Lasers Med Sci (2016) 31:289–296
DOI 10.1007/s10103-015-1854-y

ORIGINAL ARTICLE

Anti-inflammatory and lymphangiogenetic effects of low-level laser therapy on lymphedema in an experimental mouse tail model

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Received: 28 January 2015 / Accepted: 11 December 2015 / Published online: 29 December 2015

Abstract The aim of the present study was to investigate the therapeutic mechanism of low-level laser therapy (LLLT) in the mouse tail lymphedema model. Six-week-old female mice were classified into the laser treatment group, sham treatment group, and surgical control group (10 mice per group). LLLT was administered daily for 10 min from the surgical day to 11 days (12 times). Macrophage activation and lymphatic vessel regeneration were evaluated through immunohistochemical staining with anti-F4/80 and anti-LYVE-1 antibodies, respectively, at 12 days post-procedure. Quantitative real-time polymerase chain reaction (qPCR) was performed to measure messenger RNA (mRNA) expression of vascular endothelial growth factor A, B, C, R1, R2, and R3 (VEGF-A, VEGF-B, VEGF-C, VEGFR1, VEGFR2, and VEGFR3) at 12 days post-procedure. Student's *t* and one-way ANOVA tests were performed for statistical analyses. Significance was defined as $p < 0.05$. The thickness of the tail rapidly increased until 6 days in the laser and sham groups. The mice in the laser group showed a significantly decreased thickness compared with the sham group at 10 and 12 days. Immunohistochemistry assay revealed that LLLT reduced inflammation and induced new lymphatic vessel growth. qPCR showed that expressions of VEGFR3 were ($p = 0.002$) increased in the laser group. These results suggest that LLLT has anti-inflammatory and lymphangiogenetic effects for the management of lymphedema.

Keywords Lymphedema · Low-level laser therapy · Lymphangiogenesis · Vascular endothelial growth factor C

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Introduction

Recently, more attention has been paid to lymphedema as it has become a relatively common complication after treatment of cancer. Cancer-related lymphedema occurs through the accumulation of protein-rich fluid because of lymphatic flow insufficiency after lymph node dissection, cancer surgery, and radiotherapy. However, lymphedema is a chronic, debilitating condition that has traditionally been considered as an incurable disease requiring lifelong attention and management [1, 2]. As a result, the quality of life (QOL) of cancer survivors with lymphedema is decreased, and psychosocial morbidity is frequently prevalent [3]. Therefore, proper management of lymphedema is necessary to improve QOL; however, effective long-term management of lymphedema remains challenging. The management of lymphedema is usually focused on conservative interventions including complex decongestive therapy, garments, pneumatic pump therapy, and drug therapy. Recently, low-level laser therapy (LLLT) has been suggested [1, 4].

LLLT was approved by the American FDA in 2007, and several studies showed a reduction of limb volume in lymphedema [5–8]. Based on these clinical studies, LLLT has been considered as an easy-to-use treatment that may have a long-

term effect in the management of lymphedema [6, 9]. However, the exact mechanism of LLLT on lymphedema in tissues has not yet been clarified, and no experimental study of LLLT on lymphedema has been performed to date. Therefore, the aim of the present study was to investigate the therapeutic mechanism of LLLT in the mouse tail lymphedema model.

We hypothesized that LLLT may have long-term benefits through therapeutic lymphangiogenesis on lymphedema. Several reports showed that enhanced the expression vascular endothelial growth factor C (VEGF-C) and vascular endothelial growth factor receptor 3 (VEGFR3) correlate with increased lymphatic vessel growth [10–14]. We investigated the impact of LLLT on VEGF-C and VEGFR3 induced lymphangiogenesis. In addition, we assessed changes in the thickness of the mouse tail, macrophage activation, and lymphatic vessel regeneration.

Materials and methods

Mouse tail model

All protocols were approved by the Animal Care and Use Committee of the Asan Medical Center.

