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ORIGINAL ARTICLE

Effect of light-emitting diodes, platelet-rich plasma, and their combination on the activity of sheep tenocytes

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Abstract

Healthy tendons play an important role in joint movements and subjected to a group of pathologies called tendinopathy due to multiple factors. Tendons have a slowly repairing process due to the low vascularity and cellularity. Treatment options aimed at potentiating the healing response and relieving symptoms. Phototherapy and platelet-rich plasma were novel treatment modalities in tendons based on photobiomodulation and growth factors during healing, and the results were encouraging suggesting calibrating treatment parameters. This study utilizes cell culture to explore the potential effect of light-emitting diode and/or growth factors in the form of platelet-rich plasma (PRP) on the activity of tenocytes isolated from sheep Achilles tendons by measuring the cell metabolism and cell mobility using cell viability and migration assays to proof safety and confirm activity. Results showed that sheep tenocyte-cultured groups treated with 5% platelet-rich plasma alone or combined with 4 J/cm^2 light-emitting diode have increased viability significantly when compared to control group after a 48 h, while light-emitting diode treatment has not decreased cell migration significantly when compared with control. Result suggests that using platelet-rich plasma alone or combined with light-emitting diode might have potential to enhance healing response at the conditions applied. PRP could enhance proliferation while LED could enhance migration and proliferation. Further research is needed at longer durations.

Keywords Light-emitting diode · Platelet-rich plasma · Phototherapy · Tenocytes · Tissue culture model · Tendinopathy

Introduction

Tendinopathy is a pathology affecting tendons with a multifactorial etiology characterized histologically as a disruption in matrix collagen fibers and cells. It is manifested clinically with pain and dysfunction of the musculoskeletal system, and it has a significant impact on national health systems and at the indivudal level especially workers and atheltes [1]. In tendinopathy, healing phases are largely dependent on tenocyte activity; therefore, treatment options aimed at potentiating tenocyte activities, such as cell division and metabolism, recruitment of tendon cells at the injured site, to restore tendon matrix normal function with no symptoms [2].

Current treatment options demonstrate safety at the parameters used with different outcomes and with different start times of application. Eccentric exercise is the treatment choice used by many physiotherapists; however, it cannot be applied directly after injury as it may worsen the condition [3]. Other treatments used by physiotherapist include phototherapy and growth factors which can be applied immediately after injury, although it has showed safety but with different outcomes [1].

Phototherapy treatments such as light-emitting diode (LED) and laser are based on photobiomodulation, which involves the absorption of photon energy by tissue molecules which results in photochemical or photothermal reactions that lead ultimately to cellular effects [4]. LED optical devices produce a monochromatic, non-coherent light with favorite

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properties such as large spot size, high performance, and relatively safer device [5]. LED devices have showed positive effects on connective tissue disorders such as wound healing, acne treatment, and skin rejuvenation [6, 7], by modulating: inflammatory mediators, collagen synthesis, gene expression [8, 9], angiogenesis [10], uniformity of collagen fibers [11], ATP synthesis, reactive oxygen species (ROS) and nitrous oxide production, and blood flow leading to enhancement of cell enzyme activity, mitochondrial respiration which affects cell proliferation and tissue regeneration [12, 13].

LED with different energies and wavelengths has been explored for tendon disorders at cellular, preclinical animal studies and clinical human studies, and results have shown increased cellularity of chicken embryo fibroblast cultures [14], increased collagen synthesis in a low-serum fibroblast cultures [15], enhanced blood supply and reduced inflammation in a tendinitis-induced in sheep [16], modulation of gene expression and inflammatory mediators in a rat Achilles tendinitis model [8, 9], improved tissue remodeling in a rat tenotomized Achilles tendon [17], and improved pain and function in patients suffering painful knee conditions [18]. However, in other trials, no significant effect was reported, and this was attributed to different light parameters (energy delivered, duration, spot size, and frequency) [19, 20], and factors related to the patients include age, body mass index, type of tendon, and nature of lesion [6] which need further research both at clinical and preclinical level to refine parameters efficiently [21].

