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ORIGINAL ARTICLE

Evaluation of wavelength-dependent hair growth effects on low-level laser therapy: an experimental animal study

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Abstract In this study, we aimed to investigate the wavelength-dependent effects of hair growth on the shaven backs of Sprague–Dawley rats using laser diodes with wavelengths of 632, 670, 785, and 830 nm. Each wavelength was selected by choosing four peak wavelengths from an action spectrum in the range 580 to 860 nm. The laser treatment was performed on alternating days over a 2-week period. The energy density was set to 1.27 J/cm^2 for the first four treatments and 1.91 J/cm^2 for the last four treatments. At the end of the experiment, both photographic and histological examinations were performed to evaluate the effect of laser wavelength on hair growth. Overall, the results indicated that low-level laser therapy (LLLT) with a 830-nm wavelength resulted in greater stimulation of hair growth than the other wavelengths examined and 785 nm also showed a significant effect on hair growth.

Keywords Low-level laser therapy · Wavelength-dependent effect · Action spectrum · Hair growth · Hair follicle

Introduction

Since Endre Mester reported that low-level laser light induced the growth of shaved hair on the back of mice more readily than untreated shaven mice [1], research on low-level laser therapy (LLLT) for hair regrowth has been conducted [2–4]. LLLT is a process referred to as photobiomodulation, which induces a photochemical reaction in the cell, irrespective of any thermal effect [5]. The hair growth stimulation of LLLT causes more hair follicles to move from the telogen to the anagen phase [6]. This effect is mediated through a direct or an indirect increase in proliferative activity within the hair follicle epithelial matrix. Laser light irradiation results in increased cutaneous microcirculation and stimulates the secretion of endogenous growth factors, such as fibroblast growth factor and insulin-like growth factor 1. The details of the cellular mechanisms of LLLT have been reviewed elsewhere [5, 7, 8].

The exact mechanisms by which low-level laser irradiation stimulates hair growth are unknown. The HairMax LaserComb®, which is a LLLT device for the treatment of male-pattern baldness, received 510(k) clearance from the US Food and Drug Administration (FDA) in 2007 [9]. However, a recent study conducted by King Jr. et al. reported that the LaserComb treatment did not induce hair growth in C3H/HeJ mice with extensive alopecia areata (AA) in well-established spontaneous arising or full-thickness skin graft mouse models of human AA [10]. This study reported conflicting results compared with previous studies [2, 6, 11] and raised concerns about the fundamental effectiveness of LLLT devices for hair regrowth. Furthermore, there is a lack of standard wavelengths and doses of LLLT for hair regrowth treatments.

In the present study, we investigated the wavelength-dependent effects of lasers of wavelengths 632, 670, 785, and 830 nm on hair regrowth on the shaven backs of Sprague–Dawley rats. No study to date has compared the effects of near-infrared wavelengths with those of visible wavelengths [7]. The wavelengths used were selected by

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choosing four peak wavelengths from an action spectrum in the range 580 to 860 nm [12]. The action spectrum represents the relative effectiveness of different wavelengths of light in causing a particular biological response which resemble the absorption spectrum of the photoacceptor molecule [13]. We hypothesized that the hair growth rate induced by laser light of each wavelength would be dependent on the order of peak magnitude in the action spectrum, since the main photoacceptor in the action spectrum, cytochrome C oxidase, is located in the mitochondria, on which LLLT mainly acts [12]. The aim of this study was to determine the optimal wavelength of LLLT for hair regrowth.

Materials and methods

Animal preparation

All experimental procedures and protocols used in this investigation were reviewed and approved by the animal care and use committee of the university (approval no. CUD-2014-033). Twenty-five 8-week-old male Sprague–Dawley rats were used for the experiment. The rats were randomly divided into five groups as follows: normal control group (n=5), 632-nm LLLT group (n=5), 670-nm LLLT group (n=5), 785-nm LLLT group (n=5), and 830-nm LLLT group (n=5). Dorsal side pelage from the lower back was shaved using an electric clipper with a 1-mm blade. Since depilation by shaving can induce mechanical stimulation of hair growth [14], shaving was performed in the opposite direction of the slanting of the hair shaft, and the number of shavings was restricted to five, and only three to four times trimming was allowed for all animals. As shown in Fig. 1, round bracket-shaped tattoos (1-in. diameter) were drawn onto the shaven skin where laser treatment was applied. During LLLT, all animals were secured in a tail-access rodent restrainer. Anesthesia was used only at the first shaving and at the end of the experiment for euthanasia for skin tissue biopsy. All animals were housed under controlled conditions (12-h light/dark cycle, at an average temperature of 22 °C) and had access to food and water ad libitum.