A brief outline of the present study is presented in Fig. 1a. The study used 6-week-old female mice (Orient Bio, Seongnam, Korea) (10 mice per group) in the following procedures: an operation group that underwent LLLT (laser group), an operation group that underwent sham treatment (sham group), and a surgical control group (control group). The mouse tail model for lymphedema was created as previously described, but with minor modification [13, 15]. In brief, the mice were anesthetized with intraperitoneal injection with 1.2 % Avertin 0.7 ml, and the skin was circumferentially incised at 15-mm distal to the base of the tail. The major lymphatic trunks were identified through distal intradermal injection of methylene blue solution (319112; Sigma-Aldrich, St. Louis, MO, USA), and the exposed large collecting lymphatic vessels were cauterized using Bovie cautery. For surgical controls, only the skin incision was performed with methylene blue injection in the absence of lymphatic cautery.

Low-level laser therapy

LLLT with a wavelength of 904 nm, power of 5 mW, spot size of 0.2 cm^2, irradiance 25 mW/cm^2 (LTU-904; RianCorp Pty Ltd., Richmond, South Australia, Australia) and average doses of 1.5 J/cm^2 was applied continuously for 10 min, with pulse duration of 200 ns, and maximum frequency of 5000 Hz to the mice in the laser group. LLLT was applied with the similar parameters that used in previous reports [5, 6]. Before the experiment, the LLLT equipment was calibrated. LLLT was daily administered at 10 points on the subcutaneous injury site and just below the injury site from surgical day to 11 days (12 times). Each treatment point was applied for 1 min with total duration of 10 min. The laser was vertically held in contact with the skin. For the sham group, the same parameters of therapy were performed without switching on the machine.

Edema assessment of the mouse tail

Daily thickness (anterior-posterior) measurements of the tails, just below, and 2-cm below the incision were performed using a thickness gage (7301; Mitutoyo, Kawasaki, Kanagawa, Japan); each thickness was measured three times to obtain an average value.

Specimen preparation for histology

The mice were sacrificed at 12 days post-procedure. The tissue samples were prepared from incision to 1-cm below. The tissue was fixed with 4 % paraformaldehyde at 4 °C for 24 h. Specimens were decalcified in 10 % ethylenediaminetetraacetic acid solution (EDTA, 17385-0401; JUNSEI Chemical Co., Tokyo, Japan) for 2 weeks. For cryoprotection of the tissues, specimens were immersed in 30 % sucrose (S0389; Sigma-Aldrich) in phosphate-buffered saline (PBS) solution until the tissue sank to the bottom of the tube. Samples embedded in optimal cutting temperature compound (4583; Sakura Finetek USA, Inc., Torrance, CA, USA) and cut into 6-μm sections with a cryotome. For the observation of tissue swelling and histological changes, transverse sections of the tail wounds and control tails were stained by hematoxylin and eosin (H&E staining).

Immunohistochemistry assay

To examine macrophage activation and identify lymphatic vessels, immunofluorescence staining was performed with the mouse macrophage specific surface marker F4/80, and the lymphatic-specific marker lymphatic vessel endothelial hyaluronan receptor (LYVE)-1. F4/80 is well characterized and is the best known mature mouse macrophage marker. Macrophage is one of major cells involved the inflammatory response and may be related to fibrosis in chronic lymphedema [16]. LYVE-1 is a cell surface receptor on lymphatic endothelial cells and commonly used to detect lymphangiogenesis in experimental studies [17].

The tissue slides of 6 μm were washed two times with PBS for 5 min. For antigen retrieval, the sections were dipped in citrate buffer (pH 6.0) at 95 °C for 5 min. For permeabilization, the tissue slides were incubated with PBS containing 0.25 % Triton X-100 for 10 min and washed two times with PBS for 5 min each. To block unspecific binding of the antibodies, the tissue slides were incubated with 1 % bovine serum albumin in PBS-T (0.01 % Tween 20 in PBS) for 30 min. To eliminate endogenous peroxidase activity, Dako