Growth factors derived from platelet such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1, and platelet-derived growth factor (PDGF) have been used for the treatment of tendinopathy in single formulation or as a platelet-rich plasma (PRP). PRP is a source of growth factors used to potentiate the healing of tendon diseases by increased collagen expression, increased cell viability, increased fiber organization, and angiogenesis [22]. Application of PRP in in vitro studies had increased fibroblast proliferation, VEGF, and procollagen type I C-peptide expression [23], decreased inflammatory response, elevated vascularity, and increased fiber arrangement [24]. Adding plasma to ovine tenocyte 3D model enhances mitosis and attachment while adding platelet-enhanced metabolism and mitosis [25]. A recent systematic review has suggested a role of PRP in the treatment of tendinopathy; however, it suggested further research to refine the treatment regimens [26].

Recently, combined treatments were explored for tendinopathy rather than single treatment [2, 27]. Moreover, in vitro models are important research tool in investigating different treatment options to confrm safety and to provide an evidence-based treatment for clinical trials [28]. This motivates us to investigate the effect of LED and/or growth factors at the cellular level using different outcome measures with the aim to potentiate the healing response and supports the use of combined treatments. Our hypthesis is that LED and/or PRP have positive effects on the metabolism and mobility of cultured tenocyte monolayer extracted from sheep Achilles tendons.

Methodology

In vitro animal model: development and optimization

Sheep adult hind limbs ($n = 10$, age 6–10 months) were obtained from local slaughterhouse, and tissue biopsies (1 mm^3) were harvested from the Achilles tendons and cultured in 75-cm^2 flasks to allow cells to be released and sub-cultured until it reached third passage. Ten cell lines were cryopreserved ready for the experiments. Culture conditions of serum concentration, seeding density, LED parameters (energy and frequency), and PRP (dose) treatment parameters were determined based on literature and refined empirically. Viable cell number was counted using the Trypan blue method. RPMI 1640 media supplemented with 10% FBS, 5% penicillin and streptomycin, 5% HEPES buffer, 5% Amphotericin B, and 5% glutamine was used in all experiments under standard culture conditions of 5% CO_2 and 95% humidity.

Initially, a pilot study was run to optimize serum concentration in culture medium using 0, 5, and 10% *v*/*v* of fetal bovine serum (FBS). For seeding density determination, a starting concentration of 250,000 tenocytes/ml of culture media was prepared and used to prepare serial dilutions up to a concentration of 244 tenocytes/ml. Triplicates of 200 μl of each cell solution were cultured in a black 96-well plate. Cell viability was measured using an Alamar blue assay every 24 h and three readings were made at 24, 48, and 72 h of seeding.

Alamar blue assay

Tenocyte viability was determined using the Alamar blue assay (Invitrogen, USA) based on the metabolism of formazan into red fluorescent solution. Simply, 10% (*v*/*v*) Alamar blue solution was added to wells of a black 96-well clear bottom plate and incubated for 3 h under normal culture conditions in the dark, and subsequently, the fluorescence intensity was measured using a fluorescence reader (Bio-tek FLx 800, USA), at 560 nm/590 nm excitation/emission filters according to manufacturer's instructions.

Platelet-rich plasma (PRP) preparation and counting and treatment protocol

Platelet-rich plasma harvested from whole sheep blood purchased from local manufacturer and a two-spin method was used. Briefly, a fine centrifugation of 2500 rpm for 6 min was

made for the whole blood sample to separate plasma with platelet followed by a hard centrifugation (4200 rpm, 6 min) to separate platelet from plasma. Platelet pellet was mixed with 25% of the supernatant plasma to yield a platelet-rich plasma which was kept at 25 °C ready to use within 5 days. The concentrated platelet (PRP) ratio relative to the whole blood was determined. A 5% (*v*/*v*) PRP was added to the RPMi 1640 culture media and used as a treatment in all PRP protocols based on literature (Kanno et al. 2005; Choi et al. 2005; Markopoulou et al. 2009).

