

Low-level laser irradiation stimulates tenocyte proliferation in association with increased NO synthesis and upregulation of PCNA and cyclins

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Abstract Low-level laser therapy is commonly used to treat tendinopathy or tendon injury. Tendon healing requires tenocyte migration to the repair site, followed by proliferation and synthesis of the extracellular matrix. There are few evidence to elucidate that low-level laser promote tenocyte proliferation. This study was designed to determine the effect of laser on tenocyte proliferation. Furthermore, the association of this effect with secretion of nitric oxide (NO) and the expressions of proliferating cell nuclear antigen (PCNA) and cyclins D1, E, A, and B1 was investigated. Tenocytes intrinsic to rat Achilles tendon were treated with low-level laser (660 nm). Tenocyte proliferation was evaluated by MTT assay and immunocytochemistry with Ki-67 stain. NO in the conditioned medium was measured by ELISA. Western blot analysis was used to evaluate the protein expressions of PCNA and cyclins D1, E, A, and B1. The results revealed that tenocytes proliferation was enhanced dose dependently by laser. NO secretion was increased after laser treatment. PCNA and cyclins E, A, and B1 were upregulated by laser. In conclusion, low-level laser irradiation stimulates tenocyte proliferation in a process

that is mediated by upregulation of NO, PCNA, and cyclins E, A, and B1.

Keywords Laser · Tendon · Cell proliferation · Cyclin · NO · PCNA

Introduction

Low-level laser therapy (LLLT) has been used to treat musculoskeletal pain for nearly 3 decades [1]. Clinical applications show its potential of effectiveness in treating soft tissue musculoskeletal injuries, chronic pain, and wound healing [2]. Many studies have revealed the effectiveness of LLLT to treat patients with tendinopathy [3–6]. Although the clinical usage of laser suggests its efficacy, scientific evidence of its underlying molecular mechanisms for tendinopathy treatment remained limited.

In vivo studies have revealed that LLLT could enhance Achilles tendon healing by improving collagen fiber organization, preventing oxidative stress, and reducing fibrosis [7–11]. The exact mechanism is still being explored, but it is likely that the mechanism is photochemical rather than thermal [12–14]. In the regenerative phase of tendon injury, the tenocytes migrate into the repaired site, proliferate actively, and are responsible for the abundant deposition of extracellular matrix (ECM). It was demonstrated that laser irradiation could promote porcine tenocyte proliferation and upregulation of type I collagen and decorin [15]. However, to our knowledge, there are few studies exploring the molecular mechanism of laser irradiation on tenocyte proliferation.

Light irradiation can alter the redox state of cells and expression of redox-sensitive transcription factors like nuclear factor kappa B (NF- κ B), which can activate inducible form of nitric oxide synthase (iNOS) and lead to a proliferation

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increase [16, 17]. Cell proliferation is governed by the eukaryotic cell cycle [18], which is regulated by a variety of signals that act to inhibit cell cycle progression. Tenocytes, as other cells, have four cell division cycle stages: gap 1 (G1), synthesis (S), G2, and mitosis (M). This tightly controlled temporal order is imposed by the sequential activation of a number of protein kinases known as Cdks by forming complex with various cyclins [19]. Meanwhile, proliferating cell nuclear antigen (PCNA), the auxiliary protein of DNA polymerases δ and ϵ , is essential for DNA replication and repair [20]. PCNA mRNA is present in all phases of the cell cycle, with exponential cell growth and a two to threefold increase during the S phase [21]. Blocking PCNA production inhibits cell division, indicating that PCNA is pivotal in the process of cell proliferation [22]. Therapeutic ultrasound can upregulate PCNA expression, thus enhance tenocytes proliferation [23]. Whether laser can stimulate tenocyte proliferation and such effect is related to expressions of nitric oxide (NO), PCNA, or cyclins have never been investigated.

The purpose of this study is to investigate the effect of laser on tenocyte proliferation and its association with the expressions of NO, PCNA, and cyclins D1, E, A, and B1.