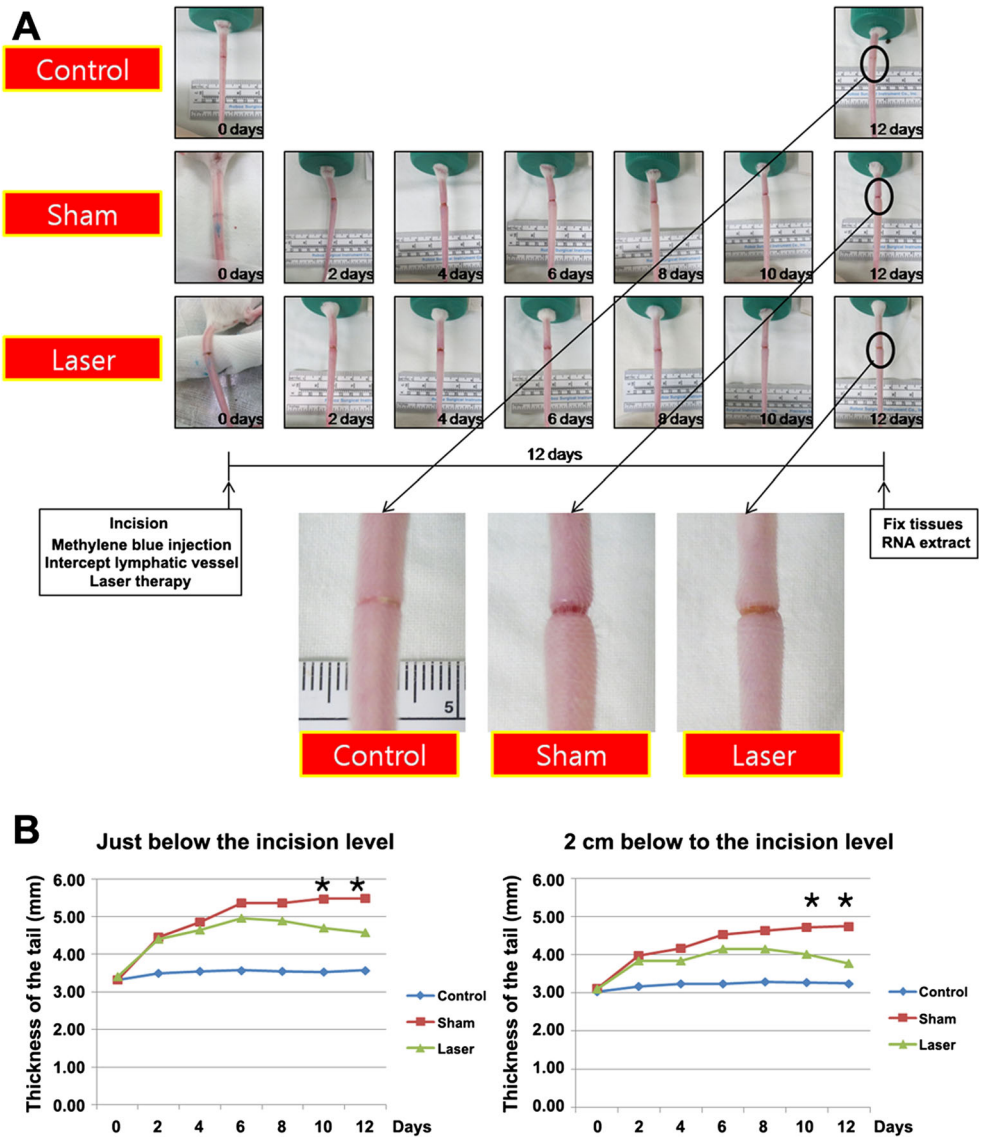

Fig. 1 **a** The outline of the present study and representative macroscopic images of changes in mouse tail thickness of the laser group and sham groups. The major lymphatic trunks were identified through distal intradermal injections of methylene blue solution. Thickness measurements of the tails were performed from surgical day to 12 days (13 times). Mice were sacrificed at 12 days post-procedure for tissue evaluations. **b** Changes of the thickness of the tail after electrocauterization of the lymphatic vessels: Just below the incision level and 2 cm below to the incision level. The laser group showed a significant decrease compared to the sham group at the 10 and 12 days ($n = 10$, $^*p < 0.05$)

