in tendon healing.

The purpose of this study was to investigate the effects of LLLT on the mRNA and protein expression of MMP-2, MMP-9, type I collagen, and type III collagen in tendon cells.

MATERIALS AND METHODS

All the procedures were approved by Chang Gung University Institutional Animal Care and Use Committee before the experiments.

Primary culture of rat Achilles tenocytes

Achilles tendons from Sprague-Dawley rats (weight, 200–250 g) were excised. The excised tendon was soaked in povidone-iodine for 3 min and washed twice in phosphate-buffered saline (PBS). Each tendon was then cut into small pieces of approximately 1.5–2.0 mm^3 (6 pieces in total), and these pieces were placed in six-well culture plates. After 5 min of air-drying for better adherence, 0.5 mL of Dulbecco's modified Eagle's medium (HyClone, Logan, Utah, USA), with 10% fetal bovine serum (Cansera, Rexdale, Ontario, Canada), 100 U/mL penicillin, and 100 μg/mL streptomycin was added to each well. The explants were then incubated at 37ºC in a humidified atmosphere of 5% CO_2/95% air. After migrating from the explants, the cells started to grow rapidly, and the confluence culture was subcultured by trypsin digestion at a 1:3 dilution ratio. Tendon cells between passages 2 and 4, with proper growth rate and normal fibroblast shape, were used in the following experiments.

Laser irradiation procedure

Laser irradiation was performed with a 660-nm laser (Konftec, Megalas-AM-800, New Taipei City, Taiwan) in continuous mode with an output power of 50 mW and unit energy density of 0.0032 J/sec-cm^2. The irradiation power was uniformly checked with a power meter before and after treatment. The laser beam irradiated the culture plate from above at a distance of 30 cm, and covered an area up to 314 cm^2. The laser beam was applied perpendicularly and evenly to the culture plates in three groups of wells for 5.2 and 10.4 min, respectively. The corresponding energy densities were 1.0 and 2.0 J/cm^2. The

irradiation was performed on a clean bench through the medium culture at room temperature. The control groups were not subjected to laser irradiation but were removed from the incubator at the same time as the laser-treated plates. The mRNA and protein expressions were assessed 24 h after laser irradiation.

Reverse transcription/real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from tendon cells using solution D (1 mL solution D/10^7 cells). Subsequently, total RNA was extracted with phenol and chloroform:isoamyl alcohol (49:1) to remove proteins and genomic DNA. Complementary (c)DNA was synthesized using 1 mg total RNA in a 20 mL volume RT reaction mixture containing 0.5 mg random primers, 0.8 mM dNTP, 0.1 M DTT, and 1 L first strand buffer. Quantitative real-time PCR was performed using an SYBR Green and MxPro-Mx3000P QPCR machine (Stratagene). Aliquots (20 ng) of cDNA were used for each quantitative PCR, and each reaction was run in triplicate. The following primers were used.

GAPDH: 5'-TTCATTGACCTCAACTACAT-3' (forward) and 5'-GAGGGGCCATCCACAGTCTT-3' (backward).

Dynamin 2: 5'-AGAACGGCAAGTGGAAAC-3' (forward) and 5'-AGCATAGGCAGCAGGTCA-3' (backward).

Relative gene expressions between experimental groups were determined using MxPro software (Stratagene), and GAPDH was used as an internal control. All real-time PCRs were performed in triplicate, and changes in gene expressions were presented as multiples of increases relative to the untreated controls.

Western blot analysis

Cell extracts were prepared in lysis buffer containing Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM PMSF, and 1% Triton X-100 followed by sonication. Protein concentration of the cell extracts was determined by Bradford assay (Bio-Rad Laboratories, CA, USA). Samples with identical protein quantities (10 μg/lane) were then separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred on to a PVDF membrane. The membrane was incubated at room temperature in blocking solution (1% BSA, 1% goat serum in PBS) for 1 h, followed by 2-h incubation in blocking solution containing an appropriate dilution of primary antibody, e.g., MMP-2, MMP-9, MMP-8, MMP-13 (NeoMarks, Fremont, CA, USA), collagen I, and collagen III (Abcam). After washing, the membrane was incubated in PBS containing goat anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) for 1 h. The membranes were washed and the positive signals developed with enhanced chemiluminescence reagent (Amershan Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). The band density of each protein was normalized to relative band density of tubulin.