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

The Achilles tendons from 16 Sprague–Dawley rats (weighing 200 to 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 mm³ (six pieces in total). These pieces were individually 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 (DMEM) (HyClone, Logan, UT, USA), with 10 % fetal bovine serum (FBS) (Cansera, Rexdale, ON, Canada), 100 U/ml penicillin, and 100 μ g/ml streptomycin, was added to each well. The explants were then incubated at 37 °C in a humidified atmosphere of 5 % CO₂/95 % air. After migrating out from the explants, the cells started to grow rapidly, and the confluence culture was subcultured by trypsin digestion at a 1:3 dilution ratio. Tenocytes from passages 2 and 4, with proper growth rate and normal fibroblast shape, were used. All the experiments were performed in triplicate.

Laser irradiation procedure

Laser irradiation was carried out with 660-nm laser (Konftec, Megalas-AM-800, New Taipei City, Taiwan) in continuous mode with output power of 50 mW and unit energy density of 0.0032 J/s/cm². The power of irradiation was uniformly checked with a power meter before and after treatment. The laser beam irradiated the culture plate from above with a distance of 30 cm and covered the area up to 314 cm². The laser beam was emitted perpendicularly and evenly to the culture plates for three groups of wells for periods of 5.2, 7.8, 10.4, and 13.0 min, respectively. The corresponding energy densities were 1.0, 1.5, 2.0, and 2.5 J/cm². The irradiation was done on a clean bench through the culture medium at room temperature. The control groups were not subjected to laser irradiation but were removed from the incubator for the same time period as laser-treated plates.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

Tenocytes without or with different doses of laser treatment were cultured for 24 h. Cell proliferation was determined by MTT assay. MTT (50 μ g/ml) was added and incubated at 37 °C for 1 h. Then, the MTT solution was discarded, and 1 ml DMSO was added to dissolve formazan crystals. Optical density (OD) at 570 nm (OD 570 nm) in aliquots was read using a spectrophotometer. Relative fold change of OD value for laser-treated tenocytes relative to control tenocytes was calculated.

Immunocytochemistry with Ki-67 stain

For the purpose of direct microscopic examination on the results of immunocytochemical analysis, tenocytes were subcultured and then seeded directly on the glass coverslips. Subconfluent tenocytes were then treated without and with 1.0 and 2.0-J/cm² laser. After 24 h, all immunostaining procedures were performed as previously described [23]. Finally, tenocytes were incubated for 1 h with rabbit monoclonal antibody against Ki-67 (Nunc, Thermo-scientific, Rockford, IL, USA) diluted in blocking solution. The signal was detected with goat antirabbit IgG fluorescein (FITC) (Leinco Technologies, Inc., St. Louis, MI, USA). After washing in 1 \times PBS, cells were stained in 1 \times PBS containing 4 μ g/ml propidium iodide (PI) and 100 μ g/ml DNase-free RNase for 30 min at 37 °C. After washing in 1 \times PBS, the fluorescent PI-stained nuclei were examined under fluorescent microscope (100 \times). The percentage of Ki-67 positive cells was defined as the number of nuclei that stained with fluorescent green/total number of cells on the coverslip \times 100 %.

Nitric oxide measurement

NO measurement was performed using NO detection kit (iNtRON Biotechnology, Seongnam, Korea), which detects NO by indirectly measuring nitrite, a byproduct of nitric oxide in the conditioned medium with or without laser treatment. This kit is based on the colorimetric change at absorbance of 490 nm, which occurs when naphthylethylenediamine is added to the byproduct of reaction between sulfanilamide and nitrite.

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 method. Protein concentration of the cell extracts was determined by Bradford assay (Bio-Rad Laboratories, CA, USA). Samples with identical protein quantities were then separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto 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., antitubulin, PCNA, and cyclins D1, E, A, and B1 (NeoMarks, Fremont, CA, USA). After washing, the membrane was incubated in PBS containing goat antimouse 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 (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). The semiquantitative measurement of the band density was calculated by Digital Analysis Software (Kodak Digital Science TM, Eastman Kodak, Rochester,

NY). The band density of each protein was normalized to relative band density of tubulin.