REAL™ Peroxidase-Blocking Solution (S2023; Dako, Glostrup, Denmark) was treated for 30 min at room temperature. After washing two times in PBS for 5 min each, the slides were incubated with a primary antibody, the anti-LYVE-1 antibody (ab14917, 1:1000; Abcam, Cambridge, MA, USA) and the anti-F4/80 antibody (ab6640, 1:100; Abcam), for 1 h at room temperature, and then washed two times with PBS for 5 min each. Dako REAL Envision Detection system Peroxidase/DAB+, Rabbit/Mouse kit (K5007; Dako) and Biotinylated Rabbit Anti-Rat IgG Antibody (BA-4000, 1:200; Vector, CA, USA) were used as secondary antibodies. Slides were treated with DAB+ substrate chromogen solution (K5007; Dako) for 5 min, after which the slides were washed with distilled water for 10 min. The slides were then dehydrated with a series of graded ethanol (50 to 100 % ethanol) and xylene and mounted with Permount solution (SP-15-100; Fisher Scientific, Bridgewater, NJ, USA). Images were recorded with a Leica DF280 and processed with Leica application suite Ver. 2. 8. 1. The percentage of DAB positive area was quantified in five fields per slide under an objective lens with a magnification power of 20 (field area 0.832 mm^2) using Image J for anti-F4/80 antibody ($n = 3$ for each experimental group). To quantify lymphatic vessels, the number of LYVE-1 positive vessel per mm^2 was counted in five randomly selected ×200 fields per slide under an objective lens with a magnification power of 10 by a blinded observer ($n = 5$ for each experimental group).

Total RNA extract and quantitative real-time polymerase chain reaction assay

Sections from 5-mm proximal to the surgical incision to 5-mm below it were harvested for RNA extract. Tissue samples were stored at −80 °C until RNA preparation. Total RNA was

extracted from tissues using the RNeasy Mini Kit (74134, QIAGEN, Hilden, Germany) according to the manufacturer's protocol. In brief, before lysis of a tissue sample, β-mercaptoethanol was added to RLT plus buffer (10-μl β-mercaptoethanol per 1-ml RLT plus buffer). A tissue sample was homogenized in 350-μl RLT plus buffer on ice. The lysate was centrifuged at 13,000 rpm, 4 °C for 5 min. The supernatant was transferred to a gDNA Eliminator column and centrifuged at 13,000 rpm, 4 °C for 1 min. Furthermore, 350 μl of 70 % ethanol was added to the flow-through and mixed well, after which the mixed solution was transferred to an RNeasy spin column and centrifuged at 13,000 rpm, 4 °C for 1 min. Next, 700 μl of buffer RW1 was added to the column and centrifuged at 13,000 rpm, 4 °C for 1 min. Buffer RPE (500 μl) was added to the column and centrifuged at 13,000 rpm, 4 °C for 1 min. The column was washed with 500 μl of buffer RW1 and centrifuged at 13,000 rpm, 4 °C for 2 min. RNA was eluted with 30 μl of RNase-free water.

Mouse GAPDH gene was used as internal control. Quantitative RT-PCR to validate gene expression changes was performed using Power SYBR® Green One-Step RNA-to-CT Reagents (# 4389986; Applied Biosystems, Carlsbad, CA, USA) according to manufacturer's protocol. As shown in Table 1, 0.1 μg of RNA and 10 pmole primer were used for quantitative real-time polymerase chain reaction (qPCR) reaction; the final volume was 20 μl. Reverse transcription step temperature was 48 °C for 30 min, and the preheating temperature was 95 °C for 10 min. The following conditions were used for qPCR: denaturation for 15 s at 95 °C, followed by annealing for 1 min at 60 °C; for a total of 40 cycles. Results are expressed as the ratio of each target gene expression relative to control group gene expression with ΔΔCT method. All experiments were performed in triplicate.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Student's *t* test was performed for the comparison of independent variables between two groups and one-way ANOVA followed by Tukey's test for more than two groups. Significance was defined as $p < 0.05$.