Statistical Analysis

All data are expressed as mean ± SEM. Comparisons between the results of spreading assays of the laser-treated and control cells were performed using Kruskal-Wallis test. A Mann-Whitney test was used to identify the difference. The level of statistical significance is set at a p value of 0.05.

RESULTS

Real-time PCR showed mRNA expression of MMP-2, MMP-8, MMP-9, MMP-13, collagen I, and collagen III (Fig 1). The mRNA expression of MMP-9 was down-regulated by LLLT dose-dependently (0.79 ± 0.03, 0.43 ± 0.02 fold for tenocytes treated by LLLT at energy densities of 1.0 J/cm^2 and 2.0 J/cm^2, respectively; p = 0.024 by Kruskal-Wallis test) (Fig. 1c). However, the mRNA expression of collagen I was up-regulated by LLLT at an energy density of 1.0 J/cm^2 (p = 0.037 by Mann–Whitney test) (Fig. 1e), and that of collagen III was up-regulated dose-dependently (p = 0.041 by the Kruskal-Wallis test) (Fig. 1f). There was no statistically significant difference in the mRNA expression of MMP-2, MMP-8, and MMP-13 (Fig. 1a, b, d).

Densitometry analysis using western blot indicated that MMP-9 protein expression was also dose-dependently down-regulated by LLLT (p = 0.006 by the Kruskal-Wallis test) (Fig. 2c), and collagen I protein expression was up-regulated by LLLT at an energy density of 1.0 J/cm^2 (p = 0.005 by the Mann–Whitney test) (Fig. 2e), which was compatible with the results of

real-time PCR (Fig. 1c, e). The protein expression of MMP-8 and collagen III was down-regulated by LLLT (Fig. 2b, f), and that of MMP-2 was up-regulated by LLLT at an energy density of 1.0 J/cm^2 (Fig. 2a).

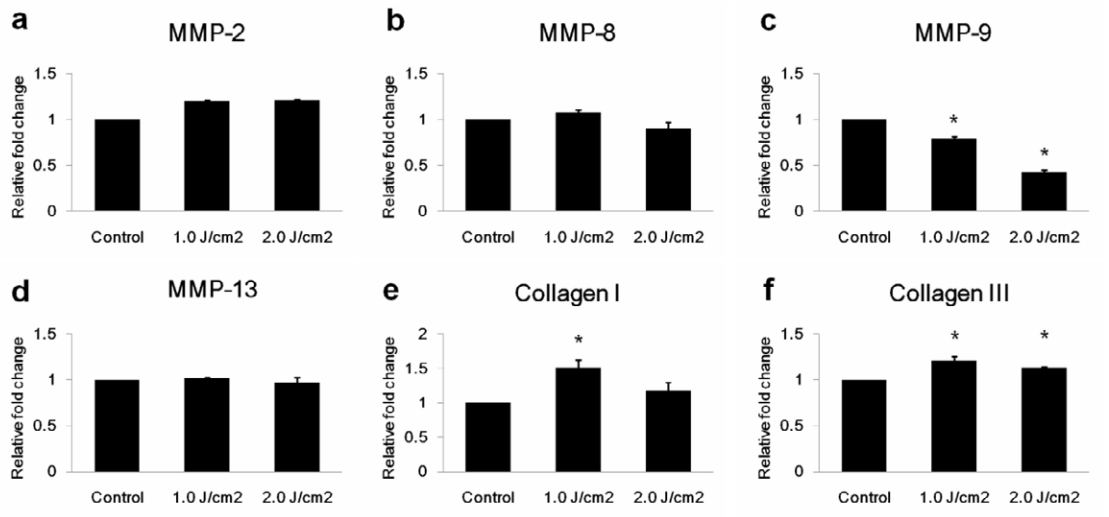

Fig 1 Real-time PCR revealed that the expression of MMP-2, MMP-8, MMP-9, MMP-13, collagen I and collagen III (* indicates $p<0.05$ between laser-treated and control tenocytes).