Statistical analysis

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

Results

Effect of laser on tenocyte proliferation

Figure 1 revealed that laser could increase the number of viable cells in a dose-dependent manner ($p=0.041$). The percentage of OD value relative to the control group was 102.2 ± 2.5 , 103.6 ± 3.0 , 112.8 ± 3.3 , and 109.6 ± 8.2 % for tenocytes treated with 1.0, 1.5, 2.0, and 2.5 J/cm², respectively (Fig. 1). Laser at 2.0 J/cm² could result in the most significant cell proliferation. Immunocytochemistry revealed much more tenocytes treated with laser stained positively with fluorescent green (Fig. 2a), which indicates that laser enhanced tenocyte proliferation. It also revealed that the percentage of Ki-67 positive tenocytes increased dose dependently after laser treatment (Fig. 2b). In the control group, the percentage of Ki-67 positive tenocytes were 53.6 ± 9.1 , 66.4 ± 10.0 , and 76.4 ± 0.7 % in control group, tenocytes treated with 1 and 2 J/cm², respectively. The difference was statistically significant ($p<0.05$).

Fig. 1 Result of MTT assay revealed that laser irradiation increased the number of viable tenocytes in a dose-dependent manner ($*p<0.05$)

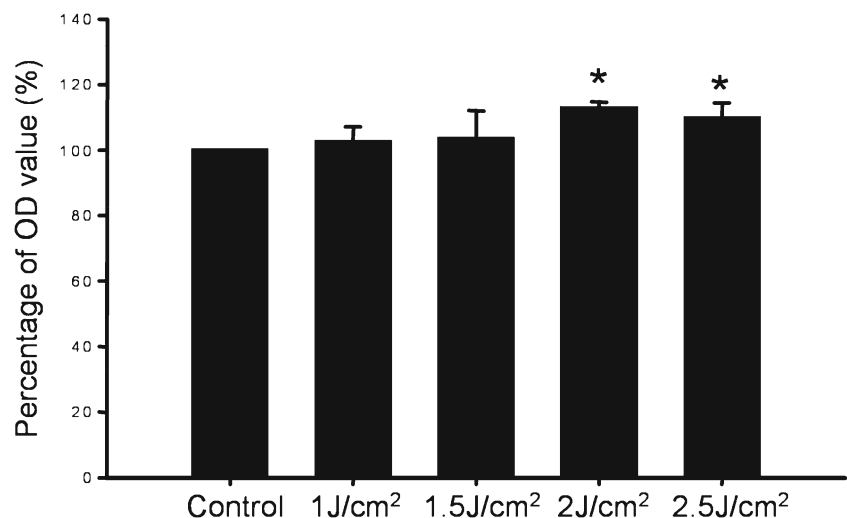
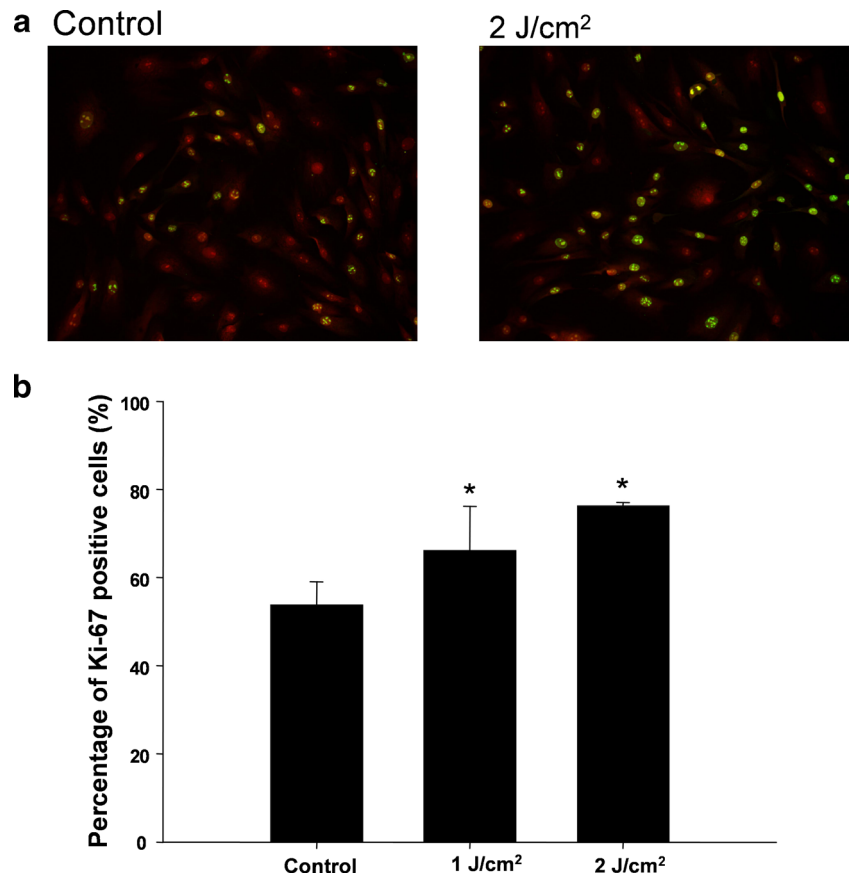