Results

Reduction of lymphedema thickness by LLLT

The thickness of the tails in the laser and sham groups rapidly increased until 6 days after electrocauterization of the lymphatic vessels. The thickness of the tail gradually decreased from day 8 in the laser group but not in the sham group. The mice in the laser group showed a significantly decreased thickness compared with the sham group on day 10 (just below the incision level: 4.70 ± 0.42 vs. 5.48 ± 0.39 mm, $p < 0.05$; 2 cm below to the incision level: 4.00 ± 0.43 vs. 4.71 ± 0.55 mm, $p < 0.05$, respectively) and day 12 (just below the incision level: 4.57 ± 0.49 vs. 5.49 ± 0.36 mm, $p < 0.05$; 2 cm below to the incision level: 3.77 ± 0.35 vs. 4.74 ± 0.49 mm, $p < 0.05$, respectively) (Fig. 1b).

Effect of LLLT on histological changes in wound tail tissues

The edema was identified in the expansion of lymphatic vessel formed at the tail lesions in sham mice. The sham group showed also greater skin thickness with highest density of infiltrating cells adjacent to the perivascular regions, representing the inflammatory changes in these tissues. In contrast, the laser group consistently revealed a decreased edema and density of infiltrating cells compared with the sham treatment group (Fig. 2).

Effect of LLLT on macrophage activation and lymphatic vessel regeneration

On staining with anti-F4/80, sham treatment group showed more noticeable brown-stained cells compared to the laser group (Fig. 3a), and quantitatively, the laser group revealed a decrease in the number of macrophages but the value was not significant (10.80 ± 5.02 vs. 18.18 ± 4.26, respectively, $p = 0.124$) (Fig. 3b). As shown in Fig. 4a, a remarkable number of LYVE-1 stained vessels were detected in the laser group. In the sham group, dilated LYVE-1-stained vessels were present. On the quantitative analysis of LYVE-1-positive vessels, the laser

Table 1 Primers for quantitative real-time PCR

Gene name	Forward primers	Reverse primers
GAPDH	TGGCCTTCCGTGTTCCTAC	GAGTTGCTGTTGAAGTCGCA
VEGF-A	CTTGTTCAGAGCGGAGAAAGC	ACATCTGCAAGTACGTTCGTT
VEGF-B	GCCAGACAGGGTTGCCATAC	GGAGTGGGATGGATGATGTCAG
VEGF-C	GTGAGGTGTGTATAGATGTGGGG	ACGTCTTGCTGAGGTAACCTG
VEGFR1 (Flt-1)	CTCAGGGTCGAAGTTAAAAGTGC	TTGCCTGTTATCCCTCCCACA
VEGFR2 (Flk-1)	CTGGAGCCTACAAGTGCTCG	GAGGTTTGAAATCGACCCTCG
VEGFR3 (Flt-4)	CCGGCCTCAACATCACACT	TGCACGACGAAGAGATTGGAA

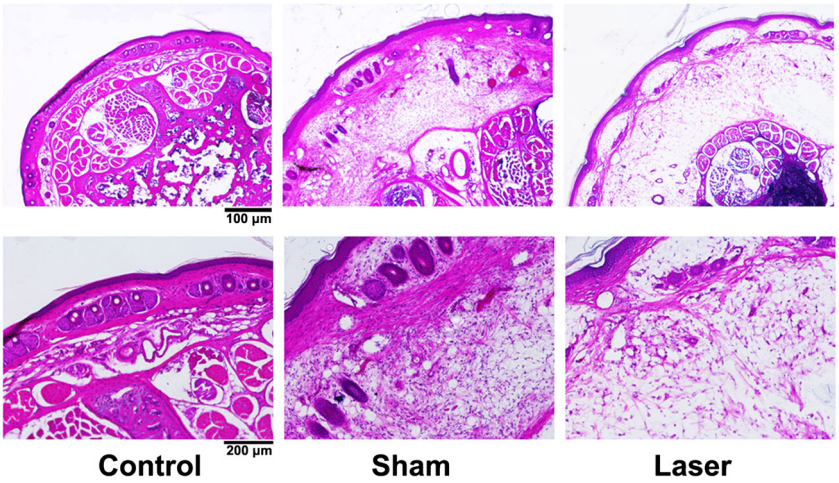

Fig. 2 An example of histological changes: The laser group revealed a decreased edema compared with the sham group. The sham group showed more inflammatory changes, hypercellularity, and greater skin thickness (H&E staining; scale bar = 100 μm and 200 μm)