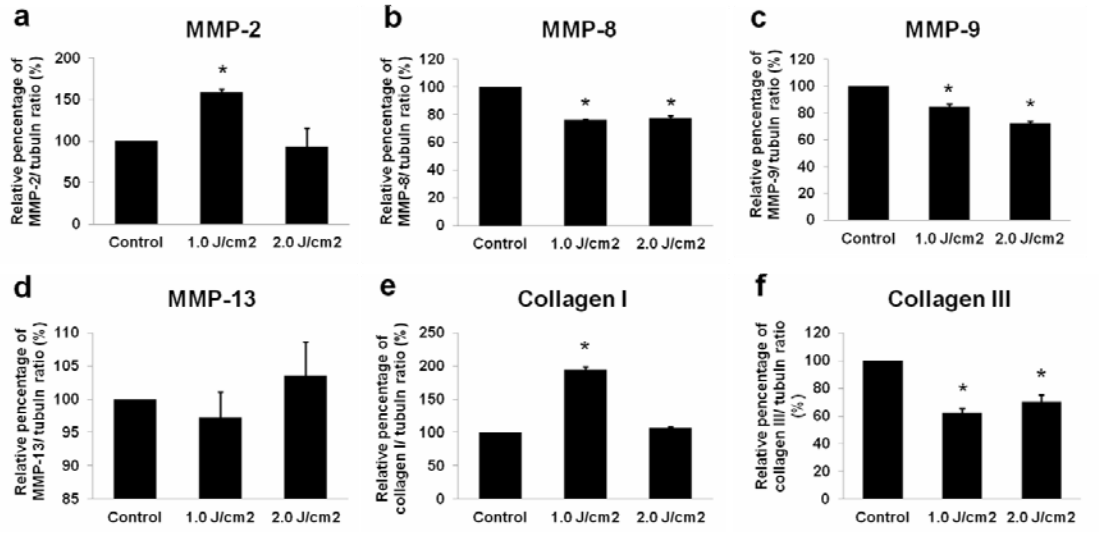

Fig 2 Densitometry analysis of western blot revealed that the expressions of MMP-2, MMP-8, MMP-9, MMP-13, collagen I and collagen III to tubulin ratio (* indicates $p<0.05$ between laser-treated and control tenocytes).

DISCUSSION

Tendon structure consists mainly of dense collagen arranged in a linear fashion along with the basic cellular component, i.e., tenocytes (tendon cells; fibroblasts). Tenocytes, which appear as stellate cells in cross-section and rows in longitudinal section, are the source of collagen production, protein mediators of repair, and matrix proteoglycans[15,25]. For an injured tendon, the healing process can be divided into three overlapping phases: (1) inflammation; (2) regeneration; and (3) remodeling and maturation[15]. In the regenerative phase of tendon injury repair, tenocytes migrate to the repaired site, proliferate

actively, and enhance abundant deposition of ECM in the tissue.

The laser intensity used in this study was between 1.0 and 2.0 J/cm^2, which is within the recommend dose range (0.1–3.0 J/cm^2) to treat tendinopathy[26]. The upper dose limit in our study was 2.0 J/cm^2 because higher doses (≥2.5 J/cm^2) would decrease cell viability and inhibit proliferation of tenocytes in this experimental setting. This finding indicated a biphasic dose response of cell viability in tenocytes treated with LLLT. A decrease in cell viability may have a negative impact on cellular behavior, including cell proliferation and motility. This finding was similar to a previous study indicated that LLLT increases proliferation of Achilles tendon fibroblasts, with the optimal dose being 2 J/cm^2. Doses higher than this optimal dose (>3 J/cm^2) did not enhance proliferation of Achilles tendon fibroblasts[12]. The experimental biphasic dose response in cell culture study using tendon fibroblasts may provide an explanation for the similar observation that low level laser may have better effect on promoting the tissue healing compared to high level laser[27].

Up-regulation of type I collagen (a major component of the tendon ECM) expression by LLLT indicates that laser may be beneficial in tendon healing. MMP-2 and MMP-9 are gelatinases that cleave soluble type IV collagen[28], as well as native and reconstituted type I collagen[23]. In this study, MMP-9 expression was down-regulated, which is known to be beneficial for tendon healing. However, MMP-2 expression was noted to be up-regulated at an energy density of 1.0 J/cm^2, which may be detrimental to tendon healing. The overall effect of MMP-2 and MMP-9 on tendon healing needs to be investigated in animal studies.