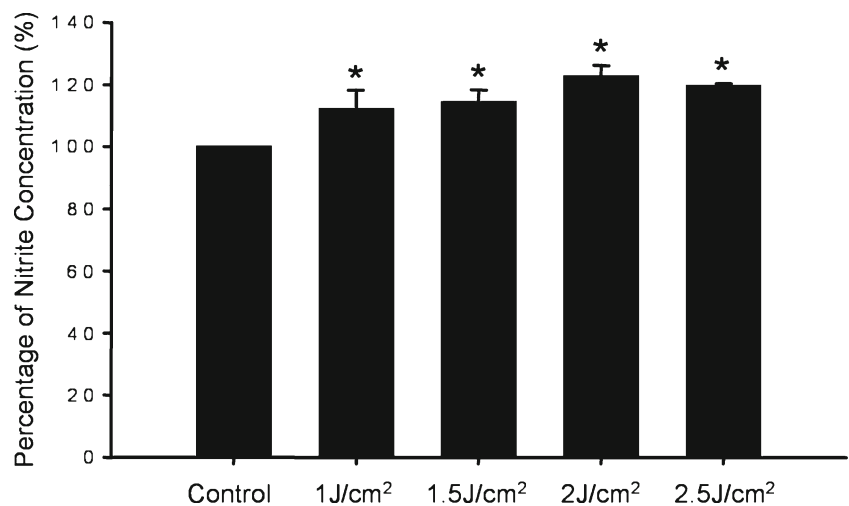
Fig. 2 **a** Immunocytochemistry with Ki-67 (green) and PI stain (red) in control tenocytes (left) and **b** tenocytes with laser treatment (2 J/cm^2 , right) ($100\times$). **b** The percentage of Ki-67 positive tenocytes were increased dose dependently ($p<0.05$)



Effect of laser on the level of secreted NO

Concentration of NO measured in the conditioned medium also revealed that laser dose dependently enhanced the level of secreted NO by tenocytes. The relative concentration of NO to control for tenocytes treated with 1.0, 1.5, 2.0, and 2.5-J/cm^2 laser was 112.1 ± 10.6 , 114.3 ± 6.9 , 122.9 ± 5.9 , and $119.6\pm 1.1\%$, respectively ($p=0.05$; Fig. 3).