group revealed a significant higher lymphatic vessel density (216.68 ± 17.98 vs. 80.43 ± 16.57, respectively, $p < 0.001$) (Fig. 4b).

Upregulation of lymphangiogenic gene expression by LLLT

As shown in Fig. 5, messenger RNA (mRNA) expression of VEGF-C for the laser group was increased but this value was not significant (2.59 ± 0.46 vs. 1.97 ± 0.37-fold, respectively, $p = 0.135$). VEGFR1, VEGFR2, and VEGFR3 mRNA expressions were increased on both laser and sham treatment groups compared with the control group. However, the laser group showed significant upregulation of VEGFR3 mRNA compared with the sham group (9.25 ± 0.39 vs. 6.89 ± 0.47-fold, respectively, $p = 0.002$).

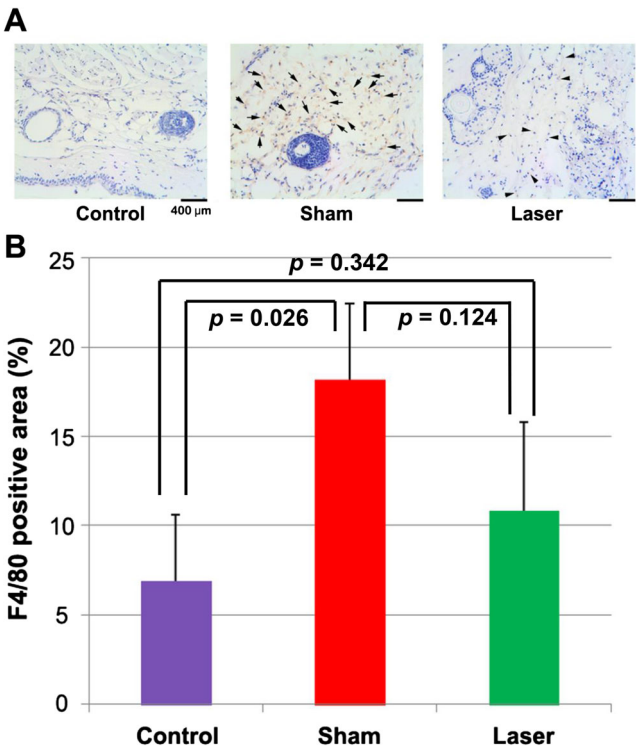

Fig. 3 An example of macrophage activation. **a** Sham treatment group showed more noticeable brown-stained cells (*arrows*) than the laser group (*arrow-heads*) (anti-F4/80 staining; scale bar = 400 μm). **b** The laser group yielded a decrease in F4/80 positive cells when compared with the sham treatment group but the value was not significant ($n = 3$)