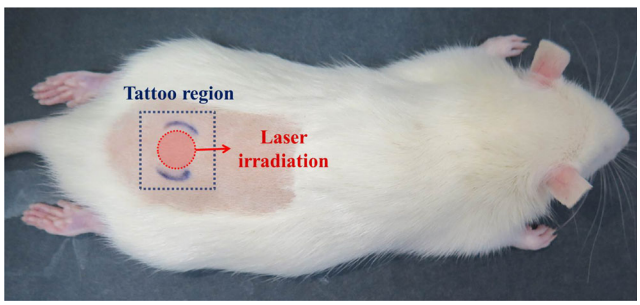

Fig. 1 Shaven back of a Sprague–Dawley rat. Laser irradiation point (*red circle*) is in a guide tattoo region (*blue round bracket*). The specified treatment region was also used for both photographic and histological examinations

Laser treatment

Laser diodes (LDs) with four different wavelengths (632 nm (HL6321G/22G, HITACHI, Japan), 670 nm (HL6756MG, HITACHI, Japan), 785 nm (L785P090, Thorlabs, USA), and 830 nm (HL8337MG/38MG, HITACHI, Japan)) were used as the LLLT sources. The LD was attached to a temperature-controlled mount (TCLDM9, Thorlabs, USA) and operated by an LD driver (LDC340, Thorlabs, USA). The operating currents differed according to the specifications of each laser diode, but the peak power for each was 10 or 15 mW. The laser beam was delivered to the tattooed skin region through direct contact using a light pipe (no. 65-838, Edmund Optics, USA), which has an aperture of 10 mm. The experiments were conducted over 2 weeks, and laser treatment was performed on alternating days for a total of eight treatments. The energy density was set to 1.27 J/cm^2 (irradiance 10 mW/cm^2, beam diameter 10 mm, exposure time 100 s) for the first four treatments and 1.91 J/cm^2 (irradiance 15 mW/cm^2, beam diameter 10 mm, exposure time 100 s) for the last four treatments. All procedures were applied to the five groups under the same conditions, with the exception of the control group, which received sham treatment.

Methods of hair growth evaluation

Photographic examination

At the end of the experiment, a handheld digital microscope (OPT-230, DIGIBIRD, Korea) was used to acquire images of the treated skin surface at ×50 magnification (Fig. 2b, c). Two images were obtained for each skin sample site. For the quantitative assessment of hair growth rate, image intensity in the L*a*b* color space was measured. The L* value of fur color is a quantitative indicator of de novo hair growth, and can be used to measure the hair growth rate in rats [15].

Histological examination

Two weeks after the start of the experiment, the rats were humanely euthanized. The light-treated dorsal skin was collected with an 8-mm biopsy punch (Integra Miltex, USA) and fixed in 10 % formalin. The skin sample was transversely sectioned for paraffin embedding. Paraffin sections (4–5 μm) were stained with hematoxylin and eosin (HE) and analyzed using an Axio Imager A1 microscope (Carl Zeiss Microimaging Inc., Thornwood, USA). During this examination, both the diameter and number of hair shafts and follicles were measured within the predefined range of the horizontal