Fig. 3 NO in the conditioned medium of tenocytes was enhanced by laser with the most significant effect at 2.0 J/cm^2 ($*p<0.05$)



Effect of laser on expression of PCNA and cyclins D1, E, A, and B1

Western blot analysis and subsequent analysis revealed that laser upregulated PCNA protein expression at optimal dosage of 2.0 J/cm^2 . The relative protein expression of PCNA of laser-treated tenocytes to control cells was 116.6 ± 1.7 , 116.7 ± 1.1 , 120.4 ± 1.2 , and $104.8\pm 0.9\%$ for tenocytes treated with

1.0, 1.5, 2.0, and 2.5-J/cm² laser, respectively ($p < 0.05$; Fig. 4a, b)

Western blot analysis revealed that protein expression of cyclin E, A, and B1 was enhanced by laser irradiation (Fig. 5a). However, the expression of cyclin D was not enhanced by laser (Fig. 5a, b). Band densitometry analysis for cyclin E, A, and B1 revealed that 2.0 (113.2±1.1 %), 2.5 (114.5±0.8 %), and 2.0 J/cm² (124.6±13.7 %), respectively, were the most optimal doses to upregulate the most significant corresponding protein expressions (Fig. 5c, d, e; $p < 0.05$).

Discussion

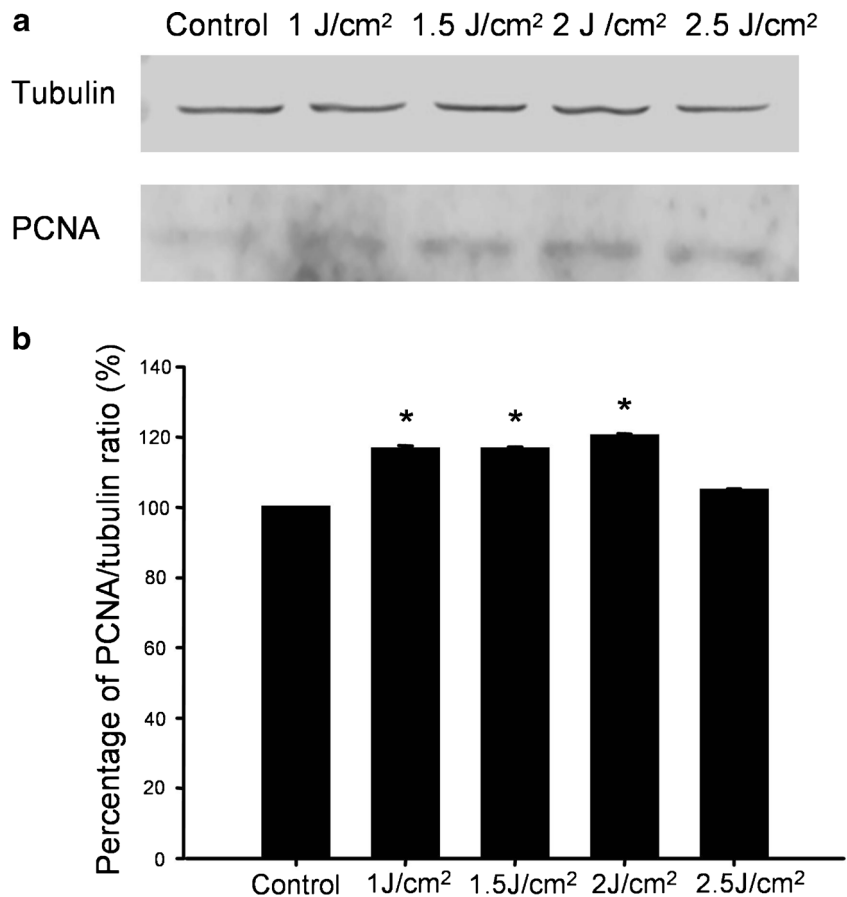
Tendon structure consists mainly of dense collagen arranged in a linear fashion and the basic cellular component, i.e., tenocytes (tendon cells; fibroblasts). Tenocytes, appearing as a stellate cell in cross sections and in rows in longitudinal section, are the source of collagen production, protein mediators of repair, and matrix proteoglycans [24, 25]. For an injured tendon, the healing process can be divided into three overlapping phases: (1) inflammation, (2) regeneration, and (3) remodeling and maturation. In the regenerative phase of tendon injury, tenocytes migrate into the repaired site, proliferate actively, and are responsible for the abundant deposition