There are some limitations to this study. First, the results of this study were analyzed after 24 h of LLLT. The accumulated effects of repeated laser treatment for longer periods on tenocytes, which is a common treatment regimen for tendinopathy or tendon injury, are unclear. Second, caution should be exercised when extrapolating these *in vitro* results to *in vivo* conditions. Therefore, further animal studies are needed to verify the findings of this study. However, a thorough understanding of the dose effects and molecular mechanism of laser on homeostasis of collagen do provide laboratory-based evidence to support the use of laser to treat tendinopathy or tendon injury.

CONCLUSION

LLLT down-regulates the MMP-9 expression in accordance with the energy density and up-regulates type I collagen expression at an energy density of 1.0 J/cm^2. These findings provide an insight into the molecular mechanisms of LLLT in enhancing injured tendon repair.

ACKNOWLEDGMENT

The authors thank the National Science Council of Taiwan for the financial support received for this research.

REFERENCES

1. Goldman JA. Investigative studies of laser technology in rheumatology and immunology In: Goldman, L. editor, The biomedical laser: technology and clinical applications. New York: Springer-Verlag; 1981. p. 293-311.
2. Basford JR BG. Therapeutic physical agents. In: Frontera, I.W. editor, Physical Medicine and Rehabilitation: Principles and Practice, fifth ed. Philadelphia: Lippincott Williams & Wilkins, 2010. p. 1706-1707.
3. Saunders L. Laser versus Ultrasound in the Treatment of Supraspinatus Tendinosis: Randomised controlled trial. Physiotherapy 2003; 89: 365-373.
4. Bjordal JM, Lopes-Martins RA, Joensen J, et al. A systematic review with procedural assessments and meta-analysis of low level laser therapy in lateral elbow tendinopathy (tennis elbow). BMC Musculoskeletal Disorders 2008; 9: 75.
5. Tumilty S, Munn J, McDonough S, et al. Low level laser treatment of tendinopathy: a systematic review with meta-analysis. Photomedicine and Laser Surgery 2010; 28: 3-16.
6. Stergioulas A, Stergioula M, Aarskog R, et al. Effects of low-level laser therapy and eccentric exercises in the treatment of recreational athletes with chronic achilles tendinopathy. Am J Sports Med 2008; 36: 881-887.

7. Casalechi HL, Nicolau RA, Casalechi VL, et al. The effects of low-level light emitting diode on the repair process of Achilles tendon therapy in rats. Lasers in Medical Science 2009; 24: 659-665.
8. Oliveira FS, Pinfildi CE, Parizoto NA, et al. Effect of low level laser therapy (830 nm) with different therapy regimes on the process of tissue repair in partial lesion calcaneous tendon. Lasers in Surgery & Medicine 2009; 41: 271-276.
9. Fillipin LI, Mauriz JL, Vedovelli K, et al. Low-level laser therapy (LLLT) prevents oxidative stress and reduces fibrosis in rat traumatized Achilles tendon. Lasers in Surgery & Medicine 2005; 37: 293-300.
10. Karu T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. Journal of Photochemistry & Photobiology. B - Biology 1999; 49: 1-17.
11. Tafur J, Mills PJ. Low-intensity light therapy: exploring the role of redox mechanisms. Photomedicine and Laser Surgery 2008; 26: 323-328.
12. Chen CH, Tsai JL, Wang YH, et al. Low-level laser irradiation promotes cell proliferation and mRNA expression of type I collagen and decorin in porcine Achilles tendon fibroblasts in vitro. J Orthop Res 2009; 27: 646-650.
13. Tsai WC, Chen JY, Pang JH, et al. Therapeutic ultrasound stimulation of tendon cell migration. Connect Tissue Res 2008; 49: 367-373.
14. Chao PH, Lu HH, Hung CT, et al. Effects of applied DC electric field on ligament fibroblast migration and wound healing. Connect Tissue Res 2007; 48: 188-197.
15. Leadbetter WB. Cell-matrix response in tendon injury. Clin Sports Med 1992; 11: 533-578.
16. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am 2005; 87: 187-202.
17. Amiel D, Frank C, Harwood F, et al. Tendons and ligaments: a morphological and biochemical comparison. J Orthop Res 1984; 1: 257-265.
18. Jones GC, Corps AN, Pennington CJ, et al. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. Arthritis Rheum 2006; 54: 832-842.
19. Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev 2000; 14: 2123-2133.
20. Ireland D, Harrall R, Curry V, et al. 2001. Multiple changes in gene expression in chronic human Achilles tendinopathy. Matrix Biol 20: 159-169.
21. Matrisian LM. The matrix-degrading metalloproteinases. Bioessays 1992; 14: 455-463.
22. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. J Biol Chem 1999; 274: 21491-21494.
23. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. J Biol Chem 1995; 270: 5872-5876.
24. Oshiro W, Lou J, Xing X, et al. Flexor tendon healing in the rat: a histologic and gene expression study. J Hand Surg Am 2003; 28: 814-823.
25. O'Brien M. Functional anatomy and physiology of tendons. Clin Sports Med 1992; 11: 505- 520.
26. Bjordal JM, CouppÃ C, Ljunggren E. Low level laser therapy for tendinopathy: Evidence of a dose-response pattern. Physical Therapy Reviews 2001; 6: 91-99.
27. Huang YY, Chen AC, Carroll JD, et al. Biphasic dose response in low level light therapy. Dose Response 2009; 7: 358-383.
28. Salo T, Turpeenniemi-Hujanen T, Tryggvason K. Tumor-promoting phorbol esters and cell proliferation stimulate secretion of basement membrane (type IV) collagen-degrading metalloproteinase by human fibroblasts. J Biol Chem 1985; 260: 8526-8531.