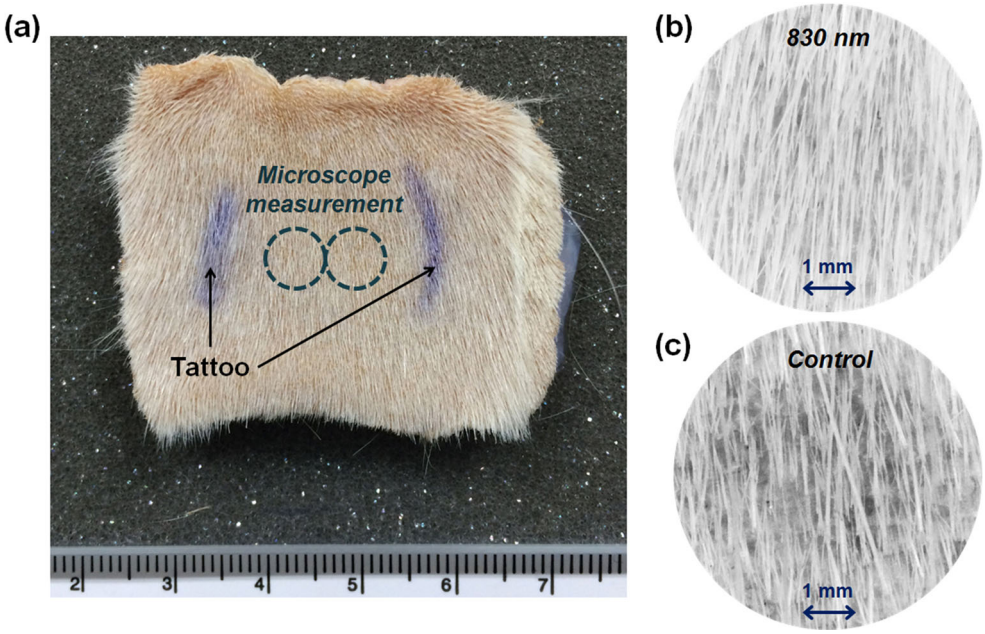

Fig. 2 **a** Photograph of an excised skin sample. Light intensity micrographs of the dorsal skin after LLLT at 830 nm (**b**) and in a control subject (**c**). This measurement was performed within the predefined tattoo region

axis (1.5 mm). Furthermore, we separated the dermis and subcutis within a specified area in the measurement, as shown in Fig. 3a.

Skin temperature measurement

Since photobiomodulation by LLLT should be independent of thermal effects [5, 16], it was necessary to determine temperature changes associated with LLLT. Thus, skin temperature was measured before and immediately after laser exposure using a near-infrared thermometer (NTF-3000, Braun, Germany).

Statistical analysis

Light intensities from photographic examination were compared by one-way analysis of variance (ANOVA). From histological examination, the diameter and number of hair follicles in both the dermis and subcutis were compared by multivariate analysis of variance (MANOVA). For post hoc comparisons, Scheffé's test was used. The values are presented as quartile plots in each figure and as the mean±standard deviation (SD) for each group in each table. All values were considered statistically significant at p values <0.05. Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 20.0 for Windows (SPSS Inc., USA).