LED device and treatment parameters

LED treatments were applied using a Photizo® device (Photon Therapy Systems LTD, 2012, South Africa)) (Table 1). LED energy parameters (i.e., energy fluence (J/cm^2) and frequency of treatment (per day)) used in this study were determined empirically by conducting a pilot study using different LED energy densities and applying LED either daily or every other day.

Experimental design

Based on the optimized culture conditions and treatment parameters, a third passage cryopreserved cell lines were used to set up all experiments in triplicate using 96-well black plates with a clear bottom for the viability assays (Alamar blue) and 24-well plates for the scratch assays. Four treatment groups were used: group 1: control group received no LED or PRP treatments; group 2: LED group received 4 J/cm^2 LED; group 3: PRP group received 5% PRP; and group 4: LED and PRP group received 4 J/cm^2 LED and 5% PRP. Culture period includes an initial 24 h to allow cells attached to the plate followed by 48-h treatment period. A seeding density of 10,000 cells/well was used for 96-well black plates and 50,000 cells/well in 24-well plates. In experiments designed for histocytochemistry, a 24-well plate was used, and coverslips were placed at the well bottoms before culturing. LED treatment (i.e., a single energy dose of 4 J/cm^2 (18 min)) and 5% PRP or their combination were applied immediately following the first 24 h of seeding. LED large probe was used and applied through the top surface of a black 96-well plate.

Table 1 LED device (Photizo®) specifications

Probe	Power output	Spot area
Standard small	630 nm/150 mW	1.2 cm^2
Large probe	625 nm and 850 nm/1200 mW	15 cm^2

Scratch assay

A simple assay-simulating wound healing in vitro, based on producing a scratch in confluent cultured monolayer cells and quantifying the cell migration during the closure of the scratch. Sheep tenocytes were cultured in a 24-well plate (Corning, USA) until it reaches confluency; at this point, a scratch line was made by 100-μl micropipette tip followed by applying optimized treatment parameters of a single dose of 4 J/cm^2 LED and/or 5%PRP. Images were taken using a phase-contrast inverted microscope at different time intervals (including zero time) until gap closed completely.

Statistical analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences software (SPSS, version 20). Distribution of data was tested using Shapiro–Wilk test. Parametric tests (i.e., ANOVA and *t* test) were employed for normally distributed data while non-parametric tests (i.e., Kruskal–Wallis tests) were applied on non-normally distributed data. Multiple comparisons were made for the different treatment groups accordingly. Statistical significance was determined at *p* value less than 0.05.

Results

Optimization of the model and culture conditions

Serum concentration in culture medium results showed that in the absence of FBS, no increase in cell number or viability was seen while adding 10% FBS increased cell numbers as well as viability significantly in the presence and absence of 5% PRP (Online Resource 1). Seeding density results are presented in Fig. 1, which shows a minimal increase in cell viability after the initial 24 h as cells are attaching to culture vessels not proliferating (i.e., lag phase). After 48 and 72 h of seeding, a gradual increase was evident with a cell density reaching the maximum growth (measured as viability) at 31,250 and 3900 cells/well, respectively. This peak represents the end of the log phase after which a gradual decline in cell viability starts which represent the stationary phase. Platelet counts in PRP were 533,000/μl of serum, and this was approximately 2.4 times that of whole blood in sheep.

LED energy and frequency optimization

LED energy and frequency optimization pilot study results are presented in Figs. 2 and 3. LED energy results showed that 4 J/cm^2 has better results than 8 and 20 J/cm^2 after 48 h of treatment (Fig. 2). Results of analysis of variances (ANOVA) and post hoc (Fisher's least significant difference (LSD) test) tests