低能量雷射治療對肌腱細胞基質金屬蛋白酶及膠原蛋白合成的影響

陳建宏[1] 洪詩惟[1] 蘇中慧[2] 蔡文鐘[1,2] 林妙穗[2] 游東陽[1,2] 張翔寧[1]

林口長庚紀念醫院復健醫學部[1] 長庚大學臨床醫學研究所[2]

目的：低能量雷射治療(low power laser therapy, LLLT)目前常用於臨床治療運動傷害造成的肌腱病變或傷害，然而對於修復肌腱病變的分子生物學機轉尚不明確，因此本研究的目的在於探討以低能量雷射治療對於肌腱細胞分泌膠原蛋白以修復受傷之肌腱，可做為低能量雷射治療肌腱病變之理論基礎。

方法：本研究使用大鼠肌腱細胞為實驗模型，來檢視低能量雷射治療(LLLT)對於肌腱細胞分泌膠原蛋白以修復受傷之肌腱，低能量雷射治療波長 660 奈米，採用連續性模式輸出功率 50 毫瓦，以不同照射時間產生不同劑量之低能量雷射去處置肌腱細胞，用即時聚合酶鏈鎖反應(real-time PCR)和西方點墨法(western blot analysis)分析基質金屬蛋白酶(matrix metalloproteinases)及膠原蛋白(collagen)的表現量和分泌量。

結果：在即時聚合酶鏈鎖反應(real-time PCR)，基質金屬蛋白酶-9 (matrix metalloproteinases-9)表現量隨著雷射劑量提升而下降，第一型膠原蛋白(type I collagen)表現量在雷射劑量 1.0 焦耳/平方公分而上升，第三型膠原蛋白(ttype III collagen)表現量隨著雷射劑量提升而上升；在西方點墨法分析中，基質金屬蛋白酶-8 (matrix metalloproteinases-8)和基質金屬蛋白酶-9 (matrix metalloproteinases-9)分泌量隨著雷射劑量提升而下降，第一型膠原蛋白(type I collagen)表現量在雷射劑量 1.0 焦耳/平方公分的上升。

結論：基質金屬蛋白酶(matrix metalloproteinases-9)表現量和分泌量隨著雷射劑量提升而下降，然而在 1.0 焦耳/平方公分的雷射劑量下，肌腱細胞的第一型膠原蛋白(type I collagen)表現量和分泌量均上升。
（台灣復健醫誌 2014；42(4)：215 - 221）

關鍵詞：雷射(laser)，肌腱(tendon)，基質金屬蛋白酶(matrix metalloproteinase)，膠原蛋白(collagen)

通訊作者：張翔寧醫師，林口長庚紀念醫院復健醫學部，桃園市 333 龜山區復興街 5 號
電話：(03) 3281200 轉 3846 E-mail：lilychang0412@gmail.com doi: 10.6315/2014.42(4)05