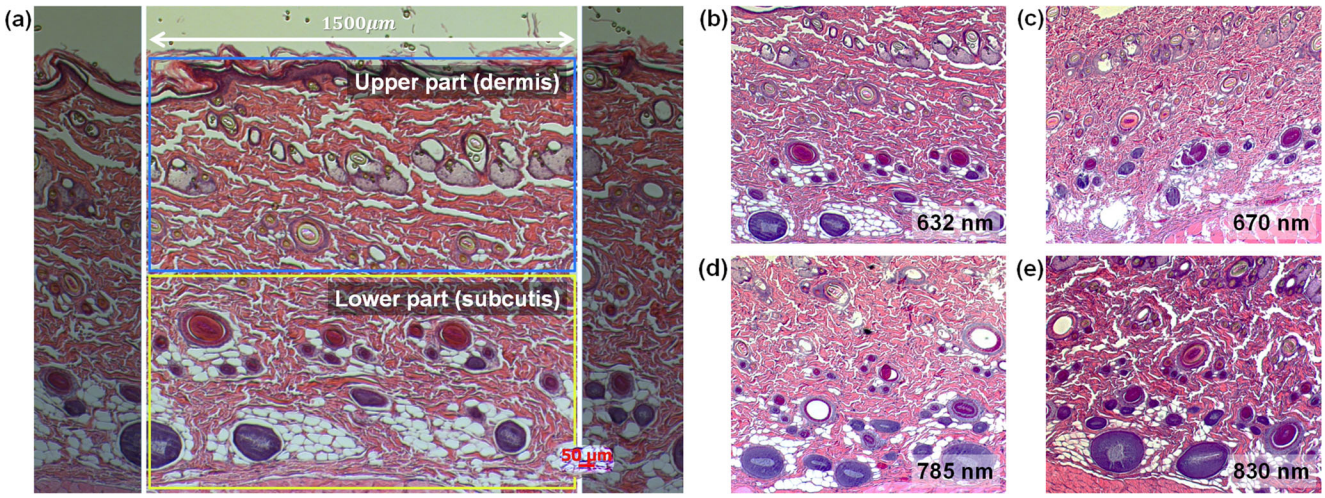

Fig. 3 Skin histology. **a** Number and diameter of follicles were individually analyzed by distinguishing the upper (dermis) and lower (subcutis) parts. The observation area was limited to a width of 1500 μm in each histological image. **b**–**e** Representative images of LLLT at each wavelength

Results

The results of descriptive statistics and one-way ANOVA of photographic examination are provided in Online Resource Tables 1 and 2, respectively. A quartile plot (Fig. 4) represents the light intensity distribution from micrographs at each wavelength. We found that the mean values of light intensity increased gradually with longer wavelengths; this result was statistically significant (F=8.775, p<0.001). The only non-significant wavelength for light intensity was 632 nm. According to Scheffé post hoc tests, 830 nm (p<0.01) and 780 nm (p<0.01) had more significant effects on hair growth rate than did 670 nm. In addition, the eta-squared value (0.438) was sufficiently high to indicate wavelength-dependent effects on hair growth. Therefore, longer wavelengths have greater effects on hair growth rate.

Figure 3b–e shows histological images of the dorsal skin of rats treated with light of four different wavelengths. Dermal and subcutis regions were visualized, and both the size and number of hair follicles were determined. The results of descriptive statistics and MANOVA of histological samples are provided in Online Resource Tables 3 and 4, respectively. A quartile plot (Fig. 5) represents the number (Fig. 5a, b) and diameter (Fig. 5c, d) of hair follicles, respectively. The size and number of hair follicles had positive correlations with follicle development and maturation. MANOVA showed a statistically significant wavelength-dependent effect on hair follicle growth rate (Pillai's trace=1.703, p<0.01). This effect is significant with an increase in the number of hair follicles in both the dermis (F=15.901, p<0.01) and subcutis (F=8.904, p<0.01) and diameter of hair follicles in the dermis (F=4.586, p<0.01). According to Scheffé post hoc tests, 830-nm (p<0.01) and 780-nm (p<0.05) light had significantly greater effects on the number of hair follicles in both the dermis and subcutis than did light of 632 and 670 nm.

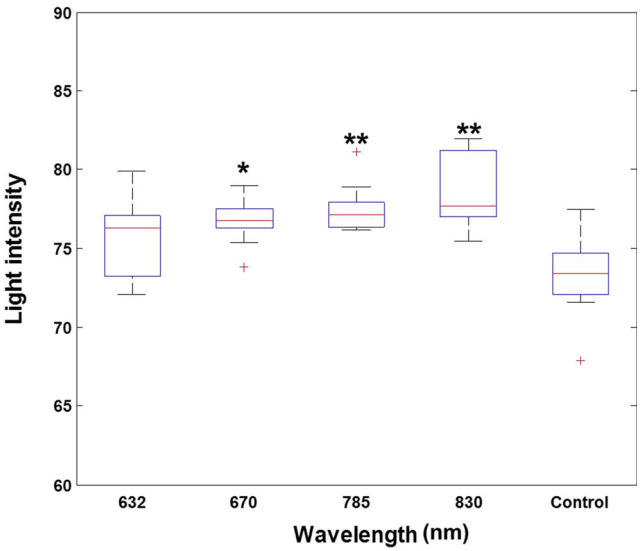

Fig. 4 Quartile plot of light-intensity distribution from analysis of photographs in the L*a*b* color space. *Red cross* represents outliers in each measurement group (*p<0.05, **p<0.01)