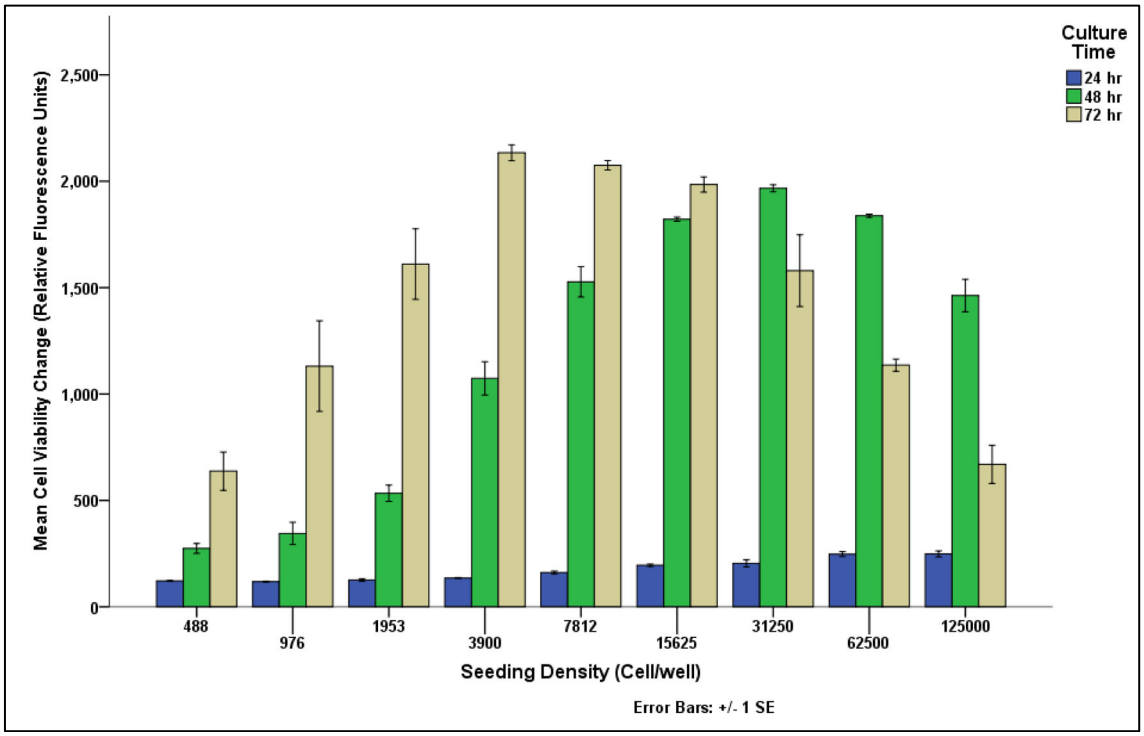

Fig. 1 Seeding density assay using serial dilutions of tenocytes after 24, 48, and 72 h of culturing. X-axis represents cell numbers and Y-axis represents cell viability change from 0 h. SE: standard error of the mean

compared to control (0 energy) revealed insignificant increase in viability at 4 J/cm^2 and insignificant decrease at 8 J/cm^2 while a significant decrease in viability at 20 J/cm^2. Frequency of LED treatment (i.e., treatment period) results showed a decrease in viability when cells were treated daily for 48 h while a slight increase in cell viability is seen after 48-h treatment period (Fig. 3). Statistical analysis showed no significant change in viability between both treatments when compared with control (independent sample t test, p value = 0.110); however, a significance difference was seen in the viability of tenocytes between the daily (24 hourly) and every other day (48 hourly) LED treatments (independent sample t test, p value = 0.017).

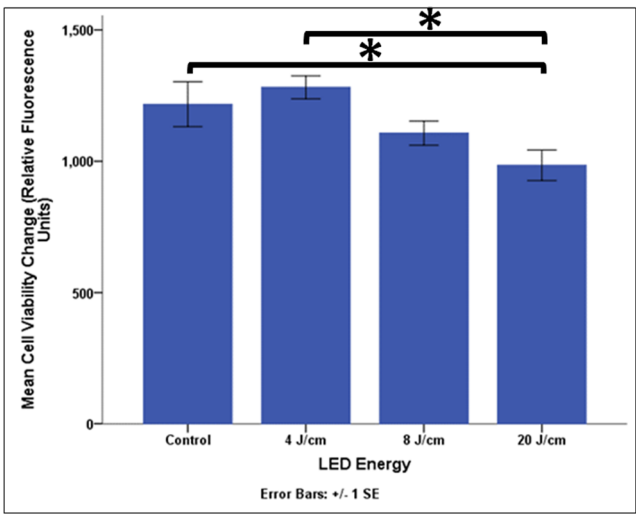

Fig. 2 LED energy optimization assay in a 48-h treatment period. X-axis presents different single LED energies in J/cm^2 and Y-axis represents cell viability change. *Significant $P < 0.05$. SE: standard error of the mean