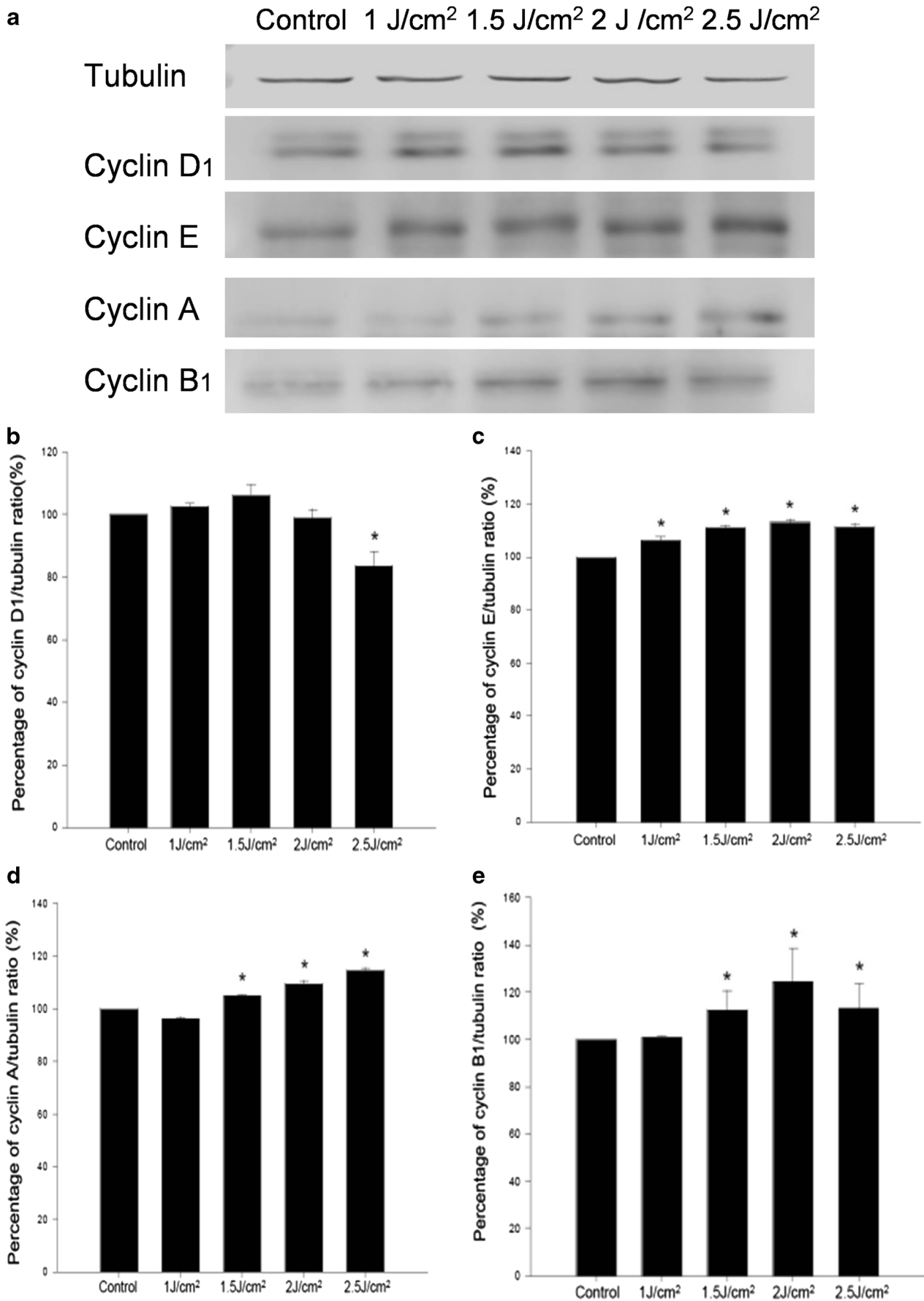
of ECM in the tissue. Tenocyte proliferation is fundamental to the healing process of an injured tendon. To our knowledge, this study was the first to demonstrate that laser, in a dose-dependent manner, can enhance tenocytes proliferation, which is associated with the upregulation of expressions of PCNA and cyclin E, cyclin A, and cyclin B1.