No statistically significant effect on the diameter of hair follicles following treatment with light of the wavelengths tested was detected on the histological images. The rationale behind this result would be due to a variation of total number of hair follicle among the five groups. Table 1 shows the follicle size distribution within three levels of the subcutis (note: a difference in the total number of hair follicle between each layer is easily detected). The effect on the number of follicles was significant for light of longer wavelengths (785 and 830 nm), compared with the control group, regardless of follicle size. Therefore, LLLT of 785 and 830 nm has a greater influence on hair follicle growth rate compared with light of visible wavelengths (632 and 670 nm).

We also measured skin temperature before and immediately after LLLT, but found no difference (data not shown). Thus, LLLT in this study was free from thermal effects.

Discussion

In this study, four wavelengths were selected by considering their peak values in the LLLT action spectrum, and the wavelength-dependent effects of LLLT on hair growth were investigated. Karu's group reported the following wavelength ranges for four peaks in the LLLT action spectrum: (1) 613.5–623.5, (2) 667.5–683.7, (3) 750.7–772.3, and (4) 812.5–846.0 nm [12]. We used laser diodes with wavelengths of 632, 670, 785, and 830 nm. Our first hypothesis was that the effects on hair growth rates of light of each wavelength would be dependent on the order of peak magnitude in the action spectrum (3>1>4>2). Overall, our results show that longer wavelengths have greater effects on the hair regrowth rate, with the effects of 830- and 785-nm light being significantly greater compared to light of shorter wavelengths.

Two factors can be referred to interpret the results. First, peak wavelengths in the action spectrum were not identical to the four selected wavelengths. Although the four selected wavelengths were within the four peak bands in the action

Table 1 Size classification of the total hair follicles in each group

Wavelength (nm)	Diameter classification			Total
	>50 μm	50–100 μm	<100 μm	
632	47	55	12	114
670	33	30	10	73
785	41	65	17	123
830	67	62	16	145
Control	33	22	7	62