Viability—Alamar blues assay

Change in the viability of cultured tenocytes measured during the LED and/or PRP treatment periods (i.e., in 48 h) was calculated as means and stander error of the mean (means ± SEM). LED treatment alone showed no change in cell viability compared with control group (Mann-Whitney test, p value = 0.304) while adding PRP to the culture media alone or combined with LED has increased cell viability significantly (Mann-Whitney test, p value = 0.013 and 0.002, respectively) (Fig. 4).

Scratch assay

Data of scratch assay extracted from images taken at different times (Online Resource 2). revealed that scratch treated with PRP tends to slow down cell migration (i.e., slow closure) while LED-treated scratch showed a tendency to accelerate cell migration (i.e., fast closure). Statistically, LED treatments increase cell migration but not significantly while treating cells with 5% PRP alone would decrease cell migration significantly but not when combined with LED (post hoc test, Tukey's HSD test; p = 0.01 and 0.073, respectively) (Fig. 5).

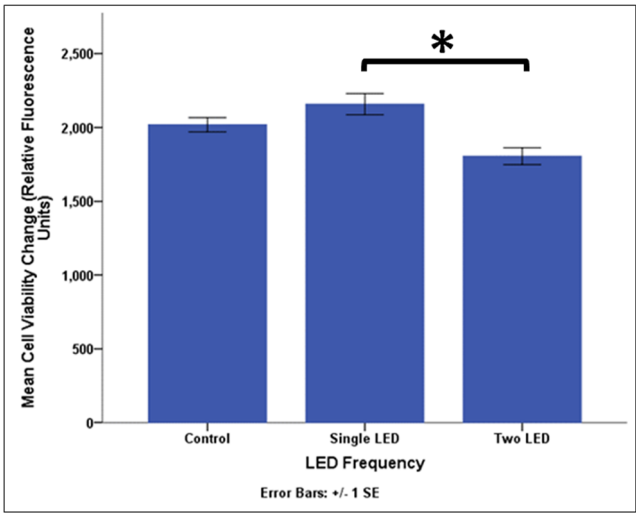

Fig. 3 LED treatment frequency assay in 48-h treatment period. X axis represents either a daily (two LED) or every other day (single LED), Y axis represents changes in cell viability. *Significant $P < 0.05$. SE: standard error of the mean

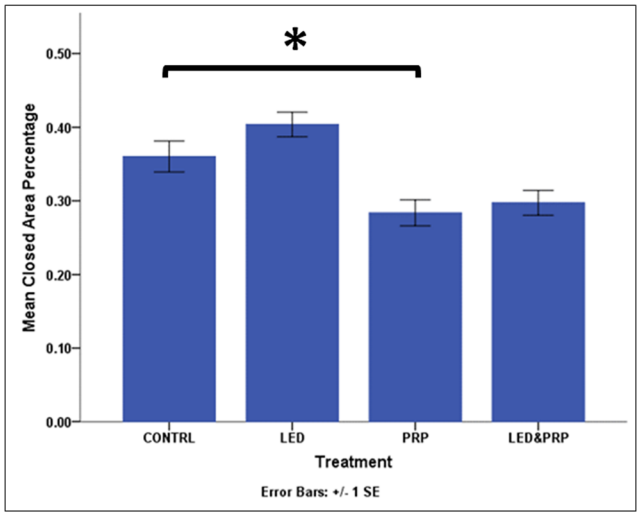

Fig. 5 Scratch assay results after 22 h. C: control, LED: light-emitting diodes, and PRP: platelet-rich plasma. *Significant $P < 0.05$. SE: standard error of the mean

Discussion

Model development and optimization

In this study, an in vitro sheep superficial Achilles tendon model was developed, optimized, and used to investigate the potential effect of different parameters of phototherapy (LED) and/or PRP on the cell viability and migration. Initially, tenocytes were mobilized from tendon explants slowly (5 days) into the culture medium without using enzymatic dissociation which prevents any injury to cells although the risk of contamination increases. A culture medium (RPMI 1640) with phenol red was used in our in vitro culture system to mimic normal conditions as tendons are vascular connective tissues and the presence of phenol red will absorb certain wavelengths similar to blood hemoglobin [29].