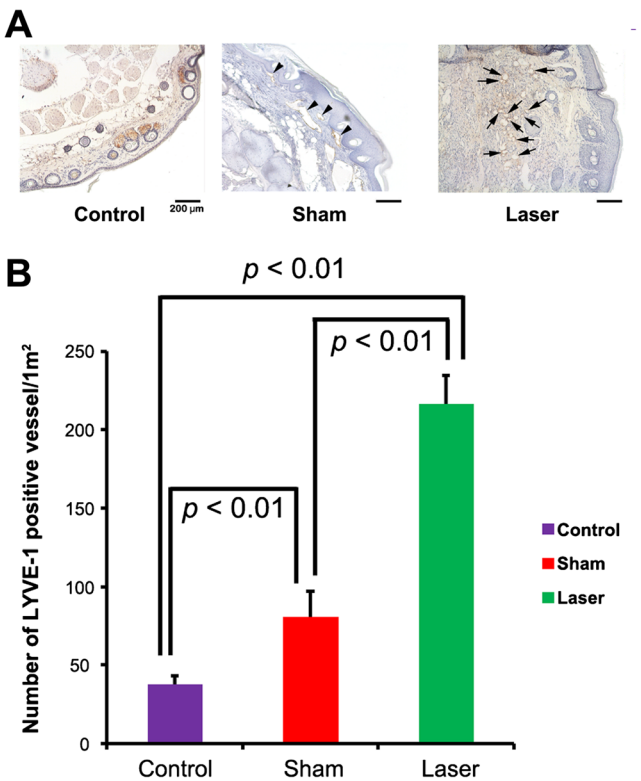

Fig. 4 An example of lymphatic vessel regeneration. **a** Note LYVE-1 stained the hyperplastic lymphatic vessels (marked by *arrow-heads*) in the sham treatment group. In the laser group, LYVE-1-stained small lymphatic vessels (*arrows*) were prominent (anti-LYVE-1 staining; scale bar = 200 μm). **b** The laser group yielded a significant higher density of LYVE-1 positive vessels when compared with the sham treatment group ($n = 5$)

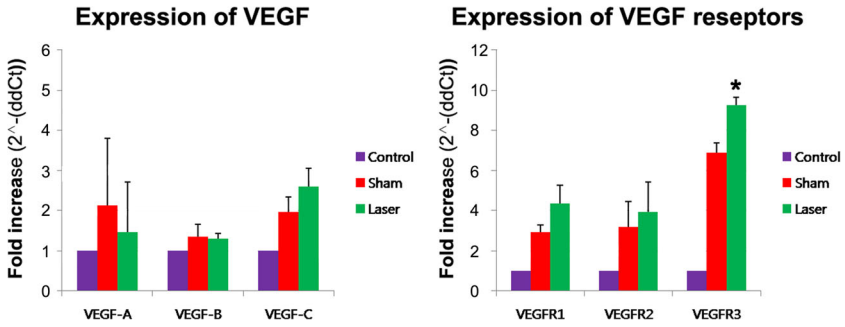

Fig. 5 Quantitative real-time PCR of VEGF-A, VEGF-B, VEGF-C, VEGFR1, VEGFR2, and VEGFR3 expression. In the laser group, VEGF-C mRNA expression was increased but the value was not significant. VEGFR1, VEGFR2, and VEGFR3 mRNA expressions were increased on both laser and sham treatment groups compared with the control group. However, the laser group showed a significant overexpression of VEGFR3 mRNA compared with the sham group ($n = 3$, $^*p < 0.05$)

Discussion

The present study showed that LLLT increased lymphatic vessel regeneration and upregulation of VEGFR3 expression and improved lymphedema in the mouse tail model. In addition, the trend toward decreased macrophage activation and upregulation of VEGF-C was indicated although these findings were not statistically significant.

The mouse tail model has been commonly used for experimental lymphedema study. Previous studies showed mouse tail swelling rapidly increased until 7 days post-procedure, reached maximum swelling from 10 to 14 days, and then slowly decreased [13, 15]. The present study showed similar results, which increased until 10 and 12 days post-procedure in the sham group.

Recently, two studies performed a systemic review on the effectiveness of LLLT in the management of breast cancer-related lymphedema [7, 8]. These systemic reviews analyzed 41 articles, including five studies of evidence level II, and suggested that LLLT demonstrated moderate to strong efficacy in the management of lymphedema. Clinically, LLLT has been used in addition to complex decongestive therapy, and further large-scale studies to more accurately assess the efficacy of LLLT are recommended. In contrast to clinical studies, the therapeutic mechanism of LLLT for lymphedema has not yet established. Hypothetically, anti-inflammation, lymphangiogenesis, and stimulation of lymphatic motoricity have been assumed [6, 9].