NO, a small free radical generated by nitric oxide synthases, is induced after tendon injury especially by healing fibroblasts [26]. In animal models, inhibition of NOS results in reduced tendon healing, and the addition of NO, on the other hand, enhances the healing of tendon [27]. Therefore, the local delivery of NO has been considered as a therapeutic option for tendon injury [28]. In the present study, we demonstrated that the level of secreted NO in tenocytes could be induced by the treatment with low-level laser in a dose-dependent manner. Mitochondria are thought to be the major photoreceptors for laser that trigger the initial events including increase of adenosine triphosphate, reactive oxygen species, intracellular calcium, and release of NO, which then leads to the expression of genes involved in the process of proliferation [29].

Cyclin E is induced at the G1/S boundary; Cyclin A is induced at a later phase of the cell cycle and is required for the cell to progress through the S phase [30]. Cyclin B1 regulated transition through the G2/M checkpoint of the cell cycle [31].

Fig. 4 **a** Western blot analysis revealed that PCNA was upregulated by laser irradiation. The tubulin (as internal control) and PCNA were identified at 57 and 36 kDa, respectively. **b** Densitometric analysis of PCNA to tubulin ratio ($*p < 0.05$)





◀ **Fig. 5 a** Western blot analysis revealed that the expressions of cyclin E, cyclin A, and cyclin B1 were upregulated by laser irradiation, whereas that of cyclin D1 was not upregulated. The tubulin (as internal control) and cyclins D1, E, A, and B1 were identified at 57, 36, 47, 60, and 62 kDa, respectively. Densitometric analysis of cyclin D1, cyclin E, cyclin A, and cyclin B1 to tubulin ratio ($*p < 0.05$) was revealed on figures **b**, **c**, **d**, and **e**, respectively

PCNA is identified as essential for the coordinated synthesis of leading and lagging DNA strands [20]. Besides involvement in the DNA synthesis and repair pathways, PCNA is shown to establish interactions with proteins required for the control of the cell cycle [32]. The findings of this study indicated that low-level laser might promote tenocytes to pass through G1 phase, enter S phase and transition through the G2/M checkpoint by affecting early cell cycle regulatory genes, and thus promotes cell proliferation.

The laser dose used in this study was between 1.0 and 2.5 J/cm², which is within the recommended dose range (0.1 to 3.0 J/cm²) to treat tendinopathy.[33] The upper limit of the laser dose in our study was 2.5 J/cm² because higher doses (>2.5 J/cm²) would decrease cell viability and damage tenocytes in this experimental setting. This finding was similar to the report that laser increases cell proliferation of Achilles tendon fibroblasts, with the optimal dose being 2.0 J/cm². Higher doses of laser stimulation (3.0 J/cm²) do not enhance cell proliferation on Achilles tendon fibroblasts [15].

There are some limitations for this study. First, the results of this study were investigated after 24 h of laser exposure. It is uncertain as to the accumulated effects of repeated laser treatments for a longer period of time on tenocytes, which is a common practice to treat patient with tendinopathy or tendon injury. Second, it should be cautious when extrapolating these in vitro results to in vivo condition. Therefore, further animal studies are mandatory to verify the findings of this study. However, a thorough understanding of the dose effects and molecular mechanism of laser on tenocyte migration does provide laboratory-based evidence to support the use of laser to treat tendinopathy or tendon injury.

Conclusion

Laser irradiation can enhance tenocyte proliferation with increased level of secreted NO, upregulation of PCNA, as well as cyclins E, A, and B1. These findings provide novel molecular mechanisms underlying LLLT for treating tendinopathy or tendon injury.

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