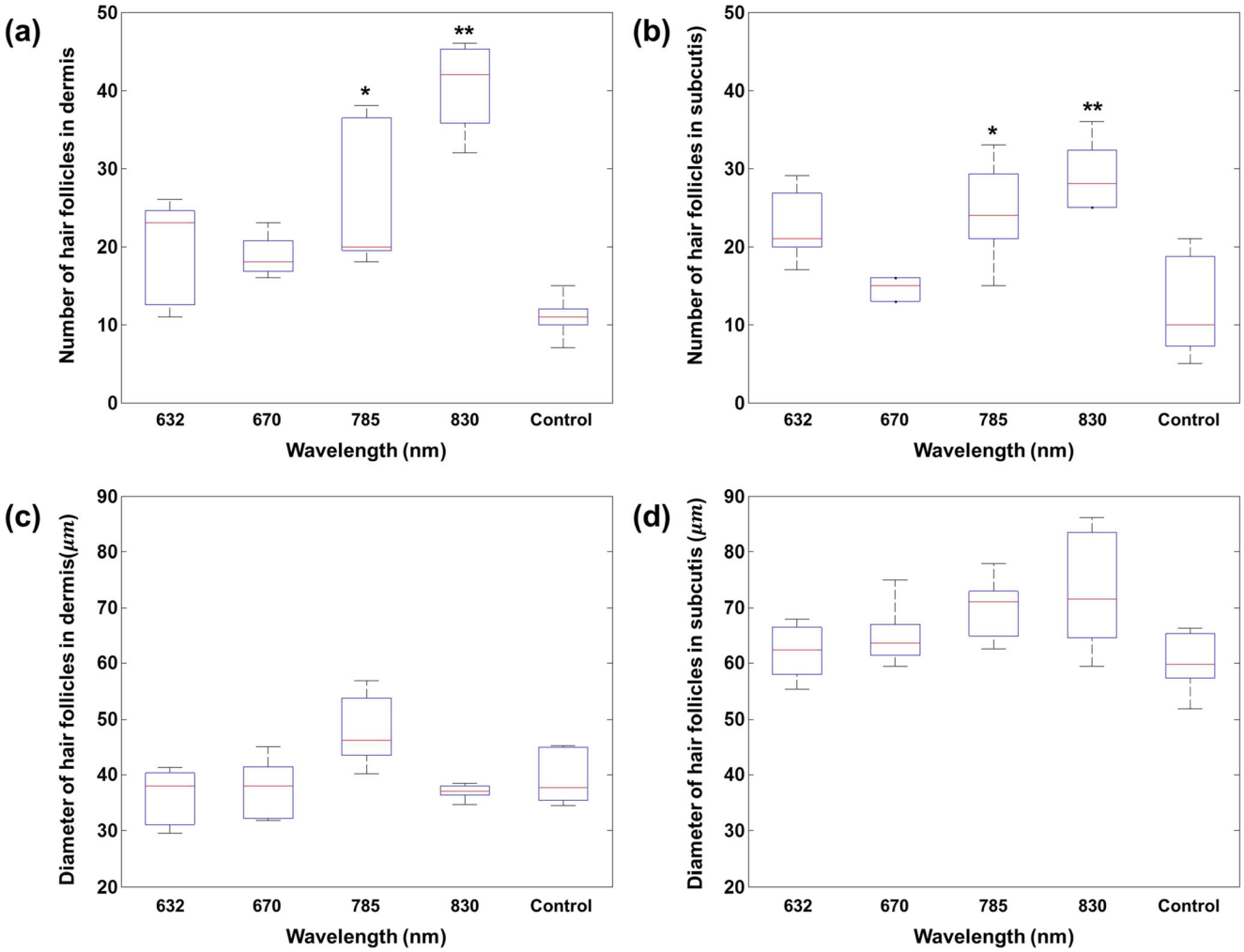

Fig. 5 Quartile plot of the number (**a**, **b**) and diameter (**c**, **d**) of hair follicles. Measurements were performed by considering the dermis and subcutis separately. **a**, **c** Dermis and **b**, **d** subcutis. There was no significant difference in the diameter of hair follicles, but the overall diameter distribution of hair follicles in the subcutis shows that the 830- and 785-nm-treated groups have more follicles of greater than 70-μm diameter (*$p<0.05$, **$p<0.01$)

spectrum, the ultimate peak wavelengths in the action spectrum differed (620, 680, 760, and 820 nm) [12]. It seems a small variation compared with actual used wavelengths but protein synthesis rate on cellular level sharply drops down when comparing 620 nm with 632 nm, as like other three wavelengths [12]. Thus, it can be inferred that no significant difference in the degree of cell stimulation would be expected among the selected four wavelengths, when precisely comparing the corresponding protein synthesis rate of each selected wavelength. Secondly, in general, a longer wavelength can penetrate deeper into the skin [17]. Since LLLT is at a very low energy density, photons have a lower probability of penetrating deeper into the skin. However, light of a longer wavelength has an advantage in terms of stimulating cells in subcutaneous tissue. We believe that deeper penetration of longer-wavelength light played an important role in facilitating the proliferation of hair follicle cells.

Almeida-Lopes et al. investigated the effect of light of wavelengths 670, 692, 780, and 786 nm on cell proliferation using the same fluence value (2 J/cm^2) [18]. Although the infrared laser induced greater cell proliferation than the visible-wavelength laser light at different power outputs, lasers of equal power output exerted similar effects on cell growth, independent of their wavelengths [18]. This study had a similar configuration to our work, but involved cultured human gingival fibroblasts, and thus did not take into account the differential ability of light of different wavelengths to penetrate the skin.

Evans and Abrahamse also investigated the effect of light of wavelengths 632.8, 830, and 1064 nm and found that 5 J/cm^2 of 632.8-nm light was more effective than light of 830 or 1064 nm [19]. They also found that a dose of 16 J/cm^2 caused DNA damage and reversible cell damage [19]. Their results are in conflict with our findings, but suggest that the

wavelength may exert different effects on the cellular responses in both wound repair and hair regrowth. In addition, Shukla et al. reported suppression of hair growth at 5 J/cm^2 of 632 nm using a He-Ne laser, while a stimulatory effect on hair growth was evident at 1 J/cm^2 [3]. Few studies have investigated the wavelength-dependent effects of light on cell stimulation and proliferation, and none has compared the effect of near-infrared wavelengths with visible wavelengths in terms of hair growth rate. Thus, a direct comparison of the results in this study with those reported previously is difficult.