LLLT has been suggested as an anti-inflammatory treatment in several diseases such as musculoskeletal disorders, heart failure, lung inflammation, and periodontitis [18–22]. Furthermore, increasing evidence has indicated that LLLT can modulate mRNA expression for pro- and anti-inflammatory mediators and reduce oxidative stress [20, 23, 24]. Pallotta et al. showed that LLLT enhanced COX-1 and COX-2 expression and inhibited pro-inflammatory mediators such as IL-1, IL-6, and PGE2 [19]. Casalechi et al. reported that LLLT increased anti-inflammatory mediators such as IL-10 expression [24]. The present study showed that macrophage activation was reduced in the laser group at 12 days post-procedure. Although our study did not reveal the activation of pro- and anti-inflammatory mediators, reduced macrophage accumulation could be explained by the anti-inflammatory process of LLLT.

Several clinical studies showed that LLLT provides the benefit of long-term effects in the management of lymphedema. These studies hypothesized that LLLT may encourage lymphangiogenesis and lymphatic motoricity [6, 25]. In the present study, we demonstrated that a remarkable number of LYVE-1-stained small vessels were detected in the laser group compared with the sham group. This result clarified that LLLT promotes the development of new lymphatic vessels and supported the long-term effect of LLLT in clinical studies. Because lymphedema is a chronic condition resulting from insufficiency of the lymphatic system, the lymphangiogenic effects of LLLT could be the ultimate benefit in the management of lymphedema.

In the present study, LLLT enhanced the expression of VEGF-C and VEFGR-3. Although several potential lymphangiogenic mediators have been indentified, VEGF-C has been considered the key regulator of lymphangiogenesis by activating VEGFR3 [12]. In several lymphedema animal models, VEGF-C administration increased the lymphatic vessel number, normalized the size of lymphatic vessels, and improved lymphedema resolution [10, 11, 13, 26]. Jin da et al. [27] showed that exogenous VEGF-C administration elicited lymphatic vascular remodeling at 11 days post-procedure, and Yoon et al. [14] reported that VEGF-C gene therapy induced new lymphatic vessel growth at 3 weeks post-procedure in the mouse lymphedema model. Our results indicated that LLLT induced new lymphatic vessel growth and improved lymphedema through enhanced expression of VEGF-C and VEFGR3 at 12 days post-procedure.

Furthermore, we believe that the anti-inflammatory effects of LLLT would promote and/or protect therapeutic lymphangiogenetic effects. Goldman et al. showed that uncontrolled excess VEGF-C caused lymphatic vessel hyperplasia without the functionality and suggested that VEGF-C by

itself cannot sustain any lasting effect of lymphangiogenesis [28]. Therefore, our results suggested that LLLT could be a promising potential treatment option for long-term management of lymphedema through synergic effects of lymphangiogenesis and anti-inflammation.

Future study is recommended to elucidate some unclear issues regarding LLLT in lymphedema management. The most commonly studied wavelength and dosage of LLLT in studies related to lymphedema, including the present study, were 904 nm and 1.5 J/cm^2, respectively. However, optimal parameters, including the timing of application, frequency, energy setting, and wavelength of LLLT for lymphedema management, are not yet established and further study is required. Although we have confirmed its effects in enhancing lymphatic drainage, the possibility that LLLT may promote metastasis in cancer patients remains unknown. Some studies showed that LLLT with low-energy density has no effect on tumor cells [29, 30]; however, further study for the safety of LLLT in lymphedema treatment is recommended.

The present study showed that LLLT improved mouse tail-modeled lymphedema. In immunohistochemistry assessment, we found that LLLT induced a decrease of inflammation and an increase of new lymphatic pathways. Considering enhanced the expression of VEGF-C and VEGFR3, LLLT may have therapeutic benefits lymphedema through lymphangiogenesis.

Acknowledgments This study was supported by a grant (2012-478) from the Asan Institute for Life Sciences, Seoul, Korea.

Compliance with ethical standards All protocols were approved by the Animal Care and Use Committee of the Asan Medical Center.

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