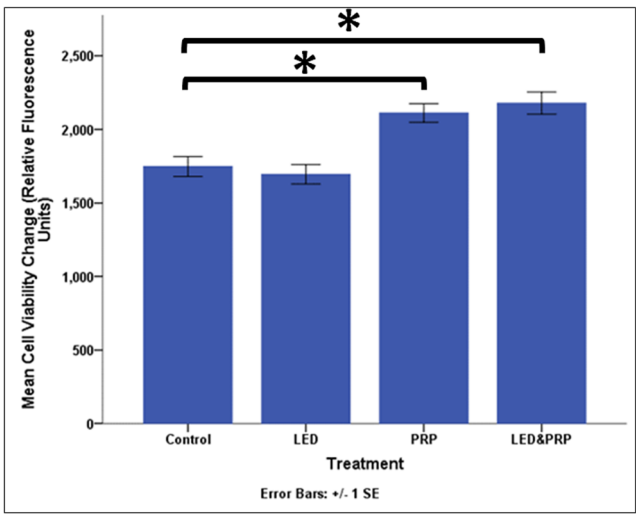

Fig. 4 Alamar blue viability assay in 48-h treatment period. LED: light-emitting diode, PRP: platelet-rich plasma. *Significant $P < 0.05$, SE: standard error of the mean

Bovine serum is essential for cell culture survival, and a concentration of 10% was selected to maintain cell viability in the log phase and allow to detect potential effects based on our pilot study results (data not shown). A cell density of 10,000 cells/well was used to determine serum concentration based on our previous study using bovine primary cell cultures [30] and in line with other in vitro studies [31]. Blank wells filled with media were added at the periphery of the tested wells to minimize the effect of evaporation due to the 48 h of culturing. Alamar blue assay was chosen over MTT assay as it measures viability of cells by fluorescence intensity units (not affected by background and depends on cell metabolism) in cell number range between 500 and 50,000 [32] which represent cells at exponential phase, while MTT assay measures viability of cells using absorbance intensity units (affected by background and depends on cell number) and readings close to 0.7 are used to determine seeding density [33]. Moreover, the change in cell viability during the treatment period was calculated which compensates for background effect, pipetting errors, and seeding number variations.

In this study, a third passage was used in all experiments which gave consistent results and represent an active cell line before a senescence started to take place [28]. Moreover, our seeding density results suggest a 10,000 cells per well of 96-well plate, and a 100,000 cells per well of a 24-well plate are appropriate seeding density to start our experiments and to allow the measurement of the treatment effect before confluence was reached and this was in accordance with other in vitro studies using seeding density ranges between 5000 and 10,000 for 96-well plates with different cell lines [32, 34]. Therefore, choosing a seeding densities which represent the midpoint of the log phase (exponential growth) of the

growth curve allowed the detection of any changes in cell growth as a result of treatment [28]. PRP used was prepared from sheep whole blood and used within 2 days which ensures a full efficiency and eliminates the potential adverse immune response [35]. A 5% (*V*/*V*) of PRP supplied with media was used as treatment for tenocytes, and this concentration was recommended in previous studies [31, 36, 37].