In this study, we used Sprague–Dawley rats with white fur, since we aimed to evaluate the effects of photobiomodulation on hair growth caused by light absorption in mainly skin tissue, which excludes melanin absorption. In fact, the 800-nm diode laser is about 30 % less absorbed by melanin than the 694-nm ruby laser [20, 21], which is correlated with the efficacy of laser hair removal treatment [21]. A difference in hair growth rate according to fur color was first reported by Mester [1]. He found differences between black and white mice: increased hair growth on the irradiated area was observed in all black animals after the fifth to seventh treatments, while in the white mice, a hair growth effect was noticed after the eighth irradiation [1]. Therefore, we believe that the use of rats with white fur was appropriate for investigation of wavelength-dependent hair growth effects without taking into consideration the influence of melanin on absorption, which might be a key variable in photobiomodulation.

Also, we designated a single region both for treatment and measurement within each animal to avoid undesirable contralateral effects. Makihara et al. demonstrated that the increase in facial temperature and blood temperature in the stimulated region by continuous laser irradiation caused an expansion of blood vessels and an increase in blood flow volume. Moreover, this process can also cause an increase in facial temperature in unstimulated regions [22]. The increase in facial temperature on the contralateral side might be caused by the vasodilator reflex via the central nervous system [23]. Although we found no temperature changes during LLLT in this study, an expansion of blood vessels and increase in blood flow could have occurred and influenced the non-treated skin regions during LLLT [22]. A recent within-subject comparison study showed that the combination of LLLT with a hair growth promoter had a greater effect on hair regrowth than did either independently [24]. However, their measurements were not immune from the influence of contralateral factors. Thus, within-subject comparisons should be avoided in studies of LLLT. The shaving procedures were also carefully accomplished on the designated treatment region due to the fact that partially, skin injury as visible scratches could influence on irregular hair re-growth [14]. Also, we did not shave the tested area of the rats before each daily therapy since the depilation by shaving can induce mechanic stimulation of hair growth. An interesting study reported by Liu et al. observed the system of linear loops formed by the regrowing hairs on rat skin and the result indicated that the linear hair regrowth was closely correlated with the shaving process [14].

Conclusion

In the present study, we endeavored to minimize the number of variables considered and the stress to animals for evaluation of the LLLT-wavelength-dependent effects on hair growth. As a result, unlike our first hypothesis, LLLT at 830 nm showed greater efficacy than did the other wavelengths, and 785 nm also exerted a statistically significant effect on hair growth. However, it should be noted that LLLT for hair regrowth is ultimately necessary to the patient with hair loss and various types of hair loss exist and those action mechanisms are also different from each case. Thus, various hair loss models (alopecia areata, androgenic alopecia, etc.) should also be used to evaluate the effects of LLLT of different wavelengths. Furthermore, a greater number of subjects should be used to ensure statistical significance. In addition, natural human hair colors are diverse (such as black, brown, blond, red, grey, and white), and the impact of LLLT of various wavelengths on hair growth is unknown. Hence, it can be postulated that the amount of melanin absorption would influence the hair regrowth induced by LLLT. Nevertheless, the results of this study will facilitate selection of the optimal wavelength of LLLT for hair regrowth. In future studies, the ultimate peak wavelengths (620, 680, 760, and 820 nm) in the action spectrum should be adopted for LLLT. Furthermore, the effect of different fur colors should also be investigated to improve our understanding of wavelength-dependent hair regrowth.

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Compliance with Ethical Standards All procedures performed in this study involving animals were in accordance with the ethical standards of the Animal Care and Use Committee of the Catholic University of Daegu (approval no. CUD-2014-33).

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted (CUD-2014-033).

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