LED energy and frequency optimization results revealed that a fluence of 4 J/cm^2 applied every other day (48-h treatment period) is expected to result in best parameters and maintain safety at the same time. A 48-h LED treatment period was in accordance with the World Association of Laser Therapy (WALT) (World Confederation for Physical Therapy, 2017) and were comparable with previous literature [38, 39]. For example, Xavier et al. (2010) use rats as a model of partial injury; LED treatment was applied every 48 h; in the other hand, Casalechi et al. (2009) used a 24-h LED treatment period; results from both in vivo animal studies improved tendon healing [9, 17]. LED treatment application was applied from the top of the plate which is comparable to tendons that are enclosed with skin and tendon sheaths. LED energy density-optimized results (4 J/cm^2) were in accordance with several previous studies investigating photobiomodulation upon healing of animal models for cartilage, tendon, and muscle disorders utilizing low energies [8, 9, 40–51].

LED and/or PRP effects on cell viability and migration

Cell proliferation, metabolism, and migration are key phases in tendon repair process [7]. Results showed that PRP alone or combined with LED increased significantly tenocyte viability while LED alone could enhance cell migration. PRP seems to enhance proliferation and metabolism and impede cell migration. These results suggested that factors released from platelets such as growth factors [22, 23] are more potent than LED-induced effect on cell proliferation and metabolism at the parameters used and under the same conditions. Previous in vitro studies applying PRP have showed similar outcomes of increased proliferation rate of tenocytes [23, 37] and fibroblast [52, 53]. In our study, cells responded to PRP with/without LED with slow migration, and this could be explained as cells were very active in proliferation and metabolism rather than migration as cells were in the initial phase of healing response actively involved in metabolism to synthesize factors such as chemotactic factors to recruit other cells simultaneously [22]. The previous study results showed that platelet factors such as histamine have different effect on cell migration and proliferation when applied at different cell types; for example, in the endothelial cell, only cell division increased while in smooth muscles, both migration and proliferation increased [57]. A recent study supports the proliferative effect of PRP but contradicts our results of slow migration effect which could be due to the human skin fibroblast used instead of sheep tenocytes [58].

Interestingly, LED treatment of skin fibroblasts and epithelial cells has increased proliferation and migration at wavelengths of 638 nm and 518 nm which supports our results [54]. However, other studies did not find significance potential effects for light therapy which could be attributed to the different light devices or different light parameters used [27, 55, 56]. Photobiomodulation-induced effects such as anti-inflammatory have been demonstrated in different animal models addressing musculoskeletal disorders involving tendons, cartilage, and muscles [8, 9, 40–51]. For example, a tendinitis model showed that 3 j of 810-nm light therapy was effective in lowering inflammatory mediators such as prostaglandins and MMPs [43, 48] and a photobiomodulation anti-inflammatory effect at the level of gene expression using a 4 J of 808 nm light upon an in vivo arthritis model; however, both treatments apply a monochromatic laser light therapy [49, 51]. Moreover, anti-inflammatory and antioxidant effects because of photobiomodulation treatment were shown in experimental models of skeletal muscle myopathy [41, 42, 44, 46] and cartilage-induced inflammation [45, 47]. Dystrophin gene and protein parameters have improved in a myopathy rat model following a 3 and 10 J of 870-nm and 640-nm LED probes combined with 905-nm laser probe [40]. Similar photobiomodulation protective effect upon a trauma-induced myopathy model at low energies of 1 and 3 J of 660-nm and 905-nm lasers was seen [50]. These experimental studies support the effect of phototherapy on disorders involving tendons, cartilage, and muscles at different energies, different wavelengths, and different optical devices and more precisely at low energies less than 10 J and within a therapeutic wavelength window of 600 to 900 nm, and these conclusions were in harmony with our in vitro results as 4 J/cm^2 of 625 nm and 850 nm LED was delivered.

In conclusion, our model safety was confirmed, and results indicate that adding PRP would significantly decrease migration (scratch assay) and increase viability (viability assay-Alamar blue), while LED could enhance migration and proliferation. Results suggested that using PRP alone or combined with LED might have the potential to enhance healing response at the conditions applied; however, this is an in vitro study which could be confirmed with future in vivo studies. In future studies, it is recommended to investigate longer culturing periods, higher passage number, and different serum levels. Further research is needed to tackle effects on metabolism especially collagen and proteoglycans.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent No informed consent is needed (in vitro